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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