UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

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AVALIAÇÃO DO POTENCIAL ANTI-INFLAMATÓRIO E ANTINOCICEPTIVO DA GEOPRÓPOLIS DE *MELIPONA SCUTELLARIS*

Dissertação de mestrado apresentada a Faculdade de Odontologia de Piracicaba da UNICAMP para obtenção do título de Mestre em Odontologia, na Área de Farmacologia, Anestesiologia e Terapêutica

Orientador: Prof. Dr. Pedro Luiz Rosalen

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"Todo conhecimento inicia-se na imaginação, no sonho; só depois desce à realidade material e terrena por meio da lógica"

Albert Einstein

RESUMO

O objetivo deste estudo foi avaliar a atividade anti-inflamatória e antinociceptiva do extrato etanólico de geoprópolis (EEGP) de *Melipona scutellaris* e frações químicas, bem como possíveis mecanismos de ação. Além disso, caracterizar quimicamente o EEGP e frações bioativas. Para avaliação da atividade anti-inflamatória foram aplicados os ensaios de recrutamento de neutrófilos na cavidade peritoneal, microscopia intravital e edema de pata induzido por carragenina. A via do óxido nítrico (NO) foi avaliada através da administração de inibidores desta via, além da avaliação da expressão das moléculas de adesão intercelular tipo 1 (ICAM-1) e quantificação de nitritos. A análise química do EEGP e fração bioativa foram realizadas pelos métodos de cromatografia gasosa com espectrometria de massas (CG/EM) e cromatografia líquida de alta eficiência em fase reversa (CLAE-FR). Para avaliação da atividade antinociceptiva, foram aplicados os ensaios de contorções abdominais induzida por ácido acético, teste da formalina, hipernocicepção inflamatória mecânica induzida por carragenina e quantificação das citocinas IL-1 β e TNF-α. A composição química do EEGP e frações bioativas foram avaliadas pela quantificação de fenóis e flavonóides totais. Conforme os resultados nos ensaios anti-inflamatórios, foi verificado que o EEGP e sua fração aquosa diminuíram a migração de neutrófilos na cavidade peritoneal induzido por carragenina, como também diminuíram a interação dos leucócitos (rolamento e adesão) com as células endoteliais. A administração de inibidores da via do NO suprimiu a atividade inibitória do EEGP e da fração aquosa sobre a migração de neutrófilos. A expressão de ICAM-1 apresentou-se diminuída, e os níveis de nitritos aumentados após o tratamento com EEGP e fração aquosa. No modelo de edema de pata induzido por carragenina, o EEGP e a fração aquosa apresentaram atividade anti-edematogênica. Nenhum padrão de ácido fenólico ou flavonóide, comumente encontrado em amostra de própolis de *Apis mellifera,* pôde ser detectado nas amostras de EEGP e da fração aquosa pelas técnicas de CG/EM e CLAE-FR. Nos ensaios de atividade antinociceptiva foi verificado que o EEGP e as frações hexânica e aquosa diminuíram o número de contorções abdominais induzida por ácido acético. No

teste da formalina, o EEGP e a fração aquosa inibiram ambas as fases (neurogênica e inflamatória), e a fração hexânica apenas a fase neurogênica. Foi evidenciada atividade do EEGP e fração aquosa na hipernocicepção inflamatória mecânica induzida por carragenina, como também foi constatado níveis diminuídos de IL-1β e TNF-α. Para as amostras de EEGP e frações hexânica e aquosa foi verificada a presença de compostos fenólicos e ausência de flavonóides. Estes resultados indicam que as frações bioativas encontradas possuem substâncias promissoras como novos agentes terapêuticos para o controle da inflamação e dor.

Palavras chaves: Geoprópolis, *Melipona scutellaris*, anti-inflamatóro, antinociceptivo, óxido nítrico, citocinas.

ABSTRACT

The aim of this study was to evaluate the anti-inflammatory and antinociceptive geopropolis of ethanolic extract (EEGP) of *Melipona scutellaris* and fractions, as well as a possible mechanism of actions. In addition, to characterize chemically EEGP and bioactive fractions. To evaluate the anti-inflammatory activity we used the recruitment of neutrophils into the peritoneal cavity, intravital microscopy and paw edema induced by carrageenan. The pathway of nitric oxide (NO) was demonstrated by administration of antagonists of this pathway, but also evaluated by the expression of intercellular adhesion molecule type 1 (ICAM-1) and quantification of nitrites. Reversed-phase high-performance liquid chromatography (RP-HPLC) and gas chromatography/mass spectrometry (GC/MS) were used the phytochemical analyses. The tests used to evaluate the antinociceptive activity were abdominal contortions induced by acetic acid, formalin test, carrageenan-induced mechanical inflammatory hypernociception and quantification of IL-1 β and TNF-α. The quantification of total phenols and flavonoids was also determined in the EEGP and the bioactive fractions. In the anti-inflammatory tests the EEGP and the aqueous fraction decreased the neutrophil migration into the peritoneal cavity induced by carrageenan and also decreased the interaction of leukocytes (rolling and adhesion) with endothelial cells. The route of administration of inhibitors of NO abolished the inhibitory activity of the aqueous fraction and EEGP on neutrophil migration. The expression of ICAM-1 was reduced and nitrite levels increased after treatment with EEGP and aqueous fraction. In the paw edema induced by carrageenan, both EEGP and aqueous fraction showed anti-edema activity. No standards of flavonoid or phenolic acid commonly found in a sample of propolis of *Apis mellifera* could be detected in any samples of aqueous fraction or EEGP by GC/MS and RP-HPLC techniques. In tests to evaluate the antinociceptive activity was determined that EEGP, hexane and aqueous fractions decreased the number of abdominal constrictions induced by acetic acid. In the formalin test, the aqueous fraction and EEGP inhibited both phases (neurogenic and inflammatory), and the hexane fraction only neurogenic phase. The EEGP and the aqueous fraction showed activity in the carrageenan-induced mechanical inflammatory hypernociception model, as also observed decreased

levels of IL-1β and TNF-α. The EEGP, hexane and aqueous fractions was verified the presence of phenolic compounds and absence of flavonoids. These results indicate that the bioactive fractions in the present study showed substances that could be promising new therapeutic agents to control inflammation and pain.

Keywords: Geopropolis, *Melipona scutellaris*, anti-inflammatory, antinociceptive, nitric oxide, cytokines.

SUMÁRIO

INTRODUÇÃO

A resposta inflamatória é um conjunto de eventos que envolvem a participação de diferentes mediadores químicos capazes de promover vasodilatação, edema e a migração de leucócitos. Dentre estas alterações, podemos destacar a migração de leucócitos para o foco inflamatório como uma estratégia fundamental na defesa do organismo contra infecções (Malech & Gallin, 1987).

Os neutrófilos são os principais leucócitos que participam na defesa do organismo durante o processo inflamatório (Mallech & Gallin, 1987). A migração destes leucócitos durante o processo inflamatório é decorrente da liberação de mediadores quimiotáxicos, como os mediadores lipídicos, citocinas e quimiocinas, que promovem o aumento da adesão dos neutrófilos com as células endoteliais (Mallech & Gallin, 1987; Dal secco et al., 2006). Este processo ocorre inicialmente, através do rolamento dos neutrófilos sobre o endotélio vênular, sendo este evento mediado por uma família de moléculas de adesão denominadas selectinas (L-, P- e E-selectinas). A seguir, ocorre uma forte adesão entre os neutrófilos e as células endoteliais, através das integrinas (β_2-integrinas), que interagem com as imunoglubulinas, tais como as moléculas de adesão intercelular tipo 1 (ICAM-1). Esta aderência permite, portanto, que os neutrófilos transmigrem para o foco inflamatório através das junções intercelulares (diapedese) presentes nas células endoteliais (Burke-Gaffney & Hellewell, 1996; Smith, 1993; Zhang et al., 2001; Pánes et al., 1999; Dinarello, 2000).

No entanto, apesar de o recrutamento de neutrófilos ser uma resposta protetora do organismo, a ocorrência de uma resposta exacerbada gera efeitos indesejáveis o que pode levar a um progressivo dano tecidual no local inflamado, podendo este fenômeno ocorrer em diferentes doenças inflamatórias. Portanto, o bloqueio do tráfego de neutrófilos durante o processo inflamatório, vem sendo um alvo promissor na descoberta de novos fármacos anti-inflamatórios (Malech & Gallin, 1987).

Aliado ao processo inflamatório, na maioria das situações ocorre o aparecimento de dor. Descrita como dor inflamatória, este evento é decorrente da liberação de mediadores inflamatórios, que provocam ativação e/ou sensibilização dos nociceptores.

No caso da sensibilização, ocorre uma diminuição do limiar de ativação do nociceptor, fazendo com o que o mesmo, seja ativado por estímulos que em condições normais seriam inócuos (Millan, 1999; Verri jr et al., 2006).

Atualmente, as prostaglandinas são conhecidas como um dos principais mediadores responsáveis pela indução da dor inflamatória. Tal fato é compreensível, devido a ação analgésica dos anti-inflamatórios não esteróides (AINEs), que inibem a síntese de prostaglandinas, consequentemente diminuindo a dor inflamatória. No entanto, devido aos muitos efeitos adversos, como por exemplo, problemas renais e gástricos atribuídos ao uso prolongado dos AINEs, o controle da dor inflamatória ainda é um desafio (Verri jr et al., 2006).

Dentre os recentes progressos dos estudos de dor inflamatória, destaca-se o papel das citocinas. Estudos têm mostrado que a liberação dos mediadores hipernociceptivos (como as prostaglandinas) durante o processo inflamatório, é decorrente de uma cascata de eventos iniciais, desencadeada por diferentes citocinas pró e anti-inflamatórias. Devido a este achado, estas moléculas se tornaram alvos na descoberta de novos fármacos analgésicos (Verri jr et al., 2006; Cunha et al., 2005).

Os produtos naturais, historicamente, levaram à descoberta de muitas drogas clinicamente úteis na terapêutica atual. De 1981 a 2002, 48 % dos novos fármacos descobertos, são de produtos naturais ou derivados dos mesmos (Newman et al., 2003). Estes dados mostram que os produtos naturais são uma fonte valiosa na descoberta de novos padrões moleculares bioativos, e os desafios dos pesquisadores, portanto, são identificar novas moléculas, elucidar o seu mecanismo de ação e propor o seu uso terapêutico (Barreiro; Viegas; Bolzani, 2006).

A própolis um produto resinoso não tóxico coletado por abelhas tem sido relatada na literatura como possuidora de diversas atividades biológicas, incluindo anti-inflamatória e antinociceptivo (Sforcin & Bankova, 2011; Paulino et al., 2003). Além disso, tem se demonstrado como uma fonte de recurso natural para a descoberta de novos compostos bioativos, como, por exemplo, o artepelim C (Ahn et al., 2007), apigenina e *tt*-farnesol (Koo et al., 2003), CAPE (Sforcin & Bankova, 2011), dentre outros.

