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Mansoa alliacea extract presents antinociceptive effect in a chronic inflammatory pain model in mice through opioid mechanisms

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ABSTRACT

In some chronic disorders, as in arthritis, the inflammatory pain persists beyond the inflammation control becoming pathological. Its treatment shows limited efficacy and adverse effects which compromises patients' quality of life. *Mansoa alliacea,* known as 'cipo alho', is popularly used as analgesic and others species of this genus show anti-inflammatory actions. We investigated the anti-inflammatory and antinociceptive potential of *M. alliacea* extract in an inflammatory pain model which presents inflammatory characteristics similar to those caused by arthritis, through of the intraplantar injection of complete Freund's adjuvant (CFA) in mice. The extract chromatographic analysis revealed the presence of ρ-coumaric, ferulic and chlorogenic acids, luteolin, and apigenin. The treatment with *M. alliacea* prevented and reversed the CFA-induced mechanical allodynia with maximum inhibition (I_{max}) of 100% and 90 ± 10%, respectively. The co-administration of *M. alliacea* extract plus morphine enhanced the anti-allodynic effect with I_{max} of 100%. The *M. alliacea* extract also reverted the CFA-induced thermal hyperalgesia with I_{max} of 3.6 times greater compared to the vehicle and reduced the thermal threshold under physiological conditions. However, *M. alliacea* extract did not reduce the CFA-induced edema and myeloperoxidase activity. Additionally, non-selective and δ-selective opioid receptor antagonists, but not κ-opioid, prevented extract anti-allodynic effect with I_{max} of 98 ± 2% and 93 ± 2%, respectively. Moreover, *M. alliacea* extract did not induce adverse effects commonly caused by opioids and other analgesic drugs, at least in the tested pharmacological doses after the acute treatment. *M. alliacea* extract presents antinociceptive activity in an inflammatory pain model, which presents inflammatory characteristics similar to those arthritis-induced, without causing adverse effects in tested pharmacological doses. These effects seem to be mediated mainly via δ-opioid receptors.

1. Introduction

Inflammatory pain occurs in response to tissue injury and the subsequent inflammatory reaction, which normally improves as inflammation resolves, a process called for physiological inflammation. However, in chronic diseases as arthritis occurs the pathological inflammation which is prolonged and dysregulated and the pain persists beyond the inflammatory control of disease (Walsh and Mcwilliams, 2014; Taneja et al., 2017; Perretti et al., 2017). Arthritis is one of the most prevalent chronic systemic inflammatory diseases which mainly affect the joints. Nearly 1% of the world's population and around 37% of the US adult population suffers from this condition which is characterized by pain, swelling, tenderness, warmth, and redness in the joints (Niu and Chen, 2014; Cuda et al., 2017; Banderas et al., 2017;

Abbreviations: M. alliacea, Mansoa alliacea; CFA, complete Freund's adjuvant; MaC, crude hydroethanolic extract of *Mansoa alliacea*; Eta, ethyl acetate fraction of *Mansoa alliacea*; But, butanolic fraction of *Mansoa alliacea*; Chl, chloroformic fraction of *Mansoa alliacea*; MPO, myeloperoxidase; PWT, paw withdrawal threshold

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Jafarzadeh and Felson, 2018). Arthritis results from immunological changes, synovial inflammation, cellular infiltration and proinflammatory cytokines production which can cause the destruction of cartilage and bones of the joint (Cuda et al., 2017; Banderas et al., 2017; Jafarzadeh and Felson, 2018; Oikonomopoulou et al., 2018).

The pain, of inflammatory characteristic, is the main arthritis symptom that arises due excitation and sensitization of peripheral nociceptors by algogenic substances present in the synovial fluid (Walsh and Mcwilliams, 2014; Boyden et al., 2016). It may occur spontaneously or be evoked when joint is normally moved or mechanically pressed triggering allodynia (pain in response to innocuous stimulus) and hyperalgesia (increased pain sensitivity) (Walsh and Mcwilliams, 2014; Boyden et al., 2016; Atzeni et al., 2015). This painful condition can impair the physical and social functioning, cause significant morbidity and mortality, and high health-care use (da Rocha Castelar Pinheiro et al., 2013; Raheel et al., 2017).

Recommended treatments for arthritis include disease-modifying antirheumatic drugs as methotrexate, leflunomide and sulfasalazine, which are partially effective (58%) or ineffective (15%), associated with glucocorticoids (Boyden et al., 2016; McWilliams et al., 2012; Smolen et al., 2017). Beyond the limited efficacy, treatments present serious adverse effects as gastrointestinal, hepatic or hematologic changes (Niu and Chen, 2014; Shea et al., 2014). Thus, search for new agents more effective and safe to treat arthritis associated-inflammatory pain is needed (Walsh and Mcwilliams, 2014). In this regard, natural products, such as plant extracts, have been an important source of new therapeutic agents, including molecules with analgesic actions on the inflammatory pain (Calixto, 2005; Mishra and Tiwari, 2011; Hamann et al., 2016; Fialho et al., 2017). The interaction of plant extracts with opioid receptors has been shown to be involved with these analgesic actions since, despite significant adverse effects, opioids are one of the main drugs used for pain control (Hamann et al., 2016; Scherrer et al., 2009; Martinez et al., 2013).

Mansoa alliacea (Lam.) A.H. Gentry, popularly known as cipo-alho, belongs to family Bignoniaceae and is native from the Brazilian Amazon region. Popular medicine has used this plant in the treatment of fever, convulsions, head and neck pain, among others (Pagani et al., 2017). To date, no biological activity study has been reported for *M. alliacea*. However, species of the same genus such as *M. hirsuta* has shown cancer chemopreventive and anti-inflammatory activities by inhibiting the COX, NF-κB and TNF-α pathway (Endringer et al., 2010; Campana et al., 2016), besides of anti-hypertensive (Castro Braga et al., 2000), antifungal (Rocha et al., 2004) and antioxidant (Pereira et al., 2017) properties. Thus, we investigated the anti-inflammatory and antinociceptive potential, as well as, possible adverse effects of *M. alliacea* extract in an inflammatory pain model with characteristics similar to those presented by patients with arthritis. We also evaluated the involvement of opioid receptors on *M. alliacea* effects.

2. Materials and methods

2.1. Plant material

The leaves of *Mansoa alliacea* (Lam.) A. H. Gentry (deposit n° IAN, 184394) were obtained from the herbal collection of the Brazilian Agricultural Research Corporation - Embrapa Amazônia Oriental, Belém/PA, Brazil. The species is from the Amazonian region and cultivated in the "Horto de Plantas Medicinais e Aromáticas". The geographical location of the collection site is 1°27′21″S latitude and 48°30′14″W longitude. The leaves were collected in September 2012 (the wet season in northern Brazil), cleaned, dried at 40 °C for 12 h, ground into a fine powder in a laboratory mill and used as a dry powdered material. CNPq (process number 010529/2014–4) issued authorization for use of the Brazilian genetic patrimony. The materials were authenticated by Dra. Silvane Rodrigues (Embrapa Amazônia Oriental, Belém/PA).

2.2. Preparation of the M. alliacea extracts

The *M. alliacea* crude hydroethanolic extract (MaC) was obtained by maceration of leaves at room temperature (25 ± 2 °C). For the maceration, 450 g of dried plant material was maintained in 70% (v/v) ethanol for seven days under occasional agitation. The solvent was renewed and the procedure was repeated three times, completing a four-week extraction process. Next, a portion of the extracts was filtered in a qualitative filter paper and evaporated under reduced pressure in a rotary evaporator to obtain the crude hydroethanolic extract.

To obtain the ethyl acetate (Eta), butanolic (But) and chloroform (Chl) fractions, the macerated extract was suspended in distilled water and transferred to a separating funnel with solvents (ethyl acetate, butanol, and chloroform). Then it was subjected to drying with anhydrous Na_2SO_4, filtration, and concentration in a rotary evaporator under reduced pressure.

2.3. Analysis of phenolic and triterpenic compounds in the M. alliacea extract

Separations were carried out on a UHPLC-MS/MS 1260 Infinity Binary system (Agilent, Santa Clara, CA, USA), which was able to operate at pressures up to 600 bar. A Zorbax SB-C18 Rapid Resolution HD column (2.1 × 50 mm, 1.8 µm, Agilent) was used at a temperature of 40 °C. The injection volume was 5 µL, and the injected aliquots were acidified to a final concentration of 0.1% acetic acid (v/v). The phenolic compounds were separated using a gradient elution composed of 0.1% acetic acid in water (A) and acetonitrile (B) as the mobile phase at a constant flow rate (0.8 mL min-1) according to the following elution program: 8% B (0.00–0.10 min); 8–25.8% B (0.10–3.45 min); 25.8–54% B (3.45–6.90 min); 54.0–100% B (6.90–7.00 min); and 100% B (7.00–9.00 min). The detection of the phenolic compounds by tandem mass spectrometry (MS/MS) was carried out by using an electrospray ionization source (ESI) as described elsewhere (Faccin et al., 2016).

The determination of triterpenes was performed using a gradient elution that consisted initially of 70% acetonitrile/water from 0 to 3.5 min followed by a ramp gradient, which achieved the end condition of 100% acetonitrile after 4.0 min. This condition was maintained until the end of the run (8 min). The flow rate was 0.6 mL/min during the first 4.5 min and was increased up to 0.8 mL/min at 5 min run. The flow rate was maintained at 0.8 mL/min until the end of the chromatographic run. The detection of the triterpenic compounds by tandem mass spectrometry (MS/MS) was carried out by using an atmospheric pressure photoionization source (APPI) as described elsewhere (Gobo et al., 2016).

