Niraldo Paulino[1]
Cristiane Teixeira[1]
Regiane Martins[1]
Amarilis Scremin[1]
Verena M. Dirsch[2]
Angelika M. Vollmar[2]
Sheila R. L. Abreu[3]
Solange L. de Castro[4]
Maria Cristina Marcucci[5]

Evaluation of the Analgesic and Anti-Inflammatory Effects of a Brazilian Green Propolis

Abstract

Phamacological activities of a standard ethanol extract **G1** from Brazilian green propolis, typified as BRP1, was evaluated in mouse models of pain and inflammation. Intraperitoneal injection (*i.p.*) of **G1** inhibited acetic acid-induced abdominal constrictions with an ID_{50} = 0.75 ± 0.05 mg/kg, and in the formalin test the ID_{50} values were 0.85 ± 0.07 mg/kg and 13.88 ± 1.12 mg/kg, respectively, for the neurogenic and inflammatory phases. The extract was ineffective when assessed in the hot-plate assay. In serotonin-induced paw edema, **G1** led to a maximal inhibition (MI) of 51.6% after 120 min when administered *i.p.* and of 36% after 15 min by the oral route (*o.r.*). When the inflammatory agent was complete Freund's adjuvant, inhibition of paw edema was also observed after administration of the extract by both routes. In the capsaicin-induced ear edema the ID_{50} values were 1.09 ± 0.08 mg/kg (*i.p.*) and 10.00 ± 0.90 mg/kg (*o.r.*). In the acute carrageenan-induced inflammatory reaction induced by carrageenan, **G1** reduced the number of neutrophils in the peritoneal cavity with IC_{50} values of 0.72 ± 0.08 mg/kg and 4.17 ± 0.50 mg/kg, by *i.p.* or *o.r.* administration, with a preferential migration of polymorphonuclear neutrophils. *In vitro*, **G1** decreased nitric oxide production in LPS-stimulated RAW 264.7 cells (IC_{50} = 41.60 μg/mL), and also the luciferase activity in TNF-α-stimulated HEK 293 cells transfected with NF-κB-luciferase reporter gene driven by the nuclear factor κB (NF-κB) (IC_{50} = 200 μg/mL). This extract, which at low concentrations induces anti-inflammatory and analgesic effects in mouse models, presents a high content of flavonoids, known to inhibit inducible NOS (iNOS) activity. These data taken together led us to reinforce the hypothesis in the literature that the anti-inflammatory effect of propolis may be a due to inhibition of iNOS gene expression, through interference with NF-κB sites in the iNOS promoter.

Key words
Typified propolis · analgesic effect · anti-inflammatory effect · NF-κB factor · nitric oxide

Abbreviations

CFA:	complete Freund adjuvant
COX-2:	inducible isoform of cyclooxygenase
Indo:	Indomethacin
iNOS:	inducible nitric oxide synthase
LPS:	Lipopolysaccharide
MI:	maximal inhibition
MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB:	nuclear factor κB
NO:	nitric oxide
TMB:	3,3′,5,5′-tetramethylbenzidine

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

Affiliation
[1] Grupo de Pesquisa e Desenvolvimento de Biofármacos (BIOFAR), Universidade do Sul de Santa Catarina, Tubarão/SC, Brazil
[2] Institute of Pharmacy, University of Munich, Munich, Germany
[3] Nectar Pharmaceuticals, Belo Horizonte, Minas Gerais, Brazil
[4] Departamento de Ultra-estrutura e Biologia Celular, IOC, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil
[5] Faculdade de Farmácia, Departamento de Pesquisa e Extensão, Universidade Bandeirante de São Paulo, São Paulo, Brazil

Correspondence
Prof. Niraldo Paulino · BIOFAR · Universidade do Sul de Santa Catarina · Av. José Acácio Moreira, 787 · PO Box 370 · 88704–900 Tubarão - SC · Brazil · Phone: +55-48-621-3283, · Fax: +55-48-621-3067 · E-mail: niraldo@unisul.br

Received February 22, 2006 · **Accepted** June 1, 2006

Bibliography
Planta Med © Georg Thieme Verlag KG Stuttgart · New York
DOI 10.1055/s-2006-947185 · Published online 2006
ISSN 0032-0943

Introduction

Propolis is a resinous material produced by bees that displays a variety of biological properties, such as activity against viruses, bacteria, fungi, patogenic protozoa, and also tumour cells [1]. This natural product also behaves as an anti-hyperalgesic and anti-inflammatory agent [2], [3], and these properties have been associated with the ability of propolis to act as a free radical scavenger.

Chemical studies have revealed the complex composition of propolis, with more than 300 constituents, including several bioactive phenolic compounds such as flavonoids and derivatives of hydroxycinnamic acids [4].

In a previous study, multivariate analysis associating ethanol extracts of different samples with the levels of bioactive compounds determined by HPLC allowed the typification of Brazilian propolis [5].

Ethanol extracts from propolis collected in Bulgaria and the South of Brazil as well as three prenylated derivatives of *p*-coumaric acid isolated from a sample from the State of Paraná, presented a potent relaxant effect on smooth muscle isolated from guinea pig trachea [6], [7], an effect associated with the release of nitric oxide (NO) [8]. The objective of the present study was to characterize the chemical composition of an ethanol extract obtained from Brazilian propolis collected in the State of Minas Gerais and investigate its anti-hyperalgesic and anti-inflammatory properties and the possible involvement in inhibition of NO production.

Materials and Methods

Preparation and chemical characterization of the ethanol extract of propolis

The propolis sample was collected in March of 2002 in the State of Minas Gerais State (Brazil) by Nectar Pharmaceuticals Ltd. and was subsequently frozen and stored in our laboratory. It was triturated and macerated with an extractive solution containing 96% (v/v) ethanol for 10 days, with a single stirring for 10 min each day. Afterwards, the solvent was evaporated to dryness and a stock solution was prepared in 96% (v/v) ethanol and named **G1**. Before use, the extract was diluted in phosphate-buffered saline (PBS) and the final concentration of ethanol in the assays did not exceed 0.5%, which had no effect on the animals or cell cultures.

