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ORIGINAL ARTICLE

Participation of Cytokines, Opioid, and Serotoninergic Systems on Antinociceptive and Anti-inflammatory Activities of *Simira grazielae* Peixoto (Rubiaceae)

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ABSTRACT

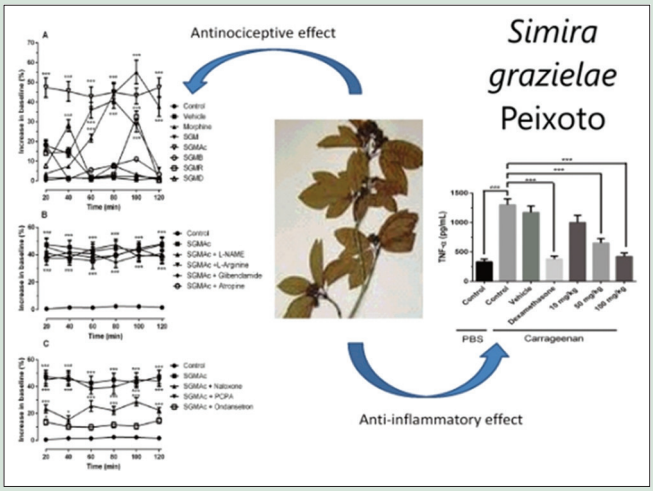
Background: *Simira grazielae* P. is widely found at Brazil. *S. grazielae* have been used to treat pain and inflammation in the Northeast of Brazil. **Objective:** This study investigated the mechanisms of the extract and partitions using pharmacological techniques in mice. **Materials and Methods:** Male Swiss mice (20–22 g) were used in models of pain (acetic acid-induced abdominal writhing, formalin, and tail-flick tests) and inflammation (edema paw and air pouch tests) as well as in model for the evaluation of motor activity (open field test). Furthermore, we evaluate the probable action mechanism of *S. grazielae* using naloxone, L-nitro-arginine methyl ester, L-arginine, glibenclamide, atropine, 4-chloro-DL-phenylalanine, and ondansetron in tail-flick test. The cytokines production was also evaluated. The methanolic extract from the *S. grazielae* and its partitions were administered orally at doses of 10–100 mg/kg. **Results:** Methanolic extract from the wood of *S. grazielae* (SGM) and its partitions showed antinociceptive properties in models of acute pain (SGM and ethyl acetate partition [SGMAc]) as well as in models of inflammation (dichloromethane partition [SGMD]). Prior administration of ondansetron and naloxone reduced the antinociceptive effect of SGMAc. SGMD reduced the production of tumor necrosis factor-α (TNF-α) induced by carrageenan. **Conclusion:** The results show that the anti-inflammatory activity showed by SGMD involves to reduction of the TNF-α, and the antinociceptive activity showed by SGMAc has relation to participation of the serotoninergic receptors and opioid system. These evidence justify the popular therapeutic use of this species in the control of pain and inflammation.

Key words: Extract, nociception, opioids, serotoninergic system, tumor necrosis factor-α

SUMMARY

- The antinociceptive and anti-inflammatory activities of the methanolic extract and partitions of *Simira grazielae* were evaluated. The ethyl acetate partition of S. *grazielae* showed antinociceptive effect related to the involvement of serotonergic and opioidergic receptors, while the partition in dichloromethane demonstrated an anti-inflammatory effect on the formation of edema and leukocyte migration probably related to inhibition in the production of tumor necrosis factor-α.

Abbreviations Used: PCPA: 4-chloro-DL-phenylalanine, SGM: Methanolic extract of *Simira grazielae,* SGMAc: Ethyl acetate partition, SGMD: Dichloromethane partition, SGMH: Hexane partition, SGMB: Butanol partition, SGMR: Residual partition, 5-HT: Serotonin, TNF-α: Tumor necrosis factor-α, n-C_6H_{14}: Hexane, CH_2Cl_2: Dichloromethane, EtOAc: Ethyl acetate, BuOH: Butanol, TLC: Thin-layer chromatography, HPLC: High-performance liquid chromatography, DAD: Diode array detector, COX-2: Cyclooxygenase-2, PGE_2: Prostaglandin E_2 eNOS: Endothelial nitric oxide synthase, NO: Nitric oxide, TRPA 1: Transient receptor potential cation, LT: Latency time, IBL: Increase in baseline, BL: Baseline.



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INTRODUCTION

Simira grazielae P. (Rubiaceae) known as "red araribа," is widely found at Brazil – Northeast of Bahia and Southeast of Espírito Santo state. Medicinal use is reported for species of *Simira* genus such as: phototoxic,[1] tonic, antifebrile, anti-inflammatory, antimalarial and antipyretic,[2] antifungal, antioxidant, larvicide,[3] neurosedative properties,[4] and anticorrosive.[5]

The presence of 3-*O*-*β*-D-glucopyranosyl sitosterol, syringaldehyde, 3,4,5-trimethoxyphenol, 6'-*O*-vanilloyltachioside, isofraxidin,

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scopoletin, 7-hydroxy-8-methoxycoumarin, 5,7-dimetoxycoumarin, harman, *N*-acetylserotonin, ophiorine B, umbelliferone, *β*-sitosterol, stigmasterol, and syringaresinol was observed on *S. grazielae*.[6] The last five compounds have been cited with anti-inflammatory and antinociceptive actions, which make relevant the investigation of the activities of *S. grazielae*.[4,7]

Inflammation is an event associated with pain that involves vascular alterations and combats to the aggressor agent and elimination of altered tissue components.[8] Although inflammation is beneficial to the organism (defense reaction), if it becomes excessive by long-term deleterious responses or by excessive acute responses or by reaction to noninjurious agents, it will cause tissue damage.[9]

The cytokines are an important component of the immune response and are linked with several inflammatory events, such as: leukocyte migration, extravasation, and recruitment of cells.[8]

