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Geopropolis from *Melipona scutellaris* decreases the mechanical inflammatory hypernociception by inhibiting the production of IL-1β and TNF-α

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ABSTRACT

Ethnopharmacological relevance: The pharmacological activity of geopropolis collected by stingless bees (important and threatened pollinators), a product widely used in folk medicine by several communities in Brazil, especially in the Northeast Region, needs to be studied.
Objective: The aim of this study was to evaluate the antinociceptive activity of *Melipona scutellaris* geopropolis (stingless bee) using different models of nociception.
Material and methods: The antinociceptive activity of the ethanolic extract of geopropolis (EEGP) and fractions was evaluated using writhing induced by acetic acid, formalin test, carrageenan-induced hypernociception, and quantification of IL-1β and TNF-α. The chemical composition was assessed by quantification of total flavonoids and phenolic compounds.
Results: EEGP and its hexane and aqueous fractions showed antinociceptive activity. Both EEGP and its aqueous fraction presented activity in the mechanical inflammatory hypernociception induced by the carrageenan model, an effect mediated by the inhibition of IL-1β and TNF-α. The chemical composition of EEGP and its hexane and aqueous fractions showed a significant presence of phenolic compounds and absence of flavonoids.
Conclusion: Our data indicate that geopropolis is a natural source of bioactive substances with promising antinociceptive activity.

1. Introduction

In recent decades, several researches have shown that analgesics represent one of the most studied therapeutic classes in the world. This fact is understandable due to the high consumption of these drugs worldwide, although it may present some adverse effects and low therapeutic efficacy. Thus, the effort to develop new drugs have been the focus in the screenings of extracts from natural sources, which historically have led to the discovery of many clinically important drugs in the current therapy (Verri et al., 2006; Newman et al., 2003; Busnardo et al., 2010).

Propolis is a resin product collected by honey bees from several parts of plants (Silva et al., 2008). For centuries propolis has been used as a popular folk medicine, due to its biological and pharmaceutical properties that include antiviral, anti-inflammatory, analgesic, anticaries, antibacterial, antioxidant and anticancer activities (Kujumgiev et al., 1999; Paulino et al., 2003; Hu et al., 2005; Koo et al., 1999, 2000, 2002; Scazzocchio et al., 2006; Kumazawa et al., 2007; Li et al., 2008). Although a multitude of studies about propolis have been published, most of them are from *Apis mellifera*. In contrast, reports about propolis from other species of bees have been sparsely studied.

The bee species, *Melipona scutellaris*, which belongs to Meliponini tribe (important and threatened pollinators) produces a variety of propolis popularly known as geopropolis. This geopropolis consists

Abbreviations: AF, aqueous fraction; CF, chloroformic fraction; EAF, ethyl acetate fraction; EEGP, ethanolic extract of geopropolis; GAE, gallic acid equivalent; HF, hexanic fraction; IL-1β, interleukin-1 beta; Indo, indomethacin; morph, morphine; PGE_2, prostaglandin E2; TNF-α, tumor necrosis factor-alpha.

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of a mixture of resin, wax and soil, providing distinctive physico-chemical characteristics (Nates-Parra, 2001; Barth, 2006). However, despite its popular use in folk medicine, very little is known about its chemical composition and biological activity.

Among the few reports, Velikova et al. (2000) analyzed 21 samples of Brazilian geopropolis from 12 different species of stingless bees, and observed the presence of compounds such as di- and triterpenes and gallic acid. The same samples showed activity against *Staphylococcus aureus*, and cytotoxic activity. Another study reported that samples of *Melipona fasciculata* geopropolis from Maranhão State showed activity against *Streptococcus mutans* (Liberio et al., 2011). Bankova et al. (2000) identified more than 50 substances, mainly phenolic compounds in Brazilian geopropolis from *Melipona compressipes*, *Melipona quadrifasciata anthidioides* and *Tetragona clavipes*. Previous investigations from our laboratory have found that geopropolis from *Melipona scutellaris* has an antimicrobial action against *Staphylococcus aureus* and antioxidant activity. These findings suggested that *Melipona scutellaris* geopropolis is highly bioactive, deserving further studies to identify other possible biological activities, as well as to elucidate its chemical composition, which would ultimately strengthen its popular use.

Thus, the aim of this study was to evaluate the antinociceptive activity of ethanolic extract of geopropolis (EEGP) of *Melipona scutellaris* and fractions using the chemical models of abdominal constrictions induced by acetic acid and formalin test. Moreover, we evaluated the activity of the EEGP and bioactive fractions in mechanical of inflammatory hypernociception model and the production of IL-1β and TNF-α. Additionally we analyzed the chemical composition of EEGP and bioactive fractions.

2. Material and methods

2.1. Geopropolis samples and fractionation

The geopropolis samples were collected from the inner parts of the beehives, more specifically in the space between the cover and supers of hives. Geopropolis was collected between June and July of 2010 in the seaside region, municipality of 'Entre Rios' (SL 11°56'31'' and WL 38°05'04''), state of Bahia, Northeast of Brazil. The geopropolis (100 g) was extracted with absolute ethanol (w/v) of proportion (1/7), at 70 °C, for 30 min, and then filtered to obtain the EEGP. The EEGP was further fractioned using a liquid–liquid extraction technique with hexane, chloroform, and ethyl acetate solvents. The final residue obtained after ethyl acetate fractionation was totally soluble in water, thus this final fraction was called aqueous fraction. The fractions obtained were monitored by a thin layer chromatography (TLC) using the anisaldehyde reagent (4-methoxy-benzaldehyde, acetic acid, sulfuric acid/1.0:48.5:0.5) and followed by incubation at 100 °C for 5 min. Fluorescent substances were visualized under UV light at the wavelengths of 254 and 366 nm (Tanaka et al., 2005). The EEGP and its hexane, chloroform, ethyl acetate, and aqueous fractions were concentrated in a rotaevaporator at 40 °C to obtain a yield of 4.33% (w/w), 1.98% (w/w), 0.23% (w/w), 0.87% (w/w), and 1.25% (w/w), respectively. The EEGP and fractions were dissolved in DMSO 1% (dissolved in PBS at 1 mM) for intraperitoneal (i.p.) administration.

