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Original Article

Antinociceptive effect and mechanism of supercritical carbon dioxide extract of *Aloysia gratissima* leaves in mice

Maryelen A. Souza [a], *Jaqueline Scapinello* [a], *João G.G. Guzatti* [b], *Mikaela Scatolin* [b], *Rafael Martello* [a], *Monica S.Z. Schindler* [a], *Jean F.F. Calisto* [a], *Bianca Alves* [b], *Letícia V. Morgan* [b], *J. Vladimir Oliveira* [c], *Jacir Dal Magro* [a], *Liz G. Müller* [a,b,*]

[a] *Stricto Sensu Postgraduate Program in Environmental Sciences, Community University of Chapeco Region (Unochapeco), Chapeco, Brazil*

[b] *Health Sciences Area, Community University of Chapeco Region (Unochapeco), Chapeco, Brazil*

[c] *Department of Chemical and Food Engineering, Federal University of Santa Catarina, Florianopolis, Brazil*

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ABSTRACT

Background: *A. gratissima* is a shrub used in folk medicine as analgesic and sedative. However, studies on its antinociceptive activity are scarce. This research aimed to evaluate the antinociceptive effect of a supercritical carbon dioxide ($SCCO_2$) extract of *A. gratissima* leaves (EAG) in mice.

Methods: *A. gratissima* leaves were subjected to extraction with supercritical CO_2 (60 °C, 200 bar). The chemical composition of EAG was determined by gas chromatography–mass spectrometry (GC–MS). The antinociceptive profile of the extract (1, 10 and 30 mg/kg, p.o.) was established using acetic acid-induced abdominal contraction tests and formalin-induced paw-licking tests. The open field and rota-rod tests were used to evaluate a possible interference of EAG on mice motor performance. The contribution of the opioid system and adenosine triphosphate (ATP) sensitive K^+ channels in the mechanism(s) of EAG action was evaluated by specific receptor blockers. EAG's acute toxicity was investigated using OECD 423 guideline.

Results: The GC–MS revealed the presence of sesquiterpenes (guaiol and pinocamphone) in the EAG. Doses of 10 mg/kg and 30 mg/kg significantly reduced the number of abdominal writhes and paw licking time in mice in the formalin test. The EAG did not affect the locomotor activity and motor coordination of the mice. The antinociceptive effect of the EAG was prevented by glibenclamide in the mice formalin test, unlike naloxone pre-treatment. The acute administration of EAG caused no mortality.

Conclusion: *A. gratissima* leaves possess antinociceptive effect, mediated by K^+ channels sensitive to ATP.

* Corresponding author. Communitarian University of Chapeco Region (Unochapeco), Servidao Anjo da Guarda, nº 295-D, Bairro Efapi, 89809-900, Chapeco, SC, Brazil.
E-mail address: lizmuller@unochapeco.edu.br (L.G. Müller).

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At a glance of commentary

Scientific background on the subject

Aloysia gratissima aerial parts are traditionally used as sedative and for the treatment of headache. The essential oil from *A. gratissima* presents sesquiterpenes, which are related to the pharmacological action of plants.

What this study adds to the field

Our study demonstrated the presence of sesquiterpenes (guaiol and pinocamphone) in the supercritical CO_2 extract of *Aloysia gratissima* leaves and confirmed its antinociceptive activity in mice. This effect is mediated by K^+ channels sensitive to ATP.

The scientific interest in researching compounds derived from plant extracts is associated with the knowledge of folk medicine [1]. The secondary metabolites present in these natural compounds can have their biological activity evaluated through in vivo experimental tests, so that their functionalities as drugs, food and cosmetics can be proven [2,3]. Among the species popularly known for their pharmacological actions, are the ones from the *Aloysia* genus, belonging to the Verbenaceae family, native to South America [4].

In Brazil, the species *Aloysia gratissima* (Gillies & Hook) Tronc. is commonly grown as an ornamental plant, being known as "Alfazema do Brasil" [5]. Aerial parts of this plant are used for the treatment of headache, bronchitis, as well as anxiety, depression and digestive disorders [6,7]. The essential oil from *A. gratissima* presents more than 70% of hydrocarbons, from which more than half are sesquiterpenes. This is particularly interesting, since the pharmacological action of plants is related to the presence of metabolites such as alkaloids, terpenes, flavonoids and phytosterols [5].

The use of natural products in the food and pharmaceutical industry, provides alternative results to current needs [8]. Additionally, obtaining compounds through pressurized fluids, such as carbon dioxide and propane, have been presented as alternatives, due to the use of non-toxic, non-flammable and low-cost solvents [8]. In this context, the extraction and study of the compounds present in these plant matrices through supercritical fluids is an interesting approach [8].

The presence of terpenes in plants, such as caryophyllene, caryophyllene oxide, guaiol, pinocamphone and spathulenol is described by several authors as the possible reason for the antinociceptive action of different extracts that present these compounds [9–12]. Furthermore, it has been demonstrated that terpenes are able to exert antinociceptive effects by opening K^+ channels [13], which leads to K^+ efflux and cell membrane hyperpolarization [14]. This effect, therefore, reduces the excitability of peripheral neurons and the release of neurotransmitters (e.g. substance P) in the spinal cord [15–17]. Different types of K^+ channels are involved in antinociception: the adenosine triphosphate (ATP)-sensitive K^+ channels, activated via several different drugs (including terpenes such as caryophyllene oxide and guaiol [11]) in both the central and the peripheral nervous system; and the G-protein-regulated inwardly rectifying K^+ channels, that open through the G-protein activation in neurons [18]. These kinds of K^+ channel activations are both involved in opioid induced antinociception [14]. Interestingly, some terpenes exert their antinociceptive activity by activating opioid receptors [19–22], thus reducing neurons' excitability and the release of neurotransmitters.

