MARCOS GUILHERME DA CUNHA

GEOPRÓPOLIS DE *MELIPONA SCUTELLARIS*: ATIVIDADE ANTIMICROBIANA, ANTIPROLIFERATIVA E AÇÃO SOBRE BIOFILME DE *STREPTOCOCCUS MUTANS IN VITRO*

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Orientador: Prof. Dr. Pedro Luiz Rosalen

Este exemplar corresponde à versão final da dissertação de mestrado defendida pelo aluno Marcos Guilherme da Cunha, e orientada pelo Prof. Dr. Pedro Luiz Rosalen.

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"A dúvida é o princípio da sabedoria"

Aristóteles

RESUMO

O presente estudo sobre a geoprópolis de Melipona scutellaris teve como objetivo avaliar suas atividades antimicrobiana contra *Streptococcus mutans* *Staphylococcus aureus*, *S. aureus* resistente à meticilina, *Enterococcus faecalis*, *Actinomyces naeslundii*, *Pseudomonas aeruginosa* e também antiproliferativa sobre linhagens celulares, além de caracterizar quimicamente o seu extrato etanólico e a fração química bioativa. Analisou-se também a capacidade desta fração bioativa de atuar sobre *S. mutans* organizado na forma de biofilme *in vitro*. Inicialmente obteve-se o extrato etanólico de geoprópolis (EEGP), que foi fracionado, resultando nas frações hexânica (FH), clorofórmica (FC) e acetato de etila (FAc). O EEGP e frações foram submetidos a testes antimicrobianos para determinação das concentrações inibitória mínima (CIM) e bactericida mínima (CBM). Verificada a ação antimicrobiana, o EEGP e fração bioativa foram avaliados quanto a sua citotoxicidade por meio da atividade antiproliferativa contra linhagens de células normais e tumorais. A caracterização química foi realizada por meio de análises por cromatografia líquida de alta eficiência em fase reversa (CLAE-FR) e cromatografia gasosa com espectrometria de massas (CG-EM). A fração bioativa selecionada teve a ação sobre biofilme de *S. mutans* testada pelos ensaios de inibição de formação de biofilme, time kill, queda de pH e por microscopia eletrônica de varredura (MEV). O EEGP foi capaz de inibir o crescimento de *S. mutans* e da maioria das cepas bacterianas, sendo a FH, que apresentou menor CIM. Com relação à atividade antiproliferativa, tanto o EEGP quanto a FH inibiram o crescimento de forma mais seletiva para as linhagens tumorais, porém a FH em concentrações mais baixas. A análise química do EEGP e FH indicou a presença de compostos de baixa polaridade, ausência de flavonóides e de derivados do ácido cinâmico. A FH foi efetiva na diminuição da biomassa do biofilme em ambas as concentrações estudadas (250 e 400 µg/mL), quando comparada com o controle ($p<0,05$), porém não alterou a viabilidade bacteriana (time kill), nem a produção de ácidos pela bactéria ($p>0,05$). As análises por MEV demonstraram uma modificação na matriz do biofilme tratado com FH, verificada pela aparente perda de homogeneidade superficial. Tais dados sugerem que a geoprópolis é uma promissora fonte de compostos ativos contra algumas bactérias, com citotoxicidade maior para células tumorais que normais e também capaz de atuar sobre biofilme de *S. mutans*, podendo ser útil no controle de doenças biofilme dependentes, relacionadas a este microrganismo.

Palavras-chaves: Geoprópolis, *Streptococcus mutans*, biofilme, atividade antiproliferativa.

ABSTRACT

The present study concerning *Melipona scutellaris* geopropolis aimed to evaluate their antimicrobial activity against *Streptococcus mutans* and other pathogens of clinical importance, the antiproliferative activity on normal and tumor cell lines and to chemically characterize the ethanol extract and its bioactive chemical fraction. Further, also it analyzed the ability of this bioactive fraction acting on *in vitro S. mutans* biofilm. Initially it was obtained the ethanolic extract of geopropolis (EEGP), which was split, resulting in the hexane (HF), chloroform (CF) and ethyl acetate (FAC) fractions. The EEGP and fractions were tested to determine the minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) against *S. mutans* UA159 and five bacterial strains of clinical interest. After checked for antimicrobial activity, the EEGP and bioactive fraction were evaluated for their cytotoxicity through antiproliferative activity against normal and also tumor cells lines. The chemical characterization was performed by reverse phase high performance liquid chromatography (RP-HPLC) and gas chromatography with mass spectrometry (GC-MS). The action of selected bioactive fraction on *S. mutans* biofilms was evaluated by inhibition of biofilm formation, time kill, drop in pH assays, and scanning electron microscopy (SEM). The EEGP and HF were able to inhibit the growth of *S. mutans* and most bacterial strains, and HF presented the lowest MIC among the tested fractions. Concerning the antiproliferative activity, both EEGP and HF selectively inhibited the growth of tumor lines, but the HF at lower concentrations. Chemical analysis of EEGP and fraction indicated the presence of bioactive compounds of low polarity and the absence of flavonoids and cinnamic acid derivatives. The HF effectively reduced the biofilm biomass at both concentrations studied (250 and 400 mg / ml) compared with control ($p<0.05$), but did not affect bacterial viability (time kill), nor acid production by bacteria ($p>0.05$). The SEM analysis showed a change in the biofilm matrix treated with FH verified by the apparent loss of surface homogeneity. These data suggest that geopropolis is a promising source of active compounds against some bacteria, with more cytotoxicity to tumor cells than normal and also able to act on *S. mutans* biofilms, which may be useful controlling biofilm dependent diseases related to this microrganism.

Keywords: Geopropolis, *Streptococcus mutans*, biofilm, antiproliferative activity.

SUMÁRIO

INTRODUÇÃO

Historicamente os produtos naturais são utilizados na medicina popular devido ao acúmulo de conhecimento empírico adquirido através de várias gerações. A eficácia de produtos naturais sobre diversas condições patológicas é amplamente descrita na literatura, sendo que tais produtos são a principal fonte de novas drogas disponibilizadas no mercado (Newmam & Cragg, 2007). Assim, estudos buscam elucidar a atividade biológica de compostos bioativos naturais a fim de apresentar alternativas terapêuticas que sejam de baixo custo, seguras e que em alguns casos possam gerar um valor agregado científico a um produto até então negligenciado, mas que pode se tornar a fonte de prevenção e cura de doenças, inclusive servindo subsistência de comunidades carentes que dependem de sua produção.

Entre esses produtos naturais, a própolis é um que tem seu uso relatado desde antigas civilizações egípcias para diversos fins, inclusive terapêuticos (Sforcin & Bankova, 2011). É uma resina vegetal não tóxica coletada por abelhas em diversas fontes vegetais, que tem como função primária proteger sua colméia (do grego, *pro*: a favor e *polis*: cidade) (Salatino *et al*., 2011). Sua composição química é variável, dependendo da biodiversidade da região visitada pelas abelhas, podendo possuir compostos da classe dos flavonóides, diterpenos, ácidos graxos e benzofenonas polipreniladas (Salatino *et al*., 2005; Duarte *et al*., 2006; Castro *et al*., 2009). Estudos sobre as propriedades biológicas de diversos tipos de própolis conhecidas descrevem uma ampla gama de atividades farmacológicas como, por exemplo, antimicrobiana, antitumoral, antiinflamatória, anti-úlcera e antioxidante (Koo *et al*., 2000; Paulino *et al.,*2003; Barros *et al*., 2007; Kumazawa *et al*., 2007; Búfalo *et al*., 2009; Jeon *et al*., 2011). Porém, a maioria das informações científicas a respeito da própolis referem-se àquelas coletadas por abelhas *Apis mellifera*, enquanto que outros tipos de própolis, coletadas por

espécies diferentes permanecem ainda sem descrição detalhada sobre sua composição e atividade biológica.

As abelhas nativas sem-ferrão, da tribo Meliponini, são reconhecidas pela sua produção de mel e seu papel na manutenção do ecossistema (Cortopassi-Laurino, *et al*., 2006). Estas abelhas habitam principalmente regiões tropicais e sub-tropicais, têm a capacidade de realizar vôos curtos e têm importância crucial na polinização de diversas espécies de plantas (Ramalho, 2004). *Melipona scutellaris*, também conhecida como uruçú, é encontrada no nordeste do Brasil e produz um tipo diferente de própolis chamada geoprópolis, que é composta por resina, cera e terra (Velikova *et al*., 2000). Existem relatos que este produto é usado empiricamente para o tratamento de gastrite e como agente antibacteriano (Quezada-Euan *et al.,* 2001).