Pain involves the regulation of peripheral and central events, derived from a harmful or innocuous stimulus.[10] The serotoninergic pathways are related to pain perception and also to the analgesic action of morphine. Studies have showed the participation of the bulbospinal serotoninergic system in pain. Other studies show that serotonin (5-HT) is involved in the brain control of nociception. The 5-HT participates in the control of nociception and pain exerted by the brainstem by acting at the spinal and supraspinal levels. The morphine analgesia can be severely affected by the destruction of the serotoninergic neurons, while increased levels of serotonin at the synapse reduce nociception.[11]

Pain is related the transduction of the noxious stimulus and the cognitive and emotional processing of the encephalon, while nociception is the neural process of decoding and processing the noxious stimulus.[10] Pain treatment is a very important area in pharmacological research because analgesic drugs used clinically cause side effects.[12]

From these observations, the present work intends to evaluate the effects of the methanolic extract and partitions from the wood of *S. grazielae* in some nociceptive and inflammatory models in mice and its possible mechanisms.

MATERIALS AND METHODS

Plant material

The wood of *S. grazielae* P. was collected by Marcelo Francisco de Araujo in 2007 at forest reserve of Vale do Rio Doce Company (VRDC), at the city of Linhares – Espírito Santo, Brazil (19° 6' 54" S, 39° 56' 20" W). The classification of the plant was realized by Domingos A. Folli and it was deposited in the herbarium of the VRDC with code CVRD 357.

Methanolic extract and partitions from the *Simira grazielae*

The wood of *S. grazielae* P. (4.5 kg) was ground and extracted with a methanol by maceration followed by filtration and further extraction. The crude methanolic extract of *S. grazielae* (SGM, 271.63 g) was obtained from the concentration of the filtrates using a rotary vacuum evaporator. The extraction efficiency was approximately 6% (w/w). The extract was stored at −20°C.

The SGM (235.13 g) was soluble in methanol/water (7:3) and successive extractions were realized with hexane, dichloromethane, ethyl acetate, and butanol (BuOH) yielding, after distillation of the solvents, SGMH (hexane partition (5.34 g), SGMD (dichloromethane partition, 11.44 g), SGMAc (ethyl acetate partition, 12.44 g), and SGMB (butanol partition, 42.25 g), respectively, in addition to residue residual partition (SGMR) (16.34 g) obtained from the methanol/water solution after distillation, also under vacuum.

Phytochemical prospecting

Phytochemical analysis of SGM extract and their partition SGMD, SGMAc, SGMB, and SGMR were performed to detect the presence of alkaloids, steroids, triterpenoids, flavonoids,[13] saponins, saccharides,[14] reducing sugars, purines, depsides, depsidonas, coumarin[15] organic acids,[16] and nonprotein amino acids.[17] The tests were based on colorimetric reactions or presence of precipitate [Table 1].

Chemical and instruments

Thin-layer chromatography was performed on silica gel 60 F254 (SiliCycle) eluted with n-BuOH/acetic acid/water (8/1/1), visualized under ultraviolet light (254 and 365 nm), and developed with ceric sulfate solution and aluminum chloride 2% ethanol solution.

High-performance liquid chromatography (HPLC) analyzes were performed using a Shimadzu liquid chromatography Prominence LC-20AT coupled to a SPD-20A diode array detector (column temperature oven-20A, communications bus module-20A). The reversed-phase column used was Betasil Thermo C18 (250 mm × 4.6 mm, 5 μm) with mobile phase consisted of water containing acetic acid 1% (A) and acetonitrile (B) and the injection volume for all samples was 20 μL using Autosample Sil 10AF. The samples were run for 23 min at a flow rate of 1 mL/min, with oven set at 30°C and absorbance monitored between 200 and 600 nm. The gradient started with 10% B and of 0–5 min (15% B), 5–10 min (15%–20% B), 10–20 min (20%–60% B), 20–22 min (60%–10% B), and 23 min stop the run. The compounds were quantified from a calibration curve of haman in triplicates of five concentrations (0.02–0.1 mg/mL). Alkaloids compounds were analyzed by matching the retention time and their spectral characteristics against those of standards. Standard of harman and ophiorine was isolated and characterized by our group.

Animals

Male Swiss mice were obtained from the Bioterium of the Federal Rural University of Rio de janeiro. The experimental protocols for utilization of the animals were approved by the Ethics Committee for Animal Research of the Federal Rural University of Rio de Janeiro (COMEP-UFRRJ) under number 22678.003457/2012-32. The mice were kept in a controlled temperature room (22°C ± 1°C) and 12 h light-dark cycle. Water and food *ad libitum,* but the food was withdrawn 8 h before oral administration of the substances to avoid interference in the absorption.

Chemicals

The following substances were used: acetic acid (Vetec, Rio de Janeiro, Brazil), formaldehyde (Merck, Darmstadt, Germany), dexamethasone (purity – 97%), L-nitro-arginine methyl ester (L-NAME) (purity – 98%), L-arginine (purity – 99%), acetylsalicylic acid (purity – 99%), λ-carrageenan, and dimethyl sulfoxide (Sigma–Aldrich, St. Louis, MO, USA), and morphine (purity – 97%) (Cristália, São Paulo, Brazil).