Por ser um produto obtido a partir de diferentes fontes vegetais, os constituintes químicos e atividades farmacológicas da própolis variam de acordo com a época do ano, tipo de vegetação existente em cada região onde é produzida e a espécie de abelha responsável pela coleta (König, 1985; Greenaway et al., 1990; Park et al., 2002). Devido a estas características, cada vez mais estudos com diferentes variedades de própolis estão sendo realizados com o objetivo de isolamento e identificação de compostos bioativos que possam ser aplicados como novos agentes terapêuticos (Sforcin & Bankova, 2011). No entanto, a maioria dos estudos científicos se refere à própolis coletadas por abelhas *Apis mellifera* (Sforcin & Bankova, 2011), enquanto que outros tipos de própolis, coletadas por outras abelhas permanecem ainda sem descrição detalhada sobre as suas atividades biológica e características químicas.

A geoprópolis uma mistura de resina, cera e terra, é uma própolis não comum, sendo coletada por abelhas nativas sem ferrão da tribo Meliponini, amplamente encontradas nas áreas tropicais e subtropicais do mundo todo (Nates-Parra, 2001; Barth, 2006; Roubem, 1989; Cortopassi-Laurino et al., 2006).

Dentre os poucos estudos com este tipo de própolis, Velikova et al. (2000) analisaram 21 amostras de geoprópolis de doze diferentes espécies de Meliponinae, e evidenciaram significativa atividade contra *Staphylococcus aureus*, além de atividade citotóxica. As mesmas amostras foram caracterizadas quimicamente, onde foi observada a presença de compostos como di e triterpenos e ácido gálico. Em outro estudo, Bankova et al. (1998) identificaram mais de cinquenta compostos, principalmente fenólicos e terpênicos em geoprópolis brasileira, produzida pelas abelhas *Melipona compressipes, Melipona quadrifasciata anthidioides* e *Tetragona clavipes*.

A geoprópolis proveniente da espécie de abelha *Melipona scutellaris,* também conhecida popularmente como “uruçu” e encontrada no Nordeste do Brasil, tem sido alvo de interesse pelo nosso grupo de pesquisa. Estudos prévios constataram atividades biológicas, como antimicrobiana contra *Staphylococcus aureus* e antioxidante. Estes resultados sugerem, portanto, que mais estudos sejam realizados para identificação de outras atividades biológicas para este tipo de própolis (anti-inflamatória e antinociceptiva), como também a identificação de substâncias químicas novas que apresentem potencial

farmacológico promissor, propiciando a geoprópolis de *Melipona scutellaris* valorização cientifica, podendo tornar-se uma fonte de subsistência para comunidades carentes que dependem de sua produção.

CAPÍTULO 1

Bioactive fraction of geopropolis decreases neutrophils migration in inflammatory process: involvement of nitric oxide pathway

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Abstract

The extensive neutrophil migration and tissue damage that accompanies it, are present in several inflammatory diseases. Therefore, the search for drugs that interfere with the traffic of neutrophils in the inflammatory process has been increasing. The aim of this study was to evaluate the activity of the ethanolic extract of geopropolis (EEGP) of *Melipona scutellaris* and its fractions on the modulation of neutrophil migration in the inflammatory process, as well as the participation of nitric oxide (NO) pathway, and to check the chemical profile of EEGP and the bioactive fraction. EEGP and its aqueous fraction decreased the neutrophil migration in the peritoneal cavity induced by carrageenan. In addition, leukocyte interaction (rolling and adhesion) with the endothelial cells decreased, which was evidenced by intravital miscroscopy. It was found that the injection of antagonists of NO pathway abolished the EEGP and the aqueous fraction inhibitory activity on the neutrophil migration. The expression of intracellular adhesion molecule type 1 (ICAM-1) was reduced, and nitrite levels increased after treatment with EEGP and aqueous fraction. In the model of paw edema induced by carrageenan, EEGP and the aqueous fraction showed anti-edema activity. No pattern of flavonoid and phenolic acid commonly found in propolis samples of *Apis mellifera* could be detected in EEGP and aqueous fraction samples. According to the results, we conclude that the EEGP and the aqueous fraction showed inhibitory activity on the neutrophils influx in the inflammatory process and this effect could be related with the NO pathway. These data indicate that the aqueous fraction found has promising bioactive substances for anti-inflammatory activity.

Keywords: Geopropolis; *Melipona scutellaris*; bioactive fraction; neutrophils; anti-inflamatory; nitric oxide.

Abbreviations: AG, aminoguanidine; cGMP, guanosine 3′5′-cyclic monophosphate; DMSO, dimethyl sulphoxide; EEGP, ethanolic extract of geopropolis; GC-MS, gas chromatography/mass spectrometry; HPLC-FR, high performance liquid chromatography reverse phase; ICAM-1 – intracellular adhesion molecule type 1; iNOS, inducible nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthases; sGC, soluble guanylate cyclase; ODQ, [1H-(1,2,4)oxadiazolo (4,3-a) quinoxalin-1-one].

1. Introduction

Neutrophils are the main leukocytes that participate in the defence of the body during the inflammatory process [1]. The process of neutrophil rolling and adhesion in endothelial cells, followed by its transmigration to the extravascular space, occurs due to the release of various chemical mediators such as cytokines, chemokines, and eicosanoids, which activate selectins, integrins, and immunoglobulin, respectively [2-6]. Although it has a protective effect, the intense neutrophil migration is related to tissue damage in several inflammatory diseases [1].

Many new drugs aim to interfere with the traffic of neutrophils in the inflammatory process, antagonizing cytokines, chemokines, integrins, selectins or immunoglobulin involved in this process, or even stimulating chemical mediators that inhibit the neutrophil migration [7-11].

Natural products have been researched for decades as a promising source in the discovery of new drugs, and *Apis mellifera* bee propolis has been reported in the literature as possessing various biological activities [12,13]. In addition, *Apis mellifera* propolis has been demonstrated as a source of natural resource for the discovery of new bioactive compounds, such as artepelim C [14], apigenin and *tt*-farnesol [15], CAPE [13], among others.

Since it is a product obtained from different plant sources, the chemical constituents and pharmacological activities of propolis vary according to the time of year, existing vegetation type in each region where it is produced, and bee species responsible for collecting bee [16-18]. Due to these characteristics, several studies with propolis are performed to isolate and identify the new chemical compounds, which exhibit promising pharmacological potential and that can be applied as new therapeutic agents [13].

The geopropolis, a mixture of resin, wax and soil, is an uncommon propolis collected by native stingless bees of the Meliponini tribe and widely found in tropical and subtropical areas worldwide [19-21]. Among the geopropolis, the one from the bee *Melipona scutellaris* bee species has been the target of interest of our research group. Previous studies with geopropolis observed significant antimicrobial activity against

Staphylococcus aureus and also an antioxidant activity, suggesting thus that should be conducted more studies to identify other biological activities as well as the elucidation of its chemical profile, with the aim of identifying promisiong chemicals with pharmacological potential.

Thus, the aim of this study was to evaluate the activity of the ethanolic extract of geopropolis (EEGP) of *Melipona scutellaris* and its fractions on the modulation of neutrophil migration in the inflammatory process, as well as the participation of NO pathway, and to check the chemical profile of EEGP and the bioactive fraction.

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 38º05'04"), state of Bahia, Northeast of Brazil. The geopropolis (100 g) was extracted with absolute ethanol (w/v) of proportion (1/7), at 70 ºC, 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. At the end of three partitions, it was obtained a residue called 'aqueous fraction'. The fractions obtained were monitored by thin layer chromatography (TLC) using the anisaldehyde reagent (4-methoxy-benzaldehyde, acetic acid, sulphuric acid/1.0:48.5:0.5) and followed by incubation at 100 ºC for 5 min. Fluorescent substances were visualized under UV light at the wavelengths of 254 and 366 nm [22]. The EEGP and its hexane, chloroform, ethyl acetate, and aqueous fractions were concentrated in a rotaevaporator at 40 ºC 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 extract and fractions were dissolved in DMSO, PBS 1 mM 1:99 for subcutaneous (s.c.) administration.

2.2. Animals

Male SPF (specific-pathogen free) Balb/c mice weighing 20-25 g were housed in temperature of 22-25 °C, with a light cycle of 12 h light/ 12 h dark, humidity of 40-60 %, and with access to water and food *ad libitum*. All procedures were performed in accordance with the Guiding Principles in the Care and Use of Animals and approved by the local animal Ethics committee (CEUA UNICAMP process number 2037-1).

2.3. Drugs and reagents

Carrageenan, aminoguanidine (AG), [1H-(1,2,4)oxadiazolo (4,3-a) quinoxalin-1-one] (ODQ) and dimethylsulphoxide (DMSO) were obtained from Sigma® Chemical Co., St. Louis, MO, USA, Indomethacin (MP Biomedicals®) and organic solvents from Merck®.

2.4. Biological protocols

2.4.1. Activity evaluation of EEGP and its fraction on the neutrophil migration in the peritoneal cavity induced by carrageenan.

The mice were pretreated with EEGP, hexane, chloroform, ethyl acetate or aqueous fractions (1, 3, 10 and 30 mg, s.c.). Vehicle was used as negative control. After 30 min of treatment, it was applied carrageenan i.p. at a dose of 500 µg/cavity. After 4 h, the animals were dead and the peritoneal cavity washed with 3 mL of PBS/EDTA (1 mM). For total cell count, it was utilized the Newbauer chamber, and for differential count, performed by preparing smears in a cytocentrifuge (citospin; Shandon Lipshaw Inc, Pittsburgh, Pennsylvania, USA), which were stained with fast panotic kit, and for differentiated cells (100 cells total), a optical microscope (1000x increase) was utilized. The results were expressed as the number of neutrophils per cavity.

2.4.2. Evaluation of EEGP activity and bioactive fraction selected on the rolling and adhesion of leukocytes in the mesenteric microcirculation by intravital microscopy

The mice were pretreated with EEGP or aqueous fraction (10 mg/kg, s.c.) 30 min before the i.p. injection of carrageenan 500 µg/cavity. The negative control group

received the vehicle. After 2 and 4 h of the inflammatory stimulus, the rolling and adhesion of neutrophils were rated as previously described [23,24]. The animals were anesthetized, and the mesenteric tissue was exposed to *in-situ* assessment by intravital microscopy. The animals were placed on a plate with a thermostat at 37 °C, where the mesenteric tissue was kept warm and moist with Ringer Locke solution (pH 7.2 to 7.4) containing 1 % of gelatin. The post-capillary venules, which had a diameter of 10-18 μm, were chosen, and the interaction of leukocytes with the luminal surface of the endothelium venule was assessed, where we counted the number of rolling leukocytes for 10 min. Leukocytes were considered adherent to the endothelium if they remained stationary for >30 s. Cells were counted, and the image was recorded using five different fields for each animal to avoid variability due to sampling. Calculations were made for each animal.

2.4.3. Effect of inhibitors of NO pathway on the EEGP inhibitory effect and bioactive fraction selected on the neutrophil migration in the peritoneal cavity induced by carrageenan.

The animals were pretreated with a selective inhibitor of iNOS (aminoguanidine 50 mg/kg, s.c.), or a selective inhibitor of soluble guanylate cyclase (ODQ 5μmol / kg, i.p.) 30 min before EEGP or aqueous fraction (10 mg/kg, s.c.) administration. The negative control group received the vehicle. After 30 min of treatment, it was applied carrageenan at a dose of 500 μg/cavity and the neutrophil migration was determined as described in Section 2.4.1.

