Universidade Estadual de Campinas
Faculdade de Odontologia de Piracicaba

SIMONE DUARTE
Cirurgiã Dentista

Avaliação do potencial anticárie da própolis proveniente da região de mata atlântica da Bahia.

Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas para a obtenção do título de Doutor em Odontologia, Área de Farmacologia, Anestesiologia e Terapêutica.

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Orientador: Prof. Dr. Pedro Luiz Rosalen
Co-orientador: Prof. Dr. Hyun Koo

Banca examinadora:
Prof. Dra. Izabel Yoko Ito
Prof. Dr. Jaime Aparecido Cury
Profa. Dra. Mary Ann Foglio
Prof. Dr. Masaharu Ikegaki
Prof. Dr. Pedro Luiz Rosalen

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PROF. DR. MASAHARU IKEGAKI

PROFa. DRa. IZABEL YOKO ITO

PROF. DR. JAIME APARECIDO CURY

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Pense profundamente.
Fale gentilmente.
Ame bastante.
Ria freqüentemente.
Trabalhe com afinco.
Dê com generosidade.
Pague pontualmente.
Ore fervorosamente.
E seja bom.

Elmer Wheeler

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RESUMO

Uma nova variedade de própolis, classificada como tipo 6, embora não apresente flavonóides em sua composição química, tem demonstrado atividade antimicrobiana e anti-glucosiltransferase *in vitro*. O objetivo deste estudo foi avaliar os efeitos anticárie e a composição química da propolis tipo 6 (EEP) e de sua fração hexano (EEH), além de duas sub-frações da EEH (Hfr-2 e Hfr-3), pré-selecionadas. Os extratos de EEP e EEH foram avaliados para determinar sua influência na viabilidade (*Time-Kill*) de biofilmes de *Streptococcus mutans* UA159 e *Streptococcus sobrinus* 6715, queda de pH glicolítico e seu efeito na translocação de prótons pela atividade da ATPase. Estes extratos também foram testados utilizando modelo experimental de cárie em ratos. Além disso, foram realizados estudos utilizando Hfr-2 e Hfr-3 para analisar seu efeito na atividade de glucosiltransferase (GTF) B e GTF C em solução (GTF-sol) e em superfície (GTF-sup), e também na composição de polissacarídeos e no acúmulo de biofilmes de *S. mutans* UA159 formados em discos de hidroxiapatita. A composição química foi analisada por cromatografia gasosa – espectrometria de massa. Os resultados demonstram que os extratos EEP e EEH não apresentaram efeito na viabilidade bacteriana dos biofilmes, entretanto reduziram a produção de ácidos orgânicos e a atividade da ATPase (60-65% de redução a 800 μg/ml). Além disso, a incidência de cárie em superfície lisa foi significativamente reduzida por EEH e EEP a 5%, p/v ($p<0,05$), sendo que o EEH foi também capaz de reduzir incidência e severidade de cárie de sulco ($p<0,05$), quando comparado ao controle. A porcentagem de infecção por *S. sobrinus* não foi afetada pelos extratos. Quanto às sub-frações Hfr-2 a Hfr-3, ambas foram potentes inibidores da atividade de GTFs, tanto em solução quanto em superfície de hidroxiapatita, sendo 52-86% de redução para GTF B-sol e 22-40% de redução para GTF B-surf, em concentrações menores que 500 μg/ml. Resultados semelhantes foram encontrados para GTF C. Nos biofilmes, o peso seco e a quantidade de polissacarídeos insolúveis e intracelulares também foram significativamente reduzidos com o tratamento de Hfr-2 ($p<0,05$), embora este extrato não tenha apresentado atividade antibacteriana. Além disso, ácidos graxos (oléico, palmítico, linoléico, esteárico) foram os principais compostos identificados na própolis tipo 6 e suas frações. Os resultados indicam que os efeitos biológicos da própolis tipo 6 podem ser atribuídos à sua alta concentração de ácidos graxos, sendo que o seu efeito anticárie pode não estar relacionado com a atividade antimicrobiana, mas com a inibição dos fatores de virulência do biofilme cariogênico.