The chemical composition of **G1** was determined by high performance liquid chromatograpy using a Merck-Hitachi apparatus (Darmstadt, Germany), equipped with a pump (model L-7100, Merck-Hitachi) and a diode array detector (model L-7455, Merck-Hitachi). Separation was achieved in a Lichrochart 125 – 4 column (Merck; Darmstadt, Germany) as previously described [7]. Detection of the components was monitored at 280 nm and standard compounds were co-chromatographed with the extract. Data analysis was performed in a Merck-Hitachi D-7000 (Chromatography Data Station – DAD Manager).

Isolation and characterization of the phenolic compounds

The crude material was dissolved in a small amount of methanol and fractionated on a Sephadex LH-20 (Pharmacia; Piscataway, NJ, USA) column using methanol as eluent. We obtained four fractions by monitoring under UV (360 nm). The extracts were evaporated and analyzed by HPLC. The main fraction was evaporated to obtain a syrup and resuspended in 60% methanol (in water) and centrifuged. The supernatant was chromatographed on a Lobar LiChroprep RP-18 Merck column (40 – 63 μm), using a Duramat pump (Merck), monitoring with UV light (360 nm). We was used a gradient system to elute (methanol:water) the sample. Ten fractions were collected and chromatographed by HPLC. Compound (**1**) (10 mg) was obtained pure in fraction 2, compound (**2**) (158 mg) in fraction 4, compound (**3**) (206 mg) in fraction 8, and compound (**4**) (42 mg) in fraction 10. Finally, these products were purified by preparative HPLC (Merck-Hitachi), equipped with a pump (model L-6000, Merck-Hitachi) and a UV detector (L-4000, Merck-Hitachi); purification was achieved on a column Spherisorb ODS-2 (25 × 1 cm, 5 μm particle size column). This purification was further tested by analytical HPLC.

Drugs and reagents

Acetic acid, formalin, indomethacin, morphine, 2,2,2- tribromoethanol, serotonin, complete Freund's adjuvant, capsaicin, λ-carrageenan (grade IV), 3,3′,5,5′-tetramethylbenzidine (TMB), histamine, sulfanilamide, *N*-(1-naphthyl)ethylenediamine dihydrocholoride, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and TNF-α were acquired from Sigma Chemical Co. (St Louis, MO, USA). Lipopolysaccharide (LPS) (*Escherichia coli*, serotype 055:B5) was purchased from Sigma (Deisenhofen, Germany). For other reagents, the suppliers are indicated below.

Animals and cell cultures

Swiss male mice (18 – 30 g) were housed at 21 ± 2 °C under a 12-h light-dark cycle. Food and water were offered *ad libitum*. The experiments were carried out in accordance with current guidelines for the care of laboratory animals [9]. In all the experiments, each group consisted of 6 mice.

RAW 264.7 cells (murine macrophage lineage; ATCC; Manassas, VA, USA) were cultured in Dulbecco's modified essential medium with 4 mM L-glutamine and 4.5 g/L glucose (DMEM, endotoxin level < 0.005 EU/mL; Bio Whittaker; Heidelberg, Germany), supplemented with 10% heat-inactivated fetal calf serum (Gibco/BRL Life Technologies; Eggenstein, Germany). Cells were maintained at 37 °C in an atmosphere with 5% CO_2 and used for experiments between the 4th and 12th passages.

HEK 293 cells (human embryonic kidney lineage, ACC 305; DSMZ – German Collection of Microorganisms and Cell Cultures; Braunschweig, Germany) were maintained in DMEM supplemented with 10% fetal bovine serum (Life Technologies; Eggenstein, Germany), 2 mM L-glutamine (Merck; Darmstadt, Germany) and 100 U/mL penicillin and 100 μg/mL streptomycin (PAN Biotech; Aidenbach, Germany). Cells were split 1 : 10 when reaching 85 – 90% confluence using 0.05% trypsin plus 0.02% EDTA in PBS.

Chemically-induced abdominal constrictions assay

The mice were treated with **G1** (1 or 10 mg/kg body weight) by the intraperitoneal route (*i.p.*) 30 min before *i.p.* injection of 0.6% acetic acid in PBS. Afterwards, the mice were placed in separate boxes and the number of abdominal constrictions was cumulatively counted, as previously described [6]. Indomethacin (1 mg/kg, *i.p.*) was used as the positive control and PBS was used as the negative one.

Formalin-induced nociception assay

The mice were treated with **G1** (1 or 10 mg/kg body weight, *i.p.*) 30 min before injection under the surface of the right hindpaw of 20 μL 2.5% formalin (0.92% formaldehyde) in PBS. Indomethacin (1 mg/kg) was used as the positive control and PBS as the negative one. One mouse of each group was observed simultaneously from 0 to 30 min following formalin injection. The amount of time spent licking the injected paw, indicative of pain, was monitored. After the first 5 minutes, the licking time monitored the neurogenic phase and the following 10 min the inflammatory phase [6].

Hot-plate test

The animals were treated with **G1** (1 or 10 mg/kg body weight, *i.p.*) 30 min before the test. Morphine (10 mg/kg, *i.p.*) was used as the positive control and PBS as the negative one. The mice were placed onto a heated surface of a glass cylinder (diameter of 24 cm), and the time until shaking or licking of the paws or jumping was recorded as response latency using a thermal analgesiometer hot plate (Ugo Basile; Comerio, Italy), according to the method described by Eddy and Leimback [10].