Treatments

Methanolic extract from the *S. grazielae* and its partitions were initially administered orally at a dose of 100 mg/kg on acetic acid-induced abdominal writhing, formalin, tail-flick, edema paw, and air pouch tests. SGMAc was administered orally (10, 50, and 100 mg/kg) on acetic acid-induced abdominal writhing and formalin tests. SGMD was administered orally at doses of 10, 50, and 100 mg/kg on air pouch test to quantification of cytokines. In the open field test, SGMAc was administered at a dose of 100 mg/kg. Morphine, acetylsalicylic acid, and dexamethasone were used as positive controls. The

Table 1: Results of phytochemical prospection on wood from *Simira grazielae*

Chemical compounds	SGM	SGMD	SGMAc	SGMB	SGMR
Alkaloids	+	+	+	+	+
Saponins	+	–	+	+	–
Steroids and triterpenoids	+	–	–	–	–
Flavonoids	–	–	–	–	–
Reducing sugars	+	+	+	+	+
Saccharides	+	+	+	+	+
Purines	–	–	–	+	+
Organic acids	–	–	–	–	–
Nonprotein amino acids	+	+	+	+	+
Depsides and depsidonas	–	–	–	–	–
Coumarins derivatives	+	+	+	+	+

prosSGM: Methanolic extract of *Simira grazielae*; SGMD: Dichloromethane partition, SGMAc: Ethyl acetate partition, SGMB: Butanol partition, SGMR: Residual partition

dose of morphine (5.01 and 8.15 mg/kg – p.o. – opioid analgesic drug), dexamethasone (2.25 mg/kg-subcutaneous administration, s.c. – steroidal anti-inflammatory), and acetylsalicylic acid (100 mg/kg – p.o.) was according to dos Santos *et al.*, 2015.[18]

Dimethyl sulfoxide solubilized in distilled water (2.5%) was used as vehicle for the dissolution of the extract and partitions. The control group was constituted by mice that received distilled water.

Acetic acid-induced abdominal writhing test

Model used to screen of the antinociceptive activity.[19] This model is based on the counts of contractions of the abdominal wall followed by extension of the hind limbs and contact of the abdomen with the floor of the counting vessel. In this model, intraperitoneal administration of 0.01 mL/g acetic acid (1.2%) was performed 60 min after administration of the substances. The count of the number of writhes was realized for a period of 30 min and it started immediately after acetic acid injection.

The formalin test

Model used in the evaluation of inflammatory and noninflammatory pain.[20] In this model, 0.02 ml of formalin solution (2.5%) was injected into the right hind paw 60 min after oral administration of the substances. Afterward, the mice were placed in a container, where the count of the time that animals remained licking the administered paw was made. The time was measured in two steps: (1) neurogenic, performed between 0 and 5 min after formalin injection and (2) inflammatory, performed between 15 and 30 min after formalin injection.

The tail-flick test

This model was used to evaluate central antinociceptive activity, as previously described by D'amour and Smith, 1941.[21] In this model, a light beam was focused on the tail of the animal and the latency time (LT) was measured. The light intensity was adjusted so that the basal LTs were between 3 and 5 s; the animals that presented basal LT outside these values were excluded of the experiment. The average of the first 2 measures is called the baseline (BL) latency. After determination of BL LT, mice received the extract and partitions, and new measurements were performed at 20-min intervals between them. The result was expressed as percentage of increase over the BL (increase in BL [IBL] %) according to the following formula:

$$\text{IBL } \% = 100 - \frac{\text{LT} \times 100}{\text{BL}}$$

Evaluation of the possible mechanism(s) of action of SGMAc in the tail-flick test.

To evaluate the participation of opioid, muscarinic, nitrergic and serotoninergic systems, and ATP-sensitive potassium (K^+ ATP) – channels in the antinociceptive effect of SGMAc, mice were pretreated with naloxone (opioid antagonist, 5 mg/kg, i.p.), atropine (muscarinic antagonist, 5 mg/kg, i.p), ondansetron (5-HT_3 serotoninergic antagonist, 0.5 mg/kg, i.p.), glibenclamide (ATP-sensitive potassium channel blocker, 1 mg/kg, i.p.), L-arginine (a substrate of nitric oxide (NO) synthase, 3 mg/kg, i.p.), or L-NAME (inhibitor of NO synthase, 3 mg/kg, i.p.), 30 min before the treatment with SGMAc (100 mg/kg, p.o.). The doses of antagonists and inhibitors were chosen from the previous data described in the literature.[18] To access the participation of the endogenous serotonin in the antinociceptive effect of SGMAc, the 4-Chloro-DL-phenylalanine (PCPA) was administered, intraperitoneally, at a dose of 100 mg/kg for 3 consecutive days.[22] The antagonists and inhibitors were evaluated in the tail-flick model, as described above.

The paw edema test

To evaluate the antiedematogenic activity, the paw edema test was performed from the subplantar injection of 0.02 mL of carrageenan (1%) into one of the hind paws. In the contralateral paw was injected 0.02 mL of distilled water. The volume of edema was evaluated using the plethysmometer,[23] allowing the measurement of small volumes of fluid displaced. Paw edema was quantified during the first 4 h after the injection of flogistic agent (carrageenan), and the results are expressed as the difference, in volume, between the two paws.

Air pouch test

The model was performed as described by Vigil *et al.*, 2008.[24] A region of the dorsum (3 cm × 2.5 cm) of the mice was disinfected and injected with 7 ml of sterile air subcutaneously at a single point. The air pouches were reinforced with sterile air on alternate days for 3 days. On the 4th day, the animals were treated with the substances and 1 h later received carrageenan (1%) administered subcutaneously, and 4 h later, they were euthanized with an overdose of pentobarbital. The subcutaneous cavity was washed with 1 ml of sterile phosphate buffer solution (PBS) (pH 7.6, containing NaCl [130 mM], Na_2PO_4 [5 mM], KH_2PO_4 [1 mM], and heparin [20 IU/mL] in distilled water) and an incision was made in the skin for material collection. The collected material was used to determine total leukocytes and quantification of cytokines (tumor necrosis factor-α [TNF-α]). The quantification of total leukocytes was obtained by the counting in the Neubauer chamber under optical microscopy.

Tumor necrosis factor-α measurements

In order to quantify TNF-α present in the exudates, they were centrifuged and the supernatants were collected and the concentration of this cytokine was determined in aliquots (50 μL) of the wash in the air pouch test. For this, enzyme immunoassay kit (TNF-α ELISA Kit mouse, Cayman Chemical®) was used to measure the concentrations of TNF-α in the samples through ELISA method.