ABSTRACT

A new variety of Brazilian propolis classified as type-6, despite having no flavonoids in its chemical composition, has shown antimicrobial and anti-glucosyltransferase activities *in vitro*. The aim of this study was to evaluate the anti-caries effect and the chemical composition of propolis type-6 (EEP) and its purified hexane fraction (EEH) and also of two selected bioactive sub-fractions of EEH (Hfr-2 and Hfr-3). The EEP and EEH were analyzed to determine their influence on Time-Kill of *Streptococcus mutans* UA159 and *Streptococcus sobrinus* 6715 biofilms, glycolytic pH drop assays and their effects on proton-translocating ATPase activity. These extracts were also tested using an experimental dental caries model in rats. Furthermore, studies using Hfr-2 and Hfr-3 were done to determine their effect on glucosyltransferase (GTF) B and GTF C both in solution (GTF-sol) and adsorbed onto a saliva-coated hydroxyapatite (GTF-surf), and also on polysaccharide composition and on accumulation of *S. mutans* biofilms formed on hydroxyapatite discs. The chemical compositions were examined by gas chromatography/mass spectrometry. The results show that the EEP and EEH did not show any major antibacterial activity on biofilms, however, inhibited the organic acid production and also the ATPase activity (60-65% inhibition at 800 μg/ml). Furthermore, the caries incidence on smooth surface was significantly reduced by both extracts at 5%, w/v ($p<0.05$), and the EEH was able to reduce the incidence and severity of sulcal surface caries ($p<0.05$) when compared to the control group. The percentage of *S. sobrinus* infection was not affected by the propolis extracts. Both sub-fractions Hfr-2 and Hfr-3 were potent inhibitors of GTFs B and C activities, in solution or adsorbed to a saliva-coated hydroxyapatite surface: GTF B-sol (52-86% of reduction) and GTF B-surf (22-40% of reduction) at concentrations as low as 500 μg/ml. Similar results were observed for GTF C. In biofilms, the dry weight and the amount of insoluble and intracellular polysaccharides of the biofilms treated with Hfr-2 were significantly lower than those treated with vehicle-control ($p<0.05$), even if this extract did not show antibacterial activity. In addition, fatty acids (oleic, palmitic, linoleic, stearic) were the main compounds identified in propolis type 6 and its fractions. The data suggest that the biological effects observed for this type of propolis could be attributed to its high content of fatty acids, and the anti-caries effect could be related not to the antibacterial activity but to the inhibition of cariogenic biofilm virulence factors.

I – INTRODUÇÃO

A formação da cárie dental é atribuída à aderência de bactérias orais específicas à superfície dos dentes, iniciando a formação do biofilme patogênico e a subseqüente produção de ácidos pela fermentação de açúcares da dieta, resultando na desmineralização do esmalte dental (GIBBONS e von HOUTE, 1975; HAMADA e SLADE, 1980; MARSH e BRADSHAW, 1993; BOWEN, 2002).

Neste biofilme, os estreptococos do grupo mutans são considerados microrganismos cariogênicos principalmente por apresentarem características acidúricas e acidogênicas, além da capacidade de sintetizarem glucanos extracelulares a partir da sacarose, através das glucosiltransferases (GTFs) (de STOPELAR *et al.*, 1971; GIBONS e van HOUTE, 1975; HAMADA e SLADE, 1980; BOWEN, 2002; MARSH, 2004).

Os glucanos promovem a aderência e o acúmulo de estreptococos mutans e outras bactérias à superfície dental, resultando na formação do biofilme cariogênico (HAMADA e SLADE, 1980; BOWEN, 2002; MARSH, 2004). Com a ingestão de açúcares, inicia-se rapidamente a produção de ácidos por este biofilme, o que contribui para a desmineralização do esmalte dental. Além disso, os estreptococos mutans têm a capacidade de sobreviver neste meio ácido, utilizando alguns mecanismos como a F-ATPase, que permite a translocação de prótons H^+ para fora da célula, mantendo o pH intracelular mais alcalino que o extracelular (BELLI e MARQUIS, 1991). A alta tolerância ao meio ácido desses microrganismos tem um papel importante na sua virulência em relação à cárie dental (QUIVEY *et al.*, 2000).