Serotonin- and CFA-induced paw edema assays

The animals were treated with **G1** by *i.p.* (1 or 10 mg/kg body weight) and *o.r.* (10 or 100 mg/kg body weight), 30 min before the injection of the inflammatory agents. Under slight anesthesia with 2,2,2-tribromoethanol (0.12 g/kg), the mice received an intradermal injection in one hindpaw of 20 μL serotonin (300 nmol/paw) or CFA (20 μL/paw) in PBS. As negative control, the contralateral paw received 20 μL PBS. At several times after injection of serotonin or CFA, edema was measured by use of an analytical balance and expressed in grams as the difference between the test and control paws [11].

Capsaicin-induced ear edema assay

The animals were treated with **G1** by *i.p.* (1 or 10 mg/kg body weight) and *o.r.* (10 or 100 mg/kg body weight). After 30 min, 20 μL capsaicin (250 μg/ear) were applied to the inner and outer surface of the right ear. The left ear received 20 μL PBS. Indomethacin.

Acute carrageenan-induced inflammatory reaction in the peritoneal cavity

The animals were treated with **G1** *i.p.* (1 or 10 mg/kg body weight) and *o.r.* (10 or 100 mg/kg body weight), 30 min before the administration of carageenan (100 μg/mL, *i.p.*). Indomethacin (1 mg/kg *i.p.* and 10 mg/kg *o.r.*) was used as the positive control. After 4 h, the peritoneal fluid was collected in sterile and heparinized PBS (2 mL), for quantification of the number of total cells using a Neubauer chamber and the differential counting of leukocytes after concentration of the exudate and staining with May-Grünwald-Giemsa. Myeloperoxidase activity was quantified by the H_2O_2-dependent oxidation of TMB, by addition of 25 μL peritoneal exudate, 25 μL 1.6 mM TMB and 100 μL 0.3 mM H_2O_2 in 96-well plates. The optical density was monitored at 450 nm, and the enzyme activity determined by comparison with a standard curve. Cell migration was quantified as previoulsy described [12].

Nitric oxide and cell viability quantification

RAW 264.7 were seeded in 96-well plates (4×10^4 cells/well), and after 2 days were stimulated or not with 1 μg/mL LPS in the absence or presence of **G1** (3 to 300 μg/mL) for 20 h. The generation of NO was assessed in the supernatant of cell cultures by quantification of nitrite using the Griess reaction [13]. Briefly, 100 μL of each supernatant were added to 96-wells plates together with 90 μL 1% sulfanilamide in 5% phosphoric acid plus 90 μL 0.1% *N*-(1-naphthyl)ethylenediamine dihydrocholoride in water, and the optical density was measured at 550 nm. Cell viability was determined by mitochondrial-dependent reduction of MTT (Sigma; St. Louis, MO, USA) to formazan quantified at 550 nm. After removal of the supernatant for nitrite determination, the cells were incubated with 0.5 mg/mL MTT at 37 °C for 45 min, the medium was aspirated, and 250 μL DMSO for 3 h in the dark for solubilization of formazan.

NF-κB transactivation activity

The transfection of HEK 293 cells was performed following the instructions of the manufacturer (Alexis Biochemicals; Grünberg, Germany). Briefly, 5×10^5 cells were seeded in Petri dishes (diameter = 6 cm) and after 24 h, they were transfected with pNF-κBluc and pRSV-β-gal plasmid using the Ca^{2+}-phosphate method [14]. The cells were plated in 24-well plates (1×10^5 cells/well) and, after incubation for 16 h, submitted to treatment for 2 h with 30 to 300 μg/mL **G1** or 10 μM MG132 (Calbiochem; La Jolla, CA, USA), a specific inhibitor of the nuclear factor κB (NF-κB). Subsequently the cells were stimulated for 6 h with 1 ng/mL TNF-α, washed with PBS and lysed with passive lysis buffer. The NF-κB activity was measured by the luciferase assay system according to the manufacturer's instructions (Promega; Heidelberg, Germany), using an AutoLumat Plus luminometer (Berthold; Bad Wildbad, Germany).

Statistical analysis

For the *in vivo* studies, the results are presented as the mean ± S.E.M., except for the ID_{50} values (i.e., the doses of extract necessary to reduce the response by 50% relative to the control value), expressed as the mean ± S.D. Differences between the *in vivo* experimental groups were evaluated using analysis of variance followed by Dunnett's multiple comparison test or by Student's *t*-test. For the *in vitro* studies, statistical analysis was carried out using unpaired Student's *t*-test. The IC_{50} values were determined from individual experiments by concentration-response curves and were expressed as mean ± S.E.M. *P* values less than 0.05 were considered significant.

Results

The chemical composition of **G1** was evaluated by HPLC analysis, showing high levels of the phenolic compounds 3,5-diprenyl-4-hydroxycinnamic acid and derivatives, 2,2-dimethyl-6-carboxy-

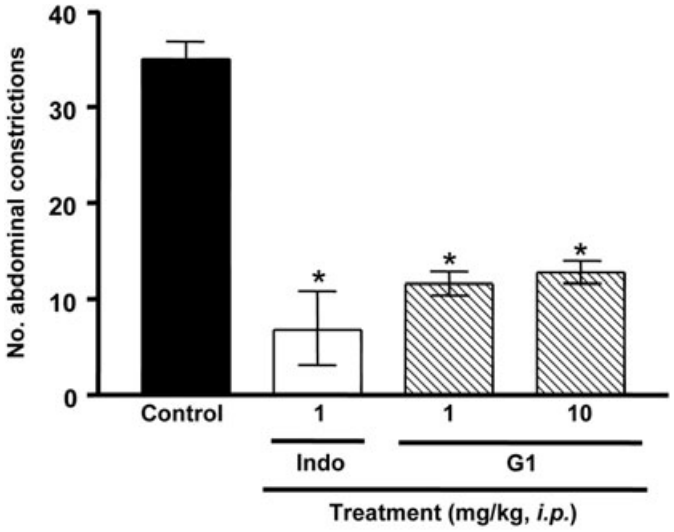

Fig. **1** Effect of the intraperitoneal administration of **G1** (1 and 10 mg/kg) or indomethacin (Indo) (1 mg/kg) in the acetic acid-induced pain model. Each value represents the mean ± S.E.M. of 6 animals, and asterisks indicate significant inhibition in the number of abdominal constrictions in relation to the untreated group, $P < 0.05$.

ethenyl-8-prenyl-2*H*-1-benzopyran, 3-prenyl-4-hydroxycinnamic, *p*-coumaric, caffeic acid, caffeoylquinic acids besides cinnamic acids and the flavonoids pinobanksin and kaempferol (Table **1**). The content of flavonoids corresponds to 22.37 mg/g dried extract.