The open field test

This model was realized to evaluate the level of locomotor activity in mice. The animals were acclimatized days before the beginning of the experiment, being placed individually in an observation chamber, daily, for a few minutes. This protocol was realized according to Barros *et al.*, 1991.[25] After the oral administration of substances, the mice were placed in observation chamber (60 min after oral administration), and the spontaneous activity was quantified by number of squares covered for a period of 5 min.

Statistical analysis

All experimental groups were formed by 6–8 animals. The results are presented as the mean ± standard error of the mean (SEM). Statistical significance between the groups was determined using one-way analysis of variance (ANOVA) followed by Bonferroni's test for the acetic acid-induced abdominal writhing, formalin, air pouch, and open field tests and two-ANOVA, followed by Bonferroni's test for the paw edema and tail-flick tests. $P < 0.05$, 0.01, and 0.001 were considered statistically significant.

RESULTS

Phytochemical prospecting and high-performance liquid chromatography profile

Phytochemical prospection of *S. grazielae* methanolic extract and partitions indicated the presence of different secondary metabolites classes [Table 1]. Many of them are known to have different therapeutic applications, including anti-inflammatory and antinociceptive activities.[26,27] The HPLC analysis led to the identification of ophiorine (2.88 min; 24.7% w/v) and harman (10.10 min; 6.1% w/v) in wood of *S. grazielae* methanolic extract. The HPLC conditions, described in the experimental section, allowed good separation for the alkaloids.

Acetic acid-induced writhing test

The administration of acetic acid (1.2%) produced 54.2 ± 6.1 writhes in a period of 30 min. The administration of 100 mg/kg of the methanolic extract from the *S. grazielae* (SGM) and its partitions inhibited writhing by approximately SGM – 83% (9.0 ± 2.0 writhes), SGMAc – 72% (15.3 ± 6.0 writhes), SGMB – 71% (15.8 ± 3.2), SGMR – 83% (9.3 ± 1.4), and SGMD – 70% (16.3 ± 2.3) [Figure 1a]. Doses of 10, 50, and 100 mg/kg of SGMAc reduced writhing by approximately 63% (20.0 ± 1.4 writhes), 69% (16.6 ± 3.6 writhes), and 81% (10.5 ± 3.3 writhes), respectively [Figure 1a].

Formalin test

The intraperitoneal injection of formalin (2.5%) produced 53.2 ± 6.3 s (first phase) and 173.9 ± 7.8 s (second phase) in the licking time. Pretreatment with the SGM and SGMAc reduced the licking time in the two phases, while SGMD only inhibited the second phase.

SGM showed 68% (17.2 ± 2.2 s) and SGMAc 56% (23.5 ± 1.9 s) inhibition in the licking time in the first phase, and in the second phase, SGM showed 63% (65.0 ± 8.2 s), SGMAc 62% (66.6 ± 7.9 s), and SGMD 48% (90.3 ± 2.3 s) inhibition at dose of 100 mg/kg [Figure 2a and b].

In the pretreatment with SGMAc, the inhibitory effect was observed in the first and second phases, only at doses of 50 and 100 mg/kg [Figure 2c]. In the first phase, the SGMAc induced 39% (32.5 ± 2.1 s) and 56% (23.5 ± 1.9 s) inhibition at doses of 50 and 100 mg/kg, respectively. In the second phase, the SGMAc induced 41% (102.8 ± 4.6 s) and 62% (66.6 ± 7.9 s) inhibition at doses of 50 and 100 mg/kg, respectively [Figure 2c]. Morphine (5.01 mg/kg) inhibited the number of licks in both the phases (1st Phase – 33.5 ± 4.6 and 2nd Phase – 84.8 ± 4.2 s). Acetylsalicylic acid (100 mg/kg) reduced the number of licks only in the second phase (79.0 ± 8.0 s).

Tail-flick test

The effect on the tail-flick test was observed only with SGM and SGMAc (100 mg/kg). Both showed effects earlier than morphine [Figure 3a]. The relation dose × effect and the evaluation of the mechanism of action were studied with SGMAc because it was the only partition to show a central antinociceptive effect on noninflammatory pain in formalin and tail-flick tests.