Assim, estratégias têm sido idealizadas no sentido de prevenir a cárie dental, eliminando seletivamente os estreptococos mutans ou inibindo seus fatores de virulência, incluindo a redução da produção de glucanos, através da inibição da atividade das GTFs, e reduzindo a capacidade desses microrganismos de produzir e tolerar ácidos.

Nas últimas décadas têm se observado um crescente interesse por medicinas alternativas e terapias naturais, e os produtos apícolas têm encontrado

grande aceitação, principalmente por suas possíveis propriedades terapêuticas (PARK *et al.*, 1997). Dentre esses produtos, a própolis tem se destacado pelas suas diversas propriedades farmacológicas tais como atividade antimicrobiana, antiinflamatória, cicatrizante, anestésica (GHISALBERTI, 1979), anticariogênica (IKENO *et al.*, 1991; PARK *et al.*, 1998; KOO *et al.*, 1999; KOO *et al.*, 2000a; KOO *et al.*, 2000b; KOO *et al.*, 2000c), antitumoral (SCHELLER *et al.*, 1977; FRENKEL *et al.*, 1993; RAO *et al.*, 1995), citotóxica (GRUNDBERGER *et al.*, 1988; MATSUNO, 1995; MATSUNO *et al.*, 1997a; MATSUNO *et al.*, 1997b; BANSKOTA *et al.*, 1998; BANSKOTA *et al.*, 2000) e antiviral (DEBIAGGI *et al.*, 1990; SERKEDJIEVA *et al.*, 1992; AMOROS *et al.*, 1992a; AMOROS *et al.*, 1992b; AMOROS *et al.*, 1994; HARISH *et al.*, 1997; KUJUMGIEV *et al.*, 1999; VYNOGRAD *et al.*, 2000).

Própolis é o nome genérico dado para uma resina de coloração e consistência variada, coletada por abelhas da espécie *Apis mellifera* de diversas partes da planta, como broto, botões florais e também dos exudatos resinosos (GHISALBERTI, 1979).

Estudos anteriores mostraram que o extrato etanólico de algumas própolis possui atividade antibacteriana bastante satisfatória (BONHEVI *et al.*, 1994; GRANGE e DAVEY, 1990; IKENO *et al.*, 1991; PARK *et al.*, 1998; STEINBERG *et al.*, 1996; KOO, *et al.*, 2000a; 2000b; 2000c; DUARTE *et al.*, 2003), assim como inibição das GTFs bruta (PARK *et al.*, 1998) e purificadas (KOO, *et al.*, 2000a; DUARTE *et al.* 2003). Em acréscimo, a composição química da própolis varia de acordo com a biodiversidade da região onde esta é coletada (KÖNIG, 1985; GREENAWAY *et al.*, 1990; GARCIA-VIGUEIRA *et al.*, 1992; BANKOVA *et al.*, 1992; TOMAS-BARBERAN *et al.*, 1993; PARK *et al.*, 1997; BANKOVA *et al.*, 1999, KUJUMUGIEV *et al.*, 1999; KOO *et al.* 2000b), a variedade da abelha rainha (KOO e PARK, 1997) e ainda, com a variação sazonal (BANKOVA *et al.*, 1996; SFORCIN *et al.*, 2000, CASTRO *et al.*, 2004).

Num estudo de própolis brasileiras foram coletadas aproximadamente 600 amostras de própolis, provenientes das regiões sudeste, sul, centro-oeste e alguns estados do nordeste, sendo a composição química extremamente variável (PARK

et al., 1997; KOO e PARK, 1997; PARK e IKEGAKI, 1998). Essas amostras foram então classificadas de acordo com o perfil químico, em 12 diferentes tipos (PARK *et al.* 2000), sendo que três amostras têm se destacado quanto à atividade contra microrganismos bucais e inibição de GTFs, sendo a do grupo 3, proveniente da região do Rio Grande do Sul; do grupo 6, da região de mata atlântica da Bahia; e do grupo 12, de Minas Gerais (PARK *et al.*, 1998; KOO *et al.*, 2000b; DUARTE *et al.*, 2003). As dos tipos 3 e 12 apresentam flavonóides em sua composição química (KOO *et al.*, 1999, KOO *et al.*, 2000c). Já a própolis do tipo 6, mostrou características químicas bastante particulares, com alta atividade antimicrobiana *in vitro* (KOO *et al.*, 2000b, DUARTE *et al.*, 2003), porém com uma composição química caracterizada principalmente por componentes apolares e ausência de flavonóides (MOURA, 2000; ALENCAR *et al.*, 2001).

