The chemical composition and pharmacological activities of geopropolis produced by *Melipona fasciculata* Smith in Northeast Brazil

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ABSTRACT

**Aim:** Geopropolis is produced by stingless bees from resinous materials of plants, adding salivary secretions, wax, mud or clay, and has been used in folk medicine for the treatment of respiratory diseases and dermatoses. Therefore, it is important to study its antibacterial, antitumor and immunomodulatory properties to confirm the ethnopharmacological applications. This work aimed to evaluate its chemical composition, and its antimicrobial, anti-tumoral and immunomodulatory activities. **Materials and Methods:** Geopropolis composition was investigated using GC-MS analysis. Antibacterial tests were performed to determine the minimum inhibitory concentration. **Results:** The major constituents of geopropolis compounds were carbohydrates and their derivatives, triterpenes, anacardic acid, alkylresorcinols, and sugar alcohols. Geopropolis alone showed no antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*, but its combination with chloramphenicol exerted a greater actions against *S. aureus* than chloramphenicol alone. Geopropolis exhibited a cytostatic action toward human laryngeal epidermoid carcinoma cells and stimulated tumor necrosis factor alpha and interleukin-10 production by human monocytes, showing an activator profile for human monocytes. **Conclusion:** The synergistic effect of geopropolis and chloramphenicol deserves further investigation due to bacterial resistance to antibiotics. Geopropolis also displayed antitumoral and immunomodulatory activity, and its biological properties may be due to triterpenes - one of its major chemical constituents.

KEY WORDS: Chemical composition, cytokine, geopropolis, human laryngeal epidermoid-2 cells, pharmacological activities

INTRODUCTION

Africanized honeybees were produced in Brazil accidentally by cross-breeding African and European honeybees, spreading throughout South, Central and North America. Stingless bees or meliponines are a large group of bees found in most tropical or subtropical regions of the world. Although they possess stingers, they are nonfunctional and cannot be used for their defense.

There are few studies on the pharmacological activities of geopropolis produced by *Melipona fasciculata* Smith, whose products have been used for centuries in Maranhão State (northeast Brazil) by the indigenous population. In addition, geopropolis composition depends on the local flora and geographic region. It is important to standardize chemical and biological assays in order to link the biological properties of geopropolis to its chemical profile.

Stingless bees may produce propolis as well as geopropolis. Propolis is a resinous product made by bees from different parts of plants, adding mandibular secretions, pollen, and wax. Propolis is used by bees to seal or fill cracks in beehives for thermal isolation, and resins found in propolis are a form of social immunity for bees protecting against pathogenic
microorganisms [1,2]. Propolis exhibits several pharmacological properties such as antimicrobial, antioxidant, hepatoprotective, anti-cancer, immunomodulatory, among others. Geopropolis composition includes soil, plant material, gland secretions, wax, and pollen. Geopropolis has been used in folk medicine for the treatment of respiratory diseases and dermatoses [3].

The anti-microbial activity of propolis produced by Africanized honeybees has been extensively investigated and in recent years there has been a great interest in the antibacterial properties of propolis and geopropolis produced by stingless bees [4,5]. Geopropolis produced by Melipona compressipes fasciculata exerted antibacterial effects in vitro against Streptococcus mutans isolated from the oral cavity of young individuals, suggesting its use as an alternative for preventing dental caries [6]. The antimicrobial action of geopropolis produced by M. fasciculata Smith was analyzed against S. mutans, Lactobacillus acidophilus and Candida albicans, confirming its potential to control or prevent infections in the oral cavity [7].

Regarding the anti-tumoral activity, Araújo et al. investigated the effects of propolis produced by Scaptotrigona aff. postica on Ehrlich tumor development in mice [8]. Propolis inhibited the tumor growth and increased the cell number in bone marrow and spleen. Borges et al. also observed the anti-proliferative action of propolis produced by Scaptotrigona spp. on human glioblastoma (U251 and U343) [9]. Previous findings of our group have shown that geopropolis exerted a cytotoxic action against canine osteosarcoma cells [10]. Nevertheless, there is no data in literature concerning the effects of geopropolis produced by M. fasciculata Smith on human laryngeal epidermoid carcinoma (HEP-2) cells, which are derived from laryngeal carcinoma cells of human nasopharyngeal mucosa [6].

