Antimicrobial and antiproliferative activities of geopropolis from stingless bee *Melipona scutellaris*

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Abstract

**Background**: Geopropolis is a resin collected by native stingless bee, containing soil and wax. Studies concerning their biological activity and chemical composition are scarce. This work evaluated the influence of Ethanoic Extract of Geopropolis (EEGP) from *Melipona scutellaris*, and its bioactive fraction against important clinical microorganisms as well as their in vitro citotoxicity and chemical profile.

**Methods**: The antimicrobial activity was examined by determining the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) on six bacteria strains and the ability to inhibit biofilm adherence by *Streptococcus mutans*. Total growth inhibition (TGI) concentration was chosen to assay antiproliferative activity on normal and tumoral cell lines. Chemical composition of geopropolis was accessed by reverse phase/high performance liquid chromatography and gas chromatography/mass spectrometry. The antimicrobial activity was examined by determining the minimal inhibitory and bactericidal concentrations on six bacteria strains and the ability to inhibit biofilm formation by *Streptococcus mutans*. Total growth inhibition concentration was chosen to assay antiproliferative activity. Chemical composition of geopropolis was accessed by RP-HPLC and CG-MS.

**Results**: EEGP significantly inhibited the *Staphylococcus aureus* strains and *S. mutans* growth at low concentrations and its hexane fraction (HF) had shown highest antibacterial activity. Further, both EEGP and HF inhibited the biofilm formation of *S. mutans* (p<0.05) and showed selectivity against human tumoral cell lines, however only HF demonstrated activity at low concentrations. Chemical analyses suggest absence of flavonoids and presence of benzophenones as major compounds.

**Conclusions**: Geopropolis from *Melipona scutellaris* presents interesting antimicrobial, anti-biofilm and antiproliferative activities. Further, the characteristics evidenced by
Chemical analysis suggest the absence of compounds commonly present in honey bee propolis and presence of benzophenones as major compounds.

**Keywords**

*Melipona scutellaris*, Geopropolis, Antimicrobial activity, Antiproliferative, Chemical profile.

**Background**

Propolis, a resin collected by bees from plants, presents a great variety of pharmacological effects described in the literature, such as the antimicrobial, anti-inflammatory, immune modulatory, anti-ulcer, and anti-tumor ones. Regarding the antimicrobial activity, several types of propolis collected by *Apis mellifera* appear active against various microorganisms, including fungi, viruses, and bacteria [1]. The variation in biological activity is directly related to its complex chemical composition, which can varies according to seasonality, region of plant resins survey [2] and also with the bee species. Most of these studies, however, are related to propolis collected by *A. mellifera*, whereas other types of propolis collected by different species of bees have been sparsely studied.

Geopropolis is a different kind of propolis by presenting wax, resin and soil in its constitution, providing unique characteristics. This propolis, collected by stingless bee, like *Melipona scutellaris*, an indigenous and endangered bee, has little description in the literature about its chemical composition and biological activity [3], thus deprived of added economic value. Velikova, et al (2000) described the antimicrobial activity of samples of Brazilian geopropolis against *Staphylococcus aureus* and *Escherichia coli*, suggesting the presence of non polar compounds as responsible for its activity [4]. Liberio et al. (2011) showed that geopropolis from Maranhão, Brazil, collected by
Melipona fasciculata has antimicrobial activity against S. aureus and Candida albicans, besides exhibited bactericidal effects on Streptococcus mutans biofilms [5]. This activity however, is attributed to samples with the highest flavonoid concentration. Thus, the geopropolis collected by these bees exhibits interesting antimicrobial profile and elucidate its biological and chemical characteristics is fundamental point to characterize the potential use of this not fully studied kind of propolis as medicine or functional food.

Bacteria that normally inhabit the oral cavity, such as S. mutans, Actinomyces naeslundii, and Enterococcus faecalis, acquire relevant clinical importance in opportunistic pathogenic situation, since they may be related to several oral infections. Among these microorganisms, S. mutans has a special attention for its unique ability to form biofilms, and thus start the process cariogenic [6]. Moreover, in some specific cases S. mutans can leads to infective endocardits by adherence to endothelial cells [7]. In addition to these organisms, bacteria such as S. aureus, methicilin resistant S. aureus (MRSA) and Pseudomonas aeruginosa are often associated with nosocomial infections and have increasing resistance to many antibiotics available [8]. Therefore, these facts stimulate the search for alternatives that effectively control infections caused by these organisms.