2.2. Animals

Male SPF (specific-pathogen free) Balb/c mice weighing 20–25 g were housed in temperature (22–25 °C), 12 h light/12 h dark and humidity (40–60%) with access to water and food *ad libitum*. In the present study were used 6 mice ($n=6$) per experimental group. Experiments reported in this study were carried out in accordance with current guidelines for the care of laboratory animals and the ethical guidelines for investigation of experimental pain in conscious animals (Zimmermann, 1983). All efforts were made to minimize the number of animals used and their suffering. The procedures described were reviewed and approved by the local Animal Ethics Committee (CEUA Unicamp process number 2037-1).

2.3. Drugs and reagents

The drugs were purchased from Sigma® Chemical Co., St. Louis, MO, USA (Carrageenan), MP Biomedicals® (Indomethacin), Merck® (Formaldehyde, Acetic acid and organic solvents) and Cristália® (Morphine).

2.4. Biological protocols

2.4.1. Evaluation of EEGP activity and fractions on abdominal constriction responses caused by acetic acid

The abdominal constriction (writhes) were induced by i.p. injection of acetic acid (1.2%) and carried out according to the procedure described previously (Koster et al., 1959; Collier et al., 1968). Mice were treated with EEGP, chloroform, ethyl acetate, aqueous (1, 3, 10 and 30 mg/kg, i.p.) or hexane fractions (0.1, 0.3, 1 and 3 mg/kg, i.p.) 30 min before irritant injection. Indomethacin (10 mg/kg, i.p.) was used as positive control and the vehicle was used as the negative one. After the challenge, the mice were individually placed in a glass cylinder of 22 cm diameter. The total numbers of writhes, which consisted in the constriction of the flank muscles associated with inward movements of the hind limb or with whole body stretching, were counted cumulatively in a period of 20 min. The antinociceptive activity was determined as the difference in number of writhes between control group and each treated group.

2.4.2. Evaluation of EEGP activity and bioactive fractions on formalin induced nociception

The method used in the present study was similar to that described by Corrêa and Calixto (1993). The mice were treated with EEGP, aqueous (1, 3, 10 and 30 mg/kg, i.p.) or hexane fractions (0.1, 0.3, 1 and 3 mg/kg, i.p.) 30 min before injection under the surface of the right hind paw of 25 μL 2.5% formalin (0.92% formaldehyde) in saline. Indomethacin (10 mg/kg, i.p.) and morphine (10 mg/kg, i.p.) were used as the positive control and vehicle was used as the negative one. Animals were observed from 0–5 min (neurogenic phase) and 15–30 min (inflammatory phase) and the time spent licking the injected paw was recorded with a chronometer and considered as indicative of nociception.

2.4.3. Evaluation of EEGP activity and bioactive fractions on carrageenan induced inflammatory hypernociception

Mechanical hypernociception was tested in mice as reported by Cunha et al. (2004). The mice were treated with EEGP, aqueous (1, 3, 10 and 30 mg/kg, i.p.) or hexane fractions (0.1, 0.3, 1 and 3 mg/kg, i.p.) 30 min before injection under the surface of the left hind paw of 25 μL carrageenan (100 μg/paw). Indomethacin (10 mg/kg, i.p.) was used as the positive control and vehicle was used as the negative one. After the challenge, in a quiet room, the mice were placed in acrylic cages ($12 \times 10 \times 17$ cm^3) with wire grid floors (0.5 cm^2), 15–30 min before the start of testing. The test consisted of evoking a hind paw flexion reflex with a hand held force transducer (Insight Scientific Equipments, SP, Brazil) adapted with a 0.5 mm^2 polypropylene tip. The investigator was

trained to apply the tip perpendicularly to the central area of the hind paw with a gradual increase in pressure. The end point was characterized by the removal of the paw followed by clear flinching movements. After the paw withdrawal, the intensity of the pressure was recorded automatically. The value for the response was an averaging of 3 measurements. The animals were tested before and after the treatments. The results are expressed by delta (Δ) withdrawal threshold (in g) calculated by subtracting the zero-time mean measurements (before carrageenan injection) from the mean measurements 3 h after stimulus (after carrageenan injection).

2.4.3.1. Cytokine assays. Based on a previous test (2.4.3) the EEGP and aqueous fraction were selected for the quantification of proinflammatory cytokines. The mice were treated with EEGP or aqueous fraction (30 mg/kg, i.p.) 30 min before injection under the surface of the left hind paw of 25 μL carrageenan (100 μg/paw). Vehicle was used as the negative control. After 3 h, the animals were killed, the plantar skin tissues were removed from the injected and control paws (saline). The samples were homogenized in 500 μl of the appropriate buffer containing protease inhibitors (Sigma®). Levels of TNF-α and IL-1β, were determined by ELISA using protocols supplied by the manufacturers (Peprotech® Inc.) from both the experiments. The results are expressed as picograms.

2.4.4. Evaluation of EEGP activity and bioactive fractions in locomotor activity

The open-field test was used to exclude the possibility that the antinociceptive action of EEGP, hexane and aqueous fractions could be resultant from non-specific disturbances in the locomotor activity of the animals. The ambulatory behavior was assessed in an open-field test as described previously Rodrigues et al. (2002) with few changes. The apparatus consisted of a plastic box measuring $45 \times 45 \times 20\ cm^3$, with the floor divided into 9 equal squares ($15 \times 15\ cm^2$). The number of squares crossed with all paws (crossing) was counted in a 6 min session. The mice were treated with EEGP (1, 3, 10 and 30 mg/kg, i.p.), hexane (1 and 3 mg/kg, i.p.) and aqueous (10 and 30 mg/kg, i.p.) fractions or vehicle for 30 min. The doses established in this test showed effect in the previous tests.