Research on the immunomodulatory activity of geopropolis is scarce. Libério et al. assessed the serum cytokine concentration of mice that received a gel prepared with geopropolis produced by M. fasciculata in the oral cavity [7]. With this, we wish to analyze the immunomodulatory activity of geopropolis on human monocytes for the first time, since natural products are a promising source for the discovery of new immunomodulatory pharmaceuticals.

Since few papers investigating the pharmacological properties of geopropolis have been published to date, we wish to present its effects against bacteria, tumors and its immunomodulatory action in humans. The goal of this work was to research geopropolis composition by gas chromatography-mass spectrometry (GC-MS), and to evaluate the antibacterial activity of geopropolis produced by M. fasciculata Smith against Staphylococcus aureus and Escherichia coli strains by determining the minimum inhibitory concentration and time kill curve. A possible interaction (synergism or antagonism) between geopropolis and antibiotics was also investigated. The cytotoxic activity of geopropolis toward HEP-2 cells was compared to carboplatin, used medically to treat several tumors, and its immunomodulatory action was assessed by analyzing cytokine (tumor necrosis factor alpha [TNF-α] and interleukin-10 [IL-10]) production in human monocytes.

**MATERIALS AND METHODS**

**Geopropolis Sample**

Geopropolis was produced by M. fasciculata Smith in Palmeirândia, Maranhão State, northeast Brazil (2° 39’ S, 44° 55’ O). Ecosystems of this region include mangroves, flooding fields, lagoons, forests and babassu fields. Geopropolis samples were kept at 4°C before extraction. A 40 g geopropolis sample was ground and macerated in 70% ethanol at room temperature under moderate shaking. After 24 h, the extract was filtered, and the dry weight of geopropolis hydroalcoholic extract was calculated (13 mg/mL) [11].

**GC-MS Analysis**

Geopropolis chemical composition was investigated using GC-MS analysis in the Institute of Organic Chemistry with Centre of Phytochemistry, Bulgaria. Analysis was performed with a Hewlett Packard Gas Chromatograph 5890 Series II Plus linked to a Hewlett Packard 5972 mass spectrometer system equipped with a 25 m long, 0.25 mm id, 0.5 µm film thickness HP5-MS capillary column. The temperature was programmed from 100°C to 310°C at a rate of 5°C/min. Helium was used as a carrier gas, flow rate 0.7 mL/min. Split ratio 1:80, injector temperature 280°C. The ionization voltage was 70 eV.

The silylation procedure was carried out mixing 5 mg of dry ethanol extract with 50 µL of dry (water-free) pyridine and 75 µL of bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and heated at 80°C for 20 min. The silylated extract was analyzed by GC-MS. The identification was accomplished using computer searches on a NIST98 MS data library. In some cases, when identical spectra have not been found, only the structural type of the corresponding component was proposed on the basis of its mass-spectral fragmentation. Reference compounds were co-chromatographed to confirm GC retention times.

**Bacterial Strains and Susceptibility Tests**

S. aureus (n = 51) and E. coli (n = 15) strains were isolated from patients of the Botucatu Medical School, UNESP. American Type Culture Collection (ATCC) strains (S. aureus ATCC 25923 and E. coli ATCC 25922) were also used in the research.

Susceptibility tests were performed according to the Clinical and Laboratory Standards Institute, and minimum inhibitory concentration (MIC<sub>min</sub>) values were determined [12,13]. Bacterial strains were inoculated in Brain Heart Infusion (BHI – Difco, USA) at 37°C for 24 h and standardized at 0.5 on the McFarland scale in sterile saline. Dilutions of each strain were performed to obtain bacterial suspensions with around 1 × 10<sup>6</sup> colony-forming units (CFU)/mL. Bacterial strains were inoculated in Petri dishes containing Mueller Hinton Agar (MHA - Difco, USA) and geopropolis hydroalcoholic extract ranging from 3% to 20% v/v (390-2600 µg/mL) using a Steer’s multiple inoculator, and incubated at 37°C for
24 h. Control plates contained only 70% ethanol in the same concentrations found in geopropolis. MIC<sub>90%</sub> was considered as the lowest concentration of geopropolis able to inhibit 90% of microorganisms, showing no visible growth or haze on the surface of the culture medium.