Considering the presence of terpenes in *A. gratissima*, it becomes important to investigate, for the first time, the effect of this plant species on animal models of nociception and its mechanisms of action. The present work evaluated the antinociceptive activity of a supercritical CO_2 extract of *A. gratissima* leaves in models that use different stimuli in mice, and also investigated the mechanisms of action involved in its antinociceptive activity.

Materials and methods

Chemicals

Carbon dioxide (99.9% non-liquid purity phase) was purchased from Air Liquide©. Acidic acid and formaldehyde were purchased from Merck©. Ketorolac and glibenclamide were obtained from EMS Sigma Pharma©. Naloxone and morphine were purchased from Cristália© and indomethacin was obtained from Sigma©.

Plant material

A. gratissima leaves were collected in December 2017 (summer), in the municipality of Erval Grande, RS, South Brazil (27°23′14.3S, 52°33′49 'W). This region presents a humid subtropical mesothermic climate. The plant specimen was deposited in the Herbarium of the Community University of Chapecó (Herbário Unochapecó, SC, Brazil) under the accession number UNO 3700. After collection, the leaves were manually separated and dried at room temperature for five days. Afterwards, they were packed in plastic bags, identified and stored at 7 °C until extraction process.

Extraction of the A. gratissima *leaves by supercritical* CO_2 *(*$SCCO_2$*)*

The experimental extraction apparatus and procedure have been described in detail in other studies of the research group [23]. *A. gratissima* leaves were extracted with $SCCO_2$ at 60 °C and 200 bar (density 724 kg/m^3). Approximately 11.03 ± 0.09 g of *A. gratissima* leaves were placed into the extraction vessel. Then, CO_2 was pumped into the bed, which had two 300 mesh wire disks at both ends and was held in contact with the sample array to allow the system to stabilize at the same condition as the experiment by 30 min. After stabilization, the extract from *A. gratissima* was then collected by opening the micrometric valve with 2 h of extraction time.

GC/MS analysis

Extract of *Aloysia gratissima* leaves (EAG's) chemical composition was analyzed by Agilent GC/MS (7890B) gas chromatography coupled to a quadripolar mass spectrometer (5977A) (Agilent Technologies, Palo Alto, CA, USA). GC/MS system's experimental condition were described by Scapinello et al. (2018) [24] with some modifications. Briefly, the system conditions were: Agilent 19091S ca-pillary column, dimension: 30 m × 250 μm × 0.25 μm. The mobile phase flow (carrier gas: He) was adjusted to 1.0 mL min^{-1}. The GC temperature program was 40.0 °C at 4 min to 240.0 °C at a rate of 10 °C min^{-1} and up to 300.0 °C at a rate of 40.0 °C min^{-1} (maintained for 5 min). The injector temperature was 280.0 °C, sample injection volume 1 μL, split ratio 1:20. The MS transferline temperature was set to 150.0 °C and the source of ions temperature was set at 230.0 °C. For GC–MS detection, an electron ionization system was used with ionization energy set at 70 eV, and mass range atm/z 40–400. The chemical components presented in the extract were identified by comparison with the equipment library (Agilent P/N G1033A). The relative amounts of each individual component were calculated using their respective peak areas in the chromatogram. The extract was solubilized in dichloromethane to be analyzed.

In vivo experiments

Animals

Male Swiss mice (25–35 g) bred at the Community University of Chapecó Region (Unochapecó) bioterium were used in all pharmacological experiments. The animals were kept in a controlled environment under standard conditions (22 ± 2 °C) with a light/dark cycle of 12 h (lights on at 6:00 a.m. to 6:00 p.m.), fed standard laboratory feed and water *ad libitum*. Animal care and experiments were conducted in accordance with the animal research ethical principles, approved by the Ethics Committee of the university (Approval number 004–18), in accordance with Brazilian law No. 11794 and Council of the European Communities; Directive of 24 November 1986 (86/609/EEC). The animals were fasted for a period of 2 h (no water restriction) prior to administration of any test substance.

Mice were treated with volumes of 10 mL/kg, according to their weight, through oral gavage (p.o.) or intraperitoneal (i.p.) routes and with 20 μL by intraplantar (i.pl.) injection, according to each experiment's specific protocol. Solubilization of the extract and substances was performed in saline (NaCl 0.9%) with the aid of 1% Tween 80 (v/v) and ultrasound. The extract's tested doses were chosen based on the study of Zeni et al. (2013) [4].

Acute toxicity

The acute toxicity study was based on Guideline 423 (2001) of the Organization for Economic Cooperation and Development (OECD). According to OECD 423, the study in female mice is recommended for the possible sensitivity to the acute toxic effects of the chemicals used in the research [25]. Female mice (n = 6) were orally treated with EAG at 2000 mg/kg. The control group (n = 3) received the administration of vehicle (0.9% NaCl, 1% Tween 80, 10 mL/kg). Thereafter, the animals were observed with special attention during the first 4 h after treatment and daily for 14 days. The occurrence of mice death, and signals such as piloerection, hypothermia, palpebral ptosis, abdominal writhing, muscle tone, shacking, hind paw paralysis, salivation, bronchial secretion and seizures were registered. Additionally, body weight and food consumption were recorded for 13 days. At the end of the experiment, the macroscopic aspect of the organs (liver, kidneys, adrenal glands, spleen, lungs, heart and brain) as well as their relative weight (%) were registered.