A atividade biológica da geoprópolis é pouco descrita na literatura. Velikova *et al*. (2000) demonstraram que amostras de geoprópolis brasileiras apresentaram forte atividade antimicrobiana contra *Staphylococcus aureus*, enquanto Dualibe *et al*. (2007) relataram que bochechos com extratos de geoprópolis podem diminuir a contagem de estreptococos orais. Recentemente, Libério *et al*. (2011) mostraram que amostras de geoprópolis do Maranhão apresentam atividade sobre *Streptococcus mutans* ATCC 25175 e não se mostraram tóxicas em modelos anti-inflamatórios *in vivo*.

Em se tratando de um produto que tem sua atividade biológica pouco estudada, e ao qual se busca agregar valor científico, é importante que inicialmente seja elucidado sua potencial capacidade de agir sobre algumas condições patológicas de destaque, tanto pela sua severidade ou prevalência na população. Dessa forma, ensaios preliminares para avaliar a atividade antimicrobiana contra microrganismos de relevância clínica, como é o caso do *S. mutans*, *Staphylococcus aureus* e *Pseudomonas aeruginosa*, por exemplo, são importantes para determinar se tal produto natural pode fornecer compostos capazes de atuar sobre infecções causadas por estas bactérias. Adicionalmente, a literatura fornece dados da atividade de vários tipos de própolis contra uma gama

considerável de microrganismos, que estão relacionados a problemas de saúde bucal até graves infecções hospitalares (Sforcin & Bankova, 2011), porém não incluem a geoprópolis.

Por ser um problema de saúde bucal ainda prevalente na população mundial, apesar das estratégias para seu controle como o uso de fluoretos, a cárie dental necessita de alternativas que possam diminuir sua incidência na população (Marsh, 2003). Pelo fato de ser uma doença biofilme dependente e associada à presença de estreptococos do grupo *mutans* (Loesch, 1986; van Houte, 1994), estudos buscam por compostos ou novos agentes, que possam ser efetivos sobre *S. mutans* e também sobre seus fatores de virulência, alterando assim a estrutura do biofilme patogênico formado por este microrganismo. Estudos indicam que produtos naturais e seus derivados podem ser aptos a realizar tal atividade (Koo & Jeon, 2009), sendo que diversos tipos de própolis demonstraram ser ativos contra *S. mutans* e capazes de alterar o equilíbrio da comunidade em biofilme, sendo efetivos inclusive contra a cárie em animais (Park *et al*., 1998; Koo *et al*., 2005; Duarte *et al*., 2006).

Entre os fatores de virulência do *S. mutans*, está a produção de glucanos a partir de carboidratos fermentáveis oriundos da dieta do hospedeiro, através de um grupo de enzimas chamadas de glucosiltransferases (GTFs) (Loesch, 1986). Estes polissacarídeos produzidos, principalmente os insolúveis extracelulares, são responsáveis pela aderência da bactéria à superfície do dente e pela arquitetura complexa da matriz do biofilme (Schilling & Bowen, 1992), permitindo a difusão do açúcar fermentável e conferindo inclusive resistência aos microrganismos a certos agentes antimicrobianos (Lewis, 2001). Além disso, os glucanos contribuiriam para a formação de uma estrutura coerente, aderente e mecanicamente estável, na qual os microrganismos estariam em sítios protegidos de influências do ambiente (Bowen & Koo, 2011) e também para diminuição da concentração de íons inorgânicos na matriz do biofilme (Cury *et al*., 2000). Já os polissacarídeos solúveis estariam relacionados, em parte, ao baixo pH do biofilme

dental, uma vez que são utilizados como reserva energética e podem ser rapidamente metabolizados pelo microrganismo (Paes Leme *et al*., 2006).

Outro importante fator de virulência relacionado ao *S. mutans* é a sua capacidade de utilizar os açúcares fermentáveis para produzir ácido (acidogenicidade) e também sobreviver em ambientes de baixo pH (aciduricidade) (Marquis *et al*., 2004). O ácido lático rapidamente produzido pelo *S. mutans* ao fermentar carboidratos, como por exemplo a sacarose, reduz o pH do biofilme dental a valores inferiores a 5, o que causa uma desmineralização do tecido dental por conta da dissociação da hidroxiapatita, levando assim ao início do processo patogênico da cárie (Bowden, 1990).

Entre as própolis brasileiras, que demonstram ser ativas sobre fatores de virulência do *S. mutans*, destaca-se a do tipo 6. Duarte *et al*. (2003, 2006) e Castro *et al*. (2009) verificaram que esta própolis, proveniente de um ambiente semelhante e próximo ao da geoprópolis (região tropical do Brasil, Mata Atlântica da Bahia) foi capaz de inibir a síntese de polissacarídeos por este microrganismo. Estes estudos também demonstraram uma característica apolar dos compostos químicos presentes neste tipo de própolis, semelhante às características encontradas para a geoprópolis de *M. scutellaris*, recentemente estudadas por nosso grupo de pesquisa.

Após a constatação de atividade biológica contra microrganismos, é importante que se conheça o potencial do produto natural em causar ou não danos às células normais do hospedeiro, para que um futuro uso terapêutico seja viável (Rodeiro *et al*., 2006). Aliado ao estudo de citoxicidade em células normais, por exemplo, por meio da atividade antiproliferativa, os produtos naturais também podem ser avaliados quanto a sua atividade citotóxica e atividade antiproliferativa específica contra células tumorais, como é o caso da doxorrubicina, reconhecido agente com propriedade antiproliferativa e que também apresenta ação antimicrobiana (Peiris & Oppenheim, 1993). A literatura científica é pródiga em demonstrar que os produtos naturais podem fontes de agentes úteis no combate a

alguns tipos de câncer, e que grande parte dos agentes antitumorais conhecidos tem sua origem ligada a produtos de origem natural (Cragg *et al*., 2009).

Assim, os objetivos desse trabalho foram: 1) avaliar a atividade da geoprópolis contra *S. mutans* e outros microrganismos de interesse clínico e também seu potencial antiproliferativo contra células normais e tumorais; 2) caracterizar quimicamente seu extrato etanólico, bem como a fração química responsável pela atividade biológica; e 3) analisar a atividade da fração bioativa sobre o biofilme de *S. mutans*.

CAPÍTULO 1

Geopropolis from stingless bee: antimicrobial and antiproliferative activities

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Summary

Geopropolis is a resin collected by native stingless bee (*Melipona scutellaris*), containing soil and wax. Studies concerning their biological activity and chemical composition are scarce. This work evaluated the influence of Ethanolic Extract of Geopropolis (EEGP), and its bioactive fraction against important medical and dentistry clinical microorganisms as well as their *in vitro* citotoxity. Also, the chemical profile of the extract and fractions were analyzed. The antimicrobial activity was examined by determining the minimal inhibitory concentration (MIC)

and minimal bactericidal concentration (MBC) on six bacteria strains. Total growth inhibition (TGI) concentration was chosen to assay antiproliferative activity. The chemical composition of geopropolis was accessed by reverse phase/high performance liquid chromatography and gas chromatography/mass spectrometry. The data showed that the EEGP significantly inhibited the *Staphylococcus aureus* strains and *Streptococcus mutans* growth at concentrations lower than 50 µg/ml. In general, the hexane fraction (non polar fraction) had shown highest antibacterial activity with lowest values of MIC and MBC. In this study, only *Pseudomonas aeruginosa* seems to be resistant to EEGP and fractions. Concerning the antiproliferative activity, EEGP and hexane fraction showed to be more selective to tumoral than normal cells tested, however only hexane fraction shows cell inhibition at low concentrations. Chemical analyses suggest the possible presence of low polarity compounds, besides the absence of flavonoids. These data indicate that geopropolis is a natural source of bioactive substances with promising antimicrobial and antiproliferative activities to be elucidated. Furthermore, the bioactive fraction acted at low concentrations, owning different chemical composition of the most common types of propolis, collected by honeybee (*Apis mellifera*).