**Time Kill Curve and Synergistic Effects of Geopropolis with Antimicrobial Drugs**

Time kill curve of *S. aureus* and *E. coli* was carried out to verify a possible bactericidal or bacteriostatic effect of geopropolis extract over time. Bacterial suspensions (1 × 10<sup>8</sup> CFU/mL) were inoculated in BHI plus Tween 80% (0.5% v/v) containing the MIC<sub>90%</sub> of geopropolis or 70% ethanol for 3, 6, 9 and 24 h at 37°C. After each period, aliquots were taken and plated on Plate Count Agar (Difco, USA) by the pour plate method. After 24 h at 37°C, CFU were counted, and the survival percentage was calculated [4].

For synergistic assays, *S. aureus* and *E. coli* (*n* = 10) and one ATCC of each strain were used. Strains were grown in BHI broth at 35°C for 18 h and, after this period, microorganisms were standardized in a sterile physiological solution using 0.5 McFarland scale. The synergistic effect of geopropolis with antibiotics was established by the disc diffusion method on MHA containing ½ or ¼ of geopropolis (MIC<sub>50µ</sub>) or ½ or ¼ of 70% ethanol (MIC<sub>40µ</sub>) and adding the discs containing chloramphenicol (50 µg), gentamicin (10 µg), tetracycline (30 µg), ciprofloxacin (5 µg) and oxacillin (1 µg) (Sigma, USA) and. Control plates contained only MHA. Plates were incubated at 37°C for 24 h and the growth inhibition zones were measured around the discs. The effects of geopropolis in association with antibiotics were considered as either synergistic (median potency was higher than antibiotic alone), antagonistic (median potency was lower than antibiotic alone), or indifferent (median not statistically different to control).

**HEp-2 Cells and Cytotoxic Assay**

HEp-2 cells were grown in 25 cm<sup>2</sup> flasks in minimum essential media (MEM) (Cultilab, Brazil) supplemented with 10% fetal bovine serum (FBS - Cultilab) and gentamicin (40 mg/mL - Gibco, UK) at 37 °C and 5% CO<sub>2</sub>. After 80% confluent monolayers, 1.2 mL of trypsin (0.2% trypsin in 5% ethylenediaminetetraacetic acid [EDTA]) were added for cell detachment. Cells were counted using a hemocytometer, adjusting to 2 × 10<sup>5</sup> cells/mL in 96 wells U-bottom plates. After 24 h at 37°C, adherent cells were incubated with geopropolis hydroalcoholic extract (5, 10, 25, and 100 µg/mL) or 70% ethanol (0.03, 0.06, 0.15, 0.29 and 0.59%) for 6, 24, 48 and 72 h [14]. Carborplatin (Darrow-Vancel® Laboratories A/S) was used as a positive control at 100, 200, 300, 400 and 500 µmol/L [15]. Before the assays, geopropolis and carboplatin were filtered using a PES membrane (pore size 0.22 µm - TTP, Switzerland). Control cells were incubated only with MEM. All experiments were performed in triplicate with 5 repetitions of the assays.

Cell viability was determined by the reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide - Sigma-Aldrich, USA). After cell incubation with the stimuli, supernatants were removed, and 100 µL of MTT (1 mg/mL) dissolved in complete MEM was added. After 3 h at 37°C, MTT was removed, and 100 µL of dimethyl sulfoxide was added. Plates were read at 540 nm, and the percentage of cell viability was calculated.