Once a natural product presents antimicrobial activity, there is interest to know whether it has compatibility with normal cells of the host to enable a possible use as harmless complementary food and/or medicine. Moreover, there are reports of natural products which act on microorganisms and also exhibit antiproliferative activity against tumor cells, increasing the range of possible uses of these products. Propolis and its constituents, like artepelin C, have their action against tumors described as promising [9]. As well as the microbial activity, studies are focused in propolis collected by A. mellifera bee, and there are no reports available regarding Melipona scutellaris geopropolis anti-tumoral potential. A similar propolis, also found in a tropical region
collected and by another stingless bee showed antiproliferative activity \textit{in vitro} against tumoral cell but not normal cell lines [10].

Thus, because of the lack of studies on this geopropolis collected by \textit{M. scutellaris} this study aimed to evaluate the antimicrobial and antiproliferative activity of ethanolic extract of geopropolis and its fractions, and characterize them chemically, thereby generating information that add value to this natural product.

**Methods**

\textit{Geopropolis sample and fractionation}. Crude samples of \textit{M. scutellaris} geopropolis were obtained from Entre Rios town, Bahia State (S 11° 57’ and W 38° 05’), Northeastern of Brazil. The geopropolis sample (100 g) was extracted with absolute ethanol (1:7, w/v), at 70 °C, for 30 min and then filtered to obtain its ethanolic extract (EEGP). The EEGP was further fractioned by liquid–liquid extraction, based on a polarity gradient, and hexane (HF), chloroform (CF), and ethyl acetate (AcF) fractions were obtained, as previously detailed [11]. The obtained fractions were monitored by thin layer chromatography (TLC) using the anisaldehyde reagent, followed by incubation at 100 °C for 5 min. Fluorescent substances were visualized under UV light at the wavelengths of 254 and 366 nm. EEGP, HF, CF, and AcF were concentrated to obtain a yield of 4.33(w/w), 1.98 (w/w), 0.23 (w/w), and 0.87 (w/w) respectively. EEGP and all the fractions were reconstituted with absolute ethanol at 3.2% (w/v) before their use. Because geopropolis presents land in its composition, it also had the antimicrobial activity of its extract evaluated, by preparing ethanolic extract at the same conditions of EEGP. Samples of biome soil around hive, and vegetation visited by bees were collected in order to be process as EEGP and finally the antimicrobial activity was evaluated, since the soil may have antimicrobial substances [12].

\textit{Bacterial strains and susceptibility testing}. The bacterial strains used in this study were: \textit{Streptococcus mutans} UA 159, \textit{Staphylococcus aureus} ATCC 25923, \textit{Staphylococcus}
aureus ATCC 33592 (Methicillin Resistant *Staphylococcus aureus*), *Enterococcus faecalis* ATCC 29212, *Actinomyces naeslundii* m104, and *Pseudomonas aeruginosa* ATCC 25619. The antimicrobial activity of EEGP and fractions were examined by determining the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (2006) [13]. MIC was performed in culture microplates (96 wells), the inoculum was 5 x 10^5 CFU/mL in BHI (Brain Heart Infusion, Difco, Franklin Lakes, NJ, USA) and EEGP and all the fractions concentrations ranged from 3.125 to 1600 μg/mL. The control vehicle was ethanol (final ethanol concentration: 5%, v/v), and positive control was chlorhexidine digluconate 0.12% (Sigma-Aldrich, St. Louis, MO, USA). The plates were incubated at 37 °C and 5% CO₂ for 24 hours and MIC was defined as the lowest concentration of EEGP or fraction that allowed no visible growth, confirmed with resazurin 0.01% dye (Sigma-Aldrich, St. Louis, MO, USA). To determine MBC, an aliquot (50 μL) of all incubated wells with concentrations higher than MIC was sub-cultured on BHI agar. MBC was defined as the lowest concentration that allows no visible growth on the agar, i.e., 99.9% kill [14]. Three separate experiments were conducted for each concentration of EEPG and each fraction.