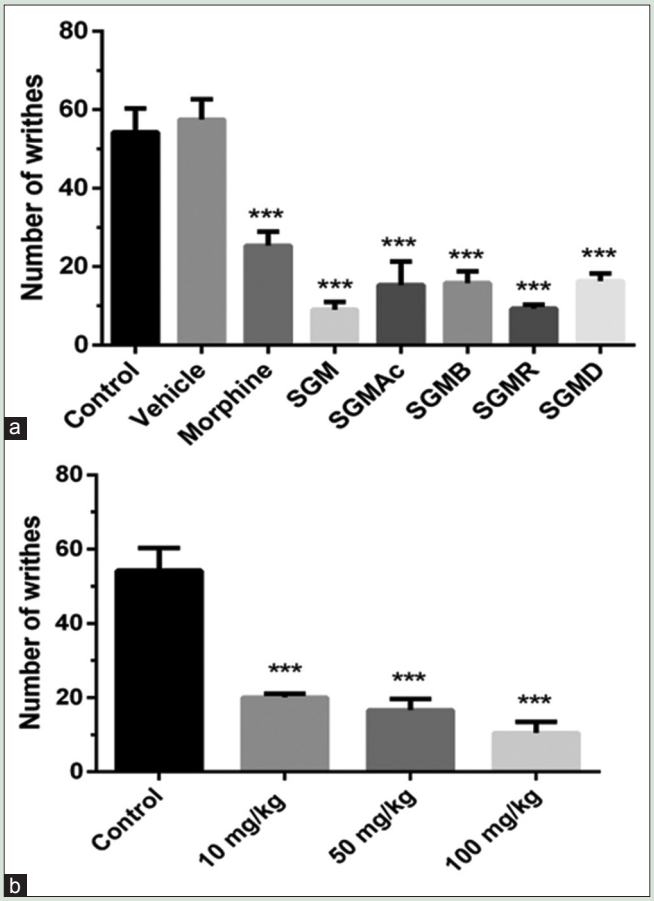

Figure 1: The effects of orally administered methanolic extract from the *Simira grazielae* and its partitions (a), and different doses of the ethyl acetate partition (b) on acetic acid-induced writhing test. In a, the mice received water, vehicle, morphine (5.01 mg/kg), methanolic extract, and its partitions (SGM, SGMAc, SGMB, SGMD, and SGMR-100 mg/kg). In b, the mice received water and SGMAc (10, 50, and 100 mg/kg). The results are showed as the mean ± standard error of the mean ($n = 8$). One-way analysis of variance followed by Bonferroni's test was used to calculate the statistical significance. In a, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ was used to compare the vehicle-, SGM-, SGMAc-, SGMB-, SGMD-, SGMR-, and morphine-treated groups with the control group. In b, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ to compare the SGMAc-treated groups with the control group. SGM: Methanolic extract, SGMAc: Ethyl acetate partition, SGMB: Butanol partition, SGMD: Dichloromethane partition and SGMR: Residual partition

Naloxone, glibenclamide, atropine, L-NAME, L-arginine, and ondansetron were previously administered; ondansetron and naloxone reduced the antinociceptive effect of SGMAc in 75% and 49%, respectively [Figure 3b and c]. The administration of PCPA intraperitoneally for 3 consecutive days did not inhibit the antinociceptive effect of SGMAc [Figure 3c].

Paw edema and air pouch test

In the paw edema test, SGMD and dexamethasone (s.c.) inhibited the paw edema induced by carrageenan by 54% and 50%, respectively [Figure 4a]. The SGMD was studied on inflammatory tests because it was the only partition to show a peripheral antinociceptive effect on inflammatory pain in formalin test.

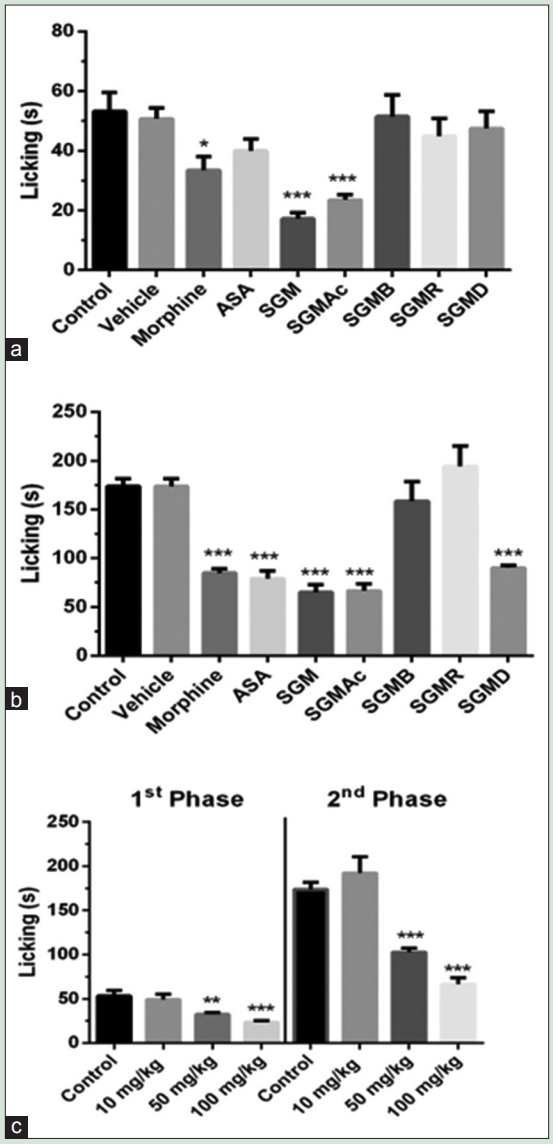

Figure 2: The effects of orally administered methanolic extract from the *Simira grazielae* and its partitions (a – First Phase and b – Second Phase), and different doses of the ethyl acetate partition (c) on formalin test. In a and b, the mice received water, vehicle, morphine (5.01 mg/kg), (ASA-100 mg/kg), methanolic extract, and its partitions. (SGM, SGMAc, SGMB, SGMD, and SGMR-100 mg/kg) In c, the mice received water and SGMAc (10, 50, and 100 mg/kg). The results are showed as the mean ± standard error of the mean ($n = 8$). One-way analysis of variance followed by Bonferroni's test was used to calculate the statistical significance. In a and b, *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ was used to compare the vehicle-, ASA-, SGM-, SGMAc-, SGMB-, SGMD-, SGMR-, and morphine-treated groups with the control group. In c, *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ to compare the SGMAc-treated groups with the control group. SGM: Methanolic extract, SGMAc: Ethyl acetate partition, SGMB: Butanol partition, SGMD: Dichloromethane partition, SGMR: Residual partition, ASA: Acetylsalicylic acid

The presence of leukocytes in the exudate, collected from the air pouch, administered with carrageenan (16.18×10^6 cells) appears increased when compared to administration of the PBS (1.88×10^6 cells).

SGMD inhibited the leukocyte migration induced by carrageenan at dose of 100 mg/kg by 48% (8.45×10^6 cells) [Figure 4b]. Subcutaneous administration of dexamethasone was reduced by 60% (6.34×10^6 cells).

Tumor necrosis factor-α measurement

SGMD at doses of 50 and 100 mg/kg inhibited TNF-α production by 50% and 67%, respectively [Figure 5]. Subcutaneous administration of dexamethasone also reduced TNF-α production by 70%.

Open field test

SGMAc (100 mg/kg) did not show effect on locomotor activity compared with the control group. Morphine significantly decreased locomotor activity [Figure 6].