**Human Monocytes and Cytokine Determination**

Ten healthy blood donors (ageing 20-50 years) were included in the present work, which was approved by the Ethics Committee of Botucatu Medical School (CEP 3599-2009). Participants were informed and signed their consent. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized (50 U/mL heparin) venous blood using Ficoll-Hypaque (density = 1.077 - Sigma, USA). Briefly, 20 mL of heparinized blood were added to an equal volume of RPMI 1640 culture medium containing 2 mM L-glutamine, 10% heat-inactivated fetal calf serum, 20 mM HEPES, and 40 µg/mL gentamicin. Samples were added to 4 mL of Ficoll-Hypaque and centrifuged at 400 g for 30 min at room temperature. The interface layer of the PBMC was taken and washed twice with phosphate buffer saline 0.1 M, pH = 7 containing 0.05 mM EDTA and once with RPMI medium at 300 g for 10 min. Cell viability, as determined by neutral red (0.02%) staining, was > 95% in all experiments. Cells were resuspended in a final concentration of 1 × 10<sup>6</sup> monocytes/mL in RPMI medium supplemented with fetal calf serum.

To assess cell viability, geopropolis was diluted in RPMI medium supplemented with 10% FBS, achieving the following concentrations: 5, 10, 25, 50 and 100 µg/mL. Extracts were filtered using a PES membrane (pore size 0.22 µm - TTP, Switzerland). The same procedure was performed with geopropolis solvent (70% ethanol) to obtain 0.03, 0.06, 0.15, 0.29 and 0.59%, which are the respective concentrations of alcohol found in geopropolis concentrations. Control cells were incubated with culture medium alone. Cell viability was assessed using the MTT assay, as previously described, and the percentage of cell viability was calculated in comparison to control (considered as 100%).

For cytokine determination, monocyte cultures (1 × 10<sup>6</sup> cells/mL) were incubated in 24-well plates at 37°C with geopropolis at nontoxic concentrations (5, 10, 25, and 50 µg/mL) or lipopolysaccharide (LPS, from *E. coli* 055:B5 - 10 µg/mL). After 18 h, supernatants were collected and stored at −70°C for cytokine determination.

TNF-α and IL-10 production were measured by enzyme-linked immunosorbent assay (ELISA), according to manufacturer’s instructions (eBiosciences). Briefly, a 96-well flat bottom microtiter plate (Nunc, USA) was coated with capture antibody specific for each cytokine and incubated overnight at 4°C in a humid chamber. The plate was washed and blocked with 0.1% bovine serum albumin before 100 µL of the supernatants, and
serially diluted specific standards were added to the respective wells. Following a series of washing, the cytokine was detected using the specific avidin-peroxidase conjugated detection antibody. The substrate was added to each well and, after color development, the plate was read at 492 nm, using an ELISA plate reader.

**Statistical Analysis**

Bacterial growth and the interaction between geopropolis and antimicrobial drugs were analyzed using nonparametric Kruskal-Wallis test to compare independent treatments. The Dunn test was used for multiple comparisons (P < 0.05).

For cytotoxic and immunomodulatory assays, analysis of variance was employed, followed by the Tukey test (P < 0.05).

**RESULTS**

**Geopropolis Chemical Analysis**

Geopropolis was a dark brown, brittle solid with a resinous odor and bitter taste. The major chemicals and chemical classes identified by GC-MS were carbohydrates and their derivatives (19.8% of TIC), triterpenes (15.9), anacardic acid (8.3%), alkylresorcinols (5.9%), and sugar alcohols (5.0%) [Table 1 and Supplementary Figure 1].

**Geopropolis Effects on S. aureus and E. coli Strains**

Geopropolis extract showed an inhibitory activity for S. aureus and E. coli only at high concentrations, and MIC90 values (% v/v) were 15.36% (1997 µg/mL) and 13.75% (1788 µg/m004C), respectively (P > 0.05). Ethanol 70% showed similar MIC90 values for both strains (15.70% and 15.77%, respectively).