2.5. Chemical composition analysis of EEGP and bioactive fractions

2.5.1. Total polyphenol and flavonoid

Total polyphenol content in EEGP, hexane and aqueous fractions were determined by the Folin–Ciocalteau colorimetric method (Singleton et al., 1999). EEGP or fractions (0.5 ml) were mixed with 2.5 ml of the Folin–Ciocalteau reagent (1:10) and 2.0 ml of 4% Na_2CO_3. Absorbance was measured at 740 nm after a 2 h incubation at room temperature, in the dark. EEGP and its fraction hexane and aqueous were evaluated at the final concentration of 90 μg/ml. Total polyphenol contents were expressed as mg/g (gallic acid equivalents).

Total flavonoid contents in the EEGP, hexane and aqueous fractions were determined using a method described by Park et al. (1995), with minor modifications. For this, 0.5 ml of EEGP, hexane fraction, and aqueous solution, 4.3 ml of 80% ethanol, 0.1 ml of 10% $Al(NO_3)_3$ and 0.1 ml of 1 M potassium acetate was added. After 40 min at room temperature, the absorbance was measured at 415 nm. EEGP, hexane fraction and aqueous were evaluated at the final concentration of 2 mg/ml. Total flavonoid contents were calculated as quercetin (mg/g) from a calibration curve.

2.6. Statistical analysis

Original untransformed data are expressed as the mean ± SEM Statistical comparisons between groups were made using analyses of variance (ANOVA) followed by Tukey test (GraphPad Prism for Windows, Version 5.0). Significance was accepted when the p value was ≤ 0.05.

3. Results

3.1. EEGP, hexane and aqueous fractions decreased the number of abdominal constrictions induced by acetic acid

The test of abdominal constrictions induced by acetic acid was initially used to evaluate the antinociceptive activity of the EEGP and their fractions. The results showed in Fig. 1A demonstrate that the EEGP (3, 10 and 30 mg/kg), produced dose-related inhibition of abdominal constrictions induced by acetic acid in the mice ($p < 0.05$), with inhibitions of 31, 56 and 75% respectively. The hexane fraction was able to inhibit the number of writhes ($p < 0.05$) in 49 and 70% at doses of 1 and 3 mg/kg, respectively (Fig. 1B), and the aqueous fraction inhibited ($p < 0.05$) in 54 and 81% at doses of 10 and 30 mg/kg, respectively (Fig. 1E). On the other hand, the chloroformic fraction (Fig. 1C) and the ethyl acetate fraction (Fig. 1D), did not show any inhibition ($p > 0.05$). Then the hexane and the aqueous fractions were selected like the bioactive fractions from EEGP.

3.2. EEGP, hexane and aqueous fractions decreased nociception induced by formalin

The results in Fig. 2A and B shows that the EEGP caused significant inhibition ($p < 0.05$) of both neurogenic (first phase: 0–5 min) and inflammatory (second phase: 15–30 min) phases of the formalin-induced licking. The calculated inhibition values for these effects were 51 and 50% for the doses of 10 and 30 mg/kg, respectively in the first phase. In the second phase the EEGP inhibited the response in 68, 52, 51 and 56% for the doses of 1, 3, 10 and 30 mg/kg, respectively. The hexane fraction showed activity only in the first phase ($p < 0.05$), with an inhibition of 65% at the dose of 1 mg/kg (Fig. 2C). The aqueous fraction caused significant inhibition of both phases of the formalin-induced licking ($p < 0.05$), with 59% at the dose of 10 mg/kg (phase 1), and 70 and 82% at the doses of 10 and 30 mg/kg (phase 2), respectively (Fig. 2E and F).

3.3. EEGP and aqueous fractions decreased mechanical inflammatory hypernociception by carrageenan

The effects of EEGP on hypernociception induced by carrageenan are shown in Fig. 3A. EEGP at the tested doses of 10 and 30 mg/kg showed an inhibition of 43 and 70%, respectively ($p < 0.05$). The aqueous fraction showed an inhibition ($p < 0.05$) of 66% only at 30 mg/kg (Fig. 3C). On the other hand, the hexane fraction was not effective even at the highest dose tested (Fig. 3B, $p > 0.05$).

3.3.1. Cytokine assay

The administration of EEGP and its aqueous fraction at a dose of 30 mg/kg, significantly reduced ($p < 0.05$) the levels of IL-1β (77 and 89%, respectively) and TNF-α (41 and 48%, respectively), in mice subjected to subplantar injection of carrageenan (Fig. 4A and B).

3.4. EEGP and bioactive fractions did not change the mice's locomotor activity

The administration of EEGP, hexane or aqueous fractions by the i.p. route did not changed the locomotor activity of animals during the 6 min of observation compared to the animals that received vehicle ($p > 0.05$). The means ± SEM crossed squared were 39 ± 4 (control group), 45 ± 8; 23 ± 2; 37 ± 11 and 42 ± 11