Keywords geopropolis; antimicrobial activity, antiproliferative, chemical profile.

Introduction

Natural products are a significant source of compounds with biological activity and potential therapeutic use.[1)] Within this source, propolis, a resin collected by bees from plants, presents a great variety of pharmacological effects described in the literature, such as antimicrobial, anti-inflammatory, immune modulatory, anti-ulcer and anti-tumor.[2)] Regarding the antimicrobial activity, several types of propolis collected by *Apis mellifera*, appear active against various microorganisms, including fungi,[3)] viruses[4)] and bacteria.[5)]

Geopropolis is a different kind of propolis by presenting wax, soil and resin in its constitution.[6] This propolis collected by native stingless bee (*Melipona scutellaris*) provides little description in the literature about its chemical composition and pharmacological activity,[7] so deprived of added economic value. Velikova *et al*. described the antimicrobial activity of samples of Brazilian geopropolis against *Staphylococcus aureus* and *Escherichia coli*, suggesting the presence of di- and triterpenes derivatives as responsible for its activity.[8] Thus, its antimicrobial potential is but promising however needs further studies.

Bacteria that normally inhabit the oral cavity, such *Streptococcus mutans*, *Actinomyces naeslundii* and *Enterococcus faecalis*, acquire relevant clinical importance in opportunistic pathogenic situation, since they may be related to the cariogenic process,[9] gingivitis[10] and endodontic infections.[11] In addition to these organisms, some Gram-positive cocci such as *Staphylococcus aureus*, are often associated with nosocomial infections and have increasingly resistant to many antibiotics available.[12] In this scenario, methicilin resistant *Staphylococcus aureus* (MRSA) has been blamed for several community-acquired infections.[13] *Pseudomonas aeruginosa*, usually associated to respiratory tract infections, and a bacterium found naturally in the environment, is an opportunistic pathogen and can cause severe infections in debilitated patients.[14] Furthermore, it is considered a difficult target for the antimicrobial treatment.

Once a substance presents antimicrobial activity, there is interest to know whether it has compatibility with normal cells of the host to enable treatment. Moreover, there are reports of natural products with activity against microorganisms that also exhibit antiproliferative activity against tumor cells.[15] Propolis and its constituents, like artepelin C, have their action against tumor described as promising.[16] As well as the microbial activity, studies are focused in propolis collected by *A. mellifera* bee, and to geopropolis there is no record regarding its anti-tumoral potential.[17]

Thus, the objective of this study was to evaluate the antimicrobial and antiproliferative activity of the ethanolic extract of geopropolis and its fractions and

characterize them chemically, thereby generate information that add value to this natural product.

Material and methods

Geopropolis sample and fractionation

Crude samples of *M. scutellaris* geopropolis were obtained between June-July 2010, from Entre Rios town, Bahia State (S 11º 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 using a liquid–liquid extraction, based on a polarity gradient, and the hexane (HF), chloroform (CF), ethyl acetate (AcF) fractions were obtained, as detailed elsewhere.[18)] The fractions obtained 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.[19)] EEGP, HF, CF and AcF were concentrated to obtain a yield of 4.33 (w/w), 1.98 (w/w), 0.23 (w/w) e 0.87 (w/w) respectively. The EEGP and all the fractions were reconstituted with absolute ethanol at 3.2 % (w/v) before using. For presenting land in its composition, it also had its antimicrobial activity of its extract evaluated, by preparing of ethanolic extract at 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.[20)]

Bacterial Strains and susceptibility testing

The bacterial strains used in this study were: *Streptococcus mutans* UA 159, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 33592 (Methicillin Resistant *Staphylococcus aureus*), *Enterococcus faecalis* ATCC 29212, *Actinomyces naeslundii* M 104 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 and Koo *et al*.[21] To determine MIC, the starting inoculum was $1\text{-}2\times10^5$ CFU/ml, 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®). The MIC was defined as the lowest concentration of EEGP or fraction that allowed no visible growth, confirmed with resazurin 0,01 % dye. To determine MBC, an aliquot (50µl) 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.[21] Three separate experiments were conducted for each concentration of the EEPG and each fraction.

Antiproliferative assay

In vitro antiproliferative assay was performed as described by Monks *et al*. [22] 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). Stock and experimental cultures were grown in medium containing 5 mL RPMI 1640 (GIBCO BRL) supplemented with 5 % fetal bovine serum (GIBCO BRL). Peniciline:Streptomicine mixture (1000 U/ml:1000 µg/ml, 1ml/l RPMI) was added to experimental cultures. Cells in 96-well plates (100 µL cells $well^{-1}$) were exposed to sample concentrations in DMSO/RPMI (0.25, 2.5, 25 and 250 µg ml^{-1}) at 37 ºC, 5 % of CO_2 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*. with some modifications.[23)] 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 EEPG 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. The column temperature was initially held at 80 °C for 1 min, and then the temperature was raised to 320 °C at a rate of 5 °C/min, followed by isothermal period of 20 min. The total run time was 69 min. The detector was set in scanning mode (*m/z* 40-800) and carrier gas (He) flow was 1.0 ml/min. Individual peaks were compared with the equipment library (Willey-138).[24)]

Results

Table 1 shows the MIC and MBC values for EEGP and fractions against the tested microorganisms. The EEGP was able to inhibit the bacterial growth of *S. mutans*, *S. aureus* and MRSA strains at a concentration below 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 the tested organisms. The soil extract from the region of geopropolis collection showed the same antimicrobial profile of vehicle, not interfering with microorganisms' growth.

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

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).

Microorganism	EEGP		HF		CF		AcF	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Streptococcus mutans UA 159	25 - 50	*	6.25 - 12.5	800 - 1600	25 - 50	*	*	*
Staphylococcus aureus ATCC 25923	6.25 - 12.5	25 - 50	6.25 - 12.5	25 - 50	25 - 12.5	50 - 100	50 - 100	100 - 200
Staphylococcus aureus ATCC 33592 (MRSA)	6.25 - 12.5	25 - 50	25 - 12.5	25 - 50	6.25 - 12.5	25 - 50	25 - 50	50 - 100
Enterococcus faecalis ATCC 29212	800 - 1600	*	100 - 200	800 - 1600	400 - 800	*	400 - 800	*
Actinomyces naeslundii m104	800 - 1600	*	200 - 400	800 - 1600	400 - 800	*	400 - 800	*
Pseudomonas aeruginosa ATCC 25619	*	*	*	*	*	*	*	*

* Value > 1600 µg/ml.

So, the most promising antimicrobial fraction from EEGP was HF, that had been chosen for further evaluations. This way, Table 2 shows the antiproliferative activity of EEGP and HF on normal and tumoral cell lines. The EEGP presented more activity against tumoral cell lines, inhibiting totally growth at low concentrations when compared to normal cell 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). TGI concentrations obtained for HF was lower than 15.00 µg/ml to most cell lines tested and 32.00 µg/ml to HaCat normal line. HF was more selective to melanoma line, presenting TGI value of 1.77 µg/ml, about six times lower than EEGP.

Table 2- Total growth inhibition (TGI) of EEGP and HF on normal and tumoral cell lines.

Cell line	TGI (µg/ml)		
	EEGP	**HF**	**Dox**[b]
Fibroblast (3T3)[a]	52.73	12.27	0.92
Keratinocytes (HaCat)[a]	43.20	32.00	0.96
Glioma (U251)	21.18	7.17	1.08
Melanoma (UACC-62)	10.90	1.77	0.22
Breast (MCF-7)	26.41	14.09	2.19
Multi drug resistant ovarian (NCI/ADR-RES)	23.92	14.34	6.19
Kidney (786-0)	32.26	8.45	1.51
Lung (NCI-H460)	26.72	9.55	0.67
Prostate (PC-3)	20.54	5.96	1.15
Ovarian (OVCAR-3)	11.93	3.93	3.78

[a] Normal cell lines; [b] Doxorubicin (positive control).

The chemical assays were performed to EEGP and HF. The chromatograms obtained by RP-HPLC analysis of EEGP and HF, shown in Figure 1 (A and B respectively), demonstrate 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.