2.4. Drugs and reagents

The following reagents were purchased from Sigma: Complete Freund's Adjuvant (CFA - 1 mg/ml of heat killed *Mycobacterium tuberculosis* oil suspension), hexadecyltrimethylammonium bromide (HTAB), 5-(N, N-diethylamino)-pentyl-3,4,5-trimethoxybenzoate (TMB), naltrindole and nor-binaltorphine. Naloxone and morphine sulfate were purchased from Cristália, São Paulo, Brazil. All other reagents were of analytical grade and were purchased from local supplier.

2.5. Animals

The present study was conducted in accordance with the national and international legislation (guidelines of Brazilian Council of Animal Experimentation and of the U.S. Public Health Service's Policy on Humane care and Use of Laboratory Animals-PHS Policy). All procedures were approved by the local Institutional Committee for Animal Care and Use (process number 116/2013) and were reported in compliance with the ARRIVE guidelines (Mcgrath et al., 2010; Mcgrath and Lilley, 2015). Moreover, the experiments were carried out in

accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). All experiments were performed using adult female or male Swiss mice (35–40 g). The animals were housed in a room with controlled temperature (22 ± 1 °C) and a 12 h light/12 h dark cycle with standard lab chow and water *ad libitum*. The number of animals and the intensity of nociceptive stimulus used were the minimum necessary to demonstrate the consistent effects of drug treatments. The behavior evaluation was performed blindly with respect to drug administration.

2.5.1. Animal treatment

The extracts obtained from *M. alliacea* leaves (crude hydroethanolic extract, and ethyl acetate, butanolic and chloroformic fractions) were dissolved in 5% Tween 80 and 95% saline (0.9% NaCl) prior oral administration by gavage (p.o.). The crude hydroethanolic extract (MaC) was administered at the dose of 10–100 mg/kg. Ethyl acetate (Eta), butanolic (But) and chloroform (Chl) fractions were administered at the dose of 10 mg/kg. Morphine sulfate (1 or 10 mg/kg, p.o.) was dissolved in saline and orally administered. Naltrindole (5 mg/kg, i.p.), nor-binaltorphine (10 mg/kg, i.p.) and naloxone (2 mg/kg, i.p.) were dissolved in saline and intraperitoneally administered (i.p.). All doses used in this study were chosen according to previous literature data (Walker et al., 2008; Koyama and Fukuda, 2010; Trevisan et al., 2013). A control group received saline with 5% Tween 80 as vehicle. The drugs were administered in a constant volume of 10 ml/kg body weight.

2.6. CFA-induced inflammatory pain model

The antinociceptive and anti-inflammatory activity of the hydroethanolic extract or *M. alliacea* fractions were evaluated in mice subjected to the CFA-induced paw inflammation, a chronic inflammatory pain model with characteristics similar to those presented by patients with arthritis. Animals were anesthetized with 2% isoflurane via a nose cone and 20 µL of CFA (1 mg/ml) or saline (0.9% NaCl; vehicle) was injected intraplantarly (i.pl.) in the right hind paw. One hour (pre-treatment protocol) or 48 h (post-treatment protocol) after CFA injection, inflammatory and nociceptive parameters were evaluated (Oliveira et al., 2014).

2.7. Nociceptive parameters

2.7.1. Assessment of mechanical allodynia

To determine the possible anti-allodynic effect of the hydroethanolic extract and *M. alliacea* fractions, mice were placed in cages with a wire mesh bottom that allowed full access to the paws. Mechanical allodynia was evaluated through the *up-and-down* method using von Frey filaments. A sequence of von Frey filaments, with different forces and logarithmic increments (0.02, 0.07, 0.16, 0.4, 1.4, 4.0 and 10 g) was used. In case of a positive response (paw withdrawal), the next filament with smaller force was applied; in case of negative response (no paw withdrawal response), the next filament with greater force was applied. This was repeated until a total of 6 applications (Oliveira et al., 2016). The mechanical paw withdrawal threshold (PWT) was then calculated according to Dixon (1980) (Dixon, 1980) and expressed in grams (g).

2.7.1.1. Assessment of the M. alliacea effect on the CFA-induced mechanical allodynia. The mechanical PWT was evaluated before CFA injection (baseline; B) in female or male mice. After baseline evaluation, a group of animals was treated (pre-treatment protocol) with vehicle (10 ml/kg, p.o.) or MaC (100 mg/kg, p.o). After 0.5 h of treatments, all animals received intraplantar CFA injection (20 µL/paw, i.pl.). The mechanical PWT was measured from 1 up to 24 h after treatments.

In other groups of animals, the mechanical PWT was measured 48 h after CFA injection (baseline; 0). The animals that presented mechanical allodynia were treated (post-treatment protocol) with vehicle (10 ml/kg, p.o.) or MaC (100 mg/kg, p.o.). The mechanical PWT was measured from 0.5 up to 24 h after treatments and after a dose-response curve (10–100 mg/kg, p.o.) at 1 h after its administrations. The mechanical PWT was also evaluated in a time-response curve after the administration of the ethyl acetate (Eta), butanolic (But) and chloroform (Chl) fractions (10 mg/kg, p.o.) from 0.5 up to 24 h after its administrations.

2.7.2. Investigation of opioid system participation in the antinociceptive effect of the M. alliacea

To investigate a possible involvement of opioid receptors in the antinociceptive effect of *M. alliacea*, opioid antagonists were used. The mechanical PWT was evaluated before (baseline; B) and 48 h after CFA injection (baseline; 0). Then, the animals that presented mechanical allodynia were pre-treated intraperitoneally with vehicle (10 ml/kg, i.p.), naloxone (2 mg/kg, i.p., non-selective opioid antagonist), naltrindole (5 mg/kg, i.p., δ-opioid antagonist) or nor-binaltorphimine (10 mg/kg, i.p., κ-opioid antagonist). After 15 min the animals received orally vehicle (10 ml/kg, p.o.) or MaC (100 mg/kg, p.o.). The mechanical PWT was measured at 1 h after the oral treatment with vehicle or MaC.

2.7.3. Assessment of thermal nociception

To determine the possible antinociceptive effect of MaC on the thermal nociception was performed the tail-flick test. The animals' tails were placed in a water bath heated to 48 ± 0.2 °C and the latency of response (in seconds; reflexive withdrawal of the distal half of the tail after its immersion in water) was measured before (baseline latency) and from 1 up to 24 h after vehicle (10 ml/kg, p.o.), MaC (100 mg/kg, p.o.) or morphine (10 mg/kg, p.o.) administrations. The cut-off time of 30 s was set to prevent tissue damage (Trevisan et al., 2013).

2.7.4. Assessment of the co-administration effect of M. alliacea and morphine on the mechanical allodynia and thermal hyperalgesia induced by CFA

The co-administration effect of *M. alliacea* and morphine (opioid agonist) on the mechanical and thermal threshold was evaluated in physiological conditions and at 48 h after the CFA-induced inflammatory pain. Animals were treated with vehicle (10 ml/kg, p.o.), MaC (10 mg/kg, p.o.; a dose 10 times lower than previous tests) or morphine (1 mg/kg, p.o.) or MaC (10 mg/kg, p.o.) plus morphine (1 mg/kg, p.o.). The mechanical PWT and latency for the tail withdrawal were measured at 1 h after treatments.

2.8. Inflammatory parameters

2.8.1. Edema evaluation

Paw thickness (in millimeter - mm) was measured before (baseline; B) and at 48 h after the intraplantar CFA injection (20 µL/paw, i.pl.) (baseline; 0) in the right hind paw using a digital caliper. Then, the animals that presented paw edema were treated with vehicle (10 ml/kg, p.o.) or MaC (100 mg/kg, p.o.) and paw thickness was again measured from 1 up to 6 h after treatments. The antiedematogenic effect was also evaluated by a dose-response curve for the MaC (10–100 mg/kg, p.o.) at 1 h after treatments. The results were expressed as the baseline and test value of the paw thickness (Fialho et al., 2017).

2.8.2. Myeloperoxidase activity

Myeloperoxidase (MPO) enzyme activity was evaluated as an indicator of neutrophil infiltration. The animals were euthanized and the paw tissue was collected at 48 h after CFA injection and at 1 h after vehicle (10 ml/kg, p.o.) or MaC (100 mg/kg, p.o.) administration. A control group (non-inflamed) received only intraplantar vehicle

administrations (20 μL/paw, i.pl.). Samples were homogenized in sodium acetate buffer (80 mM, pH 5.5) containing 0.5% HTAB in ice bath. Just before the assay, tissue homogenates were centrifuged at 16.000 × *g* for 20 min and the supernatants were collected for analysis. Then, the supernatant was mixed with sodium acetate buffer (80 mM, pH 5.5) and TMB 18.4 mM. The solution was incubated for 3 min at 37 °C and the reaction was stopped on ice by the addition of acetic acid. The absorbance was read at 630 nm in a spectrophotometer. A Fisher Biotech Microkinetics BT 2000 (Fisher Scientific, Pittsburgh, PA, USA) microplate reader was utilized and values were expressed as optical densities (OD) per ml of sample (Oliveira et al., 2014).