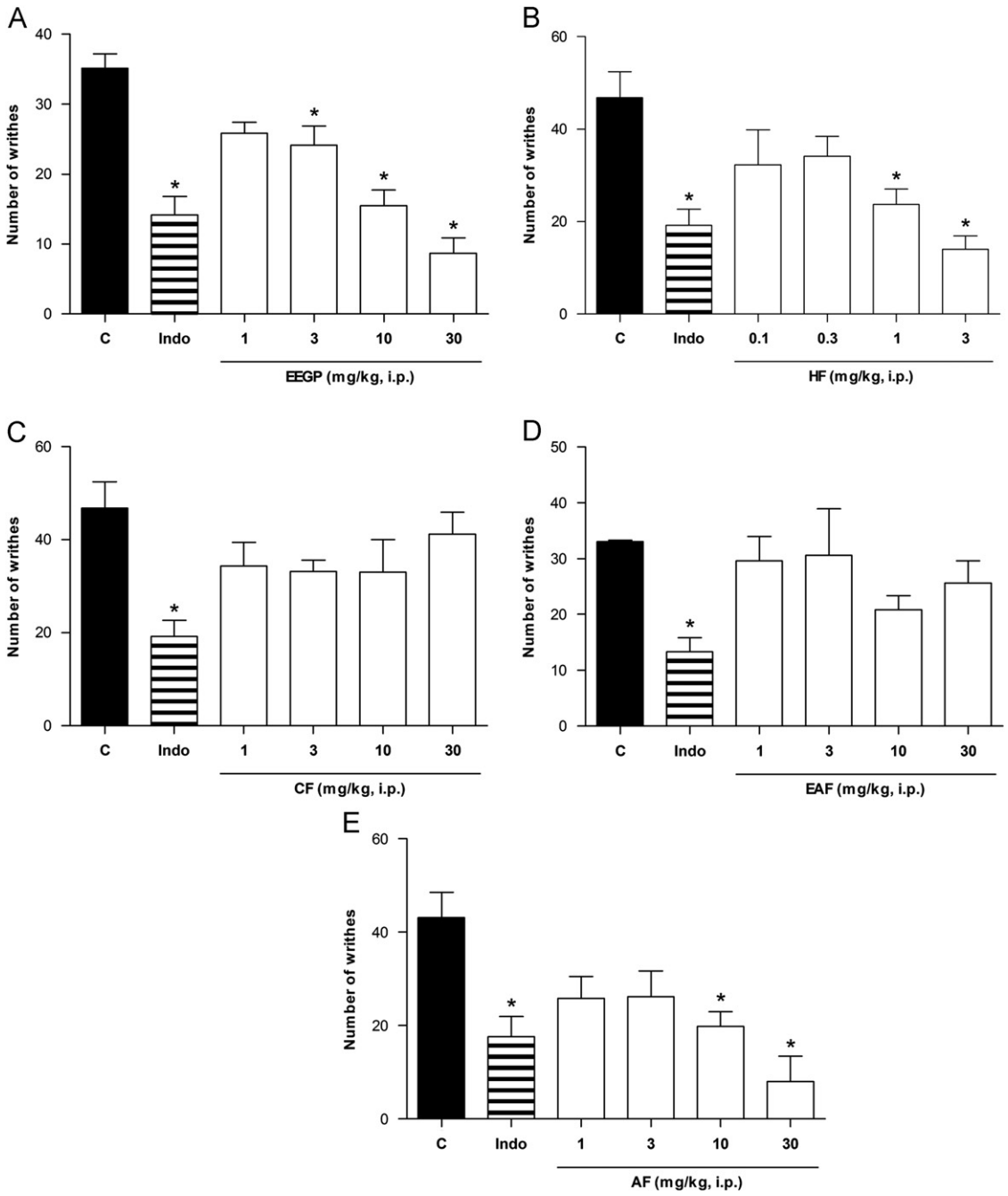

Fig. 1. Effects of i.p. injections of ethanolic extract of geopropolis (EEGP) and fraction on abdominal constriction induced by acetic acid in mice. Control (C) treated with vehicle, indomethacin 10 mg/kg (indo), EEGP (A), fractions: hexane (HF, B), choroformic (CF, C), ethyl acetate (EAF, D) and aqueous (AF, E). Data are expressed as mean ± SEM, $n=6$. Symbols indicate statistical difference ($p < 0.05$, Tukey test). *$p < 0.05$ compared to control group.

for the EEGP (at 1, 3, 10 and 30 mg/kg, respectively), 31 ± 5 and 46 ± 19 for the hexane fraction (at 1 and 3 mg/kg, respectively) and 22 ± 9 and 23 ± 7 for the aqueous fraction (at 10 and 30 mg/kg, respectively).

3.5. Total polyphenol and flavonoid contents of EEGP and bioactive fractions

The mean ± SD of the total phenols was estimated as 127 ± 1.9, 38 ± 0.7 and 138 ± 0.6 mg gallic acid equivalent/g of sample for the EEGP, hexane and aqueous fractions, respectively (Table 1). The presence of flavonoids in any sample evaluated was not observed.

4. Discussion

The aim of this study was to evaluate the antinociceptive activity of ethanolic extract of geopropolis (EEGP) from *Melipona scutellaris* and fractions in different models of nociception, as well as the mechanisms of the related action.

The ability of the EEGP as well as the hexane and aqueous fractions demonstrated in the abdominal constrictions induced by acetic acid in the mice suggests the first sign of its antinociceptive potential. The acetic acid induced constrictions test is described as a typical model of inflammatory pain. It has long been used as a screening tool for the assessment of the analgesic properties of new drugs (Le Bars et al., 2001). The nociceptive response produced in