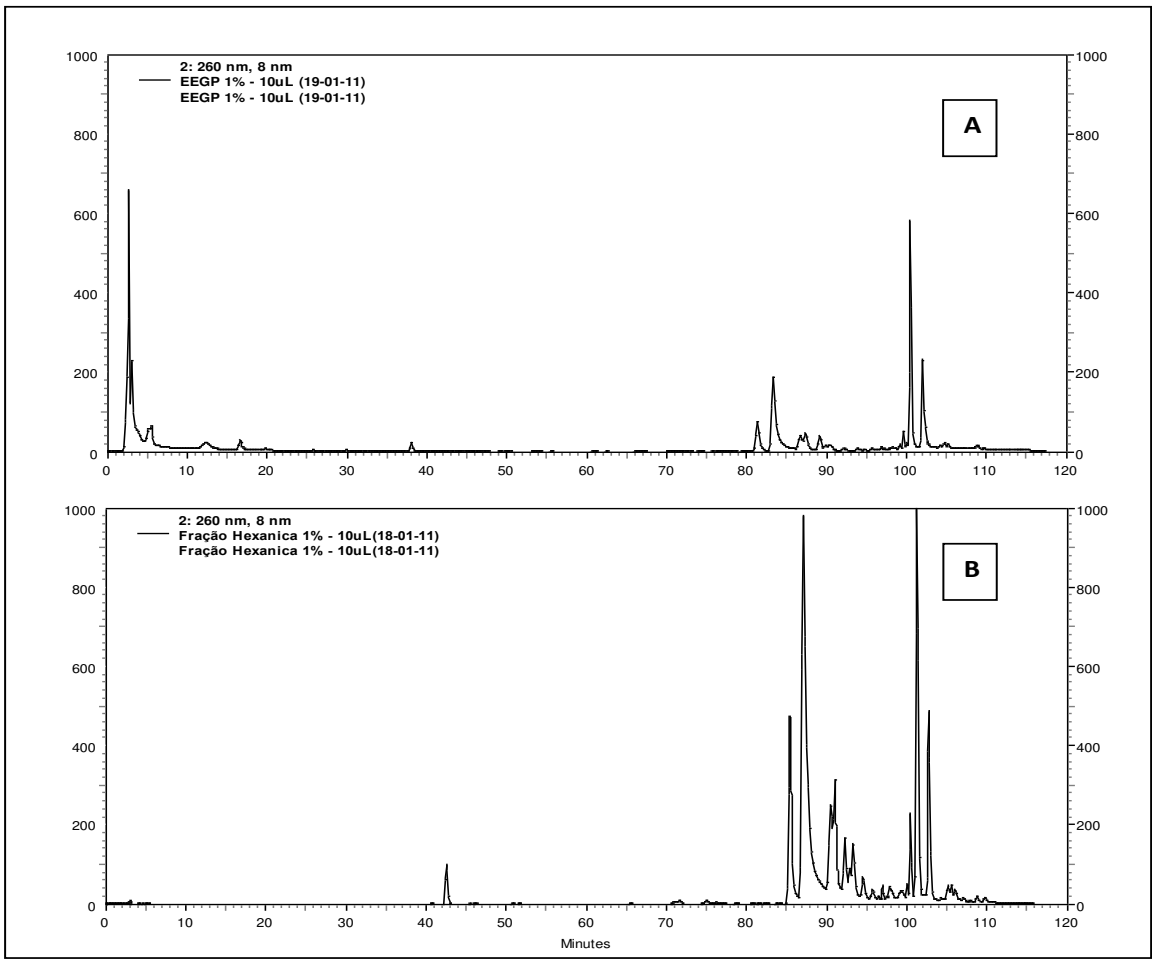

Figure 1- Chromatograms obtained by RP-HPLC of EEGP (A) and the hexane fraction (B).

Table 3 shows the identified compounds found in EEGP and HF by GC-MS analysis. Most of the 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 (TMS radical), however 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 %. Compounds 8 and 9 showed the same M^+ (*m/z* 623) with similar retention time. Moreover, 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 results of HPLC, neither flavonoid was found by this technique.

Table 3- Retention Times, Relative Area of Each Component, and Important Ions Present in the Mass Spectra of Silylated Compounds in EEGP and HF by GC-MS.

Compound	Name	RT[a] (min)	Relative area (%)		Ion (m/z, abundance in parentheses)
			EEGP	HF	
1	2-propensaeure 3-phenyl-trimethylsilylester	17.84	8.91	3.92	220 (31), 205 (100), 161 (90), 145 (33), 131 (88), 103 (60), 77 (49)
2	1,2-benzenedicarboxylic acid	36.60	-	2.24	167 (33), 149 (100), 57 (40)
3	N.I.	43.06	0.68	9.64	591 (17), 589 (80), 499 (87), 445 (40), 73 (100)
4	N.I.	43.79	6.88	8.40	591 (11), 589 (48), 501 (100), 459 (25), 445 (28), 73 (90), 57 (13)
5	N.I.	46.38	3.34	9.06	533 (2), 386 (61), 177 (70), 165 (100), 151 (67), 138 (22), 77 (18)
6	N.I.	46.94	5.79	2.30	495 (100), 459(60), 417 (21), 73 (40), 57 (21)
7	N.I.	47.29	19.17	10.35	548 (39), 533 (34), 479(17), 389(45), 73(100), 45(11)
8	N.I.	47.71	29.15	38.98	623 (66), 536 (20), 535 (52), 73 (100)
9	N.I.	47.91	6.93	4.76	623 (1), 533 (17), 551 (48), 461 (32), 407 (86), 73 (100)

[a] Retention time. Symbols: (N.I.) not identified compound; (-) not detected compound.

Discussion

Natural products have been reported as an important source of new drugs, once known its potential and variety of biological properties described in the literature.[1)] Propolis, a resin collected by bees, presents a considerable variety of well-established pharmacological activities, and its potential antimicrobial is widely studied[2,5)] especially against oral pathogens.[18,21,25-26)] Nevertheless, most of these studies describe the activity are related to propolis collected by *A. mellifera*, so the aggregate market value to 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 a low yield extracts[7)] that can partly justifies its low economic interest and the lack of studies regarding its biological activity.

In this study, EEGP showed interesting antimicrobial activity especially against *S. aureus*, *S. mutans* and MRSA strains with MIC values below 50 µg/ml. According to Duarte *et al*., a crude extract from natural products is consider promising when MIC value is below 500 µg/ml, indicating that continuation of the study is required.[27)] Velikova *et al*. reported that Brazilian geopropolis samples showed significant activity on *S. aureus* and it was weak against *E. coli*.[8)] 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 had their activity against *S. mutans* well described in literature. Duarte *et al*. showed that the ethanol extract of Brazilian propolis type 6 inhibited *S. mutans* growth in concentration between 25-100 µg/ml and Hayacibara *et al*. showed that Brazilian propolis types 3 and 12 were able to inhibit the growth at 25-50 µg/ml and 200-400 µg/ml, respectively.[18,25)] The EEGP inhibit 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 metabolic pathways of the microorganism involved in the etiology of dental caries.[28)] In the case of an infection at oral cavity, acting on microorganism´s virulence factors seems to be the best way to control the installation and pathogenesis, since that total and permanent elimination of bacteria of the oral environment is not viable. Such effect of geopropolis, whether confirmed by specific studies, should indicate 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.[29)] In this study, EEGP demonstrated 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 to all fractions tested.

Studies have reported the resistance of *A. naeslundii* and *E. faecalis* to several agents, natural or well-known antibiotics with MIC values above 1600 µg/ml.[30-33)] Our data, although showing high values to EEGP indicate that the fractionation process was able to decrease the effective concentration against these microorganisms, suggesting that the continuation of HF fraction and bioassay guided purification may lead to a compound effective in low concentrations.

In order to verify if the chemical separation was efficient, HF, CF and AcF was tested against same microorganisms and MIC values were compared to EEPG values. Hexane fraction appeared to be the most potent, reducing MIC and MBC values by 2-4 times for *S. mutans*, *E. faecalis* and *A. naeslundii* and 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 substances responsible for biological activity.

Then, EEGP and HF (active fraction) were evaluated for their antiproliferative activity. According Fouche *et al*., extracts of natural products with antiproliferative activity can be classified into follow 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).[34] 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 selectivity to cancer cell lines. On the other hand, HF maintained the weak activity on HaCat cells and was able to reduce TGI against melanoma cell line about six times, compared to EEGP. Further, HF also had potent activity against prostate and ovarian.