**Inhibition of biofilm adherence by Streptococcus mutans.** The ability of EEGP and its most active fraction to inhibit the adherence of growing cells of *S. mutans* was performed as described elsewhere [15, 16] with some modifications. Briefly, *S. mutans* cells (1.0 x 10^5 CFU/mL in BHI broth plus 1% sucrose w/v) were grown in the wells of sterile polystyrene U-bottom microtiter containing EEGP and bioactive fraction at sub-MIC concentrations or vehicle control (ethanol 5% v/v). After incubation at 37 °C for 18h, the adherent cells were dyed with colorant crystal violet and re-suspended in absolute ethanol. Biofilm formation was quantified by measuring absorbance at 575 nm.
**Antiproliferative assay.** In vitro antiproliferative assay was performed as described by Monks et al. (1991) [17]. Murine normal fibroblast (3T3) and eight human tumor cell lines [U251 (glioma), UACC-62 (melanoma), MCF-7 (breast), NCI-ADR/RES (ovarian expressing phenotype multiple drugs resistance), 786-0 (kidney), NCI-H460 (lung, non-small cells), PC-3 (prostate) and OVCAR-03 (ovarian)] were kindly provided by Frederick MA, National Cancer Institute/USA. Also, HaCat (human keratinocites) cell line was used and was kindly donated by Dr. Ricardo Della Coletta (FOP, UNICAMP, Brazil). Stock and experimental cultures were grown in medium containing 5 mL RPMI 1640 (GIBCO BRL) supplemented with 5% fetal bovine serum (GIBCO BRL). Penicilin:streptomycin mixture (1000 U/mL:1000 μg/mL, 1mL/L RPMI) was added to experimental cultures. Cells in 96-well plates (100 μL cells/well) were exposed to sample concentrations in DMSO/RPMI (0.25, 2.5, 25 and 250 μg/mL) at 37 °C, 5% of CO2 in air for 48h. Final DMSO concentration did not affect cell viability. Before (T0 plate) and after sample addition (T1 plates), cells were fixed with 50% trichloroacetic acid and cell proliferation determined by spectrophotometric quantification (540 nm) of cellular protein content using sulforhodamine B assay. Using the concentration-response curve for each cell line, TGI (concentration that produces total growth inhibition or cytostatic effect) was determined through non-linear regression analysis using software ORIGIN 8.0 (OriginLab Corporation®).

**Chemical assays.** Chemical characterization of EEGP and fractions were obtained by RP-HPC and CG-MS.

**Reverse phase high performance liquid chromatography (RP-HPLC).** The RP-HPLC analyses was performed according Alencar et al. (2007) [18] with some modifications. Samples were examined in a liquid chromatograph (Shimadzu®), equipped with two pumps (LC-6AD), an auto sample (SIL 10ADVp) coupled to a photodiode array detector (SPD-M10AVp) at 254 nm and a reverse phase column C18 (250 mm x 4,6 mm i.d.; 5 μm particle size). 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. Chemical compounds were identified by absorption spectra in the ultraviolet region, using the resources of the photodiode array detector compared with authentic standards (p-coumaric, ferulic acid, cinnamic acid, gallic acid, quercetin, kaempferol, kaempferide, apigenin, sakuranetin, isosakuranetin, pinocembrin, chrysin, acacetin and galangin) with detector.

Gas chromatography-mass spectrometry (GC-MS). Previously, the EEGP and fractions samples were silanized with N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) and performed in gas chromatography 2010, Shimadzu Co., with mass selective detector QP 2010 Plus in the electron impact ionization mode (70 eV), injector splitless, capillary column RTX5MS 30 m x 0.25 mm x 0.25 μm. Column temperature was 60 °C, 3 °C/min, 240 °C (15 min), detector in scanning mode (m/z 40-800). Carrier gas (He) was 1.0 ml/min. The individual peaks were compared with the equipment library (Willey-138) [19].