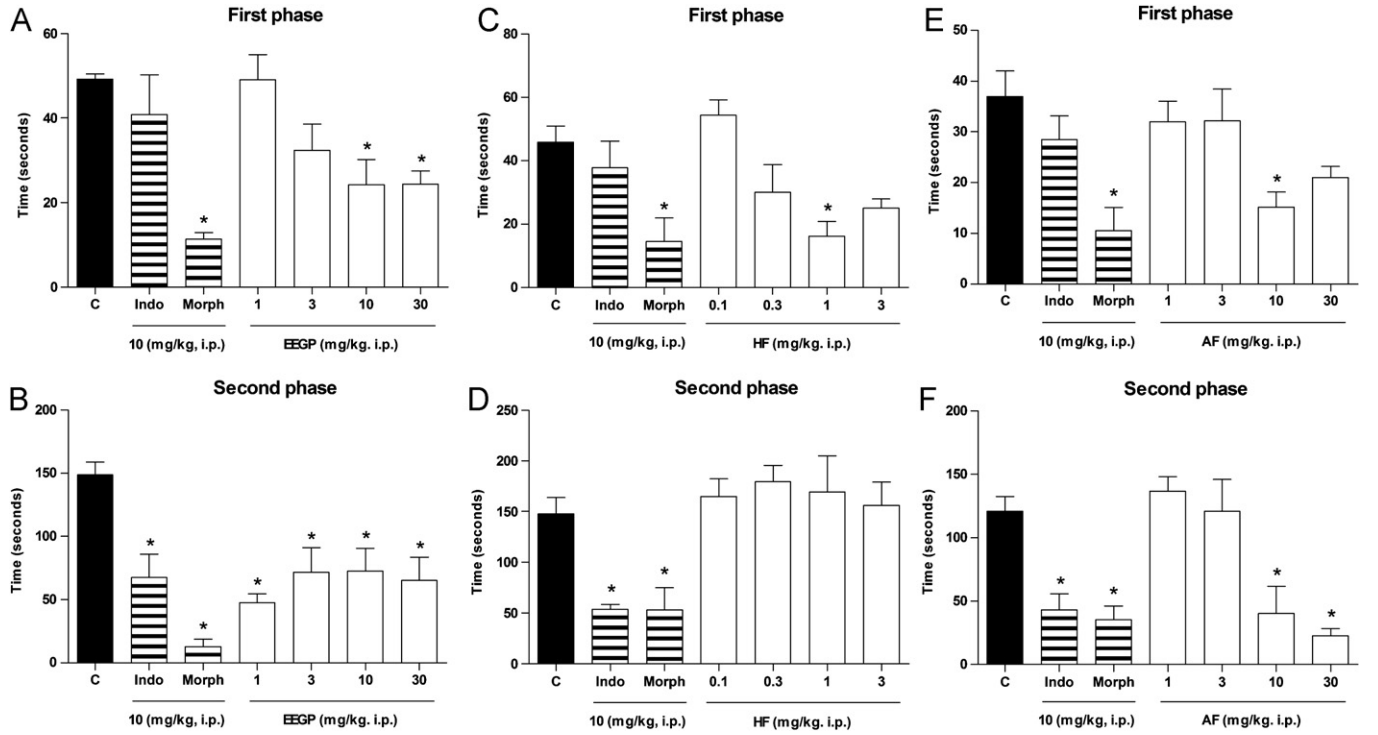

Fig. 2. Effects of i.p. injections of ethanolic extract of geopropolis (EEGP) and its bioactive fractions on the formalin-induced nociception in mice. Control (C) treated with vehicle, indomethacin 10 mg/kg (indo), morphine 10 mg/kg (morph), EEGP (A–B), hexane fraction (HF, C–D), and aqueous fraction (AF, E–F). Data are expressed as mean ± SEM, $n=6$. Symbols indicate statistical difference ($p<0.05$, Tukey test). $^{*}p<0.05$ compared to control group.

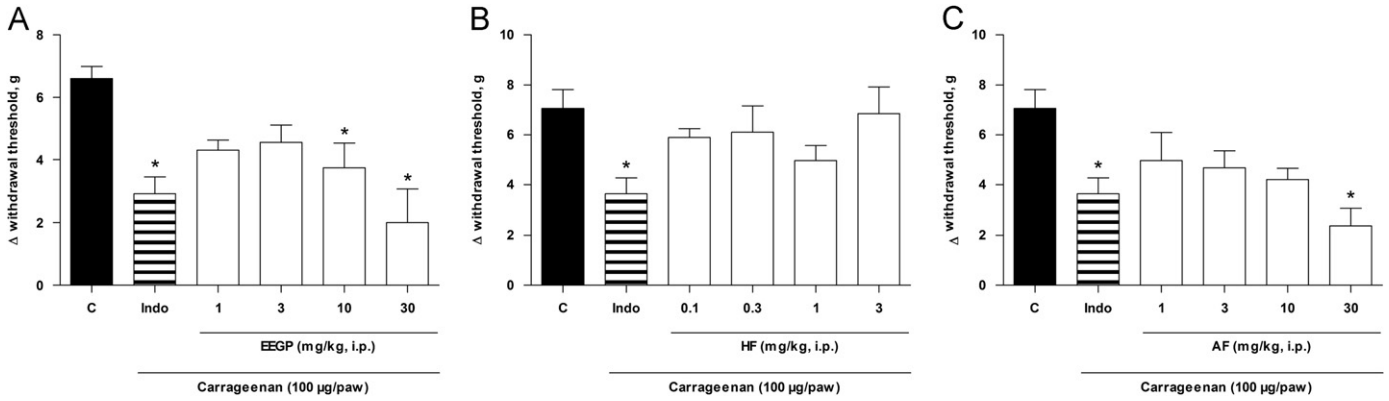

Fig. 3. Effects of i.p. injections of ethanolic extract of geopropolis (EEGP) and bioactive fraction on mechanical inflammatory hypernociception induced by carrageenan in mice. Control (C) treated with vehicle, indomethacin 10 mg/kg (indo), EEGP (A), hexane fraction (HF, B) and aqueous fraction (AF, C). Data are expressed as mean ± SEM, $n=6$. Symbols indicate statistical difference ($p<0.05$, Tukey test). $^{*}p<0.05$ compared to control group.

the constrictions test is due to the participation of several mediators such as prostaglandins, proinflammatory cytokines such as IL-1β, IL-8, TNF-α, sympathomimetic amines, acetylcholine, and substance P, among others (Kusuhara et al., 1997; Ribeiro et al., 2000; Duarte et al., 1988; Le Bars et al., 2001). However, the test of abdominal constrictions has low specificity, since several compounds, such as antihistamines, neuroleptics and adrenergic blockers may also inhibit constrictions (Le Bars et al., 2001). Thus we used the formalin test, a chemical model of nociception, which provides a more specific response compared with the model of abdominal constrictions induced by acetic acid (Tjolsen et al., 1992).