Diverse studies concerning antiproliferative activity of some kind of propolis suggest an interesting potential, like inhibition on prostate tumor[35], and laryngeal carcinoma[36], for example. Geopropolis seems to be a promising source of anti-tumoral bioactive, showing moderate or strong inhibition of a wide range of cancer 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, once the concentration that affects these normal cell lines was higher than 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 shown by the compounds, and also the concentration of substances in the hexane phase. The essential non-polar composition of Brazilian geopropolis had been described elsewhere, suggesting the presence of compounds derived from di- and triterpenes.[8)] Moreover, our findings indicate the absence of flavonoids, usually reported as responsible for pharmacological activities attributed to some types of propolis from *A. mellifera*, as well as markers of quality of Brazilian propolis.[7,37-38)]

The CG-MS data showed the presence of compounds with similar structure, indicated by similar retention time, same M^+ and fragmentation pattern. Moreover, compounds as 3 and 4 showing the same polarity feature, once both were found more concentrated at non-polar fraction. The compound 8 and 9 also seem to have similar characteristics, showed by their retention time and M^+, but compound 8 appears to be more non-polar than the other one, once it is more concentrated at HF. These chemical findings from our study corroborate to indicative of the differentiated and not fully elucidated nature of geopropolis. This stimulates the search of a detailed description of its chemical composition and pharmacological potential, and adds economic and social value to a natural product not yet fully recognized.

Recent studies on chemical composition and biological activity of Brazilian propolis type 6, collected by *A. mellifera*, from Bahia State show certain similarities to geopropolis studied here.[39-40)] Although collected by bees with completely different biology, the biome was the same and the collection sites were about 70km distant between them. These papers report the composition essentially non-polar, showing the presence mainly of unsaturated fatty acids, and absence of flavonoids. Like in the case of geopropolis, the responsible fraction for the best activity had been hexane, and in the case of propolis type 6, the biological activity was attributed to a benzophenone.

Conclusion

Geopropolis produced by *M. scutellaris* presented an interesting antimicrobial and antiproliferative activity, demonstrated by bacterial growth inhibition of ethanol extract and its active non polar fraction, and the relative selectivity to human cancer cell lines compared to normal cell. Its chemical composition appears to be essentially non-polar, which is confirmed by the concentration of activity in low polarity fractions and characteristics evidenced by chemical analysis presented. In addition, geopropolis seems to be a promise natural product for discovery of new molecules to therapeutic purposes, since its chemical characterization has not been fully described and its pharmacological potential is just in the beginning and deserve further studies.

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CAPÍTULO 2

Bioactive fraction of *Melipona scutellaris* geopropolis on *Streptococcus mutans* biofilm

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Summary

The aim of this study was to evaluate the influence of the bioactive non-polar fraction of geopropolis on *Streptococcus mutans* biofilm. The ethanolic extract of *Melipona scutellaris* geopropolis from Atlantic forest of Bahia State (Northeast Brazil) was subjected to a liquid-liquid partition, thus obtaining the bioactive hexane fraction possessing the antimicrobial activity. The effects of hexane fraction (at 250 and 400 µg/mL) on *S. mutans* UA159 biofilms generated on saliva coated hidroxyapatite discs (sHA) were analyzed by inhibition of formation, viability and glycolytic pH-drop assays. Further, biofilms treated with vehicle control and hexane fraction were analyzed by scanning electron microscopy (SEM). Statistical analyses was performed by ANOVA and Tukey-Kramer test, at 5 %. Hexane

fraction at 250 µg/mL and 400 µg/mL was able to reduce the biomass (dry weight) of biofilm at 38 and 53 %, respectively, when compared to vehicle control ($p<0.05$). Further, treatments significantly reduced the amounts of extracellular alkali-soluble glucans, intracellular iodophilic polysaccharides and proteins. No difference was observed in the number of viable cells after treatment. The killing assay showed that hexane fraction after two hours of exposure, was able to reduce the viability of cells but not significantly. Hexane fraction was not able to reduce the acid production of *S. mutans* biofilm ($p>0.05$). SEM analysis showed that hexane fraction at 250 and 400 µg/mL affected the biofilm organization formed on HA disk reducing its matrix. In conclusion, the bioactive hexane fraction of geopropolis seems to be able to control the biofilm formation, interfering with its structure by act on the extracellular polysaccharides and proteins content of *S. mutans* biofilm, without affect the bacterial viability and acid production.

Keywords: geopropolis, *Streptococcus mutans*, biofilm, virulence factors.

Introduction

Dental caries is an infectious disease, biofilm-related and is still the most prevalent oral disease (Marsh, 2003). Dental decay results mainly from the interaction between microorganisms in mouth, tooth surface and diet constituents of the host, especially fermentable carbohydrates (Bowen, 2002). Among the microorganisms present in complex oral microbiota, *Streptococcus mutans* has generally been regarded as the major etiologic agent of dental caries due to its ability to initiate the pathogenic biofilm formation (Loesch, 1986).

The key role of *S. mutans* in the origin and installation of decay is due to their physiological characteristics that allow it to metabolize fermentable substrates, leading to pathological conditions observed in the disease. One of these important virulence factors of this microorganism is the glucans production from sucrose by enzymes known as glucosyltransferases (GTFs). These polysaccharides, mainly insoluble ones, are responsible for extracellular adhesion

of bacteria to tooth surface in the initial stages of the disease onset. Furthermore, these polysaccharides are responsible by forming a complex biofilm matrix, providing stability to this microbial community as well as provide resistance to certain antimicrobial agents, and can be used as energy storage (Koo *et al*., 2002; Wunder & Bowen, 1999; Paes Leme *et al*., 2006).

Other important virulence factor is the *S. mutans* ability to produce acid from fermentable substrates and also to survive in an low pH environment (Brender *et al*., 1985). The environmental low pH is responsible for tooth demineralization, initiating the pathological process of caries (Bowden, 1990).

Although some strategies have been used in dental caries control, many compounds have shown promising activity on the virulence factors of this microorganism, providing new alternatives in the treatment and prevention of this disease (Koo & Jeon, 2009). Among these new alternatives, natural products have great merit, once about 70 % of new antimicrobials available between 1981-2002 were derived from natural sources (Newmann *et al*., 2003).

Propolis is a natural vegetal resin collected by bees and many studies had described its wide range of pharmacological effects, including activity against virulence factors of *S. mutans* (Koo *et al*., 2000; Duarte *et al*., 2003; Hayacibara *et al*., 2005; Castro *et al*., 2009; Sforcin & Bankova, 2011). Further, some bioactive isolated from Brazilian honeybee propolis had shown inhibitory capabilities on development of caries *in vivo* models (Duarte *et al*., 2006).

Most studies on biological activity and chemical composition provide added market value to various types of propolis produced by the *Apis mellifera* bee, while others remain without a detailed description of their chemical composition and pharmacological activity.

Geopropolis is a different type of propolis collected by native stingless bees, like *Melipona scutellaris*, which has resin, wax and soil contents (Dutra *et al*., 2008). However, for this type of propolis, there are few studies about its chemical and biological properties. Velikova *et al*., (2000) had described that Brazilian geopropolis samples has a strong antimicrobial activity against *Staphylococcus*

aureus and identified non-polar compounds such di- and tri-terpene in their chemical composition. Thus, further studies on its biological activity are needed to add value to this product and elucidate its potential as a source of new bioactive compounds. This study investigated the influence of non-polar fraction of ethanolic extract of geopropolis on *S. mutans* biofilm *in vitro*.

Material and methods

Propolis samples and fractionation

Crude samples of geopropolis from *Melipona scutellaris* (native stingless bee) were obtained from the "Entre Rios" town (S 11º 57´ and W 38° 05´), Bahia state, Northeast Brazil. Samples were extracted using ethanol (1:7, w/v) and dried. The ethanolic extract of geopropolis (EEGP) was subjected to chemical fractionation by a liquid–liquid extraction, based on a polarity gradient, as described by Duarte *et al*. (2003). The obtained fractions were subjected to antimicrobial testing (Clinical and Laboratory Standards Institute-CLSI, 2006) and the hexane fraction (non-polar fraction) was selected by presence of antimicrobial activity (Cunha *et al*., 2011). Before using, hexane fraction (HF) was reconstituted with absolute ethanol at 3.2 % (w/v) at concentrations based on minimal inhibitory concentration for *S. mutans*.