Table 1: Geopropolis constituents after silylation by GC-MS. TIC: Total ion chromatogram

| Compound                        | % of TIC | Compound                        | % of TIC |
|---------------------------------|----------|---------------------------------|----------|
| Carbohydrates and their derivatives | 19.8     | Alkylresorcinols                | 5.9      |
| Hexoses                         | 11.9     | Heptadecylresorcinol<sup>a</sup> | 1.5      |
| Disaccharides                   | 4.1      | Nonadecenylresorcinol (isomer)<sup>b</sup> | 1.3      |
| Glucuronic acid<sup>c</sup>     | 2.6      | Heptadecylresorcinol<sup>a</sup> | 1.2      |
| Pentoses                        | 1.2      | Pentadecylresorcinol<sup>b</sup> | 1.1      |
| Triterpenes                     | 15.9     | Nonadecenylresorcinol<sup>b</sup> | 0.9      |
| Lupeol                          | 7.3      | Heptadecadienylresorcinol<sup>b</sup> | 0.8      |
| Beta-amyrin*                    | 2.4      | Sugar alcohols                  | 5.0      |
| Triterpenic ketone (M + 424; fragment ions 69 (100%), 95, 147, 286, 355, 409) | 2.0      | Xylitol<sup>d</sup>            | 2.1      |
| Alpha-amyrite*                  | 1.8      | Glucitol<sup>d</sup>           | 1.7      |
| Beta-amyrenone<sup>e</sup>      | 1.4      | Sugar alcohol<sup>f</sup>      | 1.2      |
| Alpha-amy renone<sup>e</sup>    | 1.0      | Others                          | 5.1      |
| Anacardic acid                  | 8.3      | Inositol<sup>e</sup>           | 2.4      |
| Heptadecenyl salicylic acid isomer<sup>b</sup> | 2.8      | Glycerol<sup>d</sup>           | 2.2      |
| Nonadecenyl salicylic acid isomer<sup>e</sup> | 2.4      | Methylmalonic acid<sup>d</sup> | 0.5      |
| Nonadecenyl salicylic acid<sup>e</sup> | 1.6      |                                  |          |
| Heptadecyl salicylic acid<sup>f</sup> | 1.5      |                                  |          |
| Pentadecyl salicylic acid<sup>b</sup> | 1.0      |                                  |          |

*Identified by comparison of RT and MS fragmentation with reference substances, <sup>a</sup>Identified by comparison with literature spectra [32], <sup>b</sup>Identified by mass spectra, compared to the spectrum of reference substance pentadecyl salicylic acid, <sup>c</sup>Identified by comparison with literature spectra [33], <sup>d</sup>Identified using commercial libraries

A decreased S. aureus CFU was seen 24 h after incubation with geopropolis or 70% ethanol [Figure 1a], with an inhibitory effect after 9 h incubation using 15.36% v/v (1997 µg/mL) [Figure 1c]. E. coli was also susceptible to geopropolis using 13.75% v/v (1788 µg/mL) [Figure 1b], but a reduction of CFU was only seen after 24 h incubation. The effect of 70% ethanol was observed after 9 h incubation [Figure 1d]. A synergistic action of geopropolis was seen only with chloramphenicol on S. aureus strains, but not on E. coli [Table 2].

**Geopropolis and Carboplatin Effects on HEp-2 Cells Viability**

A significant decrease in cell viability was seen after 6 h of incubation with 50 and 100 µg/mL of geopropolis extract. After 24, 48 and 72 h of incubation, there was a significant decrease in cell viability from 25 to 100 µg/mL (P < 0.0001). There was an interaction effect between concentration and incubation time on cell viability (P < 0.0001). Control (70% ethanol) had no effect on HEp-2 cells viability [Figure 2]. Carboplatin (100, 200 and 300 µmol/L) exerted no effect on HEp-2 cells, and 400 and 500 µmol/L showed only a slight inhibitory effect, suggesting the resistance of such cells to this drug [Figure 2].

**Geopropolis Effects on Monocyte Viability and Cytokine Production**

Only the highest concentration of geopropolis extract (100 µg/mL) exerted a cytotoxic effect on monocytes (P < 0.0001) [Figure 3]. Thus, cytokine assays were carried out using only noncytotoxic concentrations. Geopropolis (10, 25 and 50 µg/mL) increased significantly TNF-α production by monocytes (P < 0.0001). An increased IL-10 production was also seen after incubation with geopropolis compared to control (P < 0.0001), and the concentrations 10 and 25 µg/mL.
presented an immunostimulatory profile similar to that of LPS (positive control) [Figure 3]. Geopropolis solvent (70% ethanol) did not interfere with cytokine production (data not shown).