The injection of formalin produces a biphasic behavioral response in which the first phase (0–5 min) is characterized by the occurrence of neurogenic pain by direct stimulation of nociceptive afferent endings and the second phase (15–30 min) is characterized by peripheral inflammation and involves a period of sensitization during which inflammatory phenomena occur (Tjolsen et al., 1992). In this study, the EEGP and its aqueous fraction showed antinociceptive activity in both phases neurogenic and inflammatory, while the hexane fraction showed activity only in the neurogenic phase.

The effect of EEGP and its bioactive fractions was also evaluated in a model of mechanical inflammatory hypernociception induced by carrageenan in mice and the production of IL-1β and TNF-α. Carrageenan is an inflammatory agent that is largely used as pharmacological tool for investigating inflammatory hypernociception in rats and mice. When injected intraplantarly in animal's hind paw, it induces an inflammatory process associated with hypernociception (Cunha et al., 2004). Tissue injury originated after the injection of carrageenan involves the release of

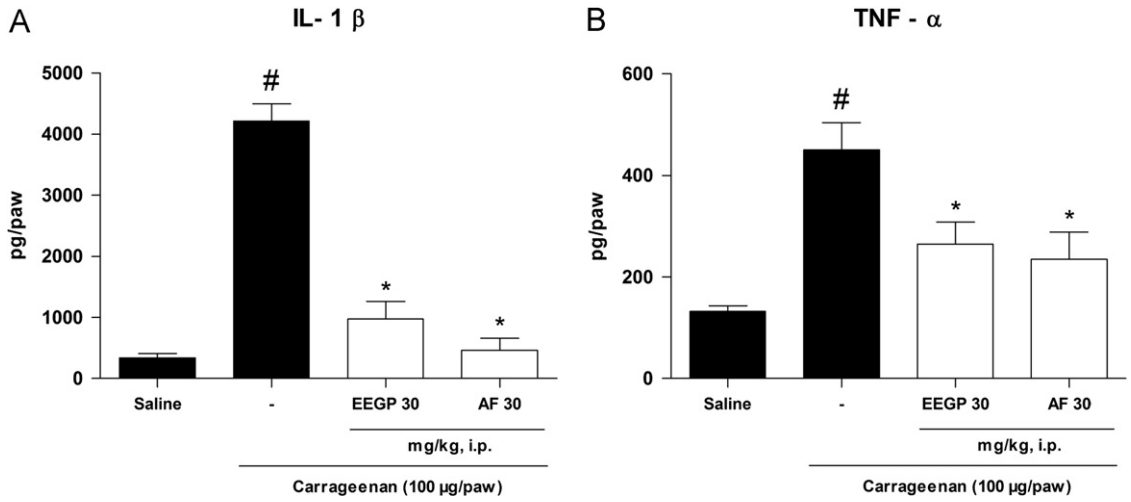

Fig. 4. Quantification of IL-1 β (A) and TNF-α (B) in the hind paw tissue of mice previously treated with vehicle (saline and carrageenan), ethanolic extract of geopropolis (EEGP) and aqueous fraction (AF) at a dose of 30 mg/kg 30 min before the carrageenan intraplantar injection. Data are expressed as mean ± SEM, $n=6$. Symbols indicate statistical difference ($p<0.05$, Tukey test). #$p<0.05$ compared to saline group; *$p<0.05$ compared to carrageenan group.

Table 1
Quantification of total phenols in EEGP, hexanic and aqueous fractions by the Folin–Ciocalteau method.

Sample	mgGAE/g sample
EEGP	127 ± 1.9
Hexanic fraction	38 ± 0.7
Aqueous fraction	138 ± 0.6

Mean ± SD, $n=03$.
Total phenolic compounds expressed in mg gallic acid equivalent (GAE)/g of sample.

different chemical mediators such as PGE_2, mast cells products histamine and serotonin, neuropeptides, and proinflamatory cytokines among others (Cunha et al., 1992; Ferreira et al., 1993). Cytokines like IL-1β and TNF-α play a crucial role in the release of prostanoids. This process occurs in the following sequence: TNF-α → IL-1β → prostanoids which sensitize nociceptors (Cunha et al., 2005). The release of proinflammatory cytokines IL-1β and TNF-α is also directly related to the migration of neutrophils in the inflammatory process, with induced rolling and adhesion of neutrophils in the vascular endothelium, and transmigration into the inflammatory site (Hogg and Walker, 1995). When neutrophils are present in inflammatory focus, they release hypernociceptive mediators such as prostaglandins (Cunha et al., 2008). In the present study, the administration of EEGP and aqueous fraction reduced the inflammatory hypernociception which could be related with the inhibition of IL-1β and TNF-α cytokines and consequent inhibition of the release of prostanoids and also the interaction neutrophils and endothelial cells. In the present study, we observed that the hexane fraction did not decrease the mechanical inflammatory hypernociception, suggesting, therefore, another course of action independent of the inflammatory process. The administration of EEGP and its fractions did not cause any significant change in the mice ambulation during the open field test, excluding non-specific disturbance in the locomotor activity.

This study was also carried out by a phytochemical analysis of EEGP and its bioactive fractions, by quantification of total phenolics and flavonoids. Regarding the bioactive fractions, the presence of phenolic compounds in both fractions (hexane and aqueous) suggests the presence of these compounds with different polarities. A higher concentration of phenolics was observed in the aqueous fraction compared with the hexane fraction. The antinociceptive activity recorded for both fractions suggests that the effect detected in the aqueous fraction may be related to this class of compounds due to the higher concentration observed. However, the fact that the phenolic content of the hexane fraction was lower than that observed in the aqueous fraction does not exclude the hypothesis that phenolic compounds may be responsible for this activity and should be chemically investigated. In addition, the absence of flavonoids in the EEGP and its bioactive fractions, which is a very common chemical class in *Apis mellifera* propolis (Kumazawa et al., 2004; Alencar et al., 2007) was observed. The absence of flavonoids found in geopropolis was also been reported in Brazilian propolis type 6 from *Apis mellifera* (Duarte et al., 2003). This particular *Apis mellifera* propolis was also collected from the Atlantic forest in the state of Bahia (Northeastern Brazil), which is not far away (about a 70 km radius) from where the geopropolis was collected for this study. Since the geographical location and vegetation are related with propolis chemical composition (Castro et al., 2007) it appears reasonable to suggest that the vegetal resin originating the propolis type 6 could have some similarity to that of the geopropolis. Additionally, propolis type 6 has a group of polyprenylated benzophenones whose biological activity was assigned to the substance hiperibone A, a novel naturally occurring biomolecule (Castro et al., 2009). These observations suggests that the geopropolis from *Melipona scutellaris* may have an unusual chemical composition devoid of significant amounts of flavonoids, instigating a better characterization aiming the isolation/identification of the bioactive compounds.