2.9. Assessment of adverse effects

2.9.1. Gastrointestinal transit

To evaluate the effect of *M. alliacea* on the gastrointestinal transit, the animals were fasted for 18 h (water *ad libitum*). After, the animals received vehicle (10 ml/kg, p.o.), MaC (100 mg/kg, p.o.) or morphine (10 mg/kg, p.o.; positive control) and 55 min later, a standard charcoal meal (5% charcoal, 20% arabic gum, 0.5 ml) was given to mice by gavage. Five minutes after administration of the standard charcoal meal, the animals were euthanized and their stomachs and small intestines were removed to measure the length of the intestine (from the pyloric sphincter to the ileum-caecal junctions, the total intestine length) and the distance traveled by the charcoal meal. Propulsive activity of the intestine was determined by the percentage of gastrointestinal traveled charcoal, calculated as: traveled% = 100 x (charcoal traveled distance/total intestine length) (Hamann et al., 2016; Trevisan et al., 2013).

2.9.2. Rectal temperature

To evaluate whether the Mac was able to change the body temperature, rectal temperature of animals was determined before (baseline), at 1 and at 2 h after administration of vehicle (10 ml/kg, p.o.), MaC (100 mg/kg, p.o.) or morphine (10 mg/kg, p.o; positive control). The results were expressed as the difference between the temperature (°C) after treatments and the baseline measurement (Δ°C) (Brusco et al., 2017).

2.9.3. Ulcerogenic activity

In order to evaluate the gastric tolerability of animals after oral administration of *M. alliacea*, mice were fasted for 18 h prior to drug exposure (water *ad libitum*). Animals were treated with vehicle (10 ml/kg, p.o.), MaC (100 mg/kg, p.o.) or diclofenac sodium (100 mg/kg, p.o.; positive control). Four hours later, animals were euthanized and their stomach were collected, opened by cutting along the greater curvature and washed with saline 4 °C. Then, the development of lesions was assessed with support of a magnifying glass. The quantification of gastric mucosal lesions was scored according to their number and size on a scale from 0 to 8 points, according to Walker et al. (2008) (Walker et al., 2008), as follows: (0) without injury, (1) color modification, (2) few petechia/alterations of villous, (3) 1–3 small injuries (≤1 mm length), (4) 1–3 large injuries (≤1 mm length), (5) 1–3 large injuries (> 1 mm), (6) more than three small injuries, (7) more than three large injuries and (8) more than three deep injuries.

2.9.4. Locomotor activity

The spontaneous locomotor activity was evaluated through the open-field test which consists of a box (20 × 20 × 20 cm) with the floor divided into 9 equal areas. The forced locomotor activity was evaluated by rotarod test, which consists of a rotatory bar (3.7 cm in diameter) divided into 3 separate compartments placed at a height of 25 cm which rotate at a fixed velocity of 8 rpm. The animals received vehicle (10 ml/kg, p.o.) or MaC (100 mg/kg, p.o.) and the locomotor activity was evaluated at 1 h after administrations. The results were expressed as the number of crossings and rearing in the open-field test and the number of falls in the rotarod test during 5 and 4 min, respectively (Brusco et al., 2017).

2.9.5. Biochemical markers of toxicity

To evaluate a possible liver or kidney injury induced by the *M. alliacea* hydroethanolic extract we measured the activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) enzymes and the urea and creatinine levels, respectively. The animals were treated with vehicle (10 ml/kg, p.o.), MaC (100 mg/kg, p.o.) or diclofenac sodium (100 mg/kg, p.o.; positive control) and at 1 h after treatments the animals were euthanized and the blood was collected. The activities of ALT and AST, and the urea and creatinine serum levels were assessed spectrophotometrically using Labtest® kits according to the manufacturer's specifications (Labtest Diagnostica, Brazil) (Oliveira et al., 2014).

2.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 software (San Diego, CA, USA). Results were expressed as the mean and standard error of the mean (SEM), except for the inhibitory doses (ID_{50}) values (i.e., the *M. alliacea* hydroethanolic extract dose that reduces mechanical allodynia to the order of 50% relative to the control value), which were expressed as geometric means accompanied by their respective 95% confidence limits, and gastric lesion scores which were reported as medians followed by their 25th and 75th percentiles. The data of mechanical PWT were log transformed before analysis to meet the parametric assumptions. The results were analyzed by Student's *t*-test or by one-way or two-way analysis of variance (ANOVA) followed by Student–Newman–Keuls's post-hoc test or Bonferroni's post-hoc test. $P < 0.05$ was considered significant.

3. Results

3.1. UHPLC-MS/MS determination of compounds present in the M. alliacea extract

The UHPLC-MS/MS of the *M. alliacea* hydroethanolic extract revealed the presence of several phenolic compounds among them, ρ-coumaric acid, ferulic acid, chlorogenic acid, vanilic acid, caffeic acid, *trans*-cinnamic acid, luteolin, apigenin and rutin (Fig. 1A). Among the twelve screened triterpenic compounds, the betulinic acid was the single found in appreciable concentrations (Fig. 1B). The phenolic compounds found in higher concentrations were ρ-coumaric acid followed by luteolin, ferulic acid, chlorogenic acid, and apigenin (Table 1; Fig. 2).

3.2. Hydroethanolic extract and M. alliacea fractions reduce CFA-induced mechanical allodynia

Mice injected with CFA developed mechanical allodynia as showed by the decrease in the PWT in response to mechanical stimuli of von Frey filaments, when compared to the intraplantar vehicle group (Fig. 3). The treatment with MaC (100 mg/kg, p.o.) in male mice prevented the development of CFA-induced mechanical allodynia from 2 up to 4 h after treatment when compared to the CFA plus vehicle group. The maximum inhibition (I_{max}) observed was of 100% at 4 h after its administration and the observed thresholds were of 2.8 ± 0.9 g to the vehicle, 0.01 ± 0.04 g to the CFA + vehicle and 4.0 ± 1.8 g to the CFA + MaC groups. (Fig. 3A). Similarly, the treatment with MaC (100 mg/kg, p.o.) in female mice also prevented the development of CFA-induced mechanical allodynia from 1 up to 6 h after treatment with I_{max} 80 ± 4% at 2 h after its administration. The observed thresholds were of 2.3 ± 0.7 g to the vehicle group, 0.05 ± 0.02 g to the CFA + vehicle group and 0.51 ± 0.09 g to the CFA + MaC group (data not shown).

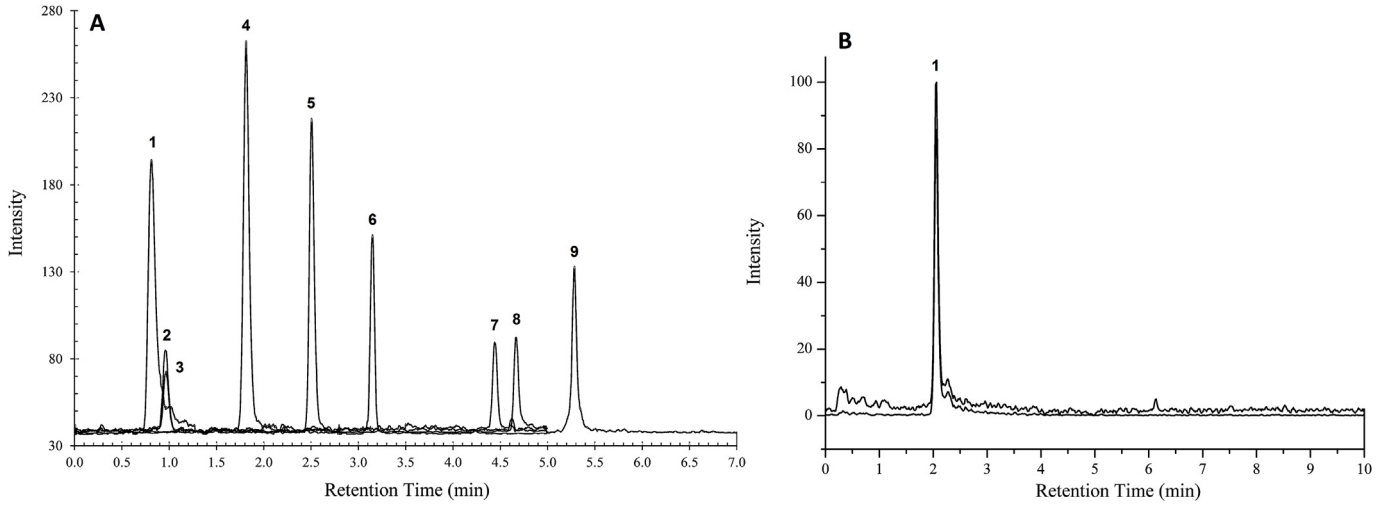

Fig. 1. Representative UHPLC-MS/MS profile of *M. alliacea* hydroethanolic extract. (1) Chlorogenic acid, (2) vanilic acid, (3) caffeic acid, (4) ρ-coumaric acid, (5) ferulic acid, (6) rutin, (7) *trans*-cinnamic acid, (8) luteolin and (9) apigenin; **(B)** (1) betulinic acid.

Table 1
Phenolic and triterpenic compounds determined in *M. alliacea* hydroethanolic extract.