2.4.4. Evaluation of ICAM-1 expression by Western blot against the EEGP treatment and the bioactive fraction in mice subjected to injection of carrageenan in the peritoneal cavity

The mice were pretreated with EEGP or aqueous fraction (10 mg/kg, s.c.). The negative control group received the vehicle. After 30 min of treatment, it was applied carrageenan i.p. at a dose of 500 μg/cavity. After 4 h, the animals were dead, then the mesenteric tissue was dissected, and the proteins were isolated. Tissues were lysed in 400 mL of buffer (1 % Triton X-100, 1 M NaF, 100 mM Nappi, 1M Na_3VO_4, 1 mg/ml aprotinin, 1 mg/ml leupeptin , 1 mg/ml PMSF) and centrifuged at 4 °C for 20 min at

12.300/g. Equal amounts of protein (50 µg) were separated by 10 % SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad). The standard molecular weight (Bio-Rad) was run in parallel to estimate the molecular weight. Membranes were blocked, overnight at 4 °C, in TBS-T (20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 0.1 % Tween 20) plus 5 % of non-fat skim milk powder. After the blocking, the membranes were incubated, overnight at 4 ° C, with rabbit anti-ICAM-1 (1:200) or α-tubulin (Santa Cruz Biotechnology), utilized as an internal control (1:1000) diluted in TBS-T containing 5 % of non-fat skim milk powder. The membranes were then incubated with rabbit anti-IgG conjugated to peroxidase (1/2000) diluted in TBS-T containing 5 % of non-fat milk powder at room temperature for 30 min. Finally, the bands recognized by the specific antibody were visualized using a chemiluminescence-based ECL system (Amersham Biosciences) and exposed to an x-ray film for 30 min (Eastman Kodak). A computer imaging system (Gel-Pro Analyzer) was utilized to measure the intensity of the OD of the bands.

2.4.5. Evaluation of nitrite levels for EEGP treatment and bioactive fraction in mice subjected to injection into the peritoneal cavity of carrageenan

The mice were pretreated with EEGP or aqueous fraction (10 mg/kg, s.c.). The negative control group received the vehicle. After 30 min of treatment, it was applied carrageenan i.p. at a dose of 500 µg/cavity. After 4 h, the animals were dead and the peritoneal cavity washed with 3 mL of PBS/EDTA (1 mM). The production of NO was determined in the peritoneal lavage using the Griees method [25] by measuring the nitrite concentration in an ELISA plate at 540 nm, and the results were expressed in micromoles of nitrite.

2.4.6. Evaluation of EEGP activity and bioactive fraction selected on the paw edema induced by carrageenan

The mice were pretreated with EEGP or aqueous fraction (1, 3, 10, and 30 mg/kg, i.p.). Indomethacin (10 mg/kg, s.c.) was used as positive control, and vehicle as negative control. After 30 min of treatment, the animals were subjected to an intraplantar injection of 50 µL carrageenan (1mg/paw) in the left hind leg. The animal's paw volume

was measured before (time 0) and after injection of carrageenan (1, 2, 3, 4, and 5 h) utilizing a plethysmometer (Ugo Basile, model 7150, Italy). The results were expressed as the threshold of the inflamed paw Δ (mL), which was calculated by subtracting the values obtained before (time 0) and after the injection of carrageenan [26].

2.5. Chemical analysis of EEGP and bioactive fraction

2.5.1. Gas chromatography with mass spectrometry

About 15 mg of EEGP and aqueous fraction were mixed with 100 µL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) in a sealed glass tube for 15 min at 60 °C to form TMS derivatives before analyzing with gas chromatography/mass spectrometry (GC-MS). After that time, the reagent's derivation was removed under nitrogen flow, and the product of the reaction was diluted again in hexane (600 µL), according to the method described by [27] with some modifications. Shimadzu gas chromatograph, model 2010 GC, coupled to mass spectrometer Shimadzu, model QP 2010 Plus, were employed for all analyses. Samples were separated on a capillary column (RTX5MS 30 m x 0.25 mm x 0.25 µm). The column temperature was initially held at 80 °C for 1 min, and then the temperature was raised to 320 °C at a rate of 20 °C min^{-1}, followed by an isothermal period of 20 min. The total run time was 69 min. Ultra-high-purity helium with an inline oxygen trap was used as carrier gas. The injector was heated to 280 °C and was on split mode with a split ratio of 1:20, and the injection volume was 0.4 µL. The integration was done in the software solution LabSolutions-GCMS, and the identification of detected compounds was performed by comparison with the data of mass spectra libraries Wiley8. The compounds that were not identified, characterized the principal molecular ions, according to the mass-charge ratio (m/z).

2.5.2. High performance liquid chromatography reverse phase

Ten microliters of each sample (EEGP or aqueous fraction) were injected into a liquid chromatograph coupled to a photodiode array detector at 260 nm, and the samples were eluted through a C18 reverse phase column (250 x 4.6 mm), with particle size of 5

μm. The mobile phase was water/acetic acid (19:1, v/v) (solvent A) and methanol (solvent B) with constant rate of 1 mL/min. The gradient started with 30 % of solvent B to 40 % of B in 15 min, 50 % of B in 30 min, 60 % of B in 45 min, 75 % of B in 65 min, 75 % of B in 85 min, 90 % of B in 95 min, 90 % of B in 110 min and 30 % of B in 120 min. The column was maintained at a constant temperature of 350 °C [28]. For the aqueous fraction, it was also utilized another mobile phase composed of water/acetic acid (98/2) (solvent A) and water/acetonitrile/acetic acid (68/30/2) with constant rate of 1 mL/min. The gradient started with 0 to 30 % of solvent B in 20 min, 30 to 50 % of B in 10 min, 50 to 70 % of B in 20 min, 70 to 100 % of B in 5 min, 100 % of B in 20 min, 100 to 0 % of B in 10 min [29]. We utilized authentic standards of flavonoids and phenolic acids to compare the retention time of substances in EEGP and aqueous fraction samples.

2.6. Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM), and statistical comparison between groups were made utilizing analysis of variance (ANOVA) followed by Tukey test. Significance was accepted when $p \leq 0.05$.

3. Results

3.1. EEGP and aqueous fraction inhibit the neutrophil migration in the peritoneal cavity

We evaluated the activity of EEGP and its aqueous fraction on the neutrophil migration in the peritoneal cavity induced by carrageenan. Regarding the results, it was found that administration of EEGP decreased the influx of neutrophils into the peritoneal cavity, compared to the carrageenan group ($p < 0.05$), where there was a reduction of 41, 61, 62, and 50 % for doses 1, 3, 10, and 30 mg/kg (Figure 1A), respectively. With regard to the chemical fractions studied, the hexane fraction (Figure 1B), the chloroform fraction (Figure 1C), and the ethyl acetate fraction (Figure 1D) showed no significant inhibition of neutrophil recruitment ($p > 0.05$). On the other hand, the aqueous fraction decreased the number of neutrophils in the peritoneal cavity after injection of carrageenan ($p < 0.05$),

where we observed an inhibition of 66 and 64 % for doses of 10 and 30 mg/kg, respectively (Figure 1E). Thus, the aqueous fraction was selected as the bioactive fraction of EEGP.

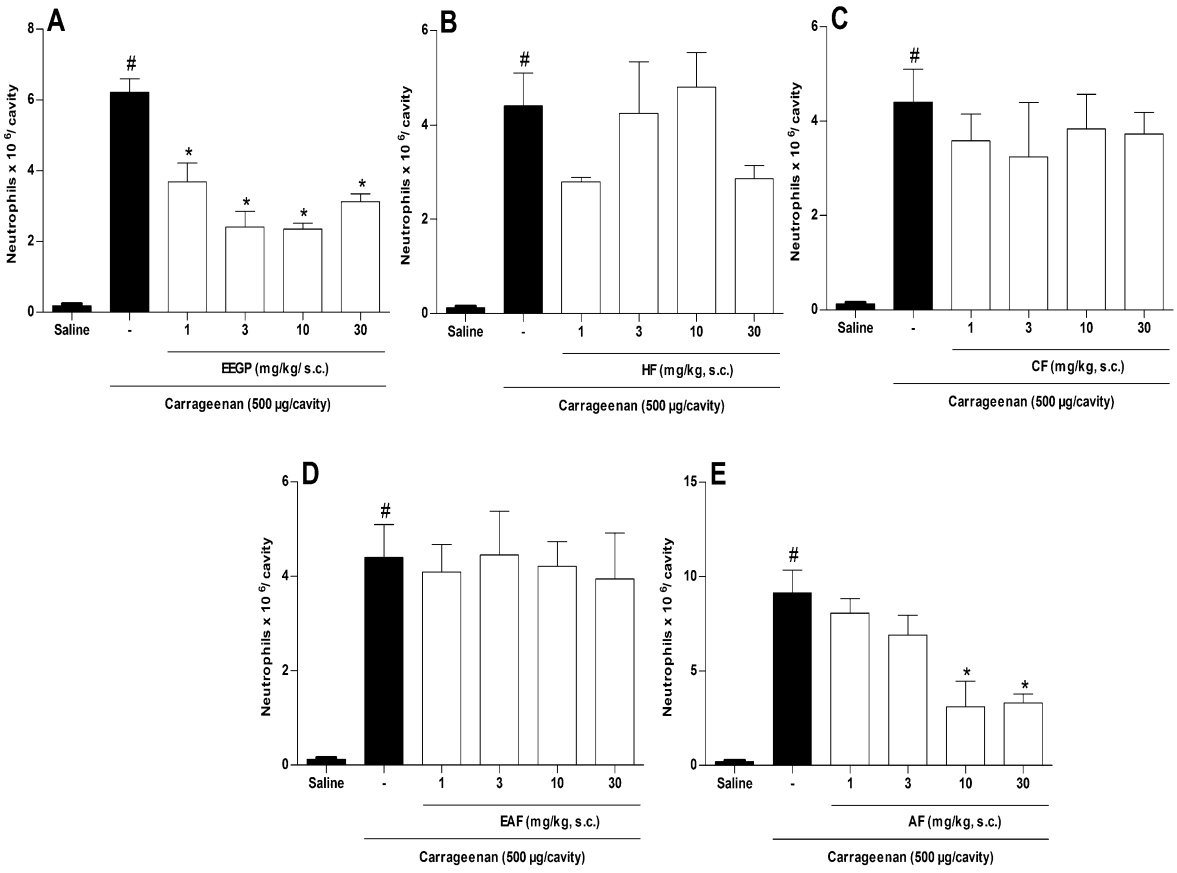

Figure 1. Inhibitory effect of ethanolic extract of geopropolis (EEGP) and aqueous fraction (AF) on the neutrophils migration into the peritoneal cavity induced by carrageenan. The neutrophil migration was determined 4 h after the injection of carrageenan 500 µg/cavity. Mice previously treated with vehicle (saline and carrageenan), EEGP (**A**), hexane fraction (FH-**B**), chloroform (FC-**C**), ethyl acetate (EAF-**D**), or aqueous (AF-**E**). The data are expressed by mean ± SEM, n = 6. Symbols indicate statistical difference ($p < 0.05$, Tukey test). # compared to the saline group; * compared to the carrageenan group.