DUARTE *et al.* (2003) analisaram amostras da própolis tipo 6, que foram fracionadas de acordo com um gradiente de polaridade em quatro frações (hexano, clorofórmio, acetato de etila e etanol). Os extratos etanólicos da própolis bruta e das frações foram estudados *in vitro* quanto às suas atividades contra estreptococos mutans e na inibição das GTFs purificadas. Os resultados confirmaram a atividade antibacteriana em células planctônicas e a inibição da aderência celular dessa própolis, e, além disso, mostraram que a própolis tipo 6 é um potente inibidor de GTFs purificadas, principalmente B e C, sendo que a sua fração apolar hexano apresentou os melhores resultados, indicando que os componentes biologicamente ativos têm características apolares.

Deste modo, baseado nesses resultados promissores *in vitro* (DUARTE *et al.* 2003), o objetivo principal deste estudo foi avaliar os efeitos anticárie e a composição química da própolis tipo 6 e suas frações, analisando também sua influência em alguns fatores de virulência do biofilme dental cariogênico.

II – PROPOSIÇÃO

1. Avaliar o potencial anticárie do extrato etanólico da própolis tipo 6 (EEP) e da sua fração hexano (EEH) através de:

- ✓ Análise da viabilidade bacteriana (*Time Kill*), da produção de ácidos e da atividade de ATPase de biofilmes de *S. mutans* e *S. sobrinus*;

- ✓ Desenvolvimento de cárie experimental em ratos.

2. Analisar o potencial anticárie das sub-frações Hfr-2 e Hfr-3 através de:

- ✓ Inibição de atividade de GTFs B e C purificadas; e

- ✓ Acúmulo e composição de biofilmes de *S. mutans*.

3. Avaliar a composição química da própolis tipo 6 (EEP) e suas frações (EEH, Hfr-2 e Hfr-3).

III – CAPÍTULOS

Esta tese está baseada na Deliberação CCPG – 001/98 – Unicamp que regulamenta o formato alternativo para tese e permite a inserção de artigos científicos de autoria do candidato.

Assim sendo, esta tese é composta de dois capítulos contendo artigos que foram ou estão sendo submetidos para publicação em revistas científicas, conforme descrito abaixo:

✓ Capítulo 1

"The influence of a novel propolis on mutans streptococci biofilms and caries development in rats." Este artigo foi submetido à publicação no periódico *Archives of Oral Biology* (Anexo 1).

✓ Capítulo 2

"Bioactive fractions of a novel Brazilian propolis on *Streptococcus mutans* glucosyltransferases and biofilm development." Este artigo está sendo submetido à publicação no periódico *FEMS Microbiol.*

Capítulo 1

The influence of a novel propolis on mutans streptococci biofilms and caries development in rats.

Running title – The influence of propolis on biofilm and caries

Simone Duarte[a], Pedro L. Rosalen[a], Mitsue F. Hayacibara[a], Jaime A. Cury[a], William H. Bowen[b], R.E. Marquis[b], Vera L. G. Rehder[c], Adilson Sartoratto[c], Masaharu Ikegaki[d], and Hyun Koo[b].

[a] Department of Physiological Sciences, Dentistry School of Piracicaba, State University of Campinas (UNICAMP), Piracicaba, SP, Brazil.

[b] Center for Oral Biology and Eastman Department of Dentistry, University of Rochester Medical Center, Rochester, NY, USA

[c] Pluridisciplinar Center of Chemical, Biological and Agricultural Researches, CPQBA/ UNICAMP, Paulínia, SP, Brazil.