Bioactive compound	Concentration (mg/kg)
Phenolic acid	
ρ-coumaric acid	372.50
Ferulic acid	268.50
Chlorogenic acid	208.10
Vanilic acid	69.10
Caffeic acid	57.50
Trans-cinnamic acid	36.40
Flavonoids	
Luteolin	355.40
Apigenin	116.40
Rutin	9.70
Triterpenes	
Betulinic acid	82.50

The treatment with MaC (100 mg/kg, p.o.) was also able to reverse the CFA-induced mechanical allodynia from 0.5 up to 4 h after its administration when compared to the CFA plus vehicle group, with I_{max} of 90 ± 10% at 1 h after its administration. The observed mechanical thresholds were of 2 ± 0.4 g to the vehicle group, 0.08 ± 0.03 g to the CFA + vehicle group and 3.2 ± 1.4 g to the CFA + MaC group. (Fig. 3B). The anti-allodynic effect of the post-treatment with MaC occurred in the three doses tested (10, 30 and 100 mg/kg) at 1 h after its administrations and the calculated inhibitory dose (ID_{50}) value was 106.2 (7.98–1413) mg/kg. Since, the dose of 100 mg/kg induced the maximal antinociceptive effect in the mechanical allodynia test, this dose of MaC was chosen for the remaining experiments (Fig. 3C). Likewise, the treatment with MaC (100 mg/kg, p.o.) in female mice was also able to reverse the CFA-induced mechanical allodynia from 0.5 up to 6 h after its administration with I_{max} of 68 ± 5% at 2 h. The observed mechanical thresholds were of 2.3 ± 0.7 g to the vehicle group, 0.09 ± 0.01 g to the CFA + vehicle group and 0.8 ± 0.01 g to the CFA + MaC group (data not shown).

The ethyl acetate fraction (Eta) (10 mg/kg, p.o.) of *M. alliacea* reversed the CFA-induced mechanical allodynia from 1 up to 6 h after its administration, when compared to the CFA plus vehicle group. The I_{max}

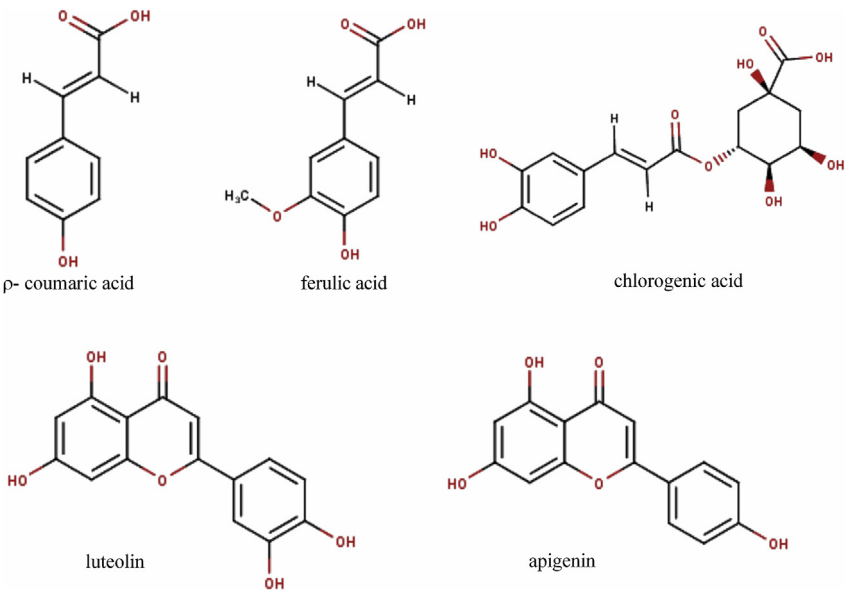

Fig. 2. Chemical structures of majority compounds found in the *M. alliacea* hydroethanolic extract. Phenolic acids (ρ-coumaric, ferulic and chlorogenic acids) and flavonoids (luteolin and apigenin).

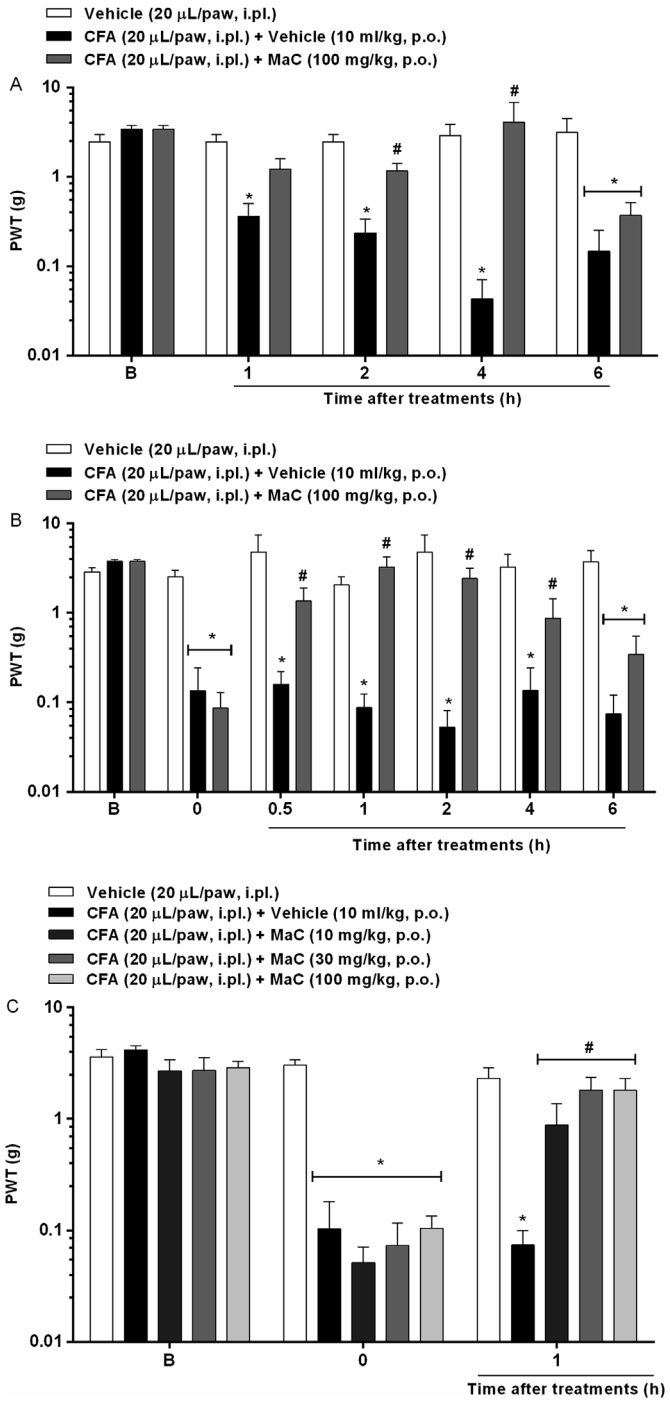

Fig. 3. Antinociceptive effect of *M. alliacea* hydroethanolic extract (MaC) on the mechanical allodynia CFA-induced (20 µL/paw) in male mice. Time-response curve in animals pretreated (A) or post-treated (B) with vehicle (10 ml/kg, p.o.) or MaC (100 mg/kg, p.o.). (C) Dose-response curve at 1 h after vehicle (10 ml/kg, p.o.) or MaC (10-30-100 mg/kg, p.o.) administrations. B in the x-axis denotes the baseline mechanical threshold before CFA injection. 0 indicates the baseline mechanical threshold at 48 h after CFA injection and before of treatments. Data are expressed as mean + SEM (n = 5–6 animals/group). *P < 0.05 when compared with the intraplantar vehicle group. #P < 0.05 when compared with the CFA plus vehicle group; two-way ANOVA (A; B) or one-way ANOVA (C) followed by Bonferroni's post-hoc test.

was of 76 ± 7% at 1 h after treatment. The butanolic fraction (But) (10 mg/kg, p.o.) was effective in reversing mechanical allodynia only at 2 h after its administration with inhibition of 42 ± 16%. The mechanical thresholds observed were of 3.1 ± 0.8 g to the vehicle group, 0.08 ± 0.03 g to the CFA + vehicle group, 1.5 ± 1.4 g to the CFA + Eta and 0.4 ± 0.1 g to the But group. On the other hand, the chloroform fraction (Chl) (10 mg/kg, p.o.) was not effective in reducing the CFA-induced mechanical allodynia in any of the evaluated times (Fig. 4).

3.3. Effect of *M. alliacea* hydroethanolic extract on the CFA-induced inflammation

The intraplantar CFA injection increased paw thickness, characterized as edema, as well as the MPO activity when compared to the vehicle group (Fig. 5). The treatment with MaC (10–100 mg/kg, p.o.) was not able to reduce paw edema at any of the doses tested when compared to CFA group treated with vehicle (Fig. 5A; B). Likewise, the MaC (100 mg/kg, p.o.) was unable to reverse the increase in CFA-induced MPO activity (Fig. 5C).

3.4. Effect of *M. alliacea* hydroethanolic extract on the thermal hyperalgesia

We determined the effects of MaC in the tail-flick test, a model sensitive to drugs that act at the central nervous system (Vanderah et al., 2008). The treatment with MaC (100 mg/kg, p.o.) increased tail withdrawal latencies from 1 up to 6 h after its administration when compared with the vehicle-treated group. The maximum increase of the thermal threshold was of 3.6 times in relation to the vehicle at 2 h after treatments. Likewise, morphine (10 mg/kg, p.o.) increased tail withdrawal latencies at 1 h and 2 h after its administration with a maximum increase of 3.5 times in relation to the vehicle at 1 h after treatments (Fig. 6).