Statistical Analysis. Inhibition of biofilm adherence by *Streptococcus mutans* data were compared using Kruskal-Wallis test (p value<0.05 was considered statistically significant). Triplicates from at least three separated experiments were conducted in each assay.

Results

Table 1 shows MIC and MBC values for EEGP and fractions against the tested microorganisms. EEGP was able to inhibit the bacterial growth of *S mutans*, *S. aureus*, and MRSA strains at concentrations lower than 50 μg/mL, while *E. faecalis* and A.
naeslundii were inhibited between 800-1600 μg/mL. Against P. aeruginosa, neither EEGP nor fractions inhibited growth at the tested concentrations. Except for S. aureus strains, which were killed between 25-50 μg/mL, the MBC values showed a bactericidal activity of EEGP over 1600 μg/mL against tested organisms. The extract of the soil from the region of geopropolis collection showed the same antimicrobial profile of vehicle, not interfering with the microorganisms’ growth.

The fractions were tested to observe whether chemical separation process was able to reduce the MIC values related to EEGP. Table 1 shows that the hexane fraction (non-polar) had MIC below 25 μg/mL for the S. mutans, S. aureus, and MRSA strains, while for E. faecalis and A. naeslundii, the value was reduced to 100-200 μg/mL and 200-400 μg/mL, respectively.

Figure 1 shows the effect of EEGP and HF on biofilm adherence of S. mutans. Both EEGP and HF were able to significantly decrease (p<0.05) the biofilm formation by the bacteria. The minimal concentration of EEGP and HF that allowed the significant reduce of biofilm formation was 25 and 6.25 μg/mL. EEGP at 25 μg/mL showed an inhibition rate of 51%, whereas HF at 6.25 μg/mL showed a rate of 86%, when compared with the vehicle control.

**Figure 1 Effect of ethanol extract of geopropolis (EEGP) and hexane fraction (HF) on adherence of growing cells of Streptococcus mutans UA159 biofilm.**

Each concentration marked with * differ significantly from vehicle control (p<0.05, ANOVA, Student-Newman-Keuls).

This way, Table 2 shows the antiproliferative activity of EEGP and HF on normal and tumoral human cell lines. EEGP presented more activity against tumoral cell lines, inhibiting the total growth at low concentrations when compared to normal lines. All tumoral cell lines tested were inhibited below 35 μg/mL, whereas the normal cells lines (3T3 and HaCat) were inhibited over 40 μg/mL (52.73 and 43.20 μg/mL, respectively). The lowest TGI value was observed against melanoma tumor (10.90
μg/mL). The TGI concentrations obtained of HF was lower than 15.00 μg/mL for most cell lines tested and 32.00 μg/mL for HaCat normal line. HF was also more selective to melanoma line, presenting TGI concentration of 1.77 μg/mL, about six times lower than EEGP.

The chemical assays were performed for EEGP and HF. Chromatograms obtained by RP-HPLC analysis of EEGP and HF, shown in Figure 2 (A and B respectively), demonstrated the presence of similar peaks, however more concentrated at active fraction (B). No pattern of flavonoid and cinnamic acid derivatives were detected, considering the detection limit of the method. The UV spectrum showed that major compounds observed, 4, 5 and 7 (Fig. 2) have similar λ_{max} at 279, 281 and 282 nm, respectively.

**Figure 2** RP-HPLC chromatograms of the ethanolic extract of geopropolis (A) and the hexane fraction (B). 1: UV λ 241 nm, RT = 2.72 min; 2: UV λ 287 nm, RT = 81.40 min; 3: UV λ 283 nm, RT = 83.31 min; 4: UV λ 279 nm, RT = 100.53 min; 5: UV λ 281 nm, RT = 101.95 min; 6: UV λ 240 nm, RT = 42.54 min; 7: UV λ 282 nm, RT = 87.14 min; 8: UV λ 284 nm, RT = 91.00 min.