[d] School of Pharmacy and Dentistry of Alfenas; Alfenas, MG, Brazil

* Hyun Koo
University of Rochester Medical Center, Center for Oral Biology
601 Elmwood Avenue Box 611,
14642, Rochester, NY, USA
Tel.: +1(585) 275-1433 / FAX +1(585) 473-2679
e-mail: hyun_koo@urmc.rochester.edu

Abstract

Since a flavanoids-free Brazilian propolis (type-6) showed in vitro activity against mutans streptococci and inhibition of glucosyltransferases activity, this study evaluated its effect on mutans streptococci biofilms and on caries development in rats. The ethanolic extract (EEP) of this propolis and its purified hexane fraction (EEH) were evaluated, and their chemical composition examined by gas chromatography/mass spectrometry. The influence of EEP and EEH on *Streptococcus mutans* UA159 and *Streptococcus sobrinus* 6715 biofilms was analyzed by time-kill, glycolytic pH drop assays, and on proton-translocating F-ATPase activity. In the animal study the rats were infected with *S. sobrinus* 6715 and treated topically twice/day with the extracts. The rats were fed with cariogenic diet 2000 and after 5 weeks they were killed; their detal plaque microbial composition and caries scores were determined. The results showed that fatty acids (oleic, palmitic, linoleic, stearic) were the main compounds identified in both EEP and EEH. These extracts did not show remarkable effect on mutans streptococci biofilms formation. However, EEP and EEH reduced significantly the acid production by the biofilms and also the activity of F-ATPase (60-65%). Furthermore, both extracts reduced significantly the incidence of smooth surface caries in rats ($p<0.05$). However, only EEH was able to reduce the incidence and severity of sulcal surface caries ($p<0.05$), but the percentage of *S. sobrinus* infection was not affected by the propolis extracts. The data suggest that the biological effects observed for this type of propolis could be attributed to its high content of fatty acids.

Keewords: propolis, dental biofilm, virulence factors, and dental caries.

INTRODUCTION

Propolis, a non-toxic resinous hive product collected by *Apis mellifera* bees from various plant sources, has been recognized to have several properties that may confer health benefits to humans, [1,2] including prevention of oral diseases.[3–6] However, the chemical composition and the pharmacological activity of propolis are highly variable depending on the geographic origin of this natural substance.[5,7–9] Thus far, twelve types of Brazilian propolis have been chemically characterized using chromatographic methods and classified from type-1 to 12;[10] propolis type-3 (from Southern Brazil), type-6 (from Northeastern Brazil) and type-12 (from Southeastern Brazil) have been shown anti-caries and anti-plaque properties *in vitro* (for types-3, -6, and -12) and *in vivo* (for types-3 and -12) .[4,5,9,11] Among them, propolis type-6 is the most intriguing sample because flavonoids were not detected. Nevertheless, its ethanolic extract showed potent inhibitory activities against mutans streptococci and glucosyltransferases (GTFs) activity.[9,11] In addition, Duarte *et al.*[9] showed that the non-polar hexane fraction of propolis type 6 was the most active extract against mutans streptococci *in vitro*. Thus far, diterpenic acids and phenolic compounds, such as flavonoid aglycones and (hydroxyl) cinnamic acid derivatives have been widely cited as the main biologically active compounds in propolis.[1,12–16] Therefore, propolis type-6 may harbor biological active compounds not previously considered.

It is known that mutans streptococci is generally regarded as a primary microbial culprit in the etiology of dental caries because of their acidogenic and aciduric properties together with their ability to synthesize extracellular glucans from sucrose catalyzed by glucosyltransferases.[17–21] Glucans promote the adherence and accumulation of mutans streptococci and other oral bacteria on the tooth surface which results in formation and

establishment of cariogenic dental biofilms.[19,22–25] Acid is formed rapidly within this biofilm following ingestion of sugars, which can contribute to demineralization of tooth enamel during caries development. Mutans streptococci catabolize multiple fermentable dietary carbohydrates, and carry out glycolysis at low biofilm pH values in the oral cavity.[26] One of the mechanisms by which mutans streptococci have developed to alleviate the influences of acidification is an increased proton-translocating F-ATPase activity in response to low pH.[26] The high acid tolerance, exhibited by these microorganisms, plays a critical role in their expression of virulence and in the pathogenesis of dental caries.[27] Therefore, there are several avenues for chemotherapeutic intervention other than attempting to eliminate mutans streptococci selectively; these include reduction of glucan production by GTFs and to reduce the ability of organisms to produce and tolerate acids.