3.5. Co-administration effect of *M. alliacea* and morphine on nociceptive parameters

The morphine, MaC or morphine plus MaC administrations did not alter mechanical threshold of the animals in physiological conditions at 1 h after treatments (Fig. 7A). However, after the establishment of inflammatory pain, MaC (10 mg/kg, p.o.) and morphine (1 mg/kg, p.o.) reduced the CFA-induced mechanical allodynia with I_{max} of 79 ± 7% and 79 ± 10%, respectively, when compared to the CFA group treated with vehicle. The co-administration of MaC (10 mg/kg, p.o.) plus morphine (1 mg/kg, p.o.) enhanced this effect with I_{max} of 100%. The mechanical thresholds observed were of 3.5 ± 0.7 g to the vehicle, 0.05 ± 0.01 g to the CFA + vehicle, 0.5 ± 0.1 g to the CFA + MaC, 0.5 ± 0.2 g to the CFA + morphine and 2.7 ± 0.2 to the CFA + MaC + morphine groups (Fig. 7B).

Moreover, the MaC (10 mg/kg, p.o.), morphine (1 mg/kg, p.o.) or MaC (10 mg/kg) plus morphine (1 mg/kg, p.o) administrations increased thermal threshold of the animals in physiological conditions at 2.06, 1.7 and 2.03 times respectively (Fig. 7C). The same treatments also reversed the CFA-induced thermal hyperalgesia at 1.7, 1.6 and 2.5 times, respectively, when compared to the CFA group treated with vehicle. The co-administration of MaC (10 mg/kg) plus morphine (1 mg/kg, p.o.) showed a higher increase of thermal threshold but was not statistically different from single administrations (Fig. 7D).

3.6. Opioid participation in the *M. alliacea* antinociceptive effect

In order to investigate some possible mechanisms of antinociceptive action of the *M. alliacea* hydroethanolic extract (MaC), animals were pre-treated with opioid receptor antagonists before of received MaC. Firstly, we showed that the CFA injection decreased the mechanical PWT of animals when compared to the baseline (B), characterizing the mechanical allodynia. The treatment with MaC (100 mg/kg, p.o.) reverted the CFA-induced mechanical allodynia in animals previously treated with vehicle (10 ml/kg, i.p.) when compared to the CFA plus

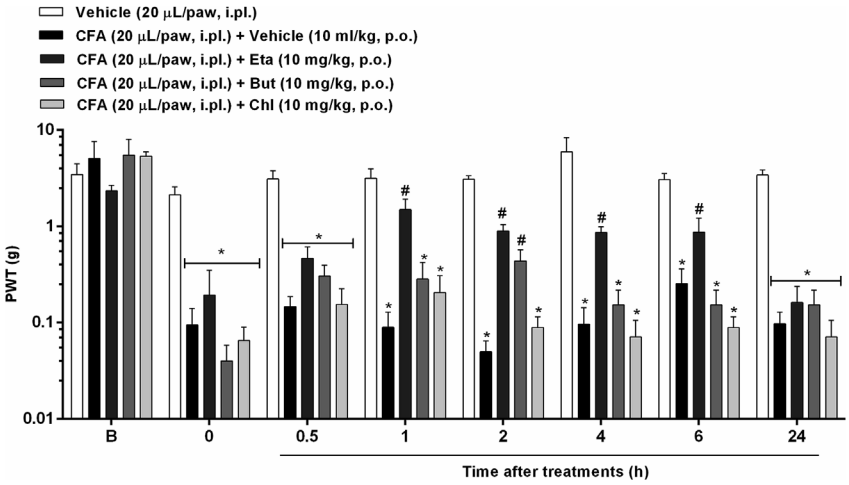

Fig. 4. Antinociceptive effect of fractions from *M. alliacea* extract on the mechanical allodynia CFA-induced (20 μL/paw) in mice. Time-response curve in animals treated with vehicle (10 ml/kg, p.o.) or ethyl acetate (Eta), butanolic (But) or chloroform (Chl) (10 mg/kg, p.o.) fractions. B in the x-axis denotes the baseline mechanical threshold before CFA injection. 0 indicates the baseline mechanical threshold at 48 h after CFA injection and before of treatments. Data are expressed as mean + SEM (n = 5 animals/group). *P < 0.05 when compared with the intraplantar vehicle group, #P < 0.05 compared with the CFA plus vehicle group; two-way ANOVA followed by Bonferroni's post-hoc test.

vehicle/vehicle group. Pre-treatment with opioid antagonists did not alter the mechanical allodynia in animals treated with vehicle (10 ml/kg, p.o.) (8A; 8B and 8C). However, pre-treatment with the non-selective opioid receptor antagonist naloxone (2 mg/kg, i.p.), and the δ-opioid receptor antagonist naltrindole (5 mg/kg, i.p.) prevented the anti-allodynic effect of MaC (100 mg/kg, p.o.) when compared to the CFA plus/vehicle/MaC group at 1 h after its administrations. The inhibitions of the anti-allodynic effect to the naloxone and naltrindole were of 98 ± 2% and 93 ± 2%, respectively (Fig. 8A and B). The mechanical thresholds observed were of 0.04 ± 0.01 g to the CFA + vehicle, 2.0 ± 0.5 g to the CFA + MaC, 0.07 ± 0.05 g to the CFA + naloxone + MaC and 0.18 ± 0.1 g to the CFA-naltrindole + MaC groups. On the other hand, pre-treatment with the κ-opioid antagonist nor-binaltorphimine (10 mg/kg, i.p.) did not prevent the anti-allodynic effect of MaC (100 mg/kg, p.o.) (Fig. 8C). These results suggest that the manly δ-opioid system is involved in the antinociceptive effect of MaC.

3.7. Assessment of the adverse effects of the M. alliacea hydroethanolic extract

Since some analgesic drugs cause adverse effects like constipation and change in body temperature (Koek et al., 1027; Els et al., 2017), we evaluated the gastrointestinal transit and rectal temperature of the animals after the administration MaC. The treatment with MaC (100 mg/kg, p.o.) did not change the gastrointestinal motility nor body temperature of the animals when compared to the vehicle group. On the other hand, morphine (10 mg/kg, p.o.; positive control) decreased the gastrointestinal transit and the body temperature of the animals when compared to the vehicle group (Fig. 9A; B).

We also evaluated the possible ulcerogenic activity of *M. alliacea,* since gastric injury induction is also a known adverse effect of analgesic drugs (Migliore et al., 2016). The treatment with MaC (100 mg/kg, p.o.) did not induce gastric mucosa lesions of the animals when compared to the vehicle group. However, diclofenac sodium (100 mg/kg, p.o., positive control) induced the formation of gastric lesions. The lesion scores were of 0.00 (0.00–0.50), 1.00 (0.00–2.00) and 4.00 (4.00–5.00) for vehicle, MaC, and diclofenac sodium, respectively.

Moreover, the MaC (100 mg/kg, p.o.) did not alter the forced or spontaneous locomotor activity of the female or male mice, assessed in the rotarod and open-field tests, respectively (Table 2). Further, MaC (100 mg/kg, p.o.) or diclofenac sodium (100 mg/kg, p.o.; positive control) caused no changes in the serum AST or ALT enzyme activities, or in the creatinine and urea levels when compared with the vehicle-treated animals (Table 3).

4. Discussion

Pathological inflammatory pain remains the most important symptom of some inflammatory injuries and diseases such as arthritis. Despite advances, novel analgesic agents with greater efficacy and lower propensity for adverse reactions are needed in order to reduce this disabling symptom (Walsh and Mcwilliams, 2014). In this study, we showed that oral administration of *M. alliacea* hydroethanolic extract caused antinociception in a CFA-induced inflammatory pain model, which presents inflammatory characteristics similar to those presented by patients with arthritis. This effect can be attributed to the active constituent presents in the *M. alliacea* and appears to occur by opioid-dependent mechanisms. Moreover, *M. alliacea* extract did not induce adverse effects commonly caused by opioids and other analgesic drugs, at least in the tested pharmacological doses after the acute treatment.

The biological activity of medicinal plants may be associated with the active metabolites present in its constitution, thus their identification and quantitation are important to explain its therapeutic effects (Hamann et al., 2016; Fialho et al., 2017; Fischer et al., 2017). Phytochemical screening of *M. alliacea* revealed the presence of several phenolic compounds as flavonoids and phenolic acids corroborating with findings of Faccin et al. (2016) (Faccin et al., 2016). The phenolic compounds found in higher concentrations were ρ-coumaric acid followed by luteolin, ferulic acid, chlorogenic acid, and apigenin. Similar to Gobo et al. (2016) (Gobo et al., 2016) among the screened triterpenic compounds only betulin acid was found in the *M. alliacea* extract. Phenolic and triterpenic compounds are active metabolites that show a variety of biological activities as anti-inflammatory and analgesic including effects on the inflammatory pain (da Silva et al., 2011; Hara et al., 2014a; Liu et al., 2016; Xu et al., 2015).

Since *M. alliacea* is rich in phenolic and triterpenic compounds, popularly used in the treatment of some pain types and species of the same genus showed anti-inflammatory actions (Pagani et al., 2017; Endringer et al., 2010; Campana et al., 2016), we verified its antinociceptive and anti-inflammatory potential in a CFA-induced inflammatory pain model. The CFA is used in animal pain models to induce inflammatory pain symptoms similar to arthritis since it is constituted by an inactivated *M. tuberculosis* suspension which may trigger autoimmune diseases as arthritis in humans (Walsh and Mcwilliams, 2014; Shen et al., 2015). The CFA, through peripheral, spinal and supraspinal mechanisms, mimics some characteristics of arthritic inflammatory pain including mechanical allodynia, thermal hyperalgesia, edema (Walsh and Mcwilliams, 2014; Banderas et al., 2017; Hamann et al., 2016) and infiltration of inflammatory cells (Cuda et al., 2017; Oliveira et al., 2014).