3.2. EEGP and aqueous fraction inhibit the rolling and adhesion of leukocytes induced

By checking the activity on the reduction of the neutrophil migration in the peritoneal cavity, we evaluated the activity of EEGP and aqueous fraction on the rolling and adhesion of leukocytes to endothelial cells. Based on the results, we found that EEGP and the aqueous fraction, at a dose of 10 mg/kg, decreased leukocyte rolling and adhesion

on endothelial cells, compared to the carrageenan group ($p < 0.05$), with an inhibition of 48 and 58 % to the rolling (Figure 2A), and 75 and 61 % for the adhesion of leukocytes (Figure 2B), respectively.

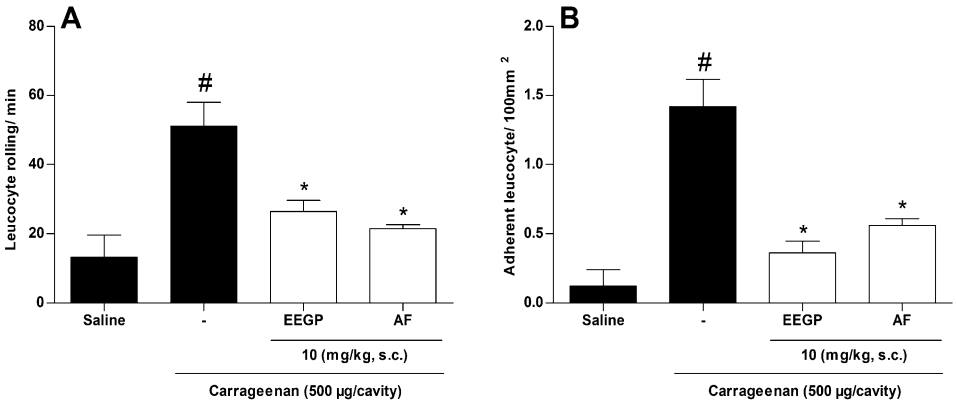

Figure 2. Inhibitory effect of ethanolic extract of geopropolis (EEGP) and aqueous fraction (AF) on the rolling (**A**) and adhesion (**B**) of leukocytes assessed by intravital microscopy in mesenteric tissue of mice, 2 and 4 h after i.p. injection of carrageenan 500 µg/cavity. Mice were pretreated with vehicle (saline and carrageenan), EEGP, or aqueous fraction (10 mg/kg). The data are expressed by mean ± SEM, n = 5. Symbols indicate statistical difference ($p < 0.05$, Tukey test). # compared to the saline group; * compared to the carrageenan group.

3.3. Inhibitors of NO pathway cause suppression on the EEGP inhibitory effect and aqueous fraction on the neutrophil migration induced by carrageenan

In order to elucidate the mechanism of action by which EEGP and aqueous fraction inhibit the neutrophil migration, we initially studied their action in the NO pathway. According to the results, we observed that the application of antagonists of NO pathway (Figure 3A, and B) abolished the inhibitory effect of EEGP and aqueous fraction, at a dose of 10 mg/kg, on the neutrophil migration induced by carrageenan ($p < 0.05$), thus confirming that the inhibitory action of recruitment of neutrophils is at least partly due to the NO pathway.

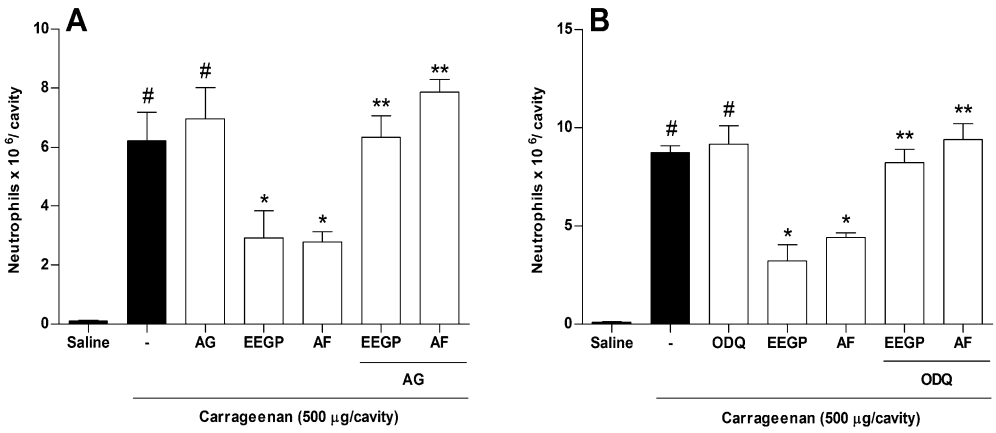

Figure 3. Inhibitory effect of ethanolic extract of geopropolis (EEGP) and aqueous fraction (AF) on the neutrophils migration into the peritoneal cavity induced by carrageenan. The neutrophil migration was determined 4 h after the injection of carrageenan 500 μg/cavity. Mice were pretreated with aminoguanidine (AG - 50 mg/kg, **A**), and ODQ (5μmol/kg, **B**) antagonists. After 30 min, the animals were pretreated with vehicle (saline and carrageenan), EEGP, or aqueous fraction (10 mg/kg). The data are expressed by mean ± SEM, n = 6. Symbols indicate statistical difference ($p<0.05$, Tukey test). # compared to the saline group; * compared to the carrageenan group; ** compared to the groups that received only EEGP and the aqueous fraction.

3.4. EEGP and aqueous fraction decrease the expression of ICAM-1 and increase nitrite levels in mice subjected to injection of carrageenan in the peritoneal cavity

Regarding the expression of adhesion molecules ICAM-1, we observed that treatment with EEGP and aqueous fraction, at a dose of 10 mg/kg, reduced its expression ($p <0.05$) when compared to the carrageenan group (Figure 4A). Besides, the treatment with EEGP and aqueous fraction (10 mg/kg) increased the nitrite levels ($p <0.05$) when compared to the carrageenan group (Figure 4B).

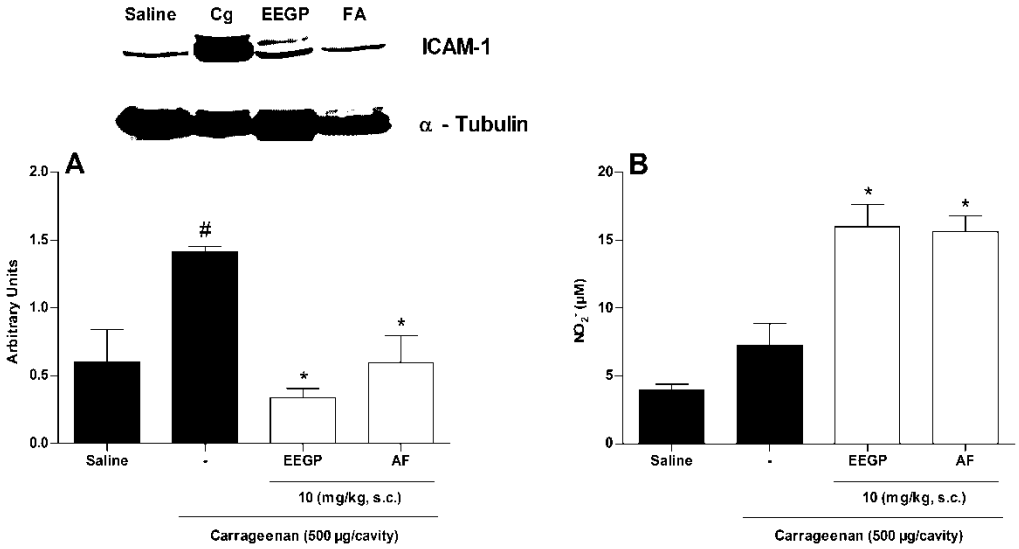

Figure 4. Effect of ethanolic extract of geopropolis (EEGP) and aqueous fraction (AF) on the expression of ICAM-1 (**A**) and NO levels (**B**) 4 h after the injection of 500 µg/cavity of carrageenan in the peritoneal cavity. Mice were pretreated with vehicle (saline and carrageenan), EEGP, or aqueous fraction (10 mg/kg, s.c.). The data are expressed by mean ± SEM, n = 5. Symbols indicate statistical difference ($p < 0.05$, Tukey test). # compared to the saline group; * compared to the carrageenan group.

3.5. Antiedematogenic activity of EEGP and aqueous fraction on paw edema

We verified that EEGP and aqueous fraction on paw edema is induced by carrageenan. According to the results, we found that EEGP showed antiedematogenic activity in inhibiting the paw edema (Table 1), and we observed an inhibition of 57, 60, 62, and 62 % for doses of 1, 3, 10, and 30 mg/kg (3 h), and 66 % (4 and 5 h after injection of carrageenan), for the 30 mg/kg dose.

Table 1. EEGP effect on the paw edema induced by carrageenan on mice

Treatment (mg/kg)	Time (h) after injection of carrageenan				
	1	**2**	**3**	**4**	**5**
Carrageenan	0.06 ± 0.01	0.08 ± 0.01	0.13 ± 0.02	0.16 ± 0.01	0.15 ± 0.01
Indomethacin 10 mg/kg	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01*	0.05 ± 0.02*	0.06 ± 0.02*
EEGP 1 mg/kg	0.04 ± 0.01	0.05 ± 0.02	0.06 ± 0.02*	0.11 ± 0.02	0.12 ± 0.02
EEGP 3 mg/kg	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.01*	0.12 ± 0.01	0.15 ± 0.01
EEGP 10 mg/kg	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.02*	0.12 ± 0.01	0.13 ± 0.01
EEGP 30 mg/kg	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01*	0.06 ± 0.01*	0.05 ± 0.02*

The data are expressed by mean ± SEM, n = 6. Symbols indicate statistical difference ($p < 0.05$, Tukey test). * compared to the carrageenan group.

According to the results presented by the aqueous fraction (Table 2), it was possible to observe a reduction of edema only for the dose of 30 mg/kg (3 and 4 h after injection of carrageenan), and we found an inhibition of 56 and 49 %, respectively.

Table 2. Aqueous fraction effect on the paw edema induced by carrageenan on mice

Treatment (mg/kg)	Time (h) after injection of carrageenan				
	1	**2**	**3**	**4**	**5**
Carrageenan	0.08 ± 0.01	0.10 ± 0.02	0.13 ± 0.01	0.16 ± 0.02	0.15 ± 0.01
Indomethacin 10 mg/kg	0.07 ± 0.01	0.06 ± 0.01	0.07 ± 0.00*	0.08 ± 0.01*	0.06 ± 0.02*
Aqueous fraction 1 mg/kg	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.14 ± 0.01	0.13 ± 0.01
Aqueous fraction 3 mg/kg	0.08 ± 0.01	0.09 ± 0.02	0.09 ± 0.01	0.13 ± 0.01	0.13 ± 0.01
Aqueous fraction 10 mg/kg	0.06 ± 0.01	0.08 ± 0.01	0.08 ± 0.02	0.11 ± 0.02	0.11 ± 0.01
Aqueous fraction 30 mg/kg	0.06 ± 0.01	0.05 ± 0.02	0.06 ± 0.02*	0.08 ± 0.03*	0.09 ± 0.02

The data are expressed by mean ± SEM, n = 6. Symbols indicate statistical difference ($p < 0.05$, Tukey test). * compared to the carrageenan group.