**DISCUSSION**

Carbohydrates and triterpenes were the major chemical constituents found in geopropolis sample collected in Palmeirândia. Libério *et al.* [7] analyzed 3 samples collected in Maranhão State: The lowest concentration of phenol content and the highest concentration of flavonoids were observed in the sample from Palmeirândia. Triterpenes were also detected in this sample. The chemical composition of Brazilian geopropolis samples produced by other stingless bees (Melipona compressipes, Melipona quadrifasciata anthidioides and Tetragonula clavipes) was evaluated by GC-MS, revealing that the main compounds were phenolics and triterpenes [16]. Geopropolis chemical composition depends on the local flora and geographic region, and this aspect is extremely important in order to link its biological properties to its chemical profile, and establish a possible standardization of the assays.

Geopropolis produced by *M. fasciculata* showed an antimicrobial activity against *S. mutans, L. acidophilus* and *C. albicans* and high flavonoids content [7]. Our geopropolis sample was not efficient for bacterial strains, although it was collected in the same region (Palmeirândia) of the sample used by Libério *et al.* [7]. Some factors may have affected the results, such as the yield of the extract, geopropolis solubility in the culture medium and different methodological approaches [13]. Biological activities of propolis or geopropolis samples depend on their chemical composition, which is related to the plant material that bees collect. In general, Africanized honeybees visit predominantly the same plants to produce propolis, and its chemical composition is qualitatively the same in the geographic region where it was produced [17]. On the other hand, propolis and geopropolis produced by stingless bees show a wide variation even among samples from the same region, since they collect material from plants near their hives, what may explain the differences in the pharmacological activities of such samples [18].

| Groups               | Chloramphenicol (30 µg) | Gentamicin (10 µg) | Tetracycline (30 µg) | Ciprofloxacin (5 µg) | Oxacillin (1 µg) |
|----------------------|-------------------------|--------------------|---------------------|----------------------|-----------------|
| **S. aureus**        |                         |                    |                     |                      |                 |
| Control              | 25.0 (24, 29)           | 22.0 (12, 22)      | 14.5 (12, 30)       | 25.0 (23, 27)        | 18.5 (0, 19)    |
| ¼ geopropolis (499 µg/mL) | 30.0* (26, 32)       | 15.0 (15, 16)      | 15.5 (14, 30)       | 24.0 (19, 25)        | 17.5 (0, 19)    |
| ½ geopropolis (998 µg/mL) | 31.5* (30, 38)       | 13.0 (13, 14)      | 19.0 (14, 32)       | 21.0 (20, 22)        | 19.5 (0, 21)    |
| ¼ 70% ethanol (3.92% v/v) | 28.5 (25, 30)        | 22.0 (12, 23)      | 14.0 (13, 31)       | 26.5 (22, 27)        | 18.5 (0, 20)    |
| ½ 70% ethanol (7.85% v/v) | 23.0 (19, 26)        | 23.0 (19, 26)      | 12.5 (10, 23)       | 9.0 (0, 12)          | 27.5 (22, 34)   |
| **E. coli**          |                         |                    |                     |                      |                 |
| Control              | 22.0 (16, 23)          | 20.5 (19, 21)      | 22.0 (7, 25)        | 31.5 (28, 32)        | 0               |
| ¼ geopropolis (447 µg/mL) | 19.0 (14, 22)        | 14.5 (14, 15)      | 19.0 (0, 22)        | 24.5 (20, 30)        | 0               |
| ½ geopropolis (894 µg/mL) | 19.0 (15, 22)        | 12.0 (12, 12)      | 18.5 (0, 21)        | 21.5 (19, 24)        | 0               |
| ¼ 70% ethanol (3.94% v/v) | 20.5 (16, 22)        | 20.5 (20, 22)      | 23.0 (8, 26)        | 31.5 (26, 35)        | 0               |
| ½ 70% ethanol (7.89% v/v) | 20.0 (18, 21)        | 23.0 (22, 24)      | 26.0 (8, 29)        | 31.5 (28, 32)        | 0               |