In the inflammatory pain occurs allodynia and hyperalgesia due to

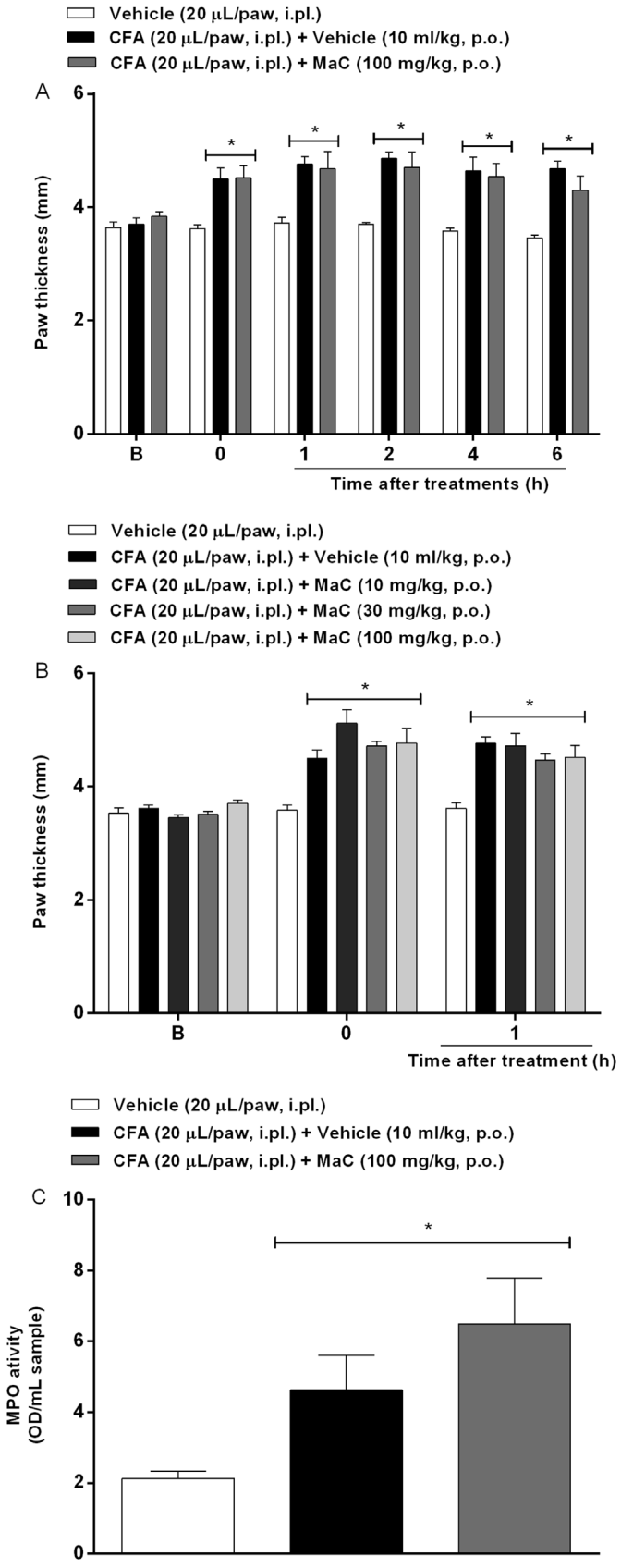

Fig. 5. Effect of the *M. alliacea* hydroethanolic extract (MaC) on the paw edema and the increase myeloperoxidase (MPO) activity CFA-induced (20 μL/paw) injection. (A) Time-response curve after the vehicle (10 ml/kg, p.o.) or MaC (100 mg/kg, p.o.) administration. (B) Dose-response curve at 1 h after vehicle (10 ml/kg, p.o.) or MaC (10-30-100 mg/kg, p.o.) administrations. (C) MPO activity at 1 h after vehicle (10 ml/kg, p.o.) or MaC (100 mg/kg, p.o.) administration. B in the x-axis denotes the baseline thickness before CFA injection. 0 indicates the baseline thickness 48 h after CFA injection and before of treatments. Data are expressed as mean + SEM (n = 5–6 animals/group). *P < 0.05 when compared with the intraplantar vehicle group two-way ANOVA (A) or one-way ANOVA (B; C) followed by Bonferroni's post-hoc test.

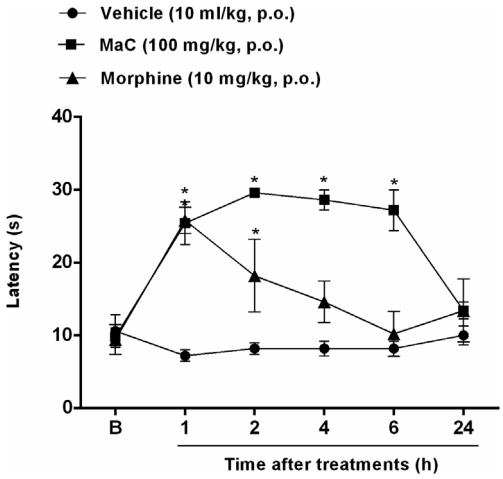

Fig. 6. Antinociceptive effect of *M. alliacea* hydroethanolic extract (MaC) on the thermal nociception in the tail-flick test. Time-response curve in animals that received vehicle (10 ml/kg, p.o.), MaC (100 mg/kg, p.o.) or morphine (10 mg/kg, p.o.) administrations. Data are expressed as mean ± SEM (n = 6 animals/group). B in the x-axis denotes the baseline thermal threshold before of treatments. *P < 0.05 when compared with the vehicle group; two-way ANOVA followed by Bonferroni's post-hoc.

peripheral mechanisms which involve activation and/or sensitization of nociceptors by algogenic substances, in addition to central mechanisms as the central sensitization (Walsh and Mcwilliams, 2014; Boyden et al., 2016). We showed that the *M. alliacea* hydroethanolic extract caused preventive (treatment before inflammatory pain induction) and curative (treatment after inflammatory pain induction) effects on the CFA-induced mechanical allodynia in mice. This result is relevant since pain is the main symptom faced by patients with a chronic inflammatory disease (Walsh and Mcwilliams, 2014). It is important to point out that this analgesic effect was similar in both female and male mice indicating that the sex did not influence the antinociceptive *M. alliacea* effect. Moreover, the ethyl acetate and butanol fractions of the extract were effective in the curative treatment when evaluated in male mice. However, fractions efficacy was lower than that of the crude extract, suggesting that the observed effect may be occurring due the combination of constituents present in the *M. alliacea* hydroethanolic extract. It is interesting to note that the effect of reversal on an already established painful hypersensitivity is quite relevant since it is similar to the pharmacological interventions clinically used which usually begin after the diagnosis is made in patients (Smolen et al., 2017).

In response to tissue damage, associated with inflammatory pain there is also activation of resident cells, release inflammatory mediators, cell infiltration, and edema (Taneja et al., 2017; Cuda et al., 2017; Banderas et al., 2017). Similar to previous findings (Hamann et al., 2016) the CFA injection caused edema and increased the MPO activity, a marker of neutrophil infiltration. Although effective on nociceptive parameters, the *M. alliacea* was not able to reduce inflammatory parameters induced by CFA injection. Thus, the antinociceptive effect of *M. alliacea*, at least in part, does not seem to be associated with a local anti-