Biofilm assays

Biofilms of *S. mutans* UA159 (ATCC 700610, serotype *c*) were formed on saliva-coated hydroxyapatite (sHA) discs (Clarkson Chromatography Products, Inc., South Williamsport, PA; surface area 1.47 cm^2). Human whole saliva was collected from one donor (Ethics Comittee in Research of the School of Dentristry of Piracicaba – State University of Campinas – Protocol # 047/2011), clarified by centrifugation (10000 *g*, 4 ºC, 10 min), sterilized and diluted (1:1) in adsorption buffer (AB – 50 mM KCl, 1 mM KPO_4, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$, pH 6.5),

supplemented with the protease inhibitor phenylmethylsulfonyl-fluoride (PMSF) at a final concentration of 1 mmol/l. The sHA discs were placed in a vertical position, in 24-well plates and inoculated with approximately 2 x 10^6 CFU/mL in buffered ultra filtered (10 kDa cutoff membrane; Prep/Scale; Millipore, MA) tryptone yeast extract (UFTYE, pH 7.0), with addition of 1 % (w/v) sucrose, a 37 ºC, 5 % CO_2. The biofilms were grown undisturbed during 24 hours and then the culture medium was replaced daily during the 5 days of each experiment (total 115 h), according Koo *et al*. (2003). To inhibition of biofilm formation assay and scanning electron microscopy, the biofilms were treated as described below. All assays were done in triplicate on at least three independent experiments.

Inhibition of biofilm formation

To access the effect of hexane fraction of geopropolis on *S. mutans* biofilm formation, 24 h-old biofilms were treated twice daily (10 a.m. and 4 p.m., total of eight treatments) with hexane fraction of geopropolis (250 and 400 µg/mL) or vehicle control (ethanol 12.5 %), both diluted in sterile AB. The biofilms were five times dip rinsed in sterile saline 0.9 % NaCl (to remove non adhered cells) exposed during one-minute to the agents, double-dip rinsed in sterile saline 0.9 % (to eliminate carry over effect) and finally returned to culture medium. At the end of the experimental period (115 h) for biochemical collection data, the biofilms were removed and subjected were subjected to ultrasound bath, sonication (30s pulse; output 7 W) to provided the maximum recoverable viable counts (Koo *et al*., 2003). The homogenized suspension was analyzed for biomass (dry weight), bacterial viability (colony forming units CFU/mL), polysaccharide and protein content. The extracellular water soluble (WSP) and alkali-soluble polysaccharides (ASP) and intracellular iodophilic polysaccharides (IPS) were extracted and quantified by colorimetric assays as detailed by Koo *et al*. (2003) and Duarte *et al*. (2008); the exopolysaccharides were quantified by the phenolsulfuric method (Dubois *et al*. 1956) using glucose as standard, whereas IPS was quantified using 0.2 % I2/2 %

KI solution and glycogen as standard, as described by DiPersio *et al*. (1974). The total protein was determined by colorimetric assays as detailed by Smith *et al*. (1985).

Killing assay

For killing assay, 5-days-old biofilms (without treatment) were exposed to HF at 250 and 400 µg/mL, vehicle (ethanol 12.5 %) and positive control (chlorhexidine digluconate 0.12 %, Sigma-Adrich®). At specific times (30, 60, 90 and 120 min) after exposure, biofilms were removed by ultrasound bath, sonication (30 s pulse; output 7 W) and then the homogenized suspension was serially diluted and plated on Brain Heart Agar. Plates were incubated in 5 % CO_2, at 37 ºC, for 48 h, when the number of colonies was determined (CFU/mL). Killing curves was constructed by plotting log10 CFU/mL versus time over 120 min (Duarte *et al*., 2006)

Glycolytic pH drop

The acid production by *S. mutans* biofilms exposed to tested agents was evaluated using a method described by Belli *et al*., (1995) with some modifications. The 5-days-old biofilms grown in sHA discs (without daily treatments) were washed in salt solution (50 mM KCl, 1 mM $MgCl_2$.6H2O, pH 7.0) and exposed to HF (250 and 400 µg/mL) or vehicle (12.5 % ethanol) control. The pH of these tested solutions were adjusted to 7.2 with 0.1 M KOH solution and glucose was then added (final concentration 1 %, w/v). The decrease in pH was monitored with an Orion® pH glass electrode attached to Orion® 290 A^+ pHmeter, during 90 min.

Scanning electron microscopy (SEM)

For SEM analyses, the treated 115 h-old-biofilms (as described to Inhibition of Biofilm Formation) were rinsed in sterile NaCl 0.9 % and then fixed with a 4 % glutaraldehyde (v/v, in PBS[1]) soltution for 24 h. After, biofilms were dehydrated in graded series of ethanol (50, 70, 90 and 100 %), dried for 24 h and sputter coated with gold-palladium. The samples were then analyzed by SEM (JSM 5600LV, JEOL® Tokyo, Japan) at 7000x (Hawser & Douglas, 1994).

Statistical analyses

The data were subjected to ANOVA and Tukey-Kramer test to adjust for multiple comparisons, using Biostat® version 5.0 software for statistical visualization. The significance level was set at 5 %.

Results

Table 1 shows the influence of bioactive HF of geopropolis on biofilm formation by *S. mutans* on saliva-coated hidroxyapatite surface. The HF was not able to reduce the recoverable viable cells when compared to the vehicle control ($p>0.05$). However it reduced significantly the formation and accumulation of biomass ($p<0.05$) of S. mutans when compared with those treated with vehicle. Treatment with HF at 250 µg/mL reduced 38 % of biomass (dry weight) when compared to vehicle treatment, whereas the treatment with HF at 400 µg/mL promoted a reduction of 53 %. The biofilms treated with HF exhibited about 51-57 % less alkali-soluble polysaccharide (ASP) and 74-80 % less intracellular iodophilic polysaccharide soluble (IPS) than those treated with the vehicle control ($p<0.05$). Excepted the water soluble polysaccharide (WSP), the other kinds of glucans

[1] Phosphate buffered saline, pH 7.4

analyzed in this study were significantly lower than the vehicle control in biofilms treated with HF at both concentration. Furthermore, the treatments also reduced significantly the total amount of protein in the biofilm, when compared o vehicle control ($p<0.05$).

Table1- Biochemical composition and bacterial viability of *Streptococcus mutans* UA 159 biofilm after 5-days treatment with vehicle or hexane fraction (HF) of geopropolis [means (± SD)].

Treatment	DW (mg)	ASP (µg)	IPS (µg)	WSP (µg)	Protein (mg)	BV (log CFU/mL)
Vehicle (Ethanol 12.5 %)	4.63 (± 0.59)	820.0 (± 82.4)	289.9 (± 44.9)	94.9 (± 31.2)	1.14 (± 0.20)	7.44 (± 0.20)
HF 250 µg/mL	2.83* (± 0.88)	403.3* (± 70.0)	73.6* (± 19.6)	75.3 (± 25.2)	0.50* (± 0.12)	7.56 (± 0.16)
HF 400 µg/mL	2.18* (± 0.28)	352.2* (± 62.1)	52.4* (± 05.7)	72.2 (± 31.3)	0.48* (± 0.06)	7.62 (± 0.27)

* $p<0.05$ when compared to vehicle control (ANOVA, Tukey-Kramer). DW: dry weight; ASP: alkali-soluble polysaccharide; IPS: intracellular iodophilic polysaccharide; WSP: water soluble polysaccharide; BV: bacterial viability.

Figure 1 shows that HF, at 250 µg/mL and 400 µg/mL, was able to reduce the cell viability of *S. mutans* on 5-days-old biofilm, but not significantly ($p>0.05$). Furthermore, the acid production by *S. mutans* was not affected by HF at both concentrations ($p>0.05$), in the same condition (Figure 2).