Therefore we conclude that the EEGP and its hexane and aqueous fractions showed antinociceptive activity. The EEGP and aqueous fraction demonstrated activity in the mechanical inflammatory hypernociception induced by the carrageenan model, and this effect is mediated by inhibition of IL-1β and TNF-α. The chemical composition of EEGP and its hexane and aqueous fractions showed a significant presence of phenolic compounds and absence of flavonoids. This study could provide a scientific basis to its popular use in the Brazilian folk medicine and suggests that more studies should be conducted for the identification and isolation of bioactive compounds.

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References

Alencar, S.M., Oldoni, T.L.C., Castro, M.L., Cabral, I.S.R., Costa-Neto, C.M., Cury, J.A., Rosalen, P.L., Ikegaki, M., 2007. Chemical composition and biological activity of a new type of Brazilian propolis: Red propolis. Journal of Ethnopharmacology 13, 278–283.

Bankova, V., De Castro, S.L., Marcucci, M.C., 2000. Propolis: recent advances in the chemistry and plant origin. Apidologie 31, 3–15.

Barth, O.M., 2006. Palynological analysis of geopropolis samples obtained from six species of Meliponinae in the Campus of the Universidade de Ribeirão Preto, USP, Brazil. Apiacta 41, 71–85.

Busnardo, T.C.P.M., Padoani, C., Mora, T.C., Biabatti, M.W., Fröde, T.S., Bürger, C., Claudino, V.D., Dalmarco, E.M., Souza, M.M., 2010. Anti-inflammatory evaluation of *Coronopus didymus* in the pleurisy and paw oedema models in mice. Journal of Ethnopharmacology 128, 519–525.

Castro, M.L., Cury, J.A., Rosalen, P.L., Alencar, S.M., Ikegaki, M., Duarte, S., Koo, H., 2007. Própolis do sudeste e nordeste do Brasil: influência da sazonalidade na atividade antibacteriana e composição fenólica. Química Nova 30, 1512–1516.

Castro, M.L., Nascimento, A.M., Ikegaki, M., Costa-Neto, C.M., Alencar, S.M., Rosalen, P.L., 2009. Identification of a bioactive compound isolated from Brazilian propolis type 6. Bioorganic & Medicinal Chemistry 17, 5332–5335.

Collier, H.O.J., Dinneen, J.C., Johnson, C.A., Schneider, C., 1968. The abdominal constriction response and its suppression by analgesic drugs in the mouse. British Journal of Pharmacology 32, 295–310.

Corrêa, C.R., Calixto, J.B., 1993. Evidence for participation of B1 and B2 kinin receptors in formalin-induced nociceptive response in the mouse. British Journal of Pharmacology 110, 193–198.

Cunha, F.Q., Poole, S., Lorenzetti, B.B., Ferreira, S.H., 1992. The pivotal role of tumour necrosis factor alpha in the development of inflammatory hyperalgesia. British Journal of Pharmacology 107, 660–664.

Cunha, T.M., Verri Jr., W.A., Vivancos, G.G., Moreira, I.F., Reis, S., Parada, C.A., Cunha, F.Q., Ferreira, S.H., 2004. An electronic pressure-meter nociception paw test for mice. Brazilian Journal of Medical and Biological Research 37, 401–407.

Cunha, T.M., Verri Jr., W.A., Silva, J.S., Poole, S., Cunha, F.Q., Ferreira, S.H., 2005. A cascade of cytokines mediates mechanical inflammatory hypernociception in mice. Proceedings of the National Academy of Sciences of the United States of America 102, 1755–1760.

Cunha, T.M., Verri Jr., W.A., Schivo Jr., I.R., Napimoga, M.H., Parada, C.A., Poole, S., Teixeira, M.M., Ferreira, S.H., Cunha, F.Q., 2008. Crucial role of neutrophils in the development of mechanical inflammatory hypernociception. Journal of Leukocyte Biology 83, 824–832.

Duarte, I.D., Nakamura, M., Ferreira, S.H., 1988. Participation of the sympathetic system in acetic acid-induced writhing in mice. Brazilian Journal of Medical and Biological Research 21, 341–433.

Duarte, S., Koo, H., Bowen, W.H., Hayacibara, M.F., Cury, J.A., Ikegaki, M., Rosalen, P.L., 2003. Effect of a novel type of propolis and its chemical fractions on glucosyltransferases and on growth and adherence of mutans *Streptococci*. Biological & Pharmaceutical Bulletin 26, 527–531.

Ferreira, S.H., Lorenzetti, B.B., Poole, S., 1993. Bradykinin initiates cytokine-mediated inflammatory hyperalgesia. British Journal of Pharmacology 110, 1227–1231.

Hogg, J.C., Walker, B.A., 1995. Polymorphonuclear leucocyte traffic in lung inflammation. Thorax 50, 819–820.

Hu, F., Hepburn, H.R., Li, Y., Chen, M., Radloff, S.E., Daya, S., 2005. Effects of ethanol and water extracts of propolis (bee glue) on acute inflammatory animal models. Journal of Ethnopharmacology 100, 276–283.

Koo, H., Rosalen, P.L., Cury, J.A., Park, Y.K., Ikegaki, M., Sattler, A., 1999. Effect of *Apis mellifera* propolis from two Brazilian regions on caries development in desalivated rats. Caries Research 33, 393–400.