Acetic acid-induced writhing response

The abdominal writhing test induced by acetic acid has been used as a screening tool to evaluate analgesic or anti-inflammatory agents, as acetic acid induces an inflammatory response in the abdominal cavity, with subsequent activation of nociceptors [26]. In this test, mice writhing is a response to the pain characterized by episodes of arching of the back, extension of hind limbs and contraction of abdominal musculature [27]. Mice (n = 6–8/group) were treated with EAG (1, 10 and 30 mg/kg, p.o.) or vehicle (0.9% NaCl + 1% Tween 80, p.o., control group) 1 h before the injection (i.p.) of acetic acid (0.6%, 10 mL/kg). Immediately after the injection, the animals were gently placed in a transparent apparatus (20 cm diameter glass box) and the number of abdominal contortions was counted cumulatively over a period of 20 min [28] by a human observer blind to the treatment. The positive control was the non-steroid anti-inflammatory indomethacin (10 mg/kg, p.o.). The dose that presented the best result in this experiment was chosen to be used in the other tests of nociception.

Open-field test

The open-field test was performed in order to evaluate the possible EAG effects on locomotor and exploratory activities of mice. The experimental protocol was based on the method described by Müller et al. (2012) [29]. The animals (n = 6/group) received orally the minimal effective dose (10 mg/kg, p.o.) of EAG that was able to reduce the nociceptive behavior in the acetic-acid writhing test, 1 h before being exposed to the open-field arena. The control group was treated with vehicle (0.9% NaCl + 1% Tween 80, p.o.). The arena consisted in an acrylic box (40 × 30 × 30 cm), with the floor divided into 24 equal squares. After the habituation (5 min), the number of squares crossed with the four paws (crossing), rearings and groomings was recorded for 10 min. The number of fecal bolus was counted after the test.

Rota-rod test

The rota-rod test was used to evaluate the EAG effects on mice motor coordination. This test was performed as described by Neves et al. (2010) [30] with minor modifications. The apparatus was a cylinder (4 cm of diameter), rotating at 3 rpm. Animals were individually habituated to the apparatus for 5 min. Twenty-four hours later, they were trained for 5 min and only the ones that were able to stay 90 s on the rotating rod were selected for testing. Immediately after, mice (n = 7/group) were orally treated with vehicle (NaCl 0.9% + 1% tween 80), indomethacin (10 mg/kg) or EAG (10 mg/kg). One hour

Table 1 Chemical composition of the *A. gratissima* leaves supercritical CO_2 extract.

Chemical compound	Area of each compound (%)
Pinocarvil	5.31
Pinocamphone	11.4
Isopinocamphone	3.52
Myrtenol	3.24
(–)- trans-pinocarvila acetate	10.5
Caryophyllene	7.63
γ – Elemene	7.28
Humelene	2.97
(–)- Spathulenol	6.17
Caryophyllene oxide	6.76
β- Cubebene	8.21
Guaiol	18.5
Bunesol	4.67
Total	96.1

later, mice performance in the rota-rod was evaluated considering the longest time of permanence on the apparatus and the number of falls, in a 5 min period.

Formalin test

The experimental procedure was similar to the one described by Santos and Calixto (1997) [31]. Briefly, the animals (n = 6/group) were orally treated with vehicle (0.9% NaCl + 1% Tween 80) or EAG (10 mg/kg) 1 h before the injection of 2% formalin (20 μL/paw, ipl) in the right hind paw. Indomethacin (administered orally 1 h before the behavioral test, 10 mg/kg) was the positive control. Immediately after the formalin injection, the time spent licking, biting or lifting the injected hind paw (nociceptive behavior) was registered during the first (0–5 min, neurogenic phase) and the second phases (15–30 min, inflammatory phase) of the test.

Involvement of the opioid system and K^+ channels sensitive to ATP

In order to assess the opioid system's involvement in the antinociceptive action mechanism of the EAG, mice were pre-treated with naloxone (a non-selective opioid receptor antagonist; 2 mg/kg, i.p.) or vehicle (0.9% NaCl, i.p.) [32,33]. After 15 min, the animals (n = 4–7/group) were treated with EAG (10 mg/kg, p.o.), morphine (opioid receptor agonist, positive control, 5 mg/kg, s.c.) or vehicle (0.9% NaCl, 1% Tween 80, p.o.).

The involvement of the ATP-sensitive K^+ channels in EAG's antinociceptive effect was evaluated according to Zapata-Morales et al. (2017) [34]. Briefly, the animals (n = 4–7/group) were pre-treated with glibenclamide (K^+ channels sensitive to ATP blocker; 20 mg/kg, i.p.) or vehicle (0.9% NaCl, i.p.). The EAG (10 mg/kg, p.o.), ketorolac (non-steroid anti-inflammatory that presents antinociceptive effects mediated by ATP-sensitive K^+ channels [35], positive control, 20 mg/kg, i.p.) or vehicle (0.9% NaCl + 1% Tween 80, p.o.) were administered to the animals 15 min after the pretreatment.

One hour after the treatments, the nociceptive behavior was evaluated in the formalin test, immediately after the i.pl. injection of formalin 2%.

Statistical analysis

Statistical analyses were carried out using Graph Pad Prism 5.0 for Windows (GraphPad Software, San Diego, California, USA). Data were analyzed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Results from the investigation of the mechanism of action were analyzed by two-way ANOVA followed by Student-Newman-Keuls test. Rota-rod test results and toxicity data were analyzed by two-way repeated measures ANOVA. The relative weight of the organs in the toxicity test was analyzed by Unpaired *t* test. The results are expressed as the mean ± S.E.M. of *n* animals per group. Values of *p* less than 0.05 ($p < 0.05$) were considered significant.