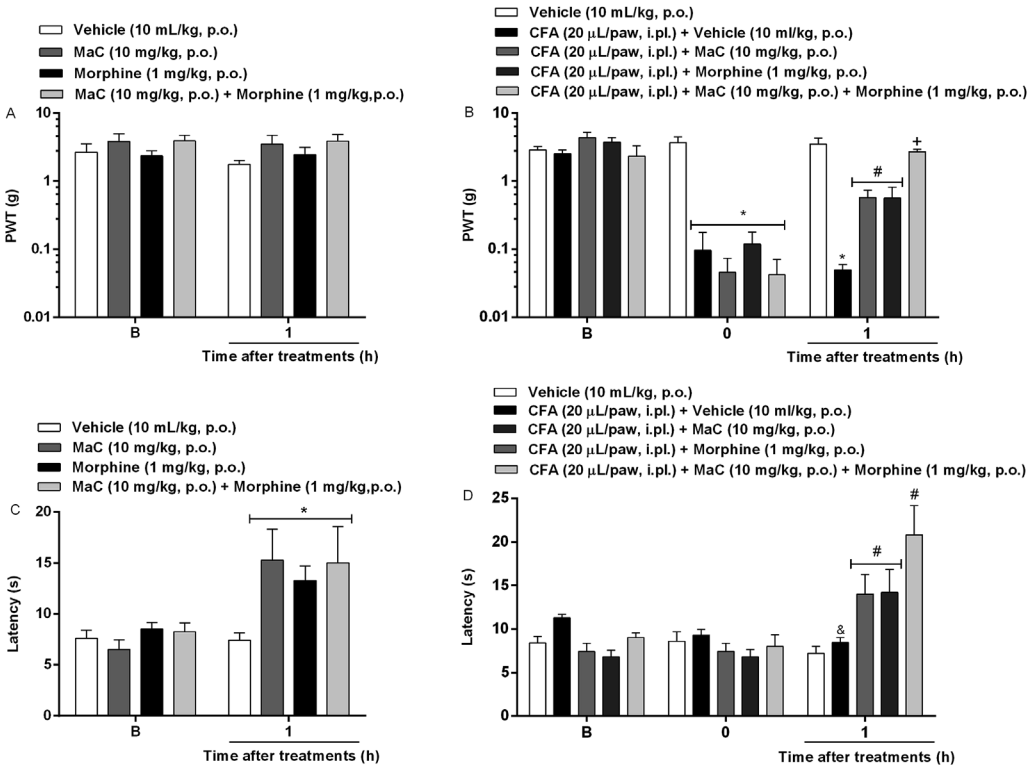

Fig. 7. Effects of morphine (1 mg/kg, p.o.) or *M. alliacea* hydroethanolic extract (MaC, 10 mg/kg, p.o.) or of co-administration of MaC (10 mg/kg) plus morphine (1 mg/kg, p.o.) on the mechanical and thermal threshold in physiological conditions (A; C) and after intraplantar CFA injection (20 µL/paw) (B; D). The antinociceptive effects were evaluated at 1 h after the oral treatments. B in the x-axis denotes the baseline threshold before of treatments (A; C) or of CFA injection (B; D). 0 indicates the baseline threshold 48 h after CFA injection and before of treatments. Data are expressed as mean + SEM (n = 5–7 animals/group). *P < 0.05 when compared with the vehicle group (B), $^{\&}$P < 0.05 when compared to the baseline latency of the CFA + vehicle group. $^{\#}$P < 0.05 compared with the CFA/vehicle group; $^{+}$P < 0.05 when compared with the CFA plus morphine or MaC alone administration; two-way ANOVA followed by Bonferroni's post-hoc test.

inflammatory action.

In addition to reducing the CFA-induced mechanical allodynia, the *M. alliacea* caused antinociceptive activity in the tail flick test under physiological conditions. The same was observed to the opioid agonist morphine. These results provide strong evidence that *M. alliacea* produce analgesic actions via central mechanisms since the tail-flick test is used to detect the antinociceptive effect of drugs acting on the central nervous system such as opioid analgesics (Vanderah et al., 2008).

M. alliacea was also able to reverse the CFA-induced thermal hyperalgesia similar to observed under physiological conditions. These results corroborate with preclinical findings where the CFA injection induced thermal hyperalgesia which was reverted by morphine (Hamann et al., 2016) and with clinical results where opioids are used to treat the arthritis-associated pain (Zamora-Legoff et al., 2017). On the other hand, neither *M. alliacea* nor morphine altered the animals' mechanical threshold under physiological conditions. However, their administrations reversed CFA-induced mechanical allodynia and this effect was enhanced by the co-administration of *M. alliacea* plus morphine. This finding is in agreement with Hamann et al. (2016) (Hamann et al., 2016), where morphine reversed the CFA-induced mechanical allodynia in an already installed painful process.

Since *M. alliacea* was able to reduce the nociception in physiological and pathological thermal pain models similar to morphine, we investigated the involvement of opioid receptors on the analgesic action of *M. alliacea* using opioid receptors antagonists. The naloxone, non-selective opioid antagonist reverted the antinociceptive effect of *M. alliacea* on the CFA-induced mechanical allodynia, as well as the selective δ-opioid receptor antagonist naltrindole, but not the κ-opioid antagonist nor-binaltorphimine. These results suggest that *M. alliacea* could interact mainly with δ-opioid receptors to produce its analgesic effects. Moreover, naloxone and naltrindole have been effective in blocking the antinociceptive effect of plant extracts (Roome et al., 2011). Likewise, other plant extracts rich in flavonoids has exerted its therapeutic effects through the activation of δ-opioid and µ-opioid receptors, but not κ-opioid (Webster et al., 2011).

Our results using opioid receptors antagonists can explain the discrepancies between the effects of *M. alliacea* and morphine on thermal and mechanical tests under physiological conditions, since µ- and δ-opioid receptors are expressed by different subsets of primary afferents. While the µ receptors are expressed on heat-sensitive peptidergic fibers contributing to the detection and regulation of thermal painful stimulus, the δ-receptors are expressed on mechanosensitive myelinated and nonpeptidergic afferents contributing to the detection and regulation of mechanical painful stimulus (Scherrer et al., 2009).

M. alliacea extract also reduced the thermal hypersensitivity in an already installed painful process, corroborating the idea that δ-opioid agonists can regulate heat pain through interactions with the µ-opioid receptor since δ-opioid agonist failed to induce thermal anti-hyperalgesic effects in µ-opioid receptor knockout mice (Scherrer et al., 2009; Gendron et al., 2007). Importantly, Gendron et al. (2007) (Gendron et al., 2007) showed that δ-opioid agonists are slightly analgesic when administrated to naïve animals, but its analgesic potency is increased following persistent inflammation. Moreover, our results are similar to previous studies which showed that δ-opioid receptor agonists decreased the mechanical hypersensitivity but not the edema induced by CFA (Scherrer et al., 2009). These data reinforce the evidence that the *M. alliacea* analgesic effects were mediated mainly via δ-opioid receptors.

As previously mentioned the biological compounds found in the *M. alliacea* could be responsible by its antinociceptive effect since ρ-coumaric acid, found in high concentrations in *M. alliacea* extract, has been responsible by the antinociceptive action of another extract which also was associated with the opioid system activation (Hirota et al., 2016). Moreover, ρ-coumaric acid was also effective in the treatment of acute gouty arthritis since it exhibited anti-inflammatory and analgesic properties against monosodium urate crystal-induced inflammation (Pragasan and Rasool, 2013).

Another phenolic acid found in high concentration in *M. alliacea* was ferulic acid, which caused antinociceptive effect in a neuropathic pain model that was mediated by δ-opioid receptors and descending monoaminergic system (Xu et al., 2015). Moreover, derivate from ferulic acid has shown anti-inflammatory and chondroprotective

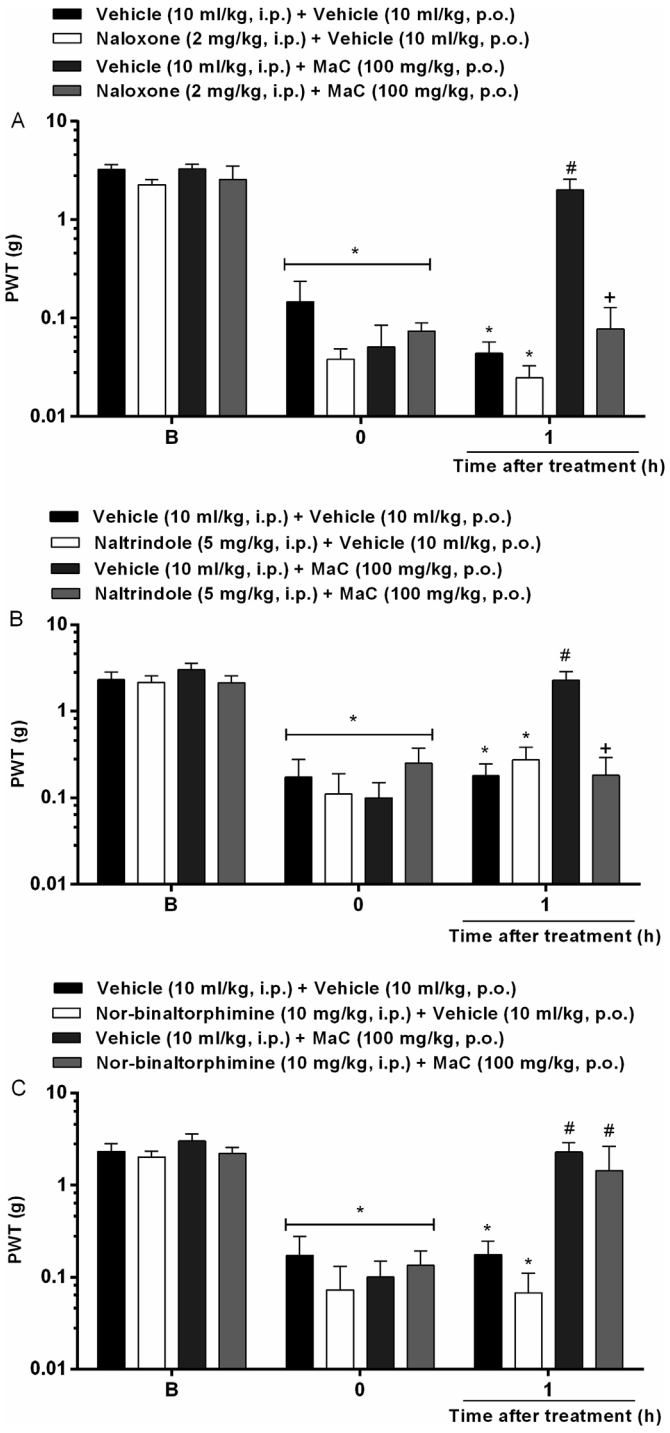

Fig. 8. Effect of the naloxone (2 mg/kg, i.p.) (A), naltrindole (5 mg/kg, i.p.) (B), or norbinaltorphimine (10 mg/kg, i.p.) (C) on the antinociceptive activity caused by *M. alliacea* hydroethanolic extract (MaC) on mechanical allodynia induced by CFA injection (20 μL/paw) in mice. The antinociceptive effect was evaluated at 1 h after the treatment with vehicle (10 ml/kg, p.o.) or MaC (100 mg/kg, p.o.). B in the x-axis denotes the baseline mechanical threshold before CFA injection. 0 indicates the baseline mechanical threshold at 48 h after CFA injection and before of treatments. Data are expressed as mean + SEM (n = 5–6 animals/group). *P < 0.05 when compared with the baseline threshold (B); $^{\#}$P < 0.05 when compared with the CFA plus vehicle/vehicle group; $^{+}$P < 0.05 when compared with the CFA plus vehicle/MaC group. Two-way ANOVA followed by Bonferroni's post-hoc test.

activities associated with TNF-α and IL-1β inhibition, as well as antioxidant activity in a CFA-induced knee monoarthritis model (Cunha et al., 2015).