The aim of this study was to analyze the chemical composition of ethanolic extract of propolis type-6 and its hexane fraction, and examine their influence on viability and acid production of mutans streptococci biofilms *in vitro*, and on caries development *in vivo*. In addition, we investigated the effects of the propolis extracts on the activity of F-ATPase, an important enzyme associated with acid tolerance of mutans streptococci.

MATERIALS AND METHODS

Propolis samples and fractionation

Crude samples of *A. mellifera* bees' propolis were obtained from Atlantic forest region of Bahia state, northeastern Brazil, and were classified as type 6 according to Park *et al.*[10] The samples were extracted using aqueous ethanol 80% (v/v) and dried. The EEP (ethanolic extract of crude propolis type 6) was prepared in aqueous ethanol (80% v/v) and

it was subjected to a chemical fractionation, as described by Duarte *et al.*[9] The EEP and the ethanolic extract of hexane fraction (EEH) were used.

Chemical analysis

Gas Chromatography / Mass Spectrometry (GC-MS)

The extracts were analyzed by Gas Chromatography coupled to a Mass Seletive Detector (GC-MS). For best results, before the GC-MS analysis, the extracts were methylated using diazomethane solution. The analyses were obtained in gas chromatography Hewlett-Packard 5890 Series II (Palo Alto, CA, USA) equipment, with mass selective detector HP-5971 in the electron impact ionization mode (70eV), injector *split/splitless*, capillary column J & W Scientific DB-5 (25 m x 0,2 mm x 0,33 μm). Temperatures: injector = 250°C, column = 60°C, 3°C/min, 240°C (7 min), detector = 300°C. Carrier gas (He) = 1,0 ml/min. The GC-MS peaks were identified by comparison with data from literature[28] and the profiles from the Wiley 138 and Nist 98 libraries.

Biofilm assays

Bacteria and biofilm growth

Streptococcus mutans UA159 and *Streptococcus sobrinus* 6715 were used in order to produce mono-organisms biofilms. *S. mutans* and *S. sobrinus* biofilms were grown on glass microscope slides in tryptone yeast extract broth with addition of 1% (w/v) of sucrose at 37° C, as described previously by Curran *et al.*[29] and Ma *et al.*[30] Each slide was transferred daily to a new fresh medium for 5 days.

Killing assays

For killing assays, a five-day-old biofilm was transferred to 1% peptone broth containing one of the following treatments: (1) EEP, (2) EEH, both at 800 μg/ml, final concentration based on the MIC results,[9] or (3) ethanol 10% (v/v), used as a vehicle-control, and incubated at 37° C. At specific time intervals (0, 30 min, 1h, 2h, 4h), entire biofilms were removed, dispersed and diluted with 1% peptone broth at pH 7.0. Biofilms were dispersed by first scraping them from glass slides into 45 ml of peptone broth. The biofilm was then sonicate on ice with a Branson Sonifier Cell Disruptor 200 at 60 W for 15 s. This procedure was sufficient to obtain suspensions with only single cells, which were used for plating and counting on tryptic soy agar (48 h, 37° C).

Glycolytic pH drop assay

Glycolytic pH drop assay was performed as previously described by Belli *et al.*[31] for intact *S. mutans* UA159 and *S. sobrinus* 6715 biofilms. Biofilms were transferred to a solution containing one of the following test agents: (1) EEP, (2) EEH, both at 800 μg/ml, or (3) ethanol 10% (v/v) The pH of the solution was adjusted to 7.2 with 2M KOH solution, and sufficient glucose was added to give a concentration of 1% (w/v). The fall in pH was assessed with glass electrode overtime (up to 3 h).