*(*P*<0.05 vs. control), S. aureus: Staphylococcus aureus, E. coli: Escherichia coli
Time kill curves were carried out to verify a possible bactericidal or bacteriostatic activity of geopropolis. A decreased S. aureus CFU was seen after 24 h incubation with geopropolis or 70% ethanol, with an inhibitory effect after 9 h incubation. The antibacterial activity of propolis produced by *Apis mellifera* was evaluated against Gram-positive and Gram-negative bacteria, and inhibitory effects on *S. aureus* were seen after 6 h incubation with propolis at a lower concentration (0.5% v/v) [4]. In our study, this effect was observed after 9 h incubation using 15.36% v/v, what suggests that geopropolis was not as efficient as propolis.

*E. coli* was also susceptible to geopropolis, but a reduction of CFU was observed after 24 h incubation. The antibacterial effect of 70% ethanol was observed after 9 h incubation. Similar results were obtained with *E. coli* using propolis produced by *A. mellifera* (MIC<sub>90</sub> = 8% v/v), with a decreased CFU only after 24 h incubation, with no inhibitory effect of 70% ethanol [4]. These authors also reported that propolis was less efficient against Gram-negative bacteria than Gram-positive ones, since the former have a more complex cell wall and higher lipid content, what may explain their resistance to the propolis extracts. Taken together, our data demonstrated that geopropolis exhibited a mild inhibitory activity for both *S. aureus* and *E. coli*; moreover, this effect was not exclusively due to geopropolis, since similar results were obtained with its solvent.

The interaction between natural products and antibiotics is very important due to increasing antibiotic resistance, and one of the strategies being employed to overcome the problem of drug resistance has been the use of drugs in combination [19,20]. Propolis has been found to potentiate the effects of some antibiotics, especially those acting on bacterial wall and ribosome [5,21]. However, there is no data in literature on the synergism of geopropolis with antibiotics. Our results demonstrated a synergistic action of geopropolis only with chloramphenicol on *S. aureus* but not on *E. coli*. This is an important finding, since one may use a lower concentration of antibiotic when combined with geopropolis in order to reduce side effects and bacterial resistance. Similar effects were found using both ½ and ⅛ of geopropolis, indicating that the lower concentration may lead to better results. Further investigations will be necessary in order to establish whether geopropolis improves absorption of chloramphenicol by the cell, improves the interaction with the ribosome or inhibits the degradation or elimination of chloramphenicol by bacteria. In particular, it would be worth testing the capacity of geopropolis to diminish resistance to chloramphenicol in resistant bacterial strains sufficiently to render this antibiotic therapeutic against such bacteria.

Geopropolis was found to be cytotoxic toward canine osteosarcoma cells at concentrations ≥ 25 µg/mL after 72 h incubation with no effects of the solvent. We also evaluated the activity of propolis produced by *A. mellifera*, concluding that the geopropolis at concentrations of 50 and 100 µg/mL were more effective after 72 h incubation than propolis at the same concentrations. These findings indicated a higher sensitivity of canine osteosarcoma cells to geopropolis than propolis [10,22]. The antiproliferative activity of aqueous and methanol extracts of propolis produced by *Trigona laeviceps* in Thailand was analyzed, revealing that the aqueous extract of propolis showed a higher antiproliferative action than methanol extract on human colon cancer cells (SW620), and both extracts induced cell death by necrosis [23]. The same authors evaluated the antiproliferative activity of ethanol, dichloromethane, hexane and methanol extracts of propolis produced by *T. laeviceps* against five neoplastic cell lines derived from lung (Chaco), stomach (KATO-III), colon (SW620), breast (BT474) and liver (Hep-G2) and on two strains of normal cells (fibroblasts-HS-27 and liver-CH). The hexane extract of propolis showed a higher cytotoxic activity against cancer cells, without affecting normal cells.