G1 caused a significant inhibition of the number of abdominal constrictions induced by acetic acid, with an ID_{50} = 0.75 ± 0.05 mg/kg and maximal inhibition (MI) of 66.9% (Fig. **1**). When assessed in the formalin test, the extract inhibited significantly the induced nociception during the neurogenic phase (ID_{50} = 0.85 ± 0.07 mg/kg; MI = 61.4%) and the inflammatory phase (ID_{50} = 13.88 ± 1.12 mg/kg; MI = 36.0%) (Fig. **2**). In the hot-plate test, **G1** produced no significant effect, while indomethacin increased by 2.2-fold the time of latency (data not shown).

Paw edema induced by serotonin was significantly inhibited by treatment with **G1** at 1 mg/kg by *i.p.*, leading to an MI = 51.6% after 120 min (Fig. **3A**) and when the extract was given orally (10 mg/kg), an MI = 36% was achieved after 15 min (Fig. **3B**). In the model of CFA-induced paw inflammation, **G1** at 1 mg/kg by *i.p.* or at 10 or 100 mg/kg by *o.r.* also led to edema inhibition (Fig. **3C, D**). In this model, **G1** at 10 mg/kg by *i.p.* led to signs of debilitation in the mice.

Fig. **4** shows that **G1** administered by both routes significantly inhibited the ear edema induced by capsaicin. When given *i.p.*, the ID_{50} was estimated to be 1.09 ± 0.08 mg/kg, and the MI was 45.8%, while by *o.r.*, the corresponding values were 10.00 ± 0.90 mg/kg and 50%.

In the model of carrageenan-induced peritonitis, the basal concentration of cells in the peritoneal cavity was 0.6×10^6 cells/mL, while after 4 h, this concentration increased to 12.9×10^6 cells/mL, considered as 100% of cell migration. The percentage of neutrophil migration (84 ± 2%) was higher than that of mononuclear cells (16 ± 3%), a pattern typical of a carrageenan-induced peritonitis (data not shown). **G1** and indomethacin caused a statistically significant decrease in the number of total cells in the peritoneal cavity, showing inhibition of the inflammatory process (Fig. **5**). The decrease in the number of neutrophils was 69.05% and 73.81% after administration by *i.p.* of, respectively, 1 and 10 mg/kg of the extract, with an estimated IC_{50} = 0.72 ± 0.08 mg/kg. When **G1** was administered by *o.r.* the decrease was 42.5%, 65.0% and 67.5% for, respectively, the doses of 1, 10 and 100 mg/kg, with an IC_{50} value of 4.17 ± 0.50 mg/kg with a preferential migration of polymorphonuclear leukocytes. Indomethacin at 1 mg/kg (*i.p.*) and 10 mg/kg (*o.r.*) caused a decrease of neutrophil migration of, respectively, 70.6% and 69.0%. By both routes of administration, **G1** and indomethacin led to a statistically significant decrease in the number of neutrophils, which was proportional to the decrease of MPO activity.

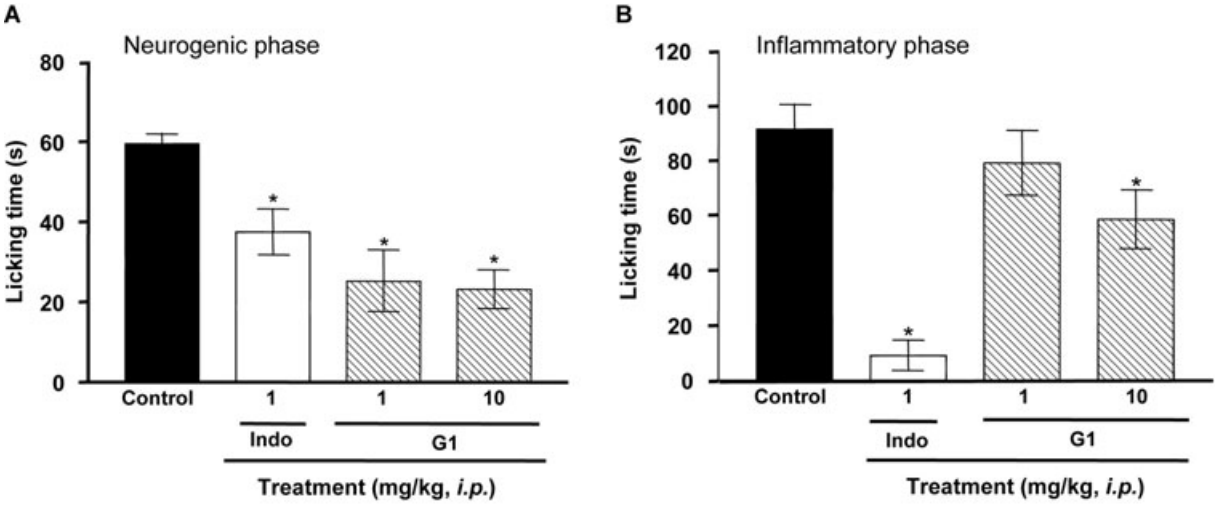

Fig. **2** Effect of the intraperitoneal administration of **G1** (1 and 10 mg/kg) or indomethacin (Indo) (1 mg/kg) in the formalin-induced nociception assay: (**A**) neurogenic phase; (**B**) inflammatory phase. Each value represents the mean ± S.E.M. of 6 animals, and asterisks indicate significant inhibition of the licking time in relation to the untreated group, $P < 0.05$.