Results

Table 1 shows the extract's chemical composition. The GC/MS analysis revealed that several terpene compounds are present in the EAG. The major compounds found in EAG were guaiol (18.50%) and pinocamphone (11.40%), in addition to the

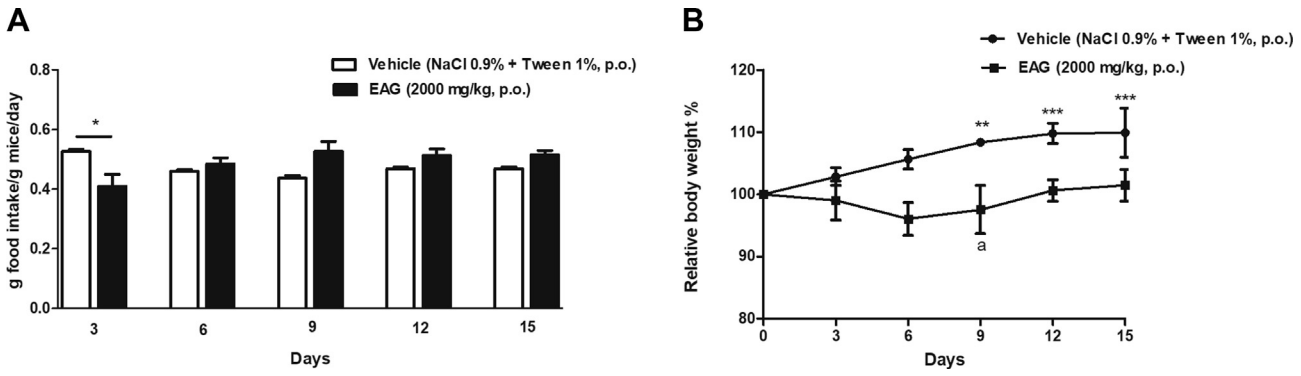

Fig. 1 Effect of the *A. gratissima* leaves supercritical extract (EAG) acute treatment (2000 mg/kg, p.o.) on food intake (A) (g food intake/g mice/day) and relative body weight (%) (B) of female Swiss mice. Data are expressed as mean ± S.E.M. (n = 3–6 mice/group). Two-way repeated measures ANOVA followed by Student-Newman-Keuls. $^{**}p < 0.01$ and $^{***}p < 0.001$ represent differences in relation to the first measure (Day 0) in the same group treatment. $^{*}p < 0.05$ different from the vehicle-treated group at the same day of treatment.