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3.6. Gas chromatography with mass spectrometry

The EEGP analysis by GC/MS is presented in Table 3. Only substances that showed a percentage of relative area greater than 5 % have been included. Most of the substances were not identified based on the standards utilized and on the library information equipment and, therefore, we did not find any flavonoid or phenolic acid commonly present in samples of propolis of *Apis mellifera*. It can be observed that

compounds 5 and 6 presented themselves as the majority, with a percentage of 19.17 and 29.15 %, respectively. Compounds 6 and 7, despite showing similarity in terms of M^+ (*m/z* 623), as well as retention time, have distinct ion fragmentation. Regarding the aqueous fraction, we did not detect any peak higher than the baseline noise of the mass detector.

Table 3. Retention time, % of relative area, and major ions present in the mass spectra of compounds silanized of the ethanolic extract of geopropolis (EEGP) of *Melipona scutellaris* by GC/MS.

Compound	Name	Rt[a] (min)	Relative area (%)	Ion (m/z, abundance in parentheses)
1	2-propensaeure 3-phenyl-trimethylsilylester	17.84	8.91	220 (31), 205 (100), 161 (90), 145 (33), 131 (88), 103 (60), 77 (49)
2	1,2-benzenedicarboxylic acid	36.60	+	167 (33), 149 (100), 57 (40)
3	-	43.79	6.88	591 (11), 589 (48), 501 (100), 459 (25), 445 (28), 73 (90), 57 (13)
4	-	46.94	5.79	495 (100), 459 (60), 417 (21), 73 (40), 57 (21)
5	-	47.29	19.17	548 (39), 533 (34), 479 (17), 389 (45), 73 (100), 45 (11)
6	-	47.71	29.15	623 (66), 536 (20), 535 (52), 73 (100)
7	-	47.91	6.93	623 (1), 551 (48), 533 (17), 461 (32) 407 (86), 73 (100)

RT[a] - Retention time; Symbols (-) not detected; + <0.1 % of relative area.

3.7. High performance liquid chromatography reverse phase

According to the chromatograms obtained by high performance liquid chromatography reverse phase (HPLC-FR) of EEGP and aqueous fraction (Figure 5A and B), utilizing the same gradient elution, we observed the existence of different chemical profiles. In the chromatogram of EEGP, we can see a great predominance of nonpolar compounds, with retention times over 70 min, which is superior to the retention time of all standards of flavonoids utilized. In the chromatogram of the aqueous fraction, we observed the predominance of a single peak and intensity greater than in EEGP. However, when the aqueous fraction was analyzed utilizing an optimized mobile phase for polar compounds (Figure 5C), we could verify the existence of two major compounds that are not compatible with any of the standards utilized, therefore demonstrating that they are highly polar molecules of low molecular weight.

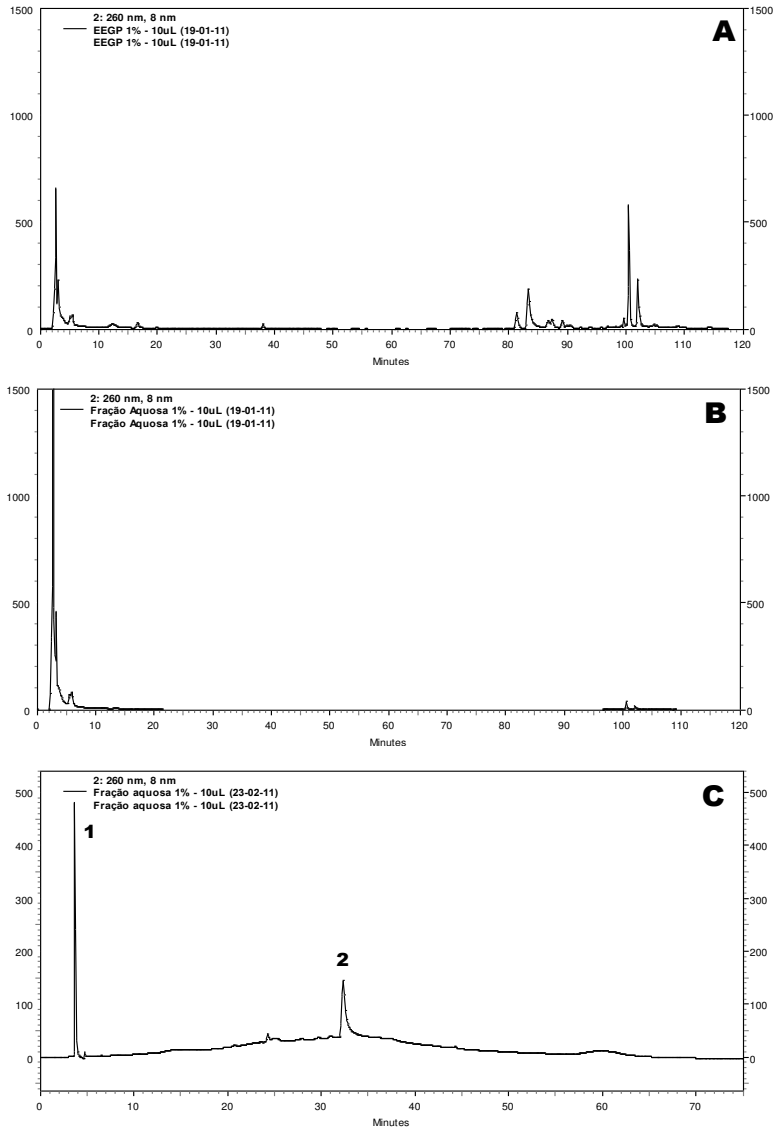

Figure 5. Chromatograms obtained by HPLC-RP of the ethanolic extract of geopropolis (EEGP-**A**) and aqueous fraction (**B-C**).

4. Discussion

The development of new anti-inflammatory drugs that interfere with the traffic of neutrophils during the inflammatory process is a great therapeutic interest. This fact is understandable due to the seminal role of neutrophils in the development of various

inflammatory diseases, as the rheumatoid arthritis, chronic obstructive pulmonary disease and periodontal disease [30-32].

In this context, it is recognized that most new drugs discovered in recent decades had natural products as the main source [12]. The present study demonstrated that the ethanolic extract of geopropolis (EEGP) and its aqueous fraction decreased leukocyte interaction (rolling and adhesion) with endothelial cells, as well as neutrophil migration into the peritoneal cavity of mice subjected to intraperitoneal injection of carrageenan through suppression of adhesion molecules ICAM-1, which is an NO pathway-dependent activity.

The inflammatory process is a set of events arising from the participation of various chemical mediators that promote vascular events, edema, and the recruitment of leukocytes [7]. NO plays different physiological functions in the body, among them as a regulator of vascular tone and modulator of leukocytes adhesion in the blood capillaries and microcapillaries. Its production involves the participation a family of enzymes known NO synthases (NOS). Among them, the inducible NOS (iNOS) is induced only in the inflammatory process [33,10,11].

Recent studies have shown that the inhibitory effect of NO (via iNOS activation) on migration of neutrophils in the inflammatory process is dependent on the activation of enzyme soluble guanylate cyclase (sGC). This inhibition of neutrophil migration by sGC mediated, generates increased levels of guanosine 3′5′-cyclic monophosphate (cGMP), and consequently inhibition of the adhesion molecules ICAM-1 [10-11].

The present study demonstrated that treatment with specific antagonists of iNOS, or the soluble guanylate cyclase suppressed the inhibition of neutrophil migration by EEGP and its aqueous fraction. These results suggest, therefore, that the inhibitory effect of EEGP and the aqueous fraction on the neutrophils migration by NO pathway is related to increased levels of NO (via iNOS activation), which can be observed by the increase in nitrite levels and consequent activation of the sGC/cGMP pathway, thus leading to the suppression of adhesion molecules ICAM-1.

As the edema is one of the key events of the inflammatory response, we also evaluated the effectiveness of EEGP and the aqueous fraction on the paw edema induced by carrageenan. The first phase (0-2.5 h) of paw edema induced by carrageenan is associated with increase in the histamine, serotonin, and kinins chemical mediators, and these are related with the increase in vascular permeability, besides the increased production of the IL-1β and TNF-α cytokines [34,35]. In the second phase (3-4 h) occurs the production of mediators derived from the inducible cyclooxygenase enzyme, such as prostaglandins, and this second phase is sensitive to anti-inflammatory cyclooxygenase inhibitors [34,35]. According to the results presented, EEGP and its aqueous fraction decreased the paw edema induced by carrageenan, where this activity was observed only in the second phase (3-4 h) of the experiment. These results therefore suggest that the activity of EEGP and the aqueous fraction may be related to the inhibition of prostaglandin formation.

In relation to chemical analysis by GC/MS, we observed the presence of chemical compounds, including two identified. Regarding the aqueous fraction, no compound has been found by the library of the equipment, which may be due to the limitations of the technique, for example, the chemical derivatization made, where resulting compounds may have been highly unstable and decayed. On the other hand, when the aqueous fraction analysis was performed by HPLC with mobile phase optimized for polar compounds, two distinct chemical substances could be identified, which shows the highly polar characteristic of bioactive chemical compounds. No flavonoid or phenolic acid was found in EEGP and the aqueous fraction. These phenolic are a class of chemicals reported in the literature as responsible for most of the biological activity of propolis from *Apis mellifera* [36,28], thus suggesting a different chemical profile of the *Melipona scutellaris* geopropolis, as evidenced in EEGP and its aqueous fraction. These chemical profile data therefore suggest the presence of chemical compounds that are not reported in the literature as to their pharmacological activities (especially the anti-inflammatory one) and that deserve special attention due to its promising mechanism of action.

Therefore, we conclude that the EEGP and its aqueous fraction decreased migration of neutrophils in the inflammatory process, and this is dependent on nitric oxide pathway. Thus, due to their distinct chemical profile and mechanism of action promising,

further studies are necessary for the isolation and identification of bioactive compounds present in the aqueous fraction, in which may enable the development of new effective anti-inflammatory drugs for the treatment of diseases inflammatory.