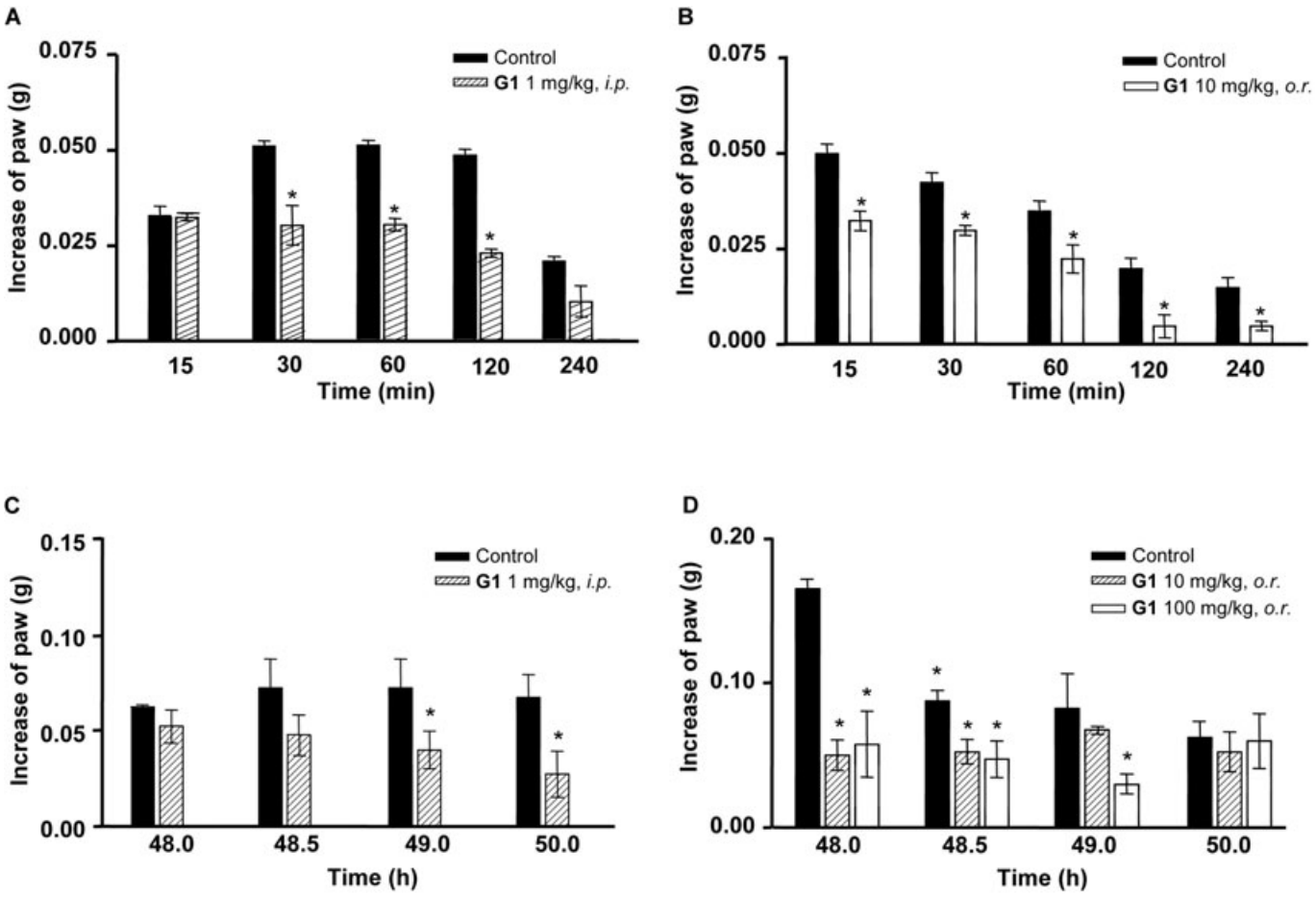

Fig. **3** Effect of **G1** in the paw oedema models induced by: (**A**) serotonin and the extract administered by *i.p.*; (**B**) serotonin and the extract administered by *o.r.*; (**C**) complete Freund's adjuvant and the extract administered by *i.p.*; (**D**) complete Freund's adjuvant and the extract administered by *o.r.* Each value represents the mean ± S.E.M. of 6 animals, and asterisks indicate significant inhibition of the paw weight in relation to the corresponding untreated groups, $P < 0.05$.