ATPase assay

ATPase assay was performed using permeabilized cells of *S. mutans* UA159 prepared as described by Belli *et al.*[31] ATPase activity was assayed in terms of the release of inorganic phosphate in 100 mmol of Tris-maleate buffer, pH 7.0, containing 10 mmol

$MgCl_2$, permeabilized cells and (1) EEP, (2) EEH, both at 800 μg/ml, or (3) ethanol 10% (v/v). The released phosphate was then determined by the method of Bencini *et al.*[32]

Animal study

To conduct the animal study, thirty-six specific pathogen-free female Wistar rats, aged 19 days, were purchased from CEMIB/UNICAMP (Campinas, Brazil). The animals were screened for indigenous mutans streptococci by means of an oral swab streaked on mitis salivarius agar (MSA) (Difco Laboratories, Detroit, Mich., USA) and mitis salivarius agar plus bacitracin (MSB) (Sigma, St. Louis, Mo., USA), according to BOWEN *et al.*[33] When aged 21, 22 and 23 days, the rats were infected with *S. sobrinus* 6715. They were fed pellet chow, Diet 2000[34] and 5 % sucrose in drinking water *ad libitum* until 25 days of age to establish the infection by *S. sobrinus*. To confirm the infection, oral swabs from the animals were streaked on mitis salivarius agar plus streptomycin (MSS) (Sigma), and, if necessary reinfected. At age 25 days, the rats were randomly divided into three groups of 12 animals each one and placed individually. From the 26th day, first day of experiment, to the 62th day, the end of the fifth week of experiment, approximately 100 μl of: (1) crude propolis (EEP), (2) hexane fraction (EEH), both at 5% (w/v), or (3) the vehicle-control ethanol 80% were applied to the molars of the rats twice daily, using a camel-hair brush, as detailed elsewhere.[5]

Diet 2000 and sterilized distilled water were provided *ad libitum*. The animals were weighed weekly, and their physical appearance noted daily. At the end of the 5-week experimental period, the rats were killed by CO_2 asphyxiation. The left jaw was aseptically dissected and transferred to 5.0 ml of 0.89% sterile NaCl solution, and sonicated using a Vibra Cell (Sonics & Material Inc; 6 pulses of 10 second with 5-second intervals at 40

watts). The suspensions obtained were used for microbial assessment. Aliquots (50 µl) of the suspensions were streaked on blood agar and on MSS (Sigma), using a spiral plater (Whitley Automatic Spiral Plater, DW Scientific), to determine the number of total microorganisms and the *S. sobrinus* recovered. All the jaws were defleshed, and the teeth were prepared for caries scoring by means of Larson's modification of Keyes' system.[35] The determination of caries score was blind by codification of the jaws and was done by one calibrated examiner.

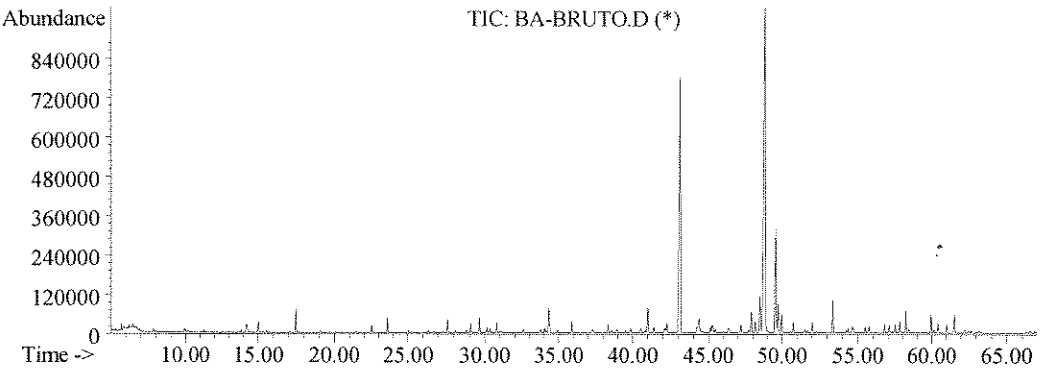
Statistical Analysis

The biofilm assays were done in three triplicates. The animal study data were subjected to ANOVA, Tukey-Kramer HSD test for all pairs. Smooth-surface and sulcal caries scores were expressed as proportions of their maximum possible values (124 and 56, respectively). The significant level was 5%.