Acknowledgements

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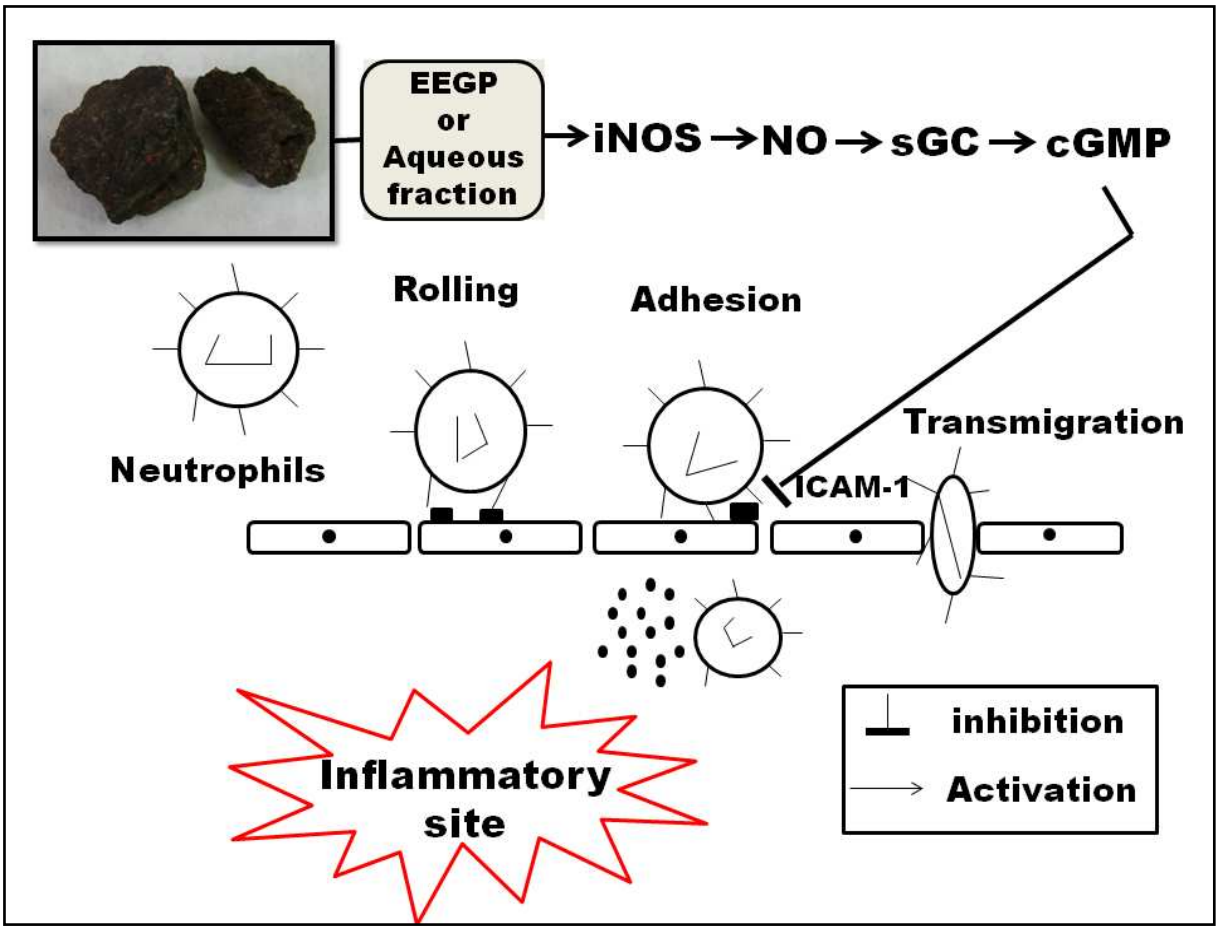

Scheme 1: Mechanism of action of the EEGP and aqueous fraction on inhibition of neutrophil migration during the inflammatory process by nitric oxide pathway

CAPÍTULO 2

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: Propolis has been widely used by folk medicine for centuries. It is described in the literature as possessing various biological activities, however, most of these reports refer to the propolis of *Apis mellifera*. The geopropolis collected by stingless bees (in extinction process) is a kind of neglected propolis by the science maybe because it does not have economic aggregated value; however it is widely used by the poor communities in Brazil especially in the Northeast region, and whose pharmacological activity needs to be studied.

Objective: The aim of this study was to evaluate the antinociceptive activity of *Melipona scutellaris* geopropolis (stingles bees) using different models of nociception.

Material, methods and results: The ethanolic extract of geopropolis (EEGP) from *Melipona scutellaris*, as well as the hexane and aqueous fractions decreased the numbers of writhes in the acetic acid induced constriction test. In the formalin test, the EEGP and the aqueous fraction clearly demonstrated antinociceptive activity in both neurogenic and inflammatory phases, while the hexane fraction suppressed only the neurogenic phase. The treatment with EEGP and aqueous fraction reduced mechanical inflammatory hypernociception induced by carrageenan and also reduced the levels of IL-1 β and TNF-α. The chemical analyses demonstrated the presence of phenolic compounds and absence of flavonoids.

Conclusion: 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 carrageenan model, and this effect is mediated by inhibition of IL-1β and TNF-α. These data indicate that geopropolis is a natural source of bioactive substances with promising antinociceptive activities.

Keywords: Geopropolis, *Melipona scutellaris*, bioactive fractions, antinociceptive, cytokines, pain.

1. Introduction

In recent decades, several studies have shown that analgesics are like one of the most studied classes of drugs 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 focus in screenings of extracts from natural sources and historically led the discovery of many clinically important drugs in the current therapy (Verri jr et al., 2006; Newman et al., 2003).

Propolis is a resin product collected by honey bees from several parts of plants (Silva *et al*., 2008). For centuries propolis has long 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; Silva et al., 2008; Awale et al., 2008). Although a multitude of studies about propolis have been published previously, most of them are from *Apis mellifera*. In contrast, reports about propolis from other species of bees have been studied sparse.

The bees species *Melipona scutellaris*, which belongs to Meliponini tribe (stingless bees in the process of extinction), produces a variety of propolis popularly known as geopropolis. This geopropolis consists of a mixture of resin, wax and soil, providing distinctive physico-chemical characteristics (Nates-Parra, 2001; Barth, 2006), however, 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 twelve 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 fifty compounds, mainly phenolic and terpene 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 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 to evaluate 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 have 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 38º05'04"), state of Bahia, Northeast of Brazil. The geopropolis (100 g) was extracted with absolute ethanol (w/v) of proportion (1/7), at 70 ºC, 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. At the end of three partitions, it was obtained a residue called 'aqueous fraction'. The fractions obtained were monitored by thin layer chromatography (TLC) using the anisaldehyde reagent (4-methoxy-benzaldehyde, acetic acid, sulphuric acid/1.0:48.5:0.5) and followed by incubation at 100 ºC 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 ºC 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, PBS 1 mM 1:99 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*. 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 was 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 Cristalia® (Morphine and Naloxone).

2.4. Biological protocols

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

The abdominal constriction (writhes) were induced by i.p. injection of acetic acid (1.2 %, v/v) 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 consist in the constriction of the flank muscles associated with inward movements of the hind limb or

with whole body stretching, were counted cumulatively 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 activity of EEGP and bioactive fractions on formalin-induced nociception

The method used in the present study was similar to that described previously by Corrêa & 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.) was 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 activity of EEGP and bioactive fractions on carrageenan-induced inflammatory hypernociception

Mechanical hypernociception was tested in mice as previously reported 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 hindpaw 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, mice were placed in acrylic cages (12×10×17 cm) 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 three measurements. The

animals were tested before and after 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 previous test (2.4.3) the EEGP and aqueous fraction were selected for the quantification of proinflammatory cytokine. 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, 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 experiments. The results are expressed as picograms.

2.4.4. Evaluation of activity of EEGP and bioactive fractions on 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 x 45 x 20 cm, with the floor divided into 9 equal squares (15 x 15 cm). The number of squares crossed with all paws (crossing) was counted in a 6-min session. Mice were treated with EEGP (1, 3, 10 and 30 mg/kg, i.p.), hexane (1 and 3 mg/kg) and aqueous (10 and 30 mg/kg) fractions or vehicle 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 contents

Total polyphenol content in EEGP, hexane and aqueous fractions was 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 2h 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 1M 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

Data were expressed as mean ± S.E.M., statistical comparisons between groups were made using analyses of variance (ANOVA) followed by Tukey test. 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 Figure 1A demonstrate that the EEGP (3, 10 and 30 mg/kg), produced dose-related inhibition of abdominal constrictions induced by acetic acid in 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 (Figure

1B), and the aqueous fraction inhibited ($p<0.05$) in 54 and 81 % at doses of 10 and 30 mg/ kg, respectively (Figura 1E). On the other hand, the chloroformic fraction (Figure 1C) and the ethyl acetate fraction (Figure 1D), did not show any inhibition ($p>0.05$). Then the hexane and the aqueous fractions were selected like the bioactive fractions from EEGP.

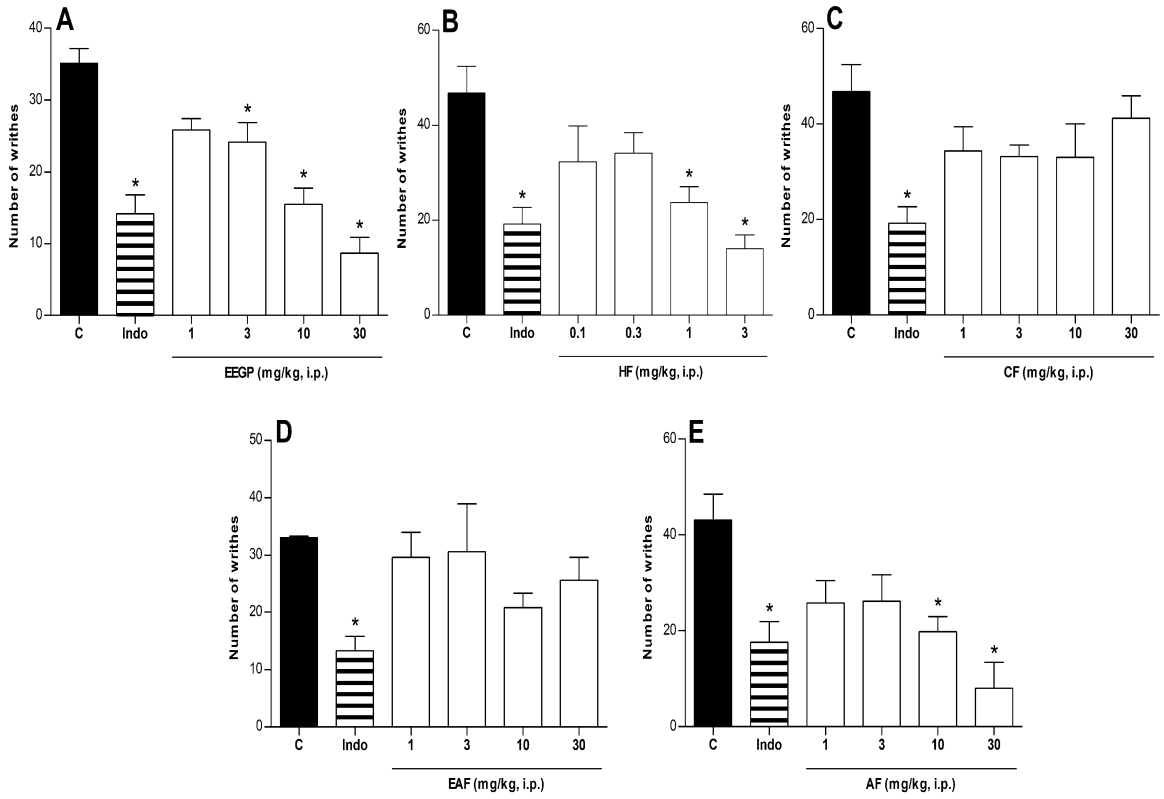

Figure 1. Effect of i.p. injection of ethanolic extract of geopropolis (EEGP) and fraction on abdominal constriction induced by acetic acid in mice. Control (C) treated with vehicle, indomethacin 10 mg/kg (indo), EEGP (**A**), fractions: hexane (HF, **B**), choroformic (CF, **C**), ethyl acetate (EAF,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

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

The results in Figure 2A and B show 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 e 56 % for the doses of 1, 3, 10 e 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 (Figure 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 (Figure 2E and F).