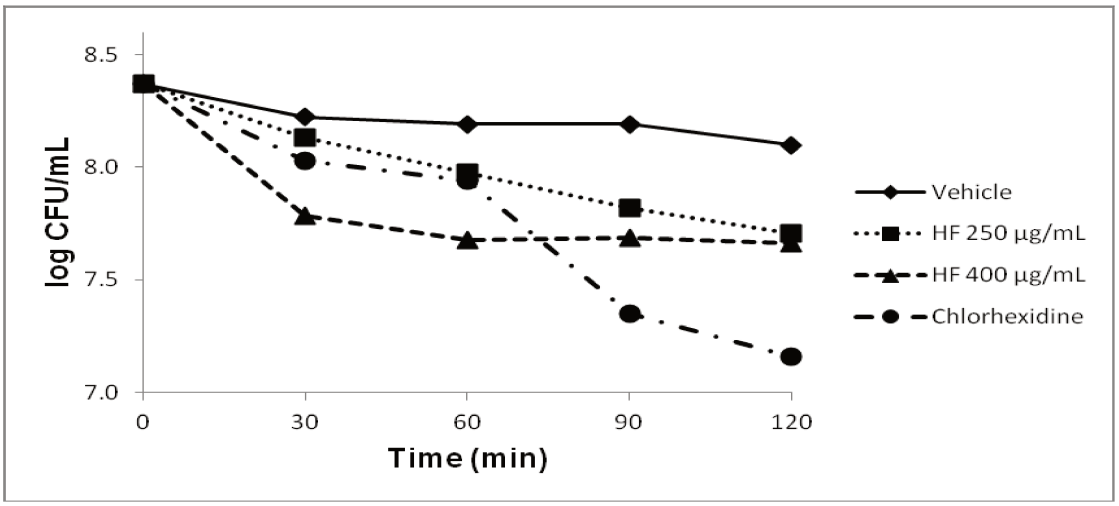

Figure 1- Time kill curves of hexane fraction on *Streptococcus mutans* biofilm (CFU – colony-forming units).

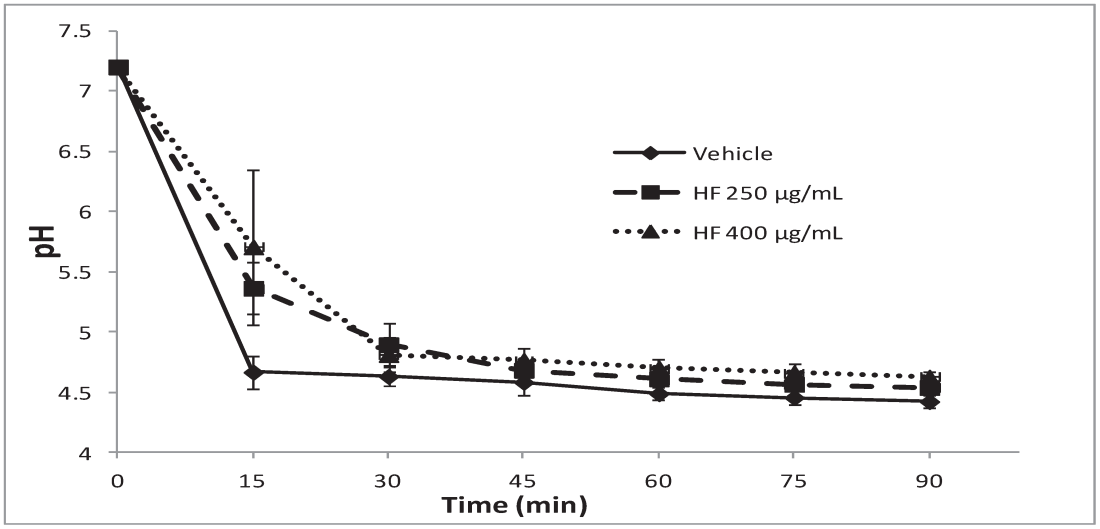

Figure 2- Influence of hexane fraction and vehicle on glycolytic pH-drop in *S. mutans* 5-days-old biofilm.

SEM analyses (Figure 3) show the effect of HF 400 µg/mL on the biofilm accumulation. The images indicate a reduction of biomass accumulation by reducing the extracellular matrix without affect the *S. mutans* growth.

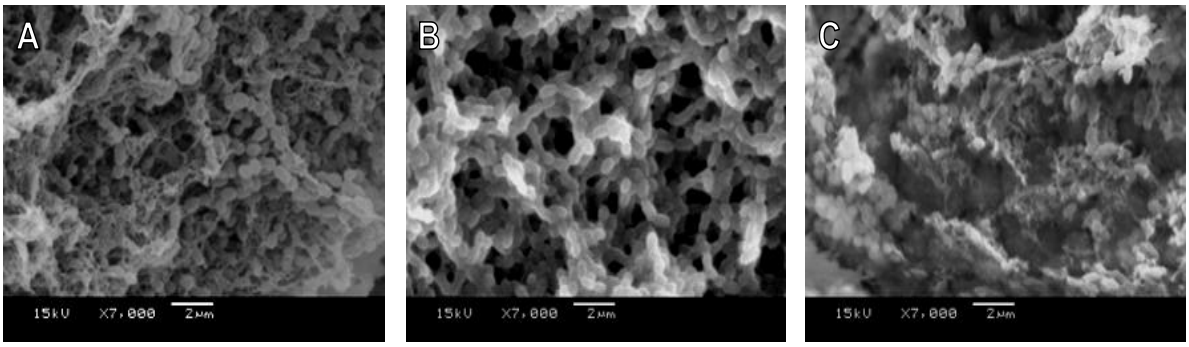

Figure 3- SEM images of *Streptococcus mutans* biofilm after treatments. The biofilms were twice daily treated during five days, with the vehicle control (A), hexane fraction at 250 (B) and 400 µg/mL (C).

Discussion

Dental caries is a multifactorial infectious disease and its origin is associated with the presence of certain bacteria, especially *S. mutans*, that is main responsible to initiate the cariogenic oral biofilm (Marsh, 2003). Thus, some alternative strategies to combat decay are focused on the control of biofilm formed by this organism, acting on their virulence factors such as acidogenicity and polysaccharides formation (Koo & Jeon, 2009). Natural products have been shown to be important source of compounds that can act on these targets. Among these products, propolis has been noted for its known action on *S. mutans*, but geopropolis, a different kind of propolis, had not been studied yet on their ability to control virulence factors of this microorganism. The aim of this study was to evaluate the activity of hexane fraction from ethanolic extract of *Melipona scutellaris* (stingless bee) geopropolis from Atlantic forest of Bahia State (Northeast Brazil) on *in vitro* biofilm of *S. mutans* UA159 (ATCC 700610) and some of its virulence factors. Libério *et al.* 2011 recently studied of *Melipona fasciculata* geopropolis of Maranhão State (Northeast Brazil) from different biomes, however differs from our study because its focus is on the viability of *S. mutans* ATCC 25175.

Geopropolis from *M. scutellaris* is active against *S. mutans* UA 159 grown in planktonic state and non-polar fraction (hexane) was selected because it has the most activity among all tested fractions. In addition, the ethanolic extract of geopropolis and its HF significantly reduced the adherence cell in biofilm *in vitro* (Cunha *et al*, 2011). In the present study, HF of ethanolic extract of geopropolis was able to reduce the biomass (dry weight) compared to vehicle control ($p<0.05$) when treated twice a day (total eight treatments). It was observed no decrease in the number of viable cells of *S. mutans*, suggesting a possible action on biofilm matrix produced by this microorganism. Therefore, the analyses of biochemical composition of biofilm indicated a significantly reduction in amounts of polysaccharides and protein. The interference on matrix formation by HF lead to a structural shift in matrix, since these biochemical compounds are responsible for a three-dimensional conformation of biofilm (Koo *et al*., 2010). Confirming the action on matrix, SEM images (Figure 3) shown a qualitative change in structure and organization of the biofilm, as well as loss of surface homogeneity. The apparent loss of homogeneity could be due to a simple rearrangement superficial, but the biochemical content data corroborate to the hypothesis that HF promoted a lower accumulation of insoluble and soluble glucans and proteins in the matrix, when compared to vehicle control. This reduction on polysaccharide accumulation can be mainly due to an inhibitory activity on bacterial GTF or by affecting the expression of *gtf* genes (Koo *et al*., 2003).