Table 3 shows the identified compounds found in EEGP and the HF by GC-MS analysis. Most of substances could not be identified based on the library device, confirming the absence of phenolic acids and flavonoids standards, at the detection limit of the used method. Compounds 3 and 4 showed M+ at m/z 591, and same fragments at m/z 589, 445 and 73, however with different base peak (73 and 501 respectively). Further, both compounds were found more concentrated at non-polar fraction (HF), with relative areas of 9.54 and 8.40%. Besides compounds 8 and 9 showed the same M+ (m/z 623) and similar retention time, they showed the same fragment m/z 105 ([C_6H_5CO]+), as well as compound 6. Also, the fragments ions of m/z 77, 69 and 55 were observed in compound 6, while compounds 8 and 9 showed
fragments m/z 69 and 77, respectively. Compound 8 was the most abundant compound found at EEGP and HF, and compound 9 was more concentrated at EEGP when compared to HF. According to results of HPLC, neither flavonoid was found by this technique.

Discussion

Propolis, a resin collected by bees, presents a considerable variety of well-established pharmacological activities, and its potential antimicrobial is widely studied, especially against oral pathogens [1, 14, 15, 20]. Nevertheless, most of these studies describe the activity are related to propolis collected by A. mellifera, so the aggregate market value of this product was result from information generate by science. Geopropolis is a type of propolis collected by native stingless bees, which, in addition to resins and wax, has soil in its composition, leading to low yield extracts that can partly justifies its low economic interest and the lack of studies regarding its biological activity [3].

In this study, EEGP showed interesting antimicrobial activity especially against S. aureus, S. mutans and MRSA strains with MIC values below 50 μg/mL, once, according Duarte, et al (2007), a crude extract from natural products is considered promising when MIC value is below 500 μg/mL [21]. Velikova et al. (2000) reported that Brazilian geopropolis samples showed significant activity on S. aureus and it was weak against E. coli [4]. Our data confirm the interesting activity on S. aureus and MRSA, also showing a weak inhibition of growth of a Gram-negative bacillus (P. aeruginosa).

Several types of A. mellifera propolis extracts have their activity against S. mutans well described in the literature. Duarte et al. (2003) showed that the ethanolic extract of Brazilian propolis type 6 inhibited S. mutans UA 159 growth in concentration between 25-100 μg/mL and Hayacibara et al. (2005) showed that Brazilian propolis types 3 and 12 were able to inhibit growth at 25-50 μg/mL and 200-400 μg/mL,
respectively [20, 22]. The EEGP inhibited the *S. mutans* UA 159 growth between 25-50 μg/mL, also demonstrating a strong inhibitory activity with bacteriostatic characteristic, suggesting an ability to act on virulence factors of the microorganism involved in dental caries etiology. In case of an infection in the oral cavity, the action on microorganism’s virulence factor seems to be the best way to control the development and pathogenesis, since that total and permanent elimination of bacteria of oral environment is not viable [23]. Such effect of geopropolis, whether confirmed by specific studies, indicates the presence of compounds that can be effective in the control and prevention of caries. *S. aureus* and MRSA infections have acquired great clinical importance, since these organisms appear to be resistant to β-lactamic, aminoglycosides, and macrolides antibiotics as well as antiseptic substances [24]. In this study, EEGP demonstrated itself to be a promising source of bioactive against this pathogen showing the lowest MIC and MBC values on both *S. aureus* strains tested. Furthermore, when compared to other strains, MRSA was the most sensitive microorganism, showing low MIC and MBC values for all fractions tested.

In order to verify if chemical separation was efficient, HF, CF, and AcF were tested against the same microorganisms and MIC values were compared with EEGP values. HF appeared to be the most potent, reducing MIC and MBC values (between 2-4 times) for *S. mutans*, *E. faecalis* and *A. naeslundii*, and it does not decrease values against *S. aureus* 25923. Against MRSA, HF was less active than EEGP and CF. In general, all other fractions showed low activity inhibiting bacterial growth when compared to HF and EEGP. Such effect suggests that non-polar compounds present in geopropolis should be the mainly the substances responsible for biological activity.