DISCUSSION

This study evaluated the methanolic extract from the *S. grazielae* and its partitions, in models of acute pain, edema formation, leukocyte migration, and TNF-α production in mice. This study aims to support the therapeutic use of this plant species in the pain and inflammation.

Phytochemical prospecting of the methanol extract and partitions of wood *S. grazielae* are rich alkaloids, saponins, steroids, triterpenoids, flavonoids, reducing sugars, saccharides, organic acids, nonprotein amino acids, and coumarin, confirming the results already presented for this species.[6] HPLC profile showed high concentration of alkaloids; harman, taxonomic markers for *Simira* genus and ophiorine are the majority compounds in *S. grazielae.*

The Rubiaceae family has a great range of chemical structures, which vary little, among the classes of secondary metabolites one can highlight iridoids, anthraquinones, triterpenes, and indole alkaloids, the latter being considered as a chemotaxonomic marker of the family.[28] According to the chemical studies of *Simira* species, there are reports of the presence of compounds that justify the positive results in all partitions tested for alkaloids and coumarins.

The acetic acid-induced abdominal writhing test is used in the evaluation of antinociceptive effects. Acetic acid induces inflammation in the abdominal cavity, with activation of nociceptors.[29] The administration of acetic acid stimulates the release of mediators such as substance P, bradykinin, prostaglandins, and cytokines (IL-1, IL-6, IL-8, and TNF-α), thus activating peripheral nociceptors and neurons.[30] The methanolic extract from the *S. grazielae* and its partitions, and different doses of ethyl acetate partition inhibited the number of writhes, showing that they presented an antinociceptive activity.

The formalin test is performed to differentiate antinociceptive activities of inflammatory and noninflammatory character. The formalin test has two different phases: the first phase (neurogenic) and the second phase (inflammatory).[31] The neurogenic phase is characterized by the direct stimulation of peripheral nociceptors, mediated by transient receptor potential cation, involving Aδ-fibers, and is also related to the involvement of preformed mediators. The second phase is related to the release of postformed mediators (bradykinin, histamine, substance P., serotonin, and prostaglandins), which interact with their respective receptors for the manifestation of inflammatory nociception.[32] Drugs that act in both phases show central antinociceptive activity, while drugs that act only in the second phase present peripheral antinociceptive activity.[33] The formalin test results showed that SGM and SGMAc (100 mg/kg), inhibited the two phases of the test, demonstrating a possible central antinociceptive activity. While the SGMD showed effect only in the second phase, suggesting peripheral antinociceptive effect, possibly due to its anti-inflammatory activity.

To confirm, the neurogenic antinociceptive activity was realized tail-flick test. The tail-flick test is a nociceptive model of thermal stimulation where radiant heat is used as a harmful stimulus. The thermal stimulus is applied to the tail of mice and tail withdrawal reflex is considered as response.[34] The spinal neurons respond to the thermal stimulus, being

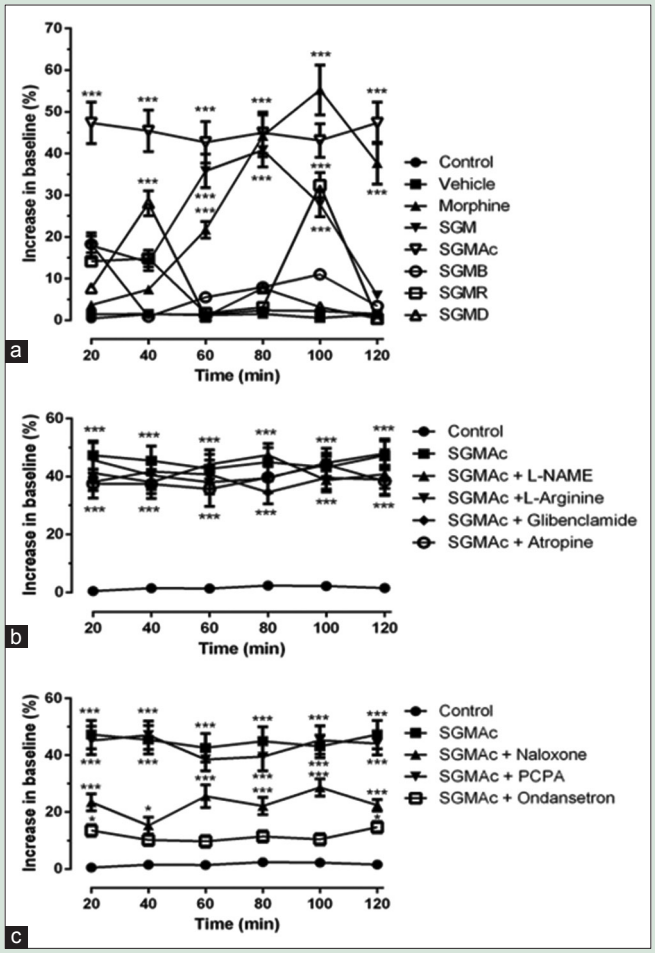

Figure 3: The effects of orally administered methanolic extract from the *Simira grazielae* and its partitions on tail-flick test (a) and evaluation of the antagonists previously administered to SGMAc in the tail-flick test (b and c). In a, the mice received with water, vehicle, morphine (8.15 mg/kg), methanolic extract, and its partitions (SGM, SGMAc, SGMB, SGMD, and SGMR-100 mg/kg). In b, the mice received L-NAME (3 mg/kg), L-arginine (3 mg/kg), glibenclamide (1 mg/kg), and atropine (5 mg/kg). In C, the mice received naloxone (5 mg/kg), ondansetron (5 mg/kg), and PCPA at a dose of 100 mg/kg for 3 consecutive days. The results are showed as the mean ± standard error of the mean (n = 8). Two-way analysis of variance followed by Bonferroni's test was used to calculate the statistical significance. In a, *P < 0.05, **P < 0.01, and ***P < 0.001 was used to compare the vehicle-, morphine- and SGMAc-treated groups with the control group. In b, *P < 0.05, **P < 0.01, and ***P < 0.001 to compare the L-NAME + SGMAc-, L-arginine + SGMAc-, glibenclamide + SGMAc-, atropine + SGMAc groups, and SGMAc-treated group with the control group. In c, *P < 0.05, **P < 0.01, and ***P < 0.001 to compare the naloxone + SGMAc-, ondansetron + SGMAc-, PCPA + SGMAc- groups, and SGMAc-treated group with the control group. SGM: Methanolic extract, SGMAc: Ethyl acetate partition, SGMB: Butanol partition, SGMD: Dichloromethane partition and SGMR: Residual partition, PCPA: 4-Chloro-DL-phenylalanine, L-NAME: L-nitro-arginine methyl ester