Once HF was able to significantly reduce the extracellular polysaccharides amounts, HF had shown an important impact on the accumulation and development of cariogenic biofilm. These polysaccharides, mainly insoluble extracellular (alkali-soluble), may represent more than 50 % of biofilm dry weight and they are considered responsible for promoting the binding and accumulation of microorganisms on the apatitic surface and to each other (Schiling & Bowen, 1992; Koo *et al*., 2010). Interfering on the synthesis of the insoluble polysaccharides, HF would be useful on attenuation of *S. mutans* virulence organized on biofilm, which protects the bacteria from environmental influences (Bowen & Koo, 2011),

including antimicrobial agents (Wunder & Bowen, 1999). With these protections affected, the bacteria would be more susceptible to host defenses, making difficult to join on the tooth surface and consequently pathogenic biofilm installation. Furthermore, a change in extracellular glucan content of the biofilm caused by HF could influence bacteria superficial adherence, which would explain the findings of Cunha *et al*. (2011) on bacterial cell adherence.

Furthermore, it was observed a reduce of IPS amount in biofilms treated with HF. This glucans are glycogen-like storage polymers with α 1-4 and α 1-6 linkages that can be fermented by bacteria under conditions in which exogenous carbohydrates are absent. The use of these polysaccharides by *S. mutans* leads to acid production that contributes to tooth demineralization (Paes Leme *et al*., 2006). This way, the long time exposure to HF could attenuate, in part, the pathogenic effects caused by cariogenic biofilm.

Besides the analysis of action on biofilm matrix, HF was evaluated on cellular viability and acid production by *S. mutans*. At the concentrations tested in this study, HF was not able to affect the *S. mutans* viability and also had no interference on acid production by microorganism. Duarte *et al*. (2006) showed a similar result for Brazilian propolis type 6, collected by *A. mellifera* bee. Although this type (6) of propolis has affected the acid production by bacteria, it did not affect *S. mutans* UA 159 viability, as well as HF in present study. Moreover, in another report, Brazilian propolis type 6 was able to inhibit the *S. mutans* growth and adherence besides to reduce the GTFs activity in solution and adsorbed onto sHA surface. Thereby, it would lead to a decrease in extracellular polysaccharides production and probably reduction on cell adherence and biofilm biomass, as observed for HF (Duarte *et al*., 2003).

Such similar results between geopropolis from *M. scutellaris* and Brazilian propolis type 6 could be explained in part by the same region of collection of these two varieties of propolis, once the biome of collect determines de chemical content of propolis (Park *et al*., 2004). Even collected by different bees, both products are obtained from the same region of Atlantic Forest on Bahia State,

Northeastern Brazil, and according to preliminary studies from our research group they appear have similar chemical profile, once geopropolis presents essentially non-polar compounds, with absence of flavonoids, as well as described for Brazilian propolis type 6 (Castro *et al*., 2007).

Libério *et al*. (2011) evaluated the activity of hydroalcoholic extract of geopropolis from *M. fasciculata* on oral pathogens. Their results indicated that this extract at 25 mg/mL, had been capable to reduce viability of *S. mutans* (ATCC 25175) on biofilm formed in cell-culture plates. The different results observed when compared to present study can be due to distinct bacterial strains tested, the active concentration (higher than 60 times of our higher concentration), biofilm model (24 wells polystyrene cell-culture plates), and models used to perform the biofilm treatment and mainly the biome (lakes and babassu palm forests) where these geopropolis were collected. Our study reports the activity of *M. scutellaris* geopropolis, collected in the Atlantic forest region of Bahia, while *M. fasciculata* geopropolis were originated from of an ecosystem composed of mangroves, wetlands, lakes and babassu palm forests, in Maranhão State, Northeastern Brazil, that probably provides a different source of vegetal resins altering geopropolis chemical composition. Furthermore, previous analyses show that *M. scutellaris* geopropolis have no flavonoids, while the activity of *M. fasciculata* geopropolis is assigned to presence of these compounds.

Finally, concerning new approaches in antimicrobial agents, Koo & Jeon (2009) describe that natural products that act on virulence factors of *S. mutans* without necessarily killing bacteria, has attracted attention as important sources of new effective drugs against dental caries. In light of this vision, our data show that geopropolis, especially its non-polar fraction (hexane fraction) are a promising source of new compounds capable to act on dental caries, and further studies are needed to confirm such activity and isolate/identify the active compound.

Conclusion

The hexane fraction of *M. scutellaris* (stingless bee) geopropolis from Atlantic forest of Bahia State (Northeast Brazil) interfered on biofilm formation, by reducing biomass, affecting the biochemical content (polysaccharides and proteins) accumulation. Also, hexane fraction has not affected the viability and acid production by *S. mutans*. Although further studies are necessary, geopropolis action on an important virulence factor of *S. mutans* appears to be a promising source of compounds that can be used on control and prevention of caries.

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CONCLUSÃO

No presente estudo, conclui-se que a geoprópolis de *M. scutellaris* apresentou uma interessante atividade antimicrobiana contra *S. mutans* e citotoxicidade mais seletiva para linhagens tumorais, sendo que a fração hexânica do extrato etanólico foi a que se mostrou mais ativa, indicando a possível composição química apolar do(s) composto(s) responsável(eis) pela atividade biológica. Além disso, a fração hexânica mostrou-se capaz de afetar o biofilme formado por *S. mutans in vitro* reduzindo a sua biomassa, pela diminuição da produção e acúmulo de componentes bioquímicos da matriz do biofilme. Embora estudos complementares sejam necessários para especificar a atividade sobre o biofilme da fração hexânica, esta se mostra promissora fonte de compostos que possam atuar no controle e prevenção da cárie dental.

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

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ANEXO 1: Informação CCPG/002/06 que trata do formato padrão das Dissertações de Mestrado e Teses de Doutorado da UNICAMP.

INFORMAÇÃO CCPG/002/06

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

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

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

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

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

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

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

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

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

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

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

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

§ 3º - A gráfica da Unicamp imprimirá os exemplares solicitados com capa padrão. Os exemplares solicitados serão retirados pelas Unidades em no máximo, cinco dias úteis para impressão preto e branco e 10 dias úteis para coloridas.

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

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

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

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

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

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

Campinas, 13 de setembro de 2006

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

ANEXO 2: Certificado de aprovação do Comitê de Ética em Pesquisa - Faculdade de Odontologia de Piracicaba/UNICAMP.

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Avaliação do potencial antimicrobiano e ação in vitro sobre o biofilme dental de compostos isolados da geoprópolis de meliponinae (Melipona scuttellaris)"**, protocolo nº 047/2011, dos pesquisadores Marcos Guilherme da Cunha e Pedro Luiz Rosalen, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 20/07/2011.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"Evaluation of antimicrobial potential and in vitro activity on the dental biofilm by isolated compounds from meliponinae geopropolis (Melipona scutellaris)"**, register number 047/2011, of Marcos Guilherme da Cunha and Pedro Luiz Rosalen, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 07/20/2011.

Profa. Dra. Lívia Maria Andaló Tenuta
Secretária
CEP/FOP/UNICAMP

Prof. Dr. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

ANEXO 3: Certificado de revisão de língua inglesa do Capítulo 1.

São Paulo, January 13th, 2012.

We hereby declare that the article:
"GEOPROPOLIS FROM STINGLESS BEE: ANTIMICROBIAL AND ANTIPROLIFERATIVE ACTIVITIES" has been reviewed by language professionals of Grupo Solución.

According to the author´s directions, we should not change the highlighted text.

Regards,

_____

Paulo de Holanda Morais

ANEXO 4: E-mail de confirmação da submissão do artigo referente ao Capítulo 1.

-------- Mensagem original --------
Assunto: [CPB-BPB] Submission Confirmation for GEOPROPOLIS FROM STINGLESS BEE: ANTIMICROBIAL AND ANTIPROLIFERATIVE ACTIVITIES
Data: 30 Jan 2012 06:43:42 -0500
De: Bulletins of the Pharmaceutical Society of Japan <bull_h@pharm.or.jp>
Para: Pedro Luiz Rosalen <rosalen@fop.unicamp.br>

Dear Rosalen,

Your submission entitled "GEOPROPOLIS FROM STINGLESS BEE: ANTIMICROBIAL AND ANTIPROLIFERATIVE ACTIVITIES" has been received by journal Bulletins of the Pharmaceutical Society of Japan

You will be able to check on the progress of your paper by logging on to Editorial Manager as an author. The URL is http://cpb-bpb.edmgr.com/.

Your manuscript will be given a reference number once an Editor has been assigned.

Thank you for submitting your work to this journal.

Kind regards,

Bulletins of the Pharmaceutical Society of Japan