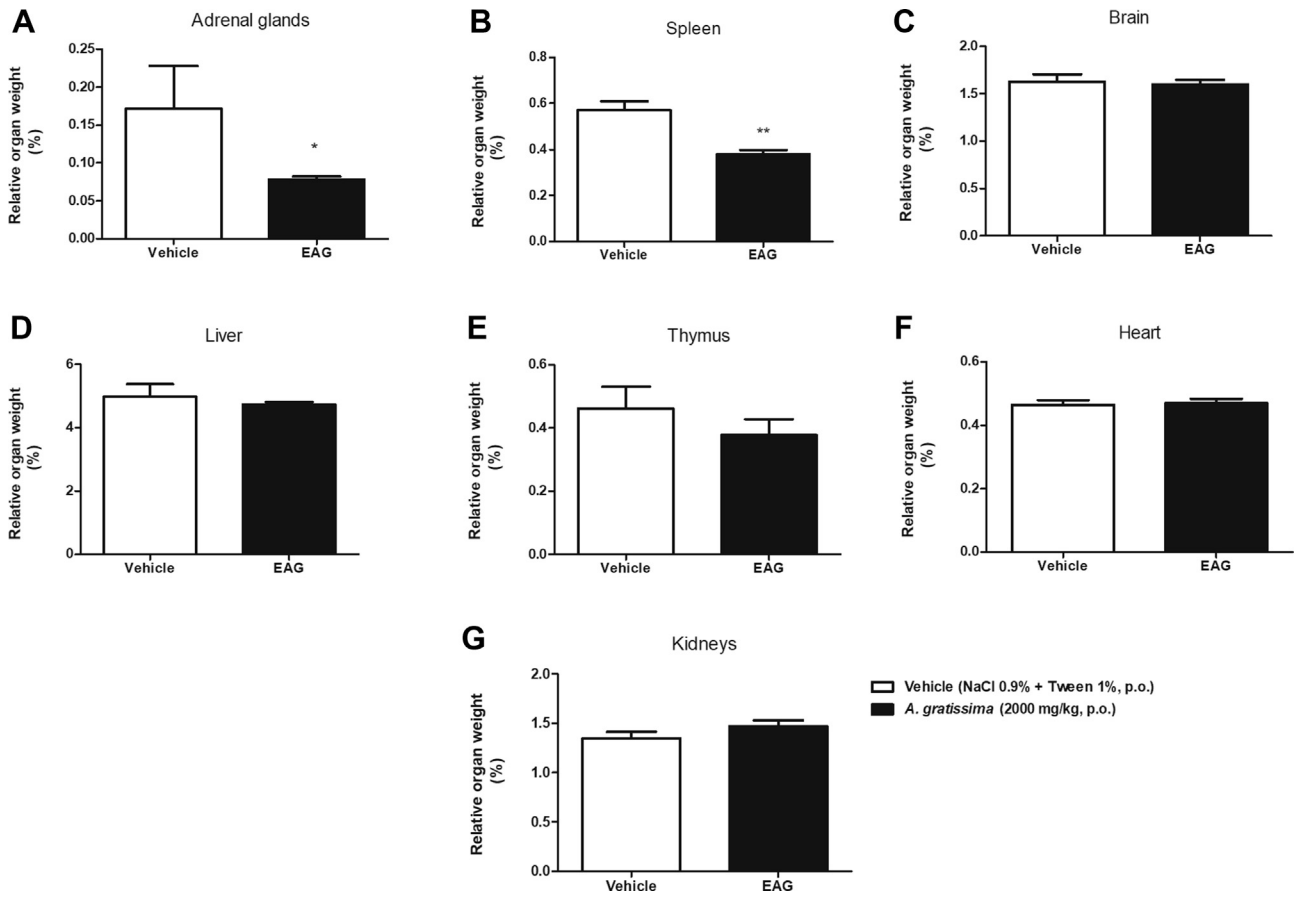

Fig. 2 Effect of *A. gratissima* leaves supercritical extract (EAG) on the relative weight of female Swiss mice organs (%) in the acute oral toxicity test. (A) adrenal glands; (B) spleen (C) brain; (D) liver; (E) thymus; (F) heart and (G) kidneys. The mice (n = 3–6) were orally treated with vehicle (NaCl 0.9% + tween 1%, 10 ml/kg) or EAG (2000 mg/kg). Unpaired *t* test, *$p < 0.05$, **$p < 0.01$.

presence of other compounds such as pinocarvil, (−)-trans-pinocarvilacetate, γ-elemene, bunesol, caryophyllene, caryophyllene oxide, (−)-spathulenol, myrtenol, isopinocamphone, β-cubebene, in smaller quantities.

The administration of EAG (2000 mg/kg, p.o.) induced an intense sedation in the mice, which lasted 45 min. After recovery, mice fed normally, with no locomotor activity changes. Food intake [Fig. 1A] of EAG-treated mice was significantly ($p < 0.05$) lower than of vehicle-treated mice at the 3rd day after treatment. Mice treated with vehicle presented a significant increase in the body weight [Fig. 1B] at the 9th ($p < 0.01$), 12th ($p < 0.001$) and 15th ($p < 0.001$) days of observation in comparison to initial weight. There were no changes in the body weight of EAG-treated animals during the period of observation. Also, no death was recorded.

Mice treatment with EAG (2000 mg/kg, p.o.) did not cause changes in the relative weight (%) of the brain, liver, kidneys, lung, heart and thymus [Fig. 2]. However, there was a significant decrease in the relative weight of the adrenal glands ($p < 0.01$) and spleen ($p < 0.05$) of EAG-treated mice in comparison to vehicle-treated mice.

The acetic acid injection evokes abdominal contractions in rodents, since it induces the peripheral production of several pro-inflammatory mediators. The EAG at 10 and 30 mg/kg ($p < 0.05$) and indomethacin at 10 mg/kg ($p < 0.01$) reduced the

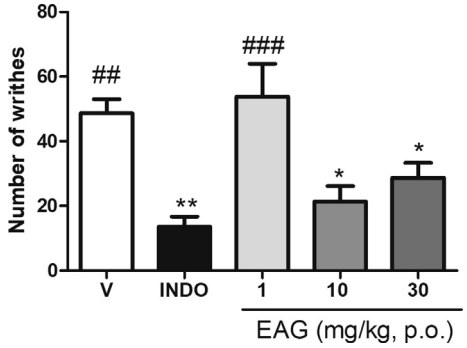

Fig. 3 Effect of a supercritical CO_2 extract of *Aloysia gratissima* leaves (EAG) in the acetic acid-induced abdominal writhing test in mice. V: vehicle treated group (0.9% NaCl + 1% Tween, 10 mL/kg p.o., n = 8). INDO: indomethacin (10 mg kg p.o., n = 6) or EAG (1, 10 and 30 mg/kg p.o., n = 6–8), 1 h prior to acetic acid administration. Each column represents the mean ± S.E.M. One-way ANOVA followed by the Student-Newman-Keuls test, **$p < 0.01$ and *$p < 0.05$ different from the vehicle group; ###$p < 0.001$ and ##$p < 0.01$ different from the INDO group.