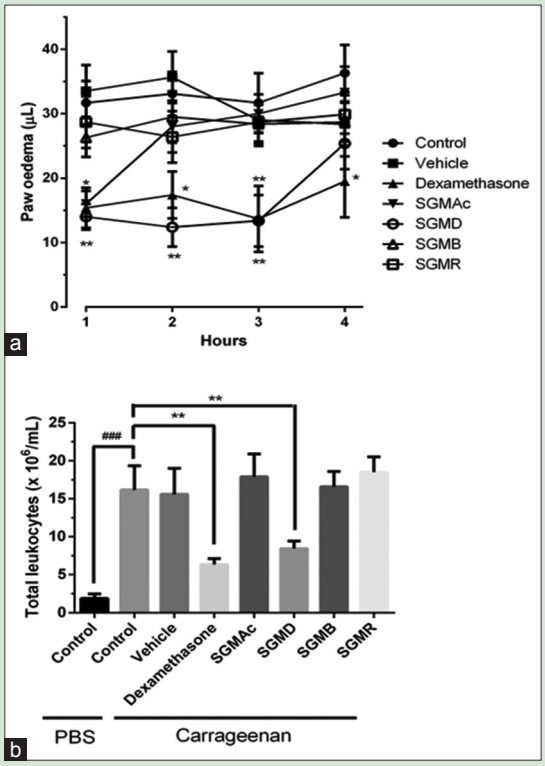

Figure 4: The effects of orally administered methanolic extract from the *Simira grazielae* and its partitions in the paw edema test (a) and quantification of total leukocytes in the air pouch test (b). The mice received water, vehicle, and dexamethasone (2.25 mg/kg; s.c.), (SGM, SGMAc, SGMB, SGMD, and SGMR-100 mg/kg). In a, carrageenan was injected in the paws of the mice. In b, PBS or carrageenan was injected in the pouch of the mice. The results are showed as the mean ± standard error of the mean (n = 8). Two-way analysis of variance (a) and one-way analysis of variance (b) followed by Bonferroni's test was used to calculate the statistical significance. *P < 0.05, **P < 0.01, and ***P < 0.001 was used to compare the SGMAc-, vehicle-, and dexamethasone-treated groups, respectively, with the control group. $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, and $^{\#\#\#}P$ < 0.001 to compare carrageenan-injected groups with the PBS-injected group. SGM: Methanolic extract, SGMAc: Ethyl acetate partition, SGMB: Butanol partition, SGMD: Dichloromethane partition, SGMR: Residual partition, PBS: Phosphate buffer solution

possible the evaluation of spinal mechanisms involved in antinociception. The reaction to this test may involve supraspinal neural structures, thus drugs such as opioids are able to increase the tail-flick LT.[35,36] SGM and SGMAc showed response in this test, suggesting central antinociceptive effect.

Cholinergic agonists act at the spinal level[37,38] demonstrating analgesic action in humans[39] and when administered intrathecally in laboratory animals reduce the withdrawal reflex.[40] The systemic actions of nicotinic and muscarinic agonists,[41] as well as acetylcholinesterase antagonists also inhibit the spinal reflex. The previous administration of atropine (nonselective muscarinic antagonist) was used to evaluate the participation of the muscarinic system in the antinociceptive effect demonstrated by SGMAc. The results confirm the absence of involvement of the muscarinic system.

The NO is produced from the isoforms of NO synthase (neuronal/nNOS, endothelial/eNOS or inducible/iNOS).[42] on L-arginine. NO is related with several physiological[43] and pathophysiological conditions.[42]

The nitrergic pathway is a pain-modulating pathway.[44] The NO is able to activate guanylate cyclase and induce intracellular cGMP increase.[45] The participation of NO in nociception is dual, studies show that administration of NO donors inhibits Prostaglandin E_2 (PGE_2)-induced hypernociception and that this effect is reversed with the use of L-NAME, a NO synthase inhibitor.[46] However, other studies have shown the relation of the nitrergic pathway on the analgesia produced by opioids.[47] In this study, the previous administration of

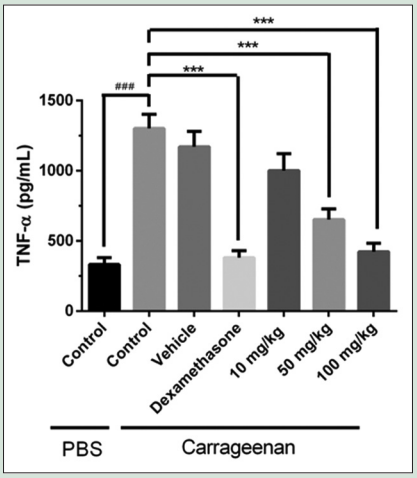

Figure 5: The effects of orally administered ethyl acetate partition of the methanolic extract from the *Simira grazielae* in the open field test. The mice received SGMAc (100 mg/kg), morphine (8.15 mg/kg), water, and vehicle. The results are showed as the mean ± standard error of the mean ($n = 8$). One-way analysis of variance followed by Bonferroni's test was used to calculate the statistical significance. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ was used to compare the SGMAc-, vehicle-, and morphine-treated groups with the control group. SGMAc: Ethyl acetate partition