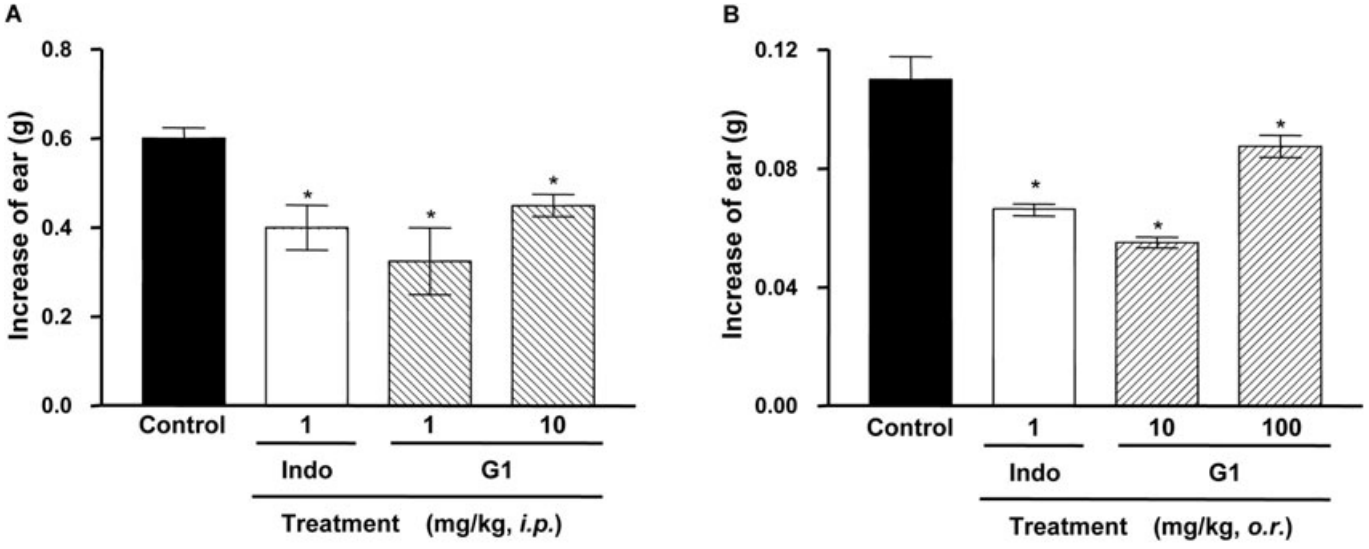

Fig. **4** Effect of the administration of **G1** in the capsaicin-induced ear oedema: (**A**) 1 or 10 mg/kg, *i.p.*; (**B**) 10 or 100 mg/kg, o.r. Each value represents the mean ± S.E.M. of 6 animals, and asterisks indicate significant inhibition of the paw weight in relation to the corresponding untreated groups, $P < 0.05$.

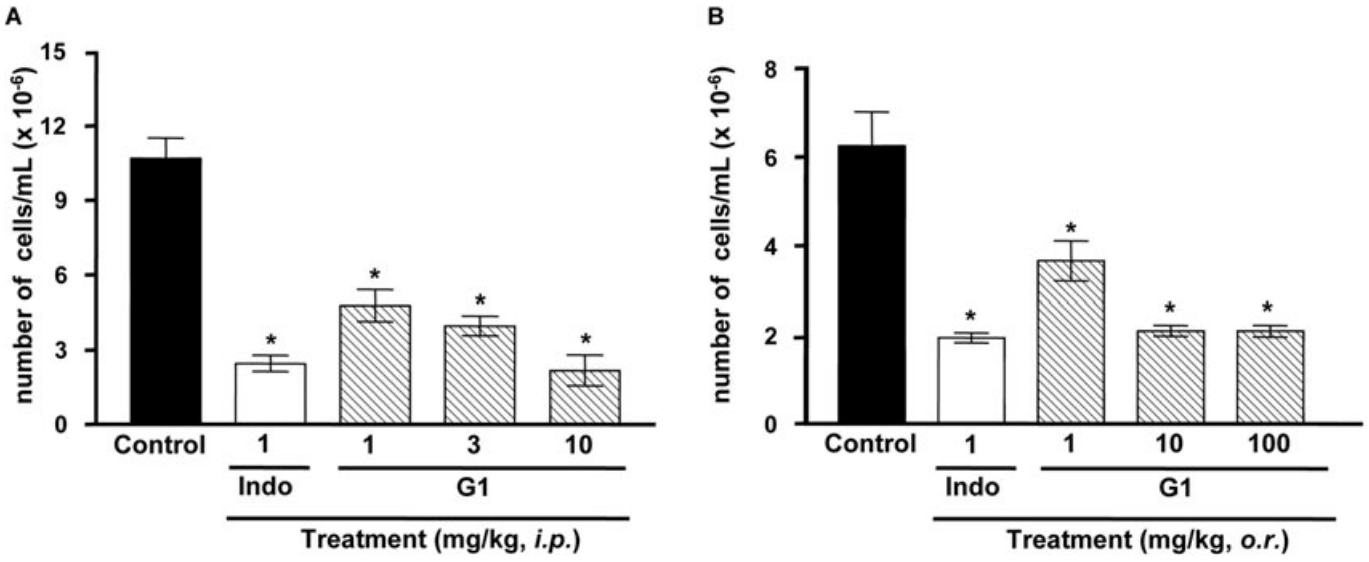

Fig. **5** Effect of the administration of **G1** (1 – 10 mg/kg, *i.p.* and 1 – 100 mg/kg, *o.r.*) and 1 mg/kg indomethacin (*i.p.* or *o.r.*) in the acute carrageenan-induced inflammatory reaction measured by the concentration of cells in the peritoneal fluid: (**A**) *i.p.*; (**B**) *o.r.* Each value represents the mean ± S.E.M. of 6 animals, and asterisks indicate significant inhibition of total number of cells in the peritoneal cavity in relation to the untreated groups, $P < 0.05$.