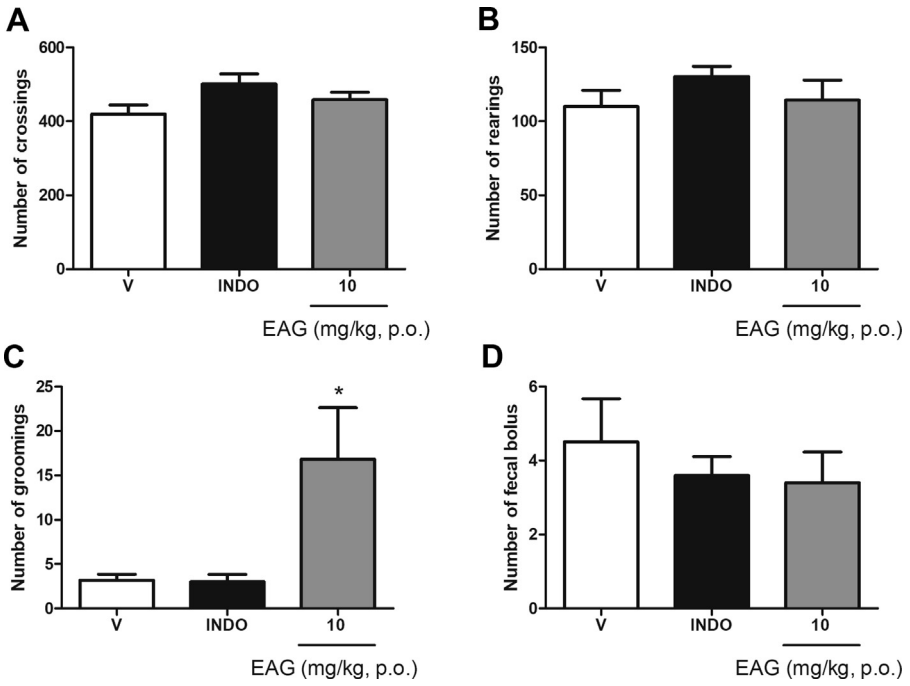

Fig. 4 Effect of a supercritical CO_2 extract of *Aloysia gratissima* leaves (EAG) on mice locomotor activity (open field test). A: number of crossings. B: number of rearings. C: number of groomings; D: number of fecal bolus. V: vehicle treated group (0.9% NaCl + 1% Tween, p.o., n = 6). INDO: indomethacin (10 mg/kg, n = 6). EAG (10 mg kg, p.o., n = 6). Each column represents the mean ± S.E.M. One-way ANOVA followed by the Student-Newman-Keuls test, *$p < 0.05$ different from the vehicle group.

number of abdominal writhes provoked by the injection of acetic acid when compared to the vehicle group. On the other hand, the EAG at the lowest dose (1 mg/kg) was not effective in reducing acetic acid-induced writhes [Fig. 3]. The abdominal contortion test was applied to investigate the lowest effective dose, in order to continue the other tests.

Considering that a non-specific effect of the EAG on animals' locomotion could influence the results of the antinociceptive tests and, therefore cause false positive or negative results, we investigated the effects of the minimal effective dose of EAG (10 mg/kg, p.o.) in the open field test. Treatment with EAG and indomethacin (10 mg/kg, p.o.) did not alter the numbers of crossings [Fig. 4A], rearings [Fig. 4B] and the number of fecal bolus [Fig. 4D]. The grooming number [Fig. 4C] for EAG-treated animals was significantly ($p < 0.05$) higher when compared to the groups treated with indomethacin or vehicle.

Table 2 Effect of a supercritical CO_2 *A. gratissima* leaves extract (EAG) on mice motor coordination (assessed by the Rota–Rod test). The animals (n = 7/group) were orally treated with vehicle (NaCl 0.9% + 1% tween 80), indomethacin (10 mg/kg) or EAG (10 mg/kg) 1 h before the test. Data are expressed as Mean ± S.E.M. Results were analyzed by Two-Way repeated measures ANOVA.

Group	Length of stay (s)	Number of falls
Vehicle	300.0	0.0
Indomethacin	300.0	0.0
EAG	299.6 ± 0.3	0.11 ± 0.03

Additionally, the treatment with EAG (10 mg/kg, p.o.) and indomethacin (10 mg/kg, p.o.) did not change mice motor coordination, compared to the vehicle group (NaCl 0.9%, p.o.) [Table 2].

The EAG at 10 mg/kg as well as indomethacin produced antinociception in the first phase ($p < 0.01$ and $p < 0.05$, respectively) – the neurogenic phase [Fig. 5A] - and in the second phase ($p < 0.001$) - inflammatory pain phase [Fig. 5B] of the formalin test. The investigation of the EAG action mechanism revealed that mice underwent pre-treatment with naloxone did not reverse the EAG antinociceptive activity in the two phases of formalin test [Fig. 6A and B], showing that the opioid system is unlikely to be involved in antinociceptive action. However, EAG's effect was prevented by mice who had pre-treatment with glibenclamide in the two formalin phases [Fig. 7A and B], thus suggesting that the K^+ channels sensitive to ATP are involved in the mechanism of antinociceptive effect of EAG.

Discussion

The solubility of chemical components obtained in the supercritical fluid is related to the fluid density. When CO_2 is used in the extraction of oils, it demonstrates good capacity for solubilization due to low polarity, without altering the chemical composition and the extract produced is free of solvent contamination [36]. The chemical composition of the *A. gratissima* leaves supercritical extract analyzed by GC/MS revealed the presence of terpene compounds, being guaiol and

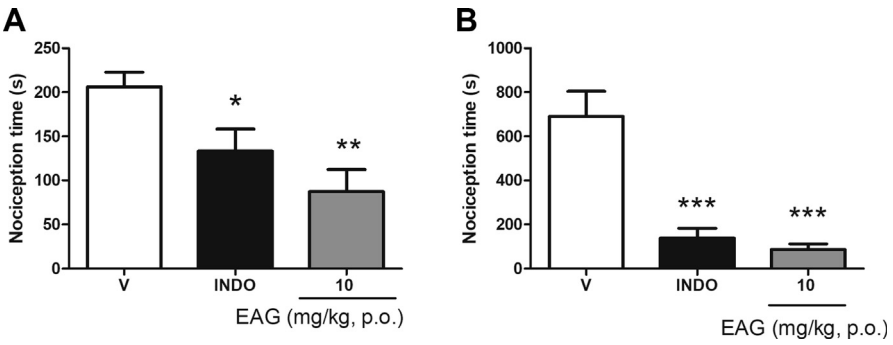

Fig. 5 Effect of a supercritical CO_2 extract of *Aloysia gratissima* leaves (EAG) on the formalin test. Nociceptive behavior was considered as the time (s) of elevation, biting or licking of the paw in the first phase (A: 0–5 min) and second phase (B: 15–30 min) of the test. Mice (n = 6 per group) were treated with vehicle (V: NaCl 0.9% + 1% tween 80, 10 ml/kg), Indomethacin (INDO: 10 mg/kg) or EAG (10 mg/kg) 1 h prior to administration of formalin 2% i.pl. One-way ANOVA followed by Student-Newman-Keuls: *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$ are different from the vehicle group. Results expressed as mean ± S.E.M.

pinocamphone the major ones. These compounds are commonly found in species from the *Aloysia* genus, and their antibacterial and antitumor activities have already been demonstrated [37,38]. Terpenes have been known to show a wide range of pharmacological activities, including effects on the central nervous system, antinociceptive, anti-inflammatory, antimicrobial and antitumor properties [39].