EEGP and HF (defined as the most active fraction) also were able to inhibit the adherence of growing cells of *S. mutans* at sub-MIC concentrations. The inhibition rates observed indicate a higher activity of HF than EEGP because a concentration of bioactive compounds at non-polar faction, and further, suggest that this fraction can own promising anti-caries agent. Other types of Brazilian propolis had shown this anti-
biofilm activity, by similar mechanism of action. Especially Brazilian propolis type 6, that showed an interesting activity on adherence of growing S. mutans cells, due to its activity on glucans synthesis, by inhibition of glucosyltransferases [14]. Further, other kinds of geopropolis collected by other bees and from different region also demonstrated similar mechanism of action against S. mutans, decreasing the cell viability of the biofilm formed by this microrganism, for example [5].

Then, EEGP and HF were evaluated for their antiproliferative activity. According to Fouche, et al. (2008), extracts of natural products with antiproliferative activity can be classified into the following categories: inactive (TGI > 50 μg/mL), weak activity (15 μg/mL < TGI < 50 μg/mL), moderate activity (6.25 μg/mL < TGI < 15 μg/mL) and potent activity (TGI < 6.25 μg/mL) [25]. EEGP showed to be inactive against normal murine fibroblast cells and a weak inhibitor of human keratinocytes. Against human cancer cell lines, EEGP showed moderate inhibition on melanoma and ovarian lines. This data indicate a non toxic profile of EEGP to normal cell besides toxicity to cancer cell lines, i.e., a selective antiproliferative activity. On the other hand, HF maintained the weak activity on HaCat cells and was able to reduce the TGI value against melanoma line about six times, compared with EEGP. Further, HF also had potent activity against prostate and ovarian tumors.

The antiproliferative activity of propolis from other stingless bee was reported by Umthong et al. (2011) that described a selective activity of Trigona laeviceps propolis against some cancer cell lines besides lowest cytotoxic activity on the normal cell lines [25]. This way, M. scutellaris geopropolis seems to be a promising source of anti-tumoral bioactive, showing moderate or strong inhibition of a wide range cell lines. Although from initial and in vitro evaluations, these results indicate that the compounds present in EEGP and HF could be used both to treat certain infections and tumors without causing significant damage to normal cells tested here, since the concentration that affects these normal cell lines was higher than the those effective ranges against some bacteria or tumoral cell lines.
The RP-HPLC analyses confirm the presence of low polarity compounds in geopropolis, evidenced by high elution times (RT between 80 and 120 min), corresponding to less polar compounds and also the concentration of substances in the hexane phase. The essential non-polar composition of other types of propolis and Brazilian geopropolis had been described elsewhere, due to a presence of terpenes and benzophenones [4]. The UV spectra of the major compounds (4, 5 and 7, Fig. 2) ranged from \( \lambda_{\text{máx}} \) 279 to \( \lambda_{\text{máx}} \) 282 nm, suggesting a possible chromophore with characteristics of polyrenylated benzophenones [26]. Further, our findings indicate the absence of flavonoids, usually reported as responsible for pharmacological activities attributed to some types of *A. mellifera* propolis, as well as markers of some type of propolis [3, 27, 28].

The CG-MS data showed the presence of compounds with similar chemical class, indicated by fragmentation pattern of the mass spectra. The fragment at m/z 105 ([C\(_6\)H\(_5\)CO]+) observed in the fragmentation pattern of compounds 6, 8 and 9 suggests the benzophenone nature of compounds and the presence of fragments m/z 55, 69 and 77 indicate the prenylated nature [26,29]. These chemical findings from our study corroborate the indicatives of the differentiated and not entirely elucidated nature of bioactive compounds of geopropolis. This stimulates the search for a detailed description of its chemical composition and potential use of the bioactives as complementary food or medicine, thus adding economic and social value to a natural product not fully recognized.

The presence of benzophenones, especially polyrenylated ones, has been describe in some types of propolis. Ishida et al. (2011) assigns the antimicrobial activity of samples from the Brazilian Amazon region to benzophenones, like *epi*-nemorosone and *7-epi*-clusianone, which are also described as typical metabolites produced by Clusiaceae (Guttifarae), a family of plants widely found in Brazil [26]. Further, studies on chemical composition and biological activity of Brazilian propolis type 6, collected by *A. mellifera*, also from the State of Bahia, showed certain similarities to geopropolis
studied here, although it is collected by bees with completely different biology [22, 30]. These papers report a composition essentially non-polar, showing the possible presence of benzophenones and absence of phenolic acids and flavonoids. Like in the case of geopropolis, the responsible fraction for the best activity is hexane, which, in the case of propolis type 6, has its biological activity attributed to hyperibone A, which also acts on S. mutans biofilm adherence [14]. These similar chemical and biological profiles between geopropolis and Brazilian propolis type 6 lead to suggest that the possible activity of geopropolis from M. scutellaris is due to a presence of a kind of benzophenone.