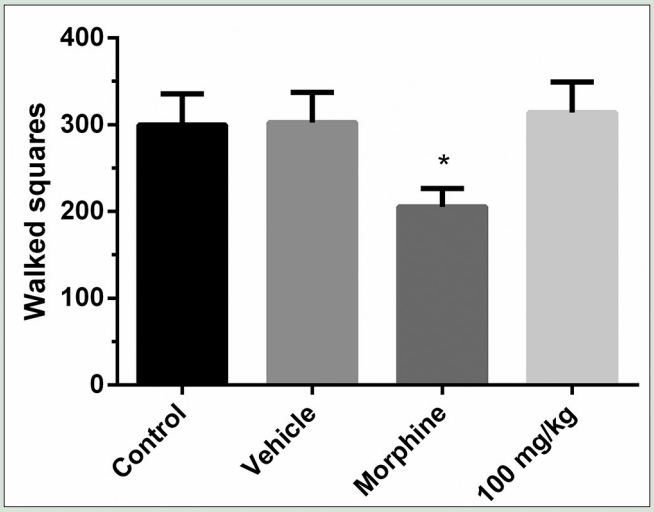

Figure 6: The effects of orally administered ethyl acetate partition of the methanolic extract from the *Simira grazileae* in the Open field test. The mice received SGMAc (100 mg/kg), morphine (8.15 mg/kg), water and vehicle. The results are showed as the mean ± SEM (n=8). One-way ANOVA followed by Bonferroni's test was used to calculate the statistical significance. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ was used to compare the SGMAc-, vehicle-, and morphine-treated groups with the control group

L-NAME and L-arginine did not induce a change in the effect produced by SGMAc, excluding the participation of nitrergic system.

Nitrergic intracellular signaling determines the antinociceptive or nociceptive action of the NO. Several studies have shown that activation of K^+ channels decreases intracellular Ca^{++} levels through cellular hyperpolarization, reducing the release of neurotransmitters, and contributing to the reduction of impulse synaptic transmission, resulting in antinociception. According to the localization, these channels can act directly or indirectly in nociceptive signaling.[48] Previous administration of glibenclamide (a specific ATP-dependent K^+ channel blocker) did not alter the effect of SGMAc, demonstrating the absence of participation of these channels in the SGMAc activity.

5-HT is considered a participant neurotransmitter in pain control mechanisms. The effects induced by 5-HT are determined by its interaction with serotonergic receptors, located in the central and peripheral nervous system.[49] Primary afferent fibers, projection neurons, and interneurons present serotoninergic receptors that are involved in nociceptive and inflammatory responses.[50] Furthermore, the release of 5-HT stimulates the periaqueductal gray matter and inhibitory interneurons, inhibiting spinal neurons through opioid peptides that are released by the inhibitory interneurons after activation of serotoninergic receptors.[51]

To evaluate the participation of the serotoninergic system was realized a cortical depletion of serotonin through an injection of PCPA. As PCPA did not change the antinociceptive effect of SGMAc, its activity may be produced by the activation of 5-HT_3 receptors, which can be confirmed by reversal of the effect of SGMAc by prior administration of ondansetron.

To evaluate the opioid system's participation in the effect of SGMAc, it was administered orally after intraperitoneal administration of naloxone in the tail-flick test. Naloxone is a nonselective opioid receptor antagonist. The downward pathway of pain control can be regulated by various systems that modulate nociception. In the process of transmission, the stimulation of cerebral areas such as periaqueductal and periventricular is able to release neuromodulating substances, such as the opioid peptides that have analgesic action, acting through their respective receptors.[52] Naloxone reduced partially the antinociceptive effect of SGMAc, showing the participation of opioid system.

To exclude a possible motor interference induced by SGMAc, the open field test was made. Agents with activity in the central nervous system interfere in nociceptive tests.[53] SGMAc did not show activity in the open field test, confirming its antinociceptive effect.

To observe a possible anti-inflammatory activity of the partitions of the methanolic extract from the *S. grazielae*, paw edema was stimulated by carrageenan. Carrageenan was used as a pro-inflammatory agent to evaluate the possible anti-inflammatory activity of the partitions.[54] The administration of carrageenan into the mouse paw induces edema resulting from the biphasic release of inflammatory mediators.[55] In the first phase, there is the participation of histamine and serotonin, while in the second phase, the participation of prostaglandins occurs.[56] In this model, the SGMD inhibited the formation of paw edema in the first 3 h after administration of carrageenan, confirming its antiedematogenic effect.

Leukocytes are cells of fundamental importance in the inflammatory process. The release of chemotactic factors and expression of adhesion molecules is responsible by recruitment of neutrophils. In this context, the air pouch test is a model characterized by the intense release of inflammatory mediators, inducing leukocyte extravasation. TNF-α is a cytokine involved in the development acute phase of inflammation. Its main actions are the activation of endothelial cells and leukocytes and induction of systemic reactions of acute phase. TNF-α is still responsible for the activation of lymphocytes, stimulation of adhesion molecules, PGE_2, platelet-activating factor, and other mediators and cytokines.[57,58] In this model was confirmed the inhibitory effect of SGMD on leukocyte extravasation and production/release of TNF-α.

CONCLUSION

The present study demonstrates that the methanolic extract from the *S. grazielae* and its partitions present antinociceptive activities in

acute pain (SGM and SGMAc) and inflammation (SGMD) models. These results suggest that the anti-inflammatory effect showed by SGMD might involve reduction of the cytokine TNF-α and that the antinociceptive effect showed by SGMAc could be expelled by involving participation of the serotoninergic receptors ($5\text{-}HT_3$ receptor) and opioid system, through the activation of serotoninergic receptors present in inhibitory interneurons releasing opioid peptides. These data show initial evidence about antinociceptive and anti-inflammatory activities of *S. grazielae*, but its mechanism of action needs to be better studied.

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Conflicts of interest

There are no conflicts of interest.

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