Mice treatment with the EAG (2000 mg/kg, p.o.) did not change the weight of the brain, liver, kidneys, lung, heart and thymus. However, there was a significant decrease in the relative weight of the spleen and adrenal glands in EAG-treated mice. Moreover, EAG-treated animals did not gain weight as vehicle-treated ones. This may suggest a sign of toxicity, which should be further investigated in a repeated dose toxicity test. The decrease in food intake of EAG-treated animals at the 3rd day after administration may be due to the sedation caused by the extract [6,7]. Zeni et al. (2013) [13] studied the toxicological effects of a hydroalcoholic extract of *A. gratissima* aerial parts and demonstrated that it induces hepatic toxicity in male mice at 2000 mg/kg. However, in the present study, we demonstrated the acute toxicity of supercritical *A. gratissima* leaves extract against mice adrenal glands and spleen. The differences between both findings may be due to the gender of mice and the type of extraction used in the experiments. Nonetheless, both extracts from the same vegetal species have proven to be harmless, since no deaths occurred after the oral administration at 2000 mg/kg. Our results show that the EAG could be included in Category 5 of the Harmonized Global Classification System (OECD Guideline 423, 2001) [25], since its LD_{50} is above 2000 mg/kg.

It is known that the intraperitoneal acetic acid injection in rodents evokes abdominal writhing, since it induces the peripheral production of several pro-inflammatory mediators, such as prostaglandins, bradykinin, substance P, prostacyclin and other cytokines, which, therefore, excite the nociceptors in the nerve endings [40]. The protective effect of substances against the noxious chemical stimulus may be an indication for a decreased production of these mediators, thus causing a reduction in the number of writhes [41]. The EAG at 30 mg/kg reduced abdominal writhing (on average 41%) compared to the vehicle group, while the dose of 10 mg/kg resulted in an average reduction of 56%, both doses being similar to the group that received indomethacin, the positive control. These results may be related to the presence of pinocamphone and

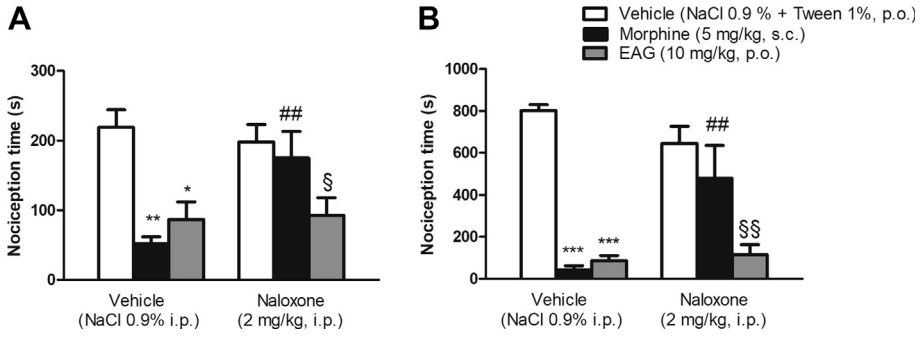

Fig. 6 Effect of mice pre-treatment with naloxone (2 mg/kg, i.p.) on the antinociceptive effect of a supercritical CO_2 extract of *Aloysia gratissima* leaves (EAG) (10 mg/kg) on the formalin test. Nociceptive behavior in the first phase (A, 0–5 min) or second phase (B, 15–30 min) of the test. Morphine (5 mg/kg, s.c.) was used as positive control. Results expressed as mean ± S.E.M. (n = 4–7 mice/group). Two-Way ANOVA followed by Student-Newman-Keuls test, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared to the vehicle plus vehicle-treated group. ##$p < 0.01$ compared to the vehicle plus morphine-treated group; §$p < 0.05$; §§$p < 0.01$ compared to the vehicle plus naloxone-treated group and morphine plus naloxone-treated group.