Chlorogenic acid, also found in *M. alliacea*, exhibits broad biological effects such as anti-inflammatory (Chauhan et al., 2012), antioxidant (Sato et al., 2011) and analgesic in neuropathic and inflammatory pain models (Liu et al., 2016). This compound exerts analgesic effects by promoting voltage-gated potassium channels activation and inactivation under an inflammatory condition, besides regulating the release of catecholamines through these channels (Liu et al., 2016). It can still inhibit acid-sensing ion channels in dorsal root ganglion neurons (Qu et al., 2014). For the anti-inflammatory effects, the chlorogenic acid reduces the pro-inflammatory cytokines production as TNF-α and IL-1β and increases anti-inflammatory cytokines as IL-4 and IL-10. Importantly, this compound reduces T cells count which active different inflammatory pathways that correlate with the extent and type of damage observed in arthritis, suggesting that chlorogenic acid is a potent antiarthritic agent (Chauhan et al., 2012).

Among flavonoids, luteolin and apigenin were mainly found in *M. alliacea* extract. Pinheiro et al. (2012) (Pinheiro et al., 2012) showed that apigenin developed central antinociceptive effect in the hot plate test and this effect was mediated by opioid and cholinergic systems. Some studies have reported anti-inflammatory potential to the apigenin since it suppresses nitric oxide production and cyclooxygenase-2 expression, in addition to inhibiting the collagenase activity involved in inflammatory arthritis (Lee et al., 2007). Similar to our finding, the presence of apigenin-7-O-glucuronide and luteolin-7-Oglucuronide has been associated with anti-arthritic and antioxidant effects of a plant extract in a CFA-induced pain model (Jeyadevi et al., 2013).

Luteolin is also reported to have an antinociceptive effect on acute and inflammatory pain besides actions on the neuropathic pain by opioid-dependent mechanisms (Hara et al., 2014b). Moreover, its association with the palmitoylethanolamine meliorates collagen-induced arthritis (Impellizzeri et al., 2013) while its co-administration with morphine potentiates the antinociceptive effects of morphine on neuropathic pain (Hashemzaei et al., 2017). The presence of luteolin has been also associated with the antinociceptive activity of others plant extracts which also demonstrated opioid-dependent action (Hamann et al., 2016). Importantly, binding assays showed that luteolin and apigenin act as agonists at μ- and δ-opioid receptors (Webster et al., 2011).

Together, our results present strong evidence that phenolic compounds as ρ-coumaric, ferulic, chlorogenic acids and luteolin and apigenin found in *M. alliacea* hydroethanolic extract contribute to its analgesic effect since these compounds present antinociceptive effects opioid-receptors dependent on the inflammatory pain as the arthritic pain.

Although largely effective, the main limitation in the use of opioids as an analgesic is the induction of adverse effects, which include constipation, dizziness, nausea, motor incoordination, hypothermia and renal failure (Koek et al., 1027; Els et al., 2017). Drugs used in the treatment of arthritis also cause adverse effects as gastrointestinal and hepatic changes (Shea et al., 2014). We showed that the *M. alliacea* did not cause constipation or hypothermia at effective doses, different from morphine that reduced the gastrointestinal transit and the animals' body temperature. In addition, the *M. alliacea* did not induce gastric lesions, as well as caused no changes in the locomotor activity or in the urea and creatinine levels and in the AST and ALT activity, which are markers of renal and hepatic lesions, respectively (Oliveira et al., 2014). Although more frequent for μ-opioid agonists, constipation also is caused by κ-opioid agonists, besides dysphoria and renal function impairment (Günther et al., 2018). It is important to note that, the δ-opioid receptors in contrast to the μ-opioid receptors are absent in visceral sensory afferents and therefore δ-opioid agonists may have a better gastrointestinal safety profile and represent an alternative to use of μ-opioid agonists (Scherrer et al., 2009). These results suggest that *M.*

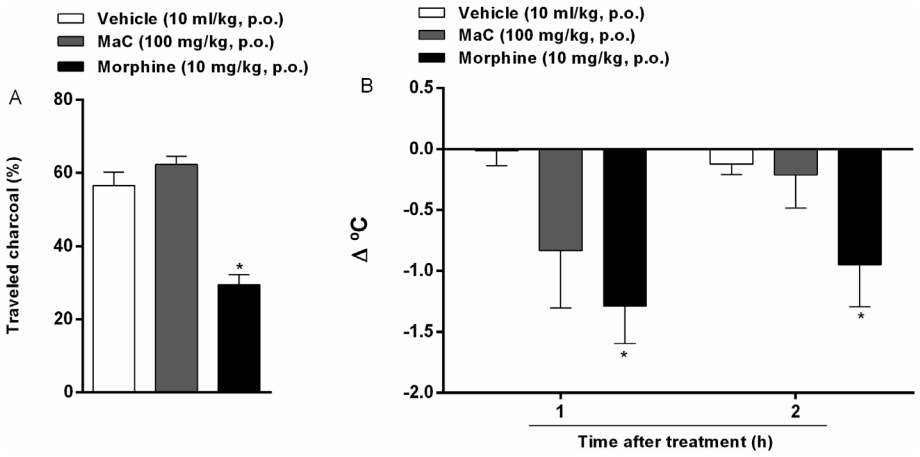

Fig. 9. Effect of the *M. alliacea* hydroethanolic extract (MaC) (100 mg/kg, p.o.) and morphine (10 mg/kg, p.o.) on the gastrointestinal transit (A) or rectal temperature (B) in mice. The traveled charcoal (%) and Δ°C were obtained at 1 h (A and B) and 2 h (B) after MaC or morphine administrations. Data are expressed as means + SEM (n = 5–8 animals/group). *P < 0.05, one-way ANOVA (A) or two-way ANOVA (B) followed by Bonferroni's post hoc test.

Table 2
Effect of the vehicle (10 ml/kg, p.o.) or MaC (100 mg/kg, p.o.) on the spontaneous and forced locomotor activity (open-field test and rota rod test) in female and male mice at 1 h after treatments.

Treatment	Open-field test		Rota rod test
	Crossing number	Rearing number	Falls number
Vehicle (male mice)	63.00 ± 6.95	51.20 ± 7.05	0.20 ± 0.20
Vehicle (female mice)	46.25 ± 4.99	17.00 ± 2.49	0.40 ± 0.24
MaC (male mice)	45.80 ± 3.59	33.80 ± 6.07	0.20 ± 0.20
MaC (female mice)	63.80 ± 7.20	19.00 ± 5.31	1.00 ± 0.31

Significant differences were not observed between groups (one-way ANOVA followed by Bonferroni's post hoc test). Data are shown as mean ± SEM of 5–6 animals.

Table 3
Effect of the vehicle (10 ml/kg, p.o.), MaC (100 mg/kg, p.o.) or diclofenac sodium (100 mg/kg, p.o.) on biochemical parameters in mice at 1 h after treatments.

Treatment	AST (U/l)	Biochemical parameters		Creatinine (mg/dl)
		ALT (U/l)	Urea (mg/dl)	
Vehicle	15.10 ± 2.11	29.28 ± 2.06	30.80 ± 2.39	1.68 ± 0.30
MaC	15.32 ± 3.66	29.64 ± 5.92	26.40 ± 1.75	1.60 ± 0.41
Diclofenac sodium	10.84 ± 3.24	23.22 ± 2.11	28.20 ± 0,73	1.60 ± 0.21

Significant differences were not observed between groups (one-way ANOVA followed by Bonferroni's post hoc test). Data are shown as mean ± SEM of 5–6 animals.

alliacea may represent a better analgesic therapy in comparison to the other centrally acting opioids.

5. Conclusion

The current study showed that the *M. alliacea* hydroethanolic extract promotes antinociception in an inflammatory pain model with characteristics similar to those presented by patients with arthritis. This analgesic effect may be attributed to compounds in its constitution and seems to be mediated by opioid system mainly via δ-opioid receptors. However, further studies are needed to confirm its exact action mechanisms. Moreover, *M. alliacea* does not cause adverse effects characteristic of classical opioid and other analgesics drugs in therapeutic doses. Our findings suggest that *M. alliacea* may represent a therapeutic alternative for treating inflammatory pain, such as arthritic pain with no detectable adverse effects.

Author contributions

Study concept and design: FRH, MAR, and SMO. Acquisition of data: FRH, GCS, and SMO. LMC, HF and LG participated in plant material and extracts preparation, analysis of phenolic and triterpenic compounds of the extract, data analysis, writing and discussion of these. Analysis and interpretation of data: FRH, IB, and SMO. Drafting of the manuscript: FRH, IB, MAR, and SMO. Study supervision: SMO.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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