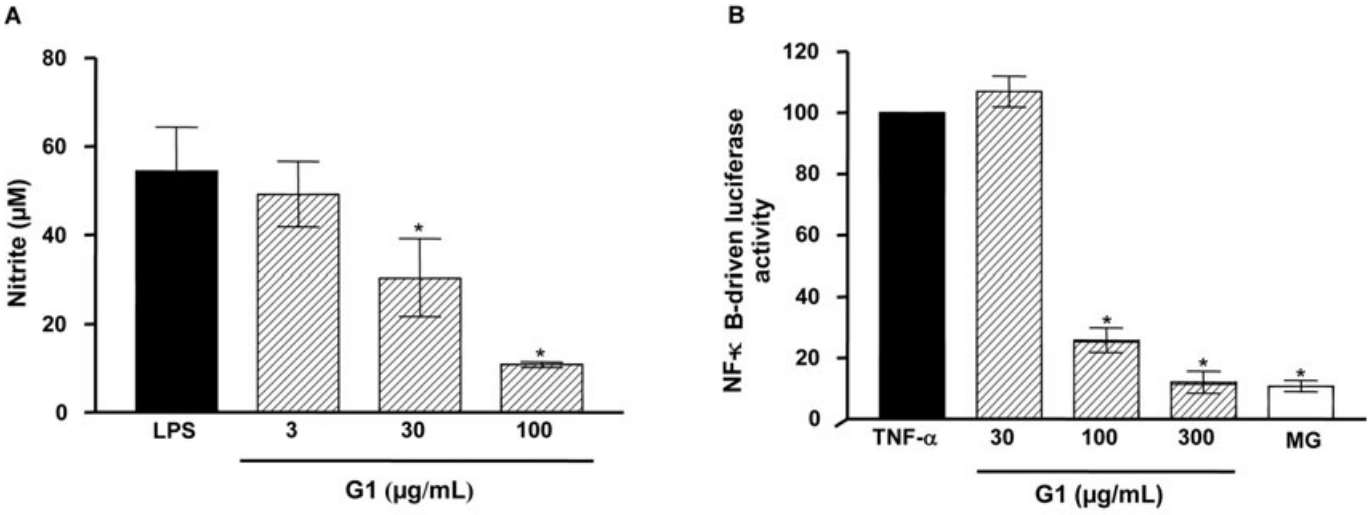

Fig. **6** Effect of treatment with **G1** of cell lineages: (**A**) nitrite production by RAW 264.7 cells stimulated with 1 μg/mL LPS for 20 h and treated with the extract (3 to 100 μg/mL); (**B**) luciferase activity in HEK 293 cell transiently transfected with a NF-κB-driven luciferase reporter gene by the extract (3 to 300 μg/mL) of 10 μM MG 132. Bars represents the mean ± S.E.M. of three independent experiments performed in triplicate, and asterisks indicate significant inhibition of the enzyme activity in relation to the untreated group, $P < 0.05$.

Treatment with **G1** led to a decrease in nitrite concentration in the supernatant of RAW 264.7 macrophages with an IC_{50} = 41.5 μg/mL, while in LPS-stimulated cells the nitrite concentration was 54.4 ± 10.0 μM (Fig. **6A**). To concentrations up to 100 μg/mL, the extract did not interfere with the viability of RAW 264.7 cells. In TNF-α-stimulated HEK 293 cells transfected with a NF-κB-driven luciferase reporter gene, **G1** reduced the luciferase activity with an IC_{50} = 200 μg/mL (Fig. **6B**).

Discussion

Brazilian propolis has been previously subdivided in four types based on a multivariate statistical analysis of the composition of a series of standardized ethanol extracts determined by HPLC [5]. The propolis sample employed in the present work is classified as a subtype of BRP(SP/MG), named BRP1, since it presents a high content of the bioactive compounds 3,5-diprenyl-4-hydroxycinnamic acid, 2,2-dimethyl-6-carboxyethenyl-8-prenyl-2*H*-1-benzopyran, 3-prenyl-4-hydroxycinnamic and *p*-coumaric acids and the absence of 2,2-dimethyl-6-carboxyethenyl-2*H*-1-benzopyran; also the flavonoids pinobanksin and kaempferol are present in high levels (22.37% of the dried extract), which is

a characteristic of green propolis samples collected in the State of Minas Gerais.

G1 showed significant analgesic and anti-inflammatory effects in mouse models. In models that induce pain by acetic acid or formalin, **G1** produced an effect similar to that of another Brazilian ethanol extract named **P1**[2], and both extracts were ineffective in the hot-plate test.

In the different models of inflammation the propolis extract was administered *i.p.* and *o.r.*, and our results indicate a satisfactory oral absorption of the bioactive components of **G1** leading to an effective anti-inflammatory activity. It is known in the literature that several vegetal extracts with important effects by *i.p.* lose their activity when administered orally [15].

The debilitation signs observed in mice injected with 10 mg/kg **G1** *i.p.*, after CFA administration, could be due to a systemic suppression of activities in neutrophils and macrophages induced by **G1** with an increase of lymphocyte activity induced by this agent. Possibly the axis of leukogenesis displaces the progenitor stem cells to differentiation to leukocytes, increasing the degenerative response induced by CFA in the joints and leading to a worsening of the animal status. On the other hand, the lower dose of 1 mg/kg **G1** showed an immunomodulatory effect, with regulation of the immunostimulatory and anti-inflammatory (suppresive) properties of propolis.

Mediators of inflammatory processes are involved in the paw or ear edema in mice induced by formalin or capsaicin [2], [16], [17]. The anti-edematogenic (vascular) effect of **G1** is possibly secondary to its anti-inflammatory activity, i.e., to the process of cell migration, involving interference of active principle(s) present in the extract with the synthesis of such mediators. Supporting this hypothesis, Mirzoeva and Calder [18] reported that an ethanol extract of propolis suppressed prostaglandin and leukotriene generation by peritoneal macrophages *in vitro* as well as during zymosan-induced acute peritoneal inflammation in mice.

The experiments with carrageenan showed clearly that propolis inhibits neutrophil mobilization to the peritoneal cavity. The decrease of MPO activity corroborates such inhibition in **G1**-treated mice, since this enzyme is absent in other types of leukocytes [19].

In vitro experiments demonstrated that **G1** inhibited the production of NO in the supernatant of RAW 264.7 macrophages and the NF-κB activity in TNF-α-stimulated HEK 293 transfected cells. Using RAW 264.7 cells, propolis was shown to inhibit inducible NOS (iNOS) expression, the enzyme activity and the binding activity of NF-κB, and, in transfected cells, to inhibit iNOS promoter activity through its NF-κB sites [20]. This nuclear factor is involved in the control of inflammatory responses, interfering with the production of iNOS and of the inducible isoform of cyclooxygenase COX-2 [15]. Flavonoids, found in high contents in **G1**, present among their broad range of properties, the ability to act as an antioxidant agent, scavenging free radicals, including nitric oxide radical, and also to interfere in inflammatory processes, inhibiting iNOS and COX-2 activities [21], [22], [23].