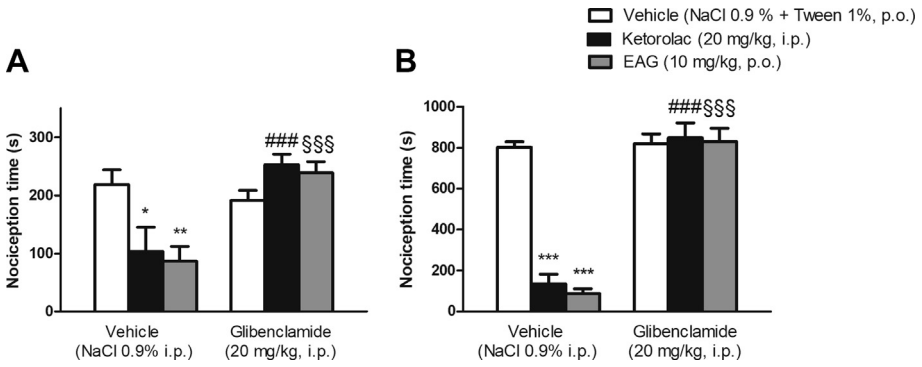

Fig. 7 Effect of mice pre-treatment with glibenclamide (20 mg/kg, i.p.) on the antinociceptive effect of a supercritical CO_2 extract of *Aloysia gratissima* leaves (EAG) (10 mg/kg, p.o.) on the formalin test. Nociceptive behavior in the first phase (A, 0–5 min) or second phase (B, 15–30 min) of the test. Ketorolac (20 mg/kg, i.p.) was used as positive control. Each column represents the mean ± S.E.M. (n = 4–7 mice/group). Two-Way ANOVA followed by the Student-Newman-Keuls test, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared to vehicle plus vehicle-treated group. ###$p < 0.001$ compared to the vehicle plus kerotolac-treated group; §§§$p < 0.01$ compared to vehicle plus EAG-treated group.

guaiol associated to caryophyllene oxide and spathulenol in EAG's chemical composition [42,43].

The acetic acid model is a preliminary test used to screen new analgesic drugs and was applied in our study to investigate the minimal antinociceptive dose of EAG, in order to continue the other tests. The lowest effective dose was used because of lower adverse effect's possibility. In order to investigate EAG's false-positive results in the nociception tests, mice behavior in the open field and rota-rod tests were evaluated [44].

The results obtained in the open field tests demonstrate that EAG (10 mg/kg) did not induce undesirable effects on the animals' locomotor activity and is not sedative or hyperstimulant. The number of groomings was the only behavior changed in comparison to the vehicle group. Considering that the grooming behavior has been used to measure pharmacologically induced anxiolytic-like effects in rodents [44,45], we may infer that the increase in mice grooming elicited by the EAG might be related to its anxiolytic property [6,7].

In order to investigate EAG's effect on mice motor coordination, we used the rota-rod test. The results of indomethacin, vehicle and EAG treated animals were not different between them in the rota-rod test, suggesting that EAG does not impair the motor coordination and, therefore does not induce false-positive results in other behavioral tests.

The formalin test aims to explore the analgesic effect of substances through central and peripheral pain mechanism [46]. The antinociceptive activity may occur in two distinct phases. In the first phase, neurological pain is induced by the chemical stimulation of afferent sensory fibers, particularly C fibers, whereas in the late phase, pain is caused by inflammatory mediators' production such as: prostaglandins, histamine, bradykinin, and serotonin [47].

The EAG elicited antinociception both in the first and second phases of the formalin test in mice and therefore, we can suggest that the EAG is effective in the treatment of neurogenic as well as inflammatory pain. Indeed, EAG's antinociceptive and anti-inflammatory activities are probably related to its main constituents. The presence of guaiol and spathulenol may be responsible for the EAG anti-inflammatory effect, as suggested by the study of Apel et al. (2010) [10], with *Myrciaria tenella* leaves extract, enriched in these compounds.

A number of authors evaluated the antinociceptive and anti-inflammatory activities of plant extracts that present the caryophyllene oxide as one of the main constituents. De Oliveira Júnior et al. (2017) [11] reported that *Croton conduplicats'* extract obtained by hydrodistillation showed antinociceptive and anti-inflammatory activities, being the caryophyllene and caryophyllene oxide the main constituents of the extract. Caryophyllene oxide is also the main component of *Myrcia pubiflora* DC leaves essential oil, obtained by hydrodistillation, and its antinociceptive and anti-inflammatory activities have been proven in *in vivo* experimental models [12].

Opioids act at the cellular level by binding to the opioid receptors present throughout the central nervous system, so the ultimate effect is the reduction of neuronal excitability, resulting in reduced neurotransmission of nociceptive impulses [48]. Pure opioid agonists (such as morphine) have high affinity for opioid receptors. To verify whether opioid receptors mediate EAG's antinociceptive effect, mice were pretreated with naloxone, an opioid antagonist. Naloxone did not prevent the EAG's antinociceptive activity in the two phases of the formalin test, suggesting that the opioid system is unlikely to be involved in its antinociceptive action.

Herein, we also investigated the involvement of ATP-sensitive K^+ channels in the mechanism of EAG's effect, by pretreating mice with glibenclamide (K^+ channel blocker). Several studies support the hypothesis that the opening of the K^+ channels mediate antinociception by inducing neurons hyperpolarization [49]. Therefore, K^+ channels, especially those sensitive to ATP, are particularly involved in the nociceptive responses [49]. Several G protein coupled receptors (including opioid receptors) that stimulate the opening of K^+ channels are involved in antinociception [49]. Our results demonstrate that the combined administration of glibenclamide (K^+ channel blocker) and EAG blocked the antinociceptive action of EAG in the two phases of the formalin test. In this context, our findings strongly suggest that the peripheral antinociceptive action of EAG might be related to the activation of K^+ channels sensitive

to ATP. Nevertheless, this activation appears to occur independently from the stimulation of opioid receptors, since naloxone did not prevent the EAG's antinociceptive activity. Indeed, the effect of terpenes as activators of K^+ channels sensitive to ATP is well known [13,50]. Corroborating our results, De Oliveira Júnior et al. (2017) [11] demonstrated that the antinociceptive effect of *Croton conduplicatus* essential oil - which contains caryophyllene oxide and guaiol - is mediated by K^+ channels sensitive to ATP.

Conclusion

Taken together, our results demonstrated the antinociceptive action of supercritical extract of *A. gratissima* leaves in mice, which is mediated by ATP-sensitive K^+ channels.

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Conflicts of interest

The authors declare no conflicts of interest.

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