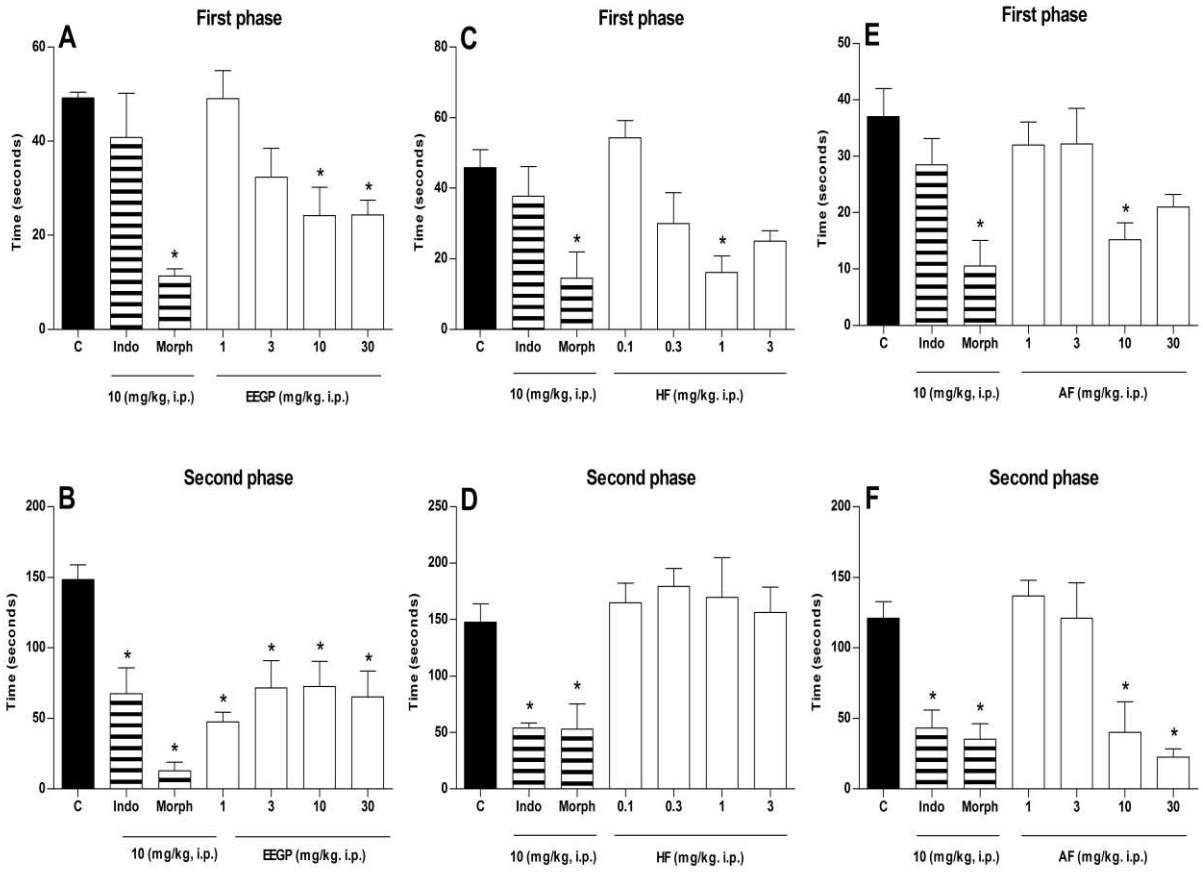

Figure 2. Effect of i.p. injection of ethanolic extract of geopropolis (EEGP) and bioactive fraction on formalin-induced nociception in mice. Control (C) treated with vehicle, indomethacin 10 mg/kg (indo), morphine 10mg/kg (morph), EEGP (**A-B**), hexane fraction (HF, **C-D**) and aqueous fraction (AF, **E-F**). The data are expressed by mean ± SEM, n = 6. Symbol indicate statistical difference ($p < 0.05$, Tukey test). * $p<0.05$ compared to control group.

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

The effects of EEGP on hypernociception induced by carrageenan are shown in Figure 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 (Figure 3C). On the other hand, the hexane fraction was not effective even at the highest dose tested (Figure 3B, $p>0.05$).

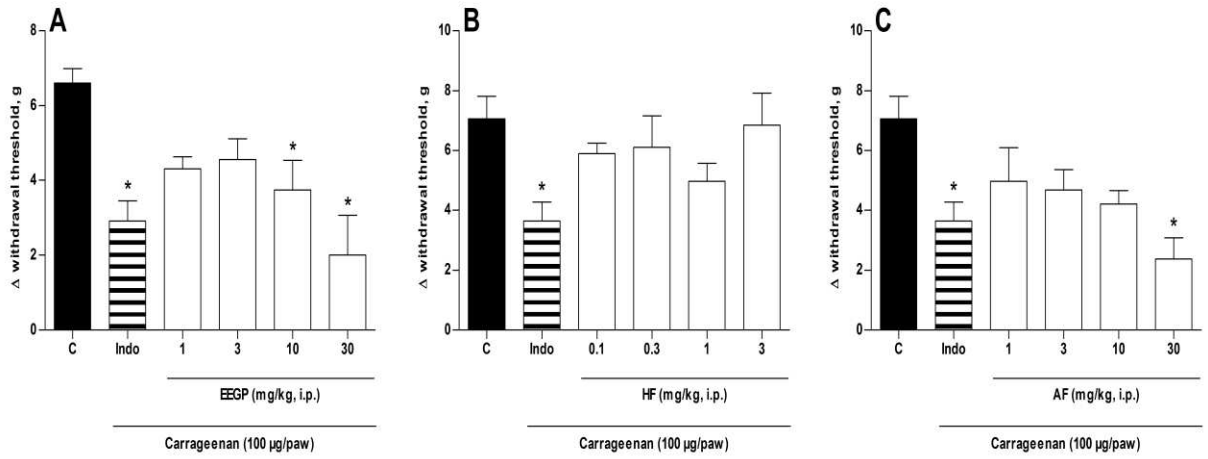

Figure 3. Effect of i.p. injection of ethanolic extract of geopropolis (EEGP) and bioactive fraction on mechanical inflammatory hypernociception induced by carrageenan in mice. Control (C) treated with vehicle, indomethacin 10 mg/kg (indo), EEGP (**A**), hexane fraction (HF, **B**) and aqueous fraction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

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 (Figure 4A and B).

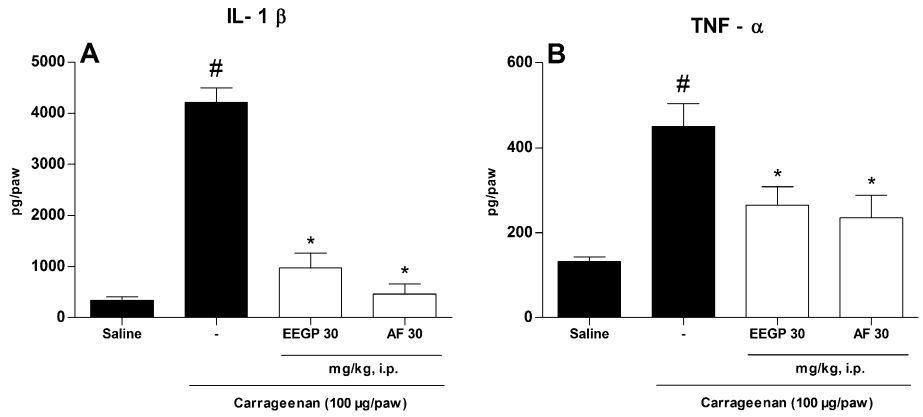

Figure 4. Quantification of IL-1 β (**A**) and TNF-α (**B**) in the hind paw tissue of mice previously treated with vehicle (saline and carrageenan), ethanolic extract of geopropolis (EEGP) and aqueous fraction (AF) at a dose of 30 mg/kg 30 min before the intraplantar injection of carrageenan. The data are expressed by mean ± SEM, n = 5. Symbols indicate statistical difference ($p < 0.05$, Tukey test). # $p<0.05$ compared to saline group; * $p<0.05$ compared to carrageenan group.

3.4. EEGP and bioactive fractions did not changed the locomotor activity of mice

The administration of EEGP, hexane or aqueous fractions by the i.p. route have not changed the locomotor activity of animals during the 6 min of observation compared with 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 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 255 ± 3.8, 76 ± 1.5 e 277 ± 1.3 w/w % gallic acid equivalents for the EEGP, hexane and aqueous fractions, respectively (Table 1). It was not observed the presence of flavonoids in any sample evaluated.

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

Sample	mgGAE/g sample
EEGP	255± 3.8
Hexanic fraction	76± 1.5
Aqueous fraction	277± 1.3

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

4. Discussion

The aim of this study was evaluate the antinociceptive activity of *Melipona scutellaris* from geopropolis in different models of nociception, as well as the mechanism of action related.

The ability of the EEGP as well as the hexane and aqueous fractions to attenuate the abdominal constrictions induced by acetic acid in mice suggests that they possess antinociceptive activity. The acetic acid induced constrictions test is described as a typical model of inflammatory pain, has long been used as a screening tool for the assessment the analgesic properties of new drugs (Le Bars et al., 2001). The nociceptive response produced by constrictions test is due to the participation of several mediators such as prostaglandins, proinflammatory cytokines such as interleukin-1β, interleukin 8, TNF-α, sympathomimetic amines, acetylcholine, 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 to 5 min) is characterized by the occurrence of neurogenic pain by direct stimulation of nociceptive afferent endings and the second phase (15 to 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 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 of 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 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 are also is 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 & 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 could be related with the inhibition of IL-1β and TNF- α cytokines and consequent inhibition 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 mechanical inflammatory hypernociception, suggesting, therefore, another course of action independent of the inflammatory process. Important, this result did not affect the locomotor activity during the open field test. The animals treated with the EEGP and the bioactive fractions did not cause any significant change in the ambulation of mice, excluding non-specific disturbance in the locomotor activity.

This study was also carried out a phytochemical analysis of EEGP and its bioactive fractions, by quantification of total phenolics and flavonoids. The concentrations of phenolic compounds in EEGP were higher than those found in propolis type 12 *Apis mellifera* (Castro et al., 2007), which is the single most studied propolis worldwide. Regarding the bioactive fractions, the presence of phenolic compounds in both fractions (hexane and aqueous) demonstrates the presence of these compounds of different polarities. In addition, it was observed the absence of flavonoids in EEGP and its bioactive fractions, which is a very common chemical class in *Apis mellifera* propolis (Kumazawa et al., 2004, Alencar et al., 2007). The absence of flavonoids in geopropolis is comparable with a unique Brazilian propolis 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 suggest that the geopropolis from *Melipona scutellaris* may have an unusual chemical composition devoid of significant amounts of flavonoids, and thereby warrants further characterization of its chemical composition and 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 carrageenan model, and this effect is mediated by inhibition of IL-1β and TNF-α. The chemical composition showed the significant presence of phenolic compounds and absence of flavonoids, thus suggesting that more studies should be conducted for the identification and isolation of bioactive compounds.