Despite the existence of several pharmacological studies with propolis extracts [1], the present work demonstrates that a typified Brazilian propolis extract, at low concentrations, induces anti-inflammatory and analgesic effects in mouse models. These observations together with the *in vitro* results led us to reinforce the hypothesis of Song and coworkers [20] that the anti-inflammatory effect of propolis may be due to inhibition of iNOS gene expression, through interference in NF-κB sites in the iNOS promoter. The propolis components responsible for the pharmacological activities are currently not known, but the flavonoids pinobanksin and kaempferol and the phenolic acids 3,5-diprenyl-4-hydroxycinnamic acid, 2,2-dimethyl-6-carboxyethenyl-8-prenyl-2*H*-1-benzopyran and 3-prenyl-4-hydroxycinnamic are strong candidates. Further experiments are now in progress aiming to investigate the influence of these compounds in these pharmacological properties of Brazilian green propolis.

Acknowledgements

This study was supported by grants from the Universidade do Sul de Santa Catarina (UNISUL) and Fundação de Amparo à Pesquisa do Estado de São Paulo (Fapesp # 95/09306 – 5) in Brazil and Deutscher Akademischer Austauschdienst (DAAD) Bonn in Germany. We like to thank Mrs. Elfriede Eppinger (University of Munich, Germany) for her excellent technical assistance.

References

[1] De Castro SL. Propolis: biological and pharmacological activities. Therapeutic uses of this bee-product. Annu Rev Biol Sci 2001; 3: 49 – 83
[2] De Campos RO, Paulino N, da Silva CH, Scremin A, Calixto JB. Anti-hyperalgesic effect of an ethanolic extract of propolis in mice and rats. J Pharm Pharmacol 1998; 50: 1187 – 93
[3] Menezes H, Alvarez JM, Almeida E. Mouse ear edema modulation by different propolis ethanol extracts. Arzneimittelforschung 1999; 49: 705 – 7
[4] Bankova VS, De Castro SL, Marcucci MC. Propolis: recent advances in the chemistry and plant origin. Apidologie 2000; 31: 3 – 15
[5] Marcucci MC. Process to typing natural products. Brazilian National Institute for Intellectual Property (INPI) PI 0105471 – 6, 2000
[6] Paulino N, Dantas AP, Bankova VS, Longhi DT, Scremin A, De Castro SL et al. Bulgarian propolis induces analgesic and anti-inflammatory effects in mice and inhibits *in vitro* contraction of airway smooth muscle. J Pharmacol Sci 2003; 93: 307 – 13
[7] Marcucci MC, Ferreres F, Garcia-Viguera C, Bankova VS, De Castro SL, Dantas AP et al. Phenolic compounds from Brazilian propolis with pharmacological activities. J Ethnopharmacol 2001; 74: 105 – 12
[8] Paulino N, Scremin FM, Raichaski LB, Marcucci MC, Scremin A, Calixto JB. Mechanisms involved in the relaxant action of the ethanolic extract of propolis in the guinea-pig trachea *in vitro*. J Pharm Pharmacol 2002; 54: 845 – 52
[9] Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983; 6: 109 – 10
[10] Eddy NB, Leimback D. Synthetic analgesics. II - Dithienylbutenyl and dithienylbutylamines. J Pharmacol Exp Ther 1953; 107: 385 – 93
[11] Calixto JB, Zanini Jr JC, Cruz AB, Yunes RA, Medeiros YS. Extract and compounds obtained from *Mandevilla velutina* inhibit arachidonic acid-induced ear oedema in mice, but not rat stomach contraction. Prostaglandins 1991; 41: 515 – 26
[12] Carvalho JC, Sertie JA, Barbosa MV, Patricio KC, Caputo LR, Sarti SJ et al. Anti-inflammatory activity of the crude extract from the fruits of *Pterodon emarginatus* Vog. J Ethnopharmacol 1999; 64: 127 – 33
[13] Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite and [^{15}N] nitrate in biological fluids. Anal Biochem 1984; 126: 131 – 8

[14] Shetty S, Brown Gladden J, Henson ES, Hu X, Villanueva J, Haney N et al. Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) up-regulates death receptor 5 (DR5) mediated by NF-κB activation in epithelial derived cell lines. Apoptosis 2002; 7: 413 – 20
[15] Calixto JB, Otuki MF, Santos AR. Anti-Inflammatory compounds of plant origin. Part I. Action on arachidonic acid pathway, nitric oxide and nuclear factor kappa (NF-κB). Planta Med 2004; 69: 973 – 83
[16] Malmberg AB, Yaksh TL. Cyclooxygenase inhibition and the spinal release of prostaglandin E_2 and amino acids evoked by paw formalin injection: A microdialysis study in unanesthetized rats. J Neurosci 1995; 15: 2768 – 76
[17] Doak GJ, Sawynok J. Formalin-induced nociceptive behavior and edema: Involvement of multiple peripheral 5-hydroxytryptamine receptor subtypes. Neuroscience 1997; 80: 939 – 49
[18] Mirzoeva OK, Calder PC. The effect of propolis and its components on eicosanoid production during the inflammatory response. Prostaglandins Leuk Essent Fatty Acids 1996; 55: 441 – 9
[19] Dallegri F, Ottonello L. Tissue injury in neutrophilic inflammation. Inflamm Res 1997; 46: 382 – 91
[20] Song YS, Park EH, Hur GM, Ryu YS, Kim YM, Jin C. Ethanol extract of propolis inhibits nitric oxide synthase gene expression and enzyme activity. J Ethnopharmacol 2002; 80: 155 – 61
[21] Olszanecki R, Gebska A, Kozlovski VI, Gryglewski RJ. Flavonoids and nitric oxide synthase. J Physiol Pharmacol 2002; 53: 571 – 84
[22] Rosenkranz HS, Thampatty BP. SAR: flavonoids and COX-2 inhibition. Oncol Res 2003; 13: 529 – 35
[23] Banerjee T, VanderVliet A, Ziboh VA. Downregulation of COX-2 and iNOS by amentoflavone and quercetin in A549 human lung adenocarcinoma cell line. Prostaglandins Leuk Essent Fatty Acids 2002; 66: 485 – 92