**Conclusion**

Geopropolis collected by M. scutellaris presented an interesting antimicrobial and antiproliferative activity. Also, it showed to be promising source of anti-biofilm agents besides the relative selectivity to human cancer cell lines compared with normal cells, at low concentrations. Its chemical composition appears to be essentially non-polar, which is confirmed by the concentration of activity in low polarity fractions. Further, the characteristics evidenced by chemical analysis presented suggest the presence of benzophenones as active compounds. Thus, geopropolis seems to be a promise natural product for discovery of new molecules of therapeutic purposes, since its chemical characterization has not been fully described and its pharmacological potential is just in the beginning and deserve further studies.

**Competing interests**

The authors have no conflicts of interest.

**Authors’ contributions**
MGC, MF and LCCG participated in the design of the study, carried out the extraction and fractionation of geopropolis, the antimicrobial tests and performed the statistical analysis. ALTGR and JEC carried out and interpreted the antiproliferative study. MI and SMA carried out and interpreted the chemical analyses. HK and PLR participated in the design of the study and prepared the article for publication. All authors read and approved the final manuscript.

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References

5. Liberio SA, Pereira AL, Dutra RP, Reis AS, Araújo MJ, Mattar NS, Silva LA, Ribeiro MN, Nascimento FR, Guerra RN, Monteiro-Neto V: Antimicrobial activity against oral pathogens and immunomodulatory effects and


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TABLE 1  Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values of ethanolic extract of geopropolis and its fractions against tested microorganisms (values in μg/mL).

<table>
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<th>Microorganism</th>
<th>EEGP MIC</th>
<th>EEGP MBC</th>
<th>HF MIC</th>
<th>HF MBC</th>
<th>CF MIC</th>
<th>CF MBC</th>
<th>AcF MIC</th>
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<td>25 - 50</td>
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<td>12.5 - 25</td>
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* Value > 1600 μg/mL
**TABLE 2** Total growth inhibition (TGI) of EEGP and HF on human normal and tumoral cell lines.

| Cell line                                      | TGI (μg/mL) |  |
|-----------------------------------------------|-------------|--|-------------------|-------------------|-------------------|-------------------|
|                                               | EEGP        | HF | Dox†              | Fibroblast (3T3)  | Keratinocytes (HaCaT) | Glioma (U251) |
|                                               | 52.73       | 12.27 | 0.92              | 43.20             | 32.00              | 0.96          |
|                                               | 21.18       | 7.17 | 1.08              | 10.90             | 1.77               | 0.22          |
|                                               | 32.26       | 8.45 | 1.51              | 20.54             | 5.96               | 1.15          |
|                                               | 11.93       | 3.93 | 3.78              |                   |                    |              |

*Normal cell lines; † Doxorubicin (positive control).
<table>
<thead>
<tr>
<th>Compound</th>
<th>RT (min)</th>
<th>Relative area (%)</th>
<th>Major MS peaks: m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>EEGP</td>
<td>HF</td>
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<tr>
<td>2-propensaeure 3-phenyl-trimethylsilylester</td>
<td>17.84</td>
<td>8.91</td>
<td>3.92</td>
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<tr>
<td>1,2-benzenedicarboxylic acid</td>
<td>36.60</td>
<td>ND†</td>
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<td>47.91</td>
<td>6.93</td>
<td>4.76</td>
</tr>
</tbody>
</table>

* Retention time; † Not detected compound
Figure 1

The graph depicts the effect of varying concentrations (μg/mL) on O.D. 575 nm for EEGP and HF. The x-axis represents concentration, while the y-axis represents O.D. 575 nm. The data points are marked with error bars, indicating the variability in the measurements. Asterisks denote statistically significant differences between the groups.