Acknowledgements

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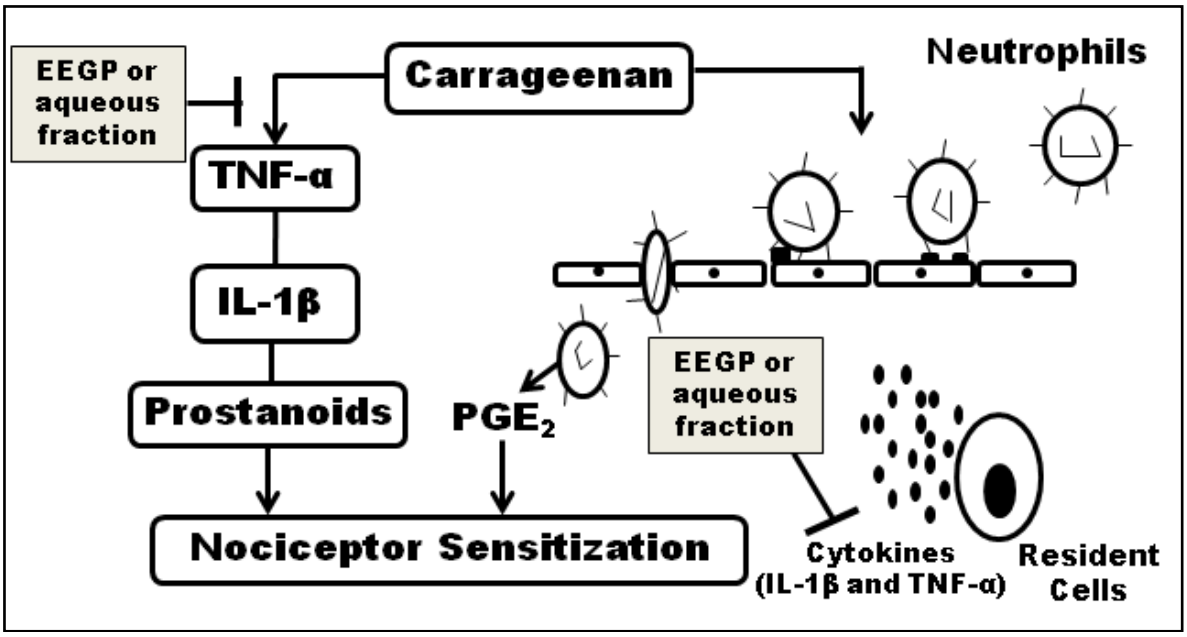

Scheme 2: Mechanism of action of EEGP and aqueous fraction of the decrease in mechanical inflammatory hypernociception by inhibiting the production of cytokine IL-1β and TNF-α.

CONCLUSÃO

Portanto conclui-se:

- O EEGP e sua fração aquosa inibiram a migração de neutrófilos no processo inflamatório, sendo está atividade dependente da via do NO;

- O EEGP e suas frações hexânica e aquosa apresentaram atividade antinociceptiva. Foi constatada atividade para o EEGP e fração aquosa na hipernocicepção inflamatória mecânica induzida por carragenina, sendo está atividade mediada pela inibição das citocinas IL-1β e TNF-α;

- Foi verificada uma característica química diferenciada das frações bioativas, quando comparada à maioria das outras variedades de própolis amplamente estudadas pela comunidade científica, sugerindo, portanto, que mais estudos sejam conduzidos para o isolamento e identificação dos compostos biologicamente ativos presentes nas frações bioativas.

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* De acordo com a norma da UNICAMP/FOP, baseada na norma do International Comitee of Medical Journal Editors – Grupo de Vancouver. Abreviatura dos periódicos em conformidade com o Medline.

ANEXO 1: Certificado de aprovação do Comitê de Ética em Pesquisa no Uso de animais – Faculdade e Odontologia de Piracicaba/UNICAMP

UNICAMP

CEUA/Unicamp

Comissão de Ética no Uso de Animais
CEUA/Unicamp

CERTIFICADO

Certificamos que o Protocolo nº **2037-1**, sobre **"Avaliação do potencial anti-inflamatório e analgésico da própolis de melipolínea (*Melipona scutellaris*)"**, sob a responsabilidade de **Prof. Dr. Pedro Luiz Rosalen / Marcelo Franchin**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética no Uso de Animais – CEUA/Unicamp em **14 de dezembro de 2009**.

CERTIFICATE

We certify that the protocol nº **2037-1**, entitled **"Evaluation of anti-inflammatory and analgesic potential of melipolinea propolis (*Melipona scutellaris*)"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - Unicamp) on **December 14, 2009**.

Campinas, 14 de dezembro de 2009.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

Fátima Alonso
Secretária Executiva

CEUA/UNICAMP
Caixa Postal 6109
13083-970 Campinas, SP – Brasil

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E-mail: comisib@unicamp.br
http://www.ib.unicamp.br/ceea/

ANEXO 2: Informação CCPG/002/06 – Trata do Formato Padrão das Dissertações de Mestrado e Teses de Doutorado da UNICAMP

INFORMAÇÃO CCPG/002/06

Tendo em vista a necessidade de revisão da regulamentação das normas sobre o formato e a impressão das dissertações de mestrado e teses de doutorado e com base no entendimento exarado no Parecer PG nº 1985/96, que trata da possibilidade do formato alternativo ao já estabelecido, a CCPG resolve:

Artigo 1º - O formato padrão das dissertações e teses de mestrado e doutorado da UNICAMP deverão obrigatoriamente conter:

I. Capa com formato único ou em formato alternativo que deverá conter informações relativas ao nível (mestrado ou doutorado) e à Unidade de defesa, fazendo referência à Universidade Estadual de Campinas, sendo o projeto gráfico das capas definido pela PRPG.
II. Primeira folha interna dando visibilidade à Universidade, a Unidade de defesa, ao nome do autor, ao título do trabalho, ao número de volumes (quando houver mais de um), ao nível (mestrado ou doutorado), a área de concentração, ao nome do orientador e co-orientador, ao local (cidade) e ao ano de depósito. No seu verso deve constar a ficha catalográfica.
III. Folha de aprovação, dando visibilidade à Comissão Julgadora com as respectivas assinaturas.
IV. Resumo em português e em inglês (ambos com no máximo 500 palavras).
V. Sumário.
VI. Corpo da dissertação ou tese dividido em tópicos estruturados de modo característico à área de conhecimento.
VII. Referências, formatadas segundo normas de referenciamento definidas pela CPG da Unidade ou por critério do orientador.
VIII. Todas as páginas deverão, obrigatoriamente, ser numeradas, inclusive páginas iniciais, divisões de capítulos, encartes, anexos, etc... As páginas iniciais poderão ser numeradas utilizando-se algarismos romanos em sua forma minúscula.
IX. Todas as páginas com numeração "ímpar" serão impressas como "frente" e todas as páginas com numeração "par" serão impressas como "verso".

§ 1º - A critério do autor e do orientador poderão ser incluídos: dedicatória; agradecimento; epígrafe; lista de: ilustrações, tabelas, abreviaturas e siglas, símbolos; glossário; apêndice; anexos.

§ 2º - A dissertação ou tese deverá ser apresentada na língua portuguesa, com exceção da possibilidade permitida no artigo 2º desta Informação.

§ 3º - As dissertações e teses cujo conteúdo versar sobre pesquisa envolvendo seres humanos, animais ou biossegurança, deverão apresentar anexos os respectivos documentos de aprovação.

Artigo 2º - A critério do orientador e com aprovação da CPG da Unidade, os capítulos e os apêndices poderão conter cópias de artigos de autoria ou de co-autoria do candidato, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, escritos no idioma exigido pelo veículo de divulgação.

§ único - O orientador e o candidato deverão verificar junto às editoras a possibilidade de inclusão dos artigos na dissertação ou tese, em atendimento à legislação que rege o direito autoral, obtendo, se necessária, a competente autorização, deverão assinar declaração de que não estão infringindo o direito autoral transferido à editora.

Artigo 3º - Dependendo da área do conhecimento, a critério do orientador e com aprovação da CPG da Unidade, a dissertação ou tese poderá ser apresentada em formato alternativo, desde que observados os incisos I, II, III, IV, V e VII do artigo 1º.

Artigo 4º - Para impressão, na gráfica da Unicamp, dos exemplares definitivos de dissertações e teses defendidas, deverão ser adotados os seguintes procedimentos:

§ 1º - A solicitação para impressão dos exemplares de dissertações e teses poderá ser encaminhada à gráfica da Unicamp pelas Unidades, que se responsabilizarão pelo pagamento correspondente.

§ 2º - Um original da dissertação ou tese, em versão definitiva, impresso em folha tamanho carta, em uma só face, deve ser encaminhado à gráfica da Unicamp acompanhado do formulário "Requisição de Serviços Gráficos", onde conste o número de exemplares solicitados.

§ 3º - A gráfica da Unicamp imprimirá os exemplares solicitados com capa padrão. Os exemplares solicitados serão encaminhados à Unidade em, no máximo, cinco dias úteis.

§ 4º - No formulário "Requisição de Serviços Gráficos" deverão estar indicadas as páginas cuja reprodução deva ser feita no padrão "cores" ou "foto", ficando entendido que as demais páginas devam ser reproduzidas no padrão preto/branco comum.

§ 5º - As dissertações e teses serão reproduzidas no padrão frente e verso, exceção feita às páginas iniciais e divisões de capítulos; dissertações e teses com até 100 páginas serão reproduzidas no padrão apenas frente, exceção feita à página que contém a ficha catalográfica.

§ 6º - As páginas fornecidas para inserção deverão ser impressas em sua forma definitiva, ou seja, apenas frente ou frente/verso.

§ 7º - O custo, em reais, de cada exemplar produzido pela gráfica será definido pela Administração Superior da Universidade.

Artigo 5º - É obrigatória a entrega de dois exemplares para homologação.

Artigo 6º - Esta Informação entrará em vigor na data de sua publicação, ficando revogadas as disposições em contrário, principalmente as Informações CCPG 001 e 002/98 e CCPG/001/00.

Campinas, 13 de setembro de 2006

Profa. Dra. Teresa Dib Zambon Atvars
Presidente
Comissão Central de Pós-Graduação

ANEXO 3: Prêmio concedido pela Sociedade Brasileira de Pesquisa Odontológica (SBPqO).

28ª Reunião Anual
3 a 6 de Setembro de 2011

Certificado

SBPqO
SOCIEDADE BRASILEIRA DE PESQUISA ODONTOLÓGICA
Divisão Brasileira da IADR

Certificamos que

o trabalho **PNc068** Atividade antinociceptiva do extrato etanólico e frações da geoprópolis de Melipona scutellaris. Franchin M*, Cunha MG, Denny C, Napimoga MH, Alencar SM, Ikegaki M, Rosalen PL foi o vencedor da área 3 da Sessão C de painéis

na 28ª Reunião Anual da Sociedade Brasileira de Pesquisa Odontológica no período de 3 a 6 de Setembro de 2011 em Águas de Lindóia - SP - Brasil.

Giuseppe Alexandre Romito
Presidente

Saul Martins de Paiva
Secretário

Adriana Bona Matos
Tesoureira

ANEXO 4: Comprovante de submissão do artigo referente ao Capítulo 1 ("Bioactive fraction of geopropolis decreases neutrophils migration in inflammatory process: involvement of nitric oxide pathway").

-------- Mensagem original --------
Assunto: Submission Confirmation
Data: 20 Jan 2012 19:54:36 +0000
De: Pharmacological Research <pharmacolres@snv.jussieu.fr>
Para: rosalen@fop.unicamp.br

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