Carboplatin is a chemotherapeutic agent used for the treatment of various cancers, sometimes in combination with other
Figure 3: Cell viability (%), tumor necrosis factor alpha and interleukin-10 production (pg/mL) by monocytes incubated with geopropolis (5, 10, 25 and 50 µg/mL) or lipopolysaccharide (10 µg/mL) for 18 h. Data represent mean and standard deviation of 10 similar assays (*P < 0.0001 vs. control).

Supplementary Figure 1: Mass chromatogram (TIC) of ethanol extract of geopropolis (after silylation): a: Pentadecylresorcinol, b: Pentadecyl salicylic acid, c: Heptadecadienylresorcinol, d: Heptadecenylresorcinol, e: Heptadecylresorcinol, f: Heptadecenyl salicylic acid, g: Heptadecenyl salicylic acid isomer, h: Nonadecenylresorcinol, i: Nonadecenylresorcinol isomer, j: Nonadecenyl salicylic acid, k: Nonadecenyl salicylic acid isomer, l: α-amyrenone, m: α-amyrine, n: β-amyrenone, o: α-amyrine, p: Lupeol
anticancer agents. This drug has a similar spectrum of activity to cisplatin but with fewer side effects [24,25]. However, in our study, the concentrations of 100 and 200 µmol/L recommended in the literature [14] exerted no effect on HEp-2 cells. Thus, higher concentrations of carboplatin were investigated. Concentrations of 400 and 500 µmol/L exhibited only a slight inhibitory effect, suggesting that HEp-2 cells were either resistant or had low sensitivity toward this drug. Cinegaglia et al. observed no carboplatin effects (100 and 200 µmol/L) on canine osteosarcoma cells [21]. The effectiveness of platinum agents against neoplastic cells may be due to the formation of DNA-platinum adducts, resulting in a conformational change in the structure of DNA that results in the inhibition of replication and/or DNA repair mechanisms [26,27]. Cells with a higher capacity for repair mechanisms are resistant to platinum compounds [26]. Clinically, restricted blood flow to the tumor can also restrict the availability of carboplatin, resulting in lower drug availability and lower sensitivity to platinum agents. Our data demonstrated that HEp-2 cells were not sensitive to carboplatin, suggesting resistance or low susceptibility to this chemotherapeutic agent, even at very high concentrations.

There is little data in literature regarding the immunomodulatory action of geopropolis produced by stingless bees. The highest concentration of Geo showed cytotoxic effects toward monocytes, and the non-cytotoxic concentrations increased TNF-α and IL-10 production by these cells. TNF-α is a pro-inflammatory cytokine produced by macrophages, monocytes, and other cells, and is the main mediator of acute inflammatory response to Gram-negative bacteria and other infectious agents. One of the physiological functions of TNF-α is to stimulate the recruitment of neutrophils and monocytes to infection sites and to activate these cells to kill microorganisms [29]. IL-10 is secreted by T cells, monocytes and macrophages to regulate both innate and adaptive immunity [30]. In our study, both TNF-α and IL-10 production were elevated after incubation with geopropolis, suggesting its activator profile.

The serum cytokine concentration of mice that received a gel with geopropolis produced by M. fasciculata in the oral cavity for a minute during four days was assessed and high concentrations of IL-4 and IL-10 were found after 7 days, with no changes in IFN-γ and TNF-α production [7]. Although several mechanisms of action of propolis produced by Africanized honeybees have been proposed [31], geopropolis, immunomodulatory action deserves further investigation. New assays should also evaluate geopropolis effects in the production of other inflammatory mediators and in the microbicidal activity of monocytes.

CONCLUSION

Geopropolis showed a promising effect in combination with chloramphenicol, and it deserves further investigation due to bacterial resistance to antibiotics. Geopropolis also inhibited HEp-2 cells and showed an activator profile on human monocytes. One may speculate that the pharmacological properties of geopropolis may be due to triterpenes, its major chemical constituents. Since few works dealing with geopropolis may be found in literature, our findings contribute to the elucidation of its properties and potential use for humans and animals.

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