Koo, H., Gomes, B.P., Rosalen, P.L., Ambrosano, G.M., Park, Y.K., Cury, J.A., 2000. In vitro antimicrobial activity of propolis and *Arnica montana* against oral pathogens. Archives of Oral Biology 45, 141–148.

Koo, H., Rosalen, P.L., Cury, J.A., Park, Y.K., Bowen, W.H., 2002. Effects of compounds found in propolis on *Streptococcus mutans* growth and on glucosyltransferase activity. Antimicrobial Agents Chemotherapy 46, 1302–1309.

Koster, R., Anderson, M., Beer, E.J., 1959. Acetic acid for analgesic screening. in: Federation Proceedings, vol. 18, pp. 412–416.

Kujumgiev, A., Tsvetkova, I., Serkedjieva, Y., Bankova, V., Christov, R., Popov, S., 1999. Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. Journal of Ethnopharmacology 64, 235–240.

Kumazawa, S., Hamasaka, T., Nakayama, T., 2004. Antioxidant activity of propolis of various geographic origins. Food Chemistry 84, 329–339.

Kumazawa, S., Ueda, R., Hamasaka, T., Fukumoto, S., Fujimoto, T., Nakayama, T.M., 2007. Antioxidant prenylated flavonoids from propolis collected in Okinawa, Japan. Journal of Agricultural and Food Chemistry 55, 7722–7725.

Kusuhara, H., Fukunari, A., Matsuyuki, H., Okumoto, T., 1997. Principal involvement of cyclooxygenase-1-derived prostaglandins in the c-fos expression of the rat hind brain following visceral stimulation with acetic acid. Molecular Brain Research 52, 151–156.

Le Bars, D., Gozariu, M., Cadden, S.W., 2001. Animal models of nociception. Pharmacologial Reviews 54, 597–652.

Li, F., Awale, S., Tezuka, Y., Kadota, S., 2008. Cytotoxic constituents from Brazilian red propolis and their structure–activity relationship. Bioorganic & Medicinal Chemistry 16, 181–189.

Liberio, S.A., Pereira, A.L., Dutra, R.P., Reis, A.S., Araujo, M.J., Mattar, N.S., Silva, L.A., Ribeiro, M.N., Nascimento, F.R., Guerra, R.N., Monteiro-Neto, V., 2011. Antimicrobial activity against oral pathogens and immunomodulatory effects and toxicity of geopropolis produced by the stingless bee *Melipona fasciculata* Smith. BMC Complementary and Alternative Medicine 4, 108.

Nates-Parra, G., 2001. Las Abejas sin aguijón (Hymenoptera: Apidae: Meliponini) de Colômbia. Biota Colombiana 2, 233–248.

Newman, D.J., Cragg, G.M., Snader, K.M., 2003. Natural products as sources of new drugs over the period 1981–2002. Journal of Natural Product 66, 1022–1037.

Park, Y.K., Koo, M.H., Sato, H.H., Contado, J.L., 1995. Survey of some components of propolis which were collected by *Apis mellifera* in Brazil. Arquivos de Biologia e Tecnologia 38, 1253–1259.

Paulino, N., Dantas, A.P., Bankova, V., Longhi, D.T., Scremin, A., Castro, S.L., Calixto, J.B., 2003. Bulgarian propolis induces analgesic and anti-inflammatory effects in mice and inhibits in vitro contraction of airway smooth muscle. Journal of Pharmacological Sciences 93, 307–313.

Ribeiro, R.A., Vale, M.L., Thomazzi, S.M., Paschoalato, A.B., Poole, S., Ferreira, S.H., Cunha, F.Q., 2000. Involvement of resident macrophages and mast cells in the writhing nociceptive response induced by zymosan and acetic acid in mice. European Journal of Pharmacology 387, 111–118.

Rodrigues, A.L.S., Da Silva, G.L., Mateussi, A.S., Fernandes, E.S., Miguel, O.G., Yunes, R.A., Calixto, J.B., Santos, A.R.S., 2002. Involvement of monoaminergic system in the antidepressant-like effect of the hydroalcoholic extract of *Siphocampylus verticillatus*. Life Sciences 70, 1347–1358.

Scazzocchio, F., D'auria, F.D., Alessandrini, D., Pantanella, F., 2006. Multifactorial aspects of antimicrobial activity of propolis. Microbiological Research 161, 327–333.

Silva, B.B., Rosalen, P.L., Cury, J.A., Ikegaki, M., Souza, V.C., Esteves, A., Alencar, S.M., 2008. Chemical composition and botanical origin of red propolis, a new type of brazilian propolis. Evidence-Based Complementary and Alternative Medicine 5, 313–316.

Singleton, V.L., Orthofer, R., Lamuela-Raventos, R.M., 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteau reagent. Methods of Enzymology 299, 152–178.

Tanaka, J.C.A., Silva, C.C., Dias Filho, B.P., Nakamura, C.V., Carvalho, J.E., Foglio, M.A., 2005. Chemical constituents of *Luehea divaricata* Mart (Tiliaceae). Quimica Nova 28, 834–837.

Tjolsen, A., Berge, O.G., Hunskaar, S., Rosland, J.H., Hole, K., 1992. The formalin test: an evaluation of the method. Pain 51, 5–17.

Velikova, M., Bankova, V., Marcucci, M.C., Tsvetkova, I., Kujumgiev, A.Z., 2000. Chemical composition and biological activity of propolis from Brazilian Meliponinae. Zeitschrift für Naturforsch Naturforsch. C. Journal of Biosciences 55, 785–789.

Verri Jr., W.A., Cunha, T.M., Parada, C.A., Poole, S., Cunha, F.Q., Ferreira, S.H., 2006. Hypernociceptive role of cytokines and chemokines: targets for analgesic drug development? Pharmacology & Therapeutics 112, 116–138.

Zimmermann, M., 1983. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 6, 109–110.