RESULTS

The chromatograms of GC-MS of propolis type 6 and its hexane fraction are shown in Figures 1; Table 1 shows the relative percentage of the identified compounds in propolis type 6 and its non-polar hexane fraction. The main constituents of propolis type 6 are fatty acids, especially oleic (37.2%) and palmitic (23.1%) acids; these compounds can be also found in a high relative percentage in the non-polar bioactive hexane fraction (43.9% of oleic and 26.6% of palmitic acids). Other fatty acids that are present in significant amounts are linoleic and stearic acids. Flavonoids were not found.

A.



B.

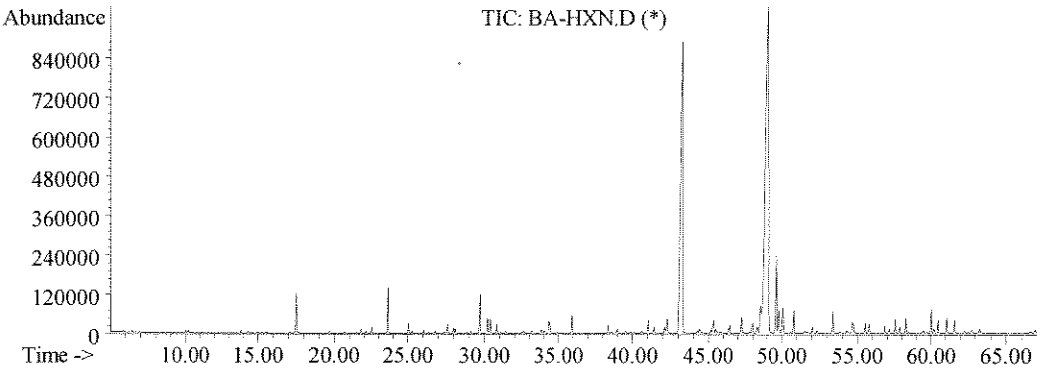


Figure 1 – **A.** GC-MS of the Ethanolic Extract of Propolis type 6. **B.** GC-MS of the non-polar bioactive Hexane Fraction of propolis type 6

Table 1 – Chemical composition of crude propolis type 6 and its non-polar hexane fraction, by GC/MS.

Time (min)	Identification	Relative %	
		Crude propolis	Hexane fraction
17.47	Benzenepropanoic acid, methyl ester	1.17	1.36
23.66	*trans*-caryophyllene	-	1.62
25.07	α-humulene	-	0.35
29.66	Nerolidol	0.89	-
35.93	Myristic acid	-	0.60
41.02	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	1.50	0.90
43.15	Palmitic acid, methyl ester	23.06	26.59
44.39	Hexadecanoic acid, methyl ester	1.99	-
48.56	Linoleic acid, methyl ester	-	1.92
48.87	Oleic acid, methyl ester	37.19	43.91
49.59	Stearic acid, methyl ester	7.06	3.24
59.40	Behenic acid, methyl ester	-	0.60
62.01	1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	1.36	0.59

Myristic acid, methyl ester = tetradecanoic acid methyl ester = methyl myristate; Palmitic acid, methyl ester = hexadecanoic acid, methyl ester = methyl palmitate; Linoleic acid, methyl ester = (Z,Z)-9,12- octadecadienoic acid, methyl ester = methyl linoleate; Oleic acid, methyl ester = (Z)-9-octadecnoic acid, methyl ester = methyl oleate; Stearic acid, methyl ester = octadecanoic acid, methyl ester = methyl stearate; behenic acid, methyl ester = docosanoic, methyl ester = methyl docosanoate.

The results of the influence of EEP and EEH on mutans streptococci biofilms are shown in Figures 2-4. The propolis extracts were devoid of any major killing activity against either *S. mutans* UA159 or *S. sobrinus* 6715 biofilms at a concentration of 800 μg/ml (Figure 2). In contrast, the acid production of the biofilms was remarkably reduced by EEP and especially EEH (Figure 3). Figure 4 illustrates the effects of EEP and EEH on the activity of F-ATPases. Clearly, both extracts (at 800 μg/ml) inhibited the activity of this enzyme showing more than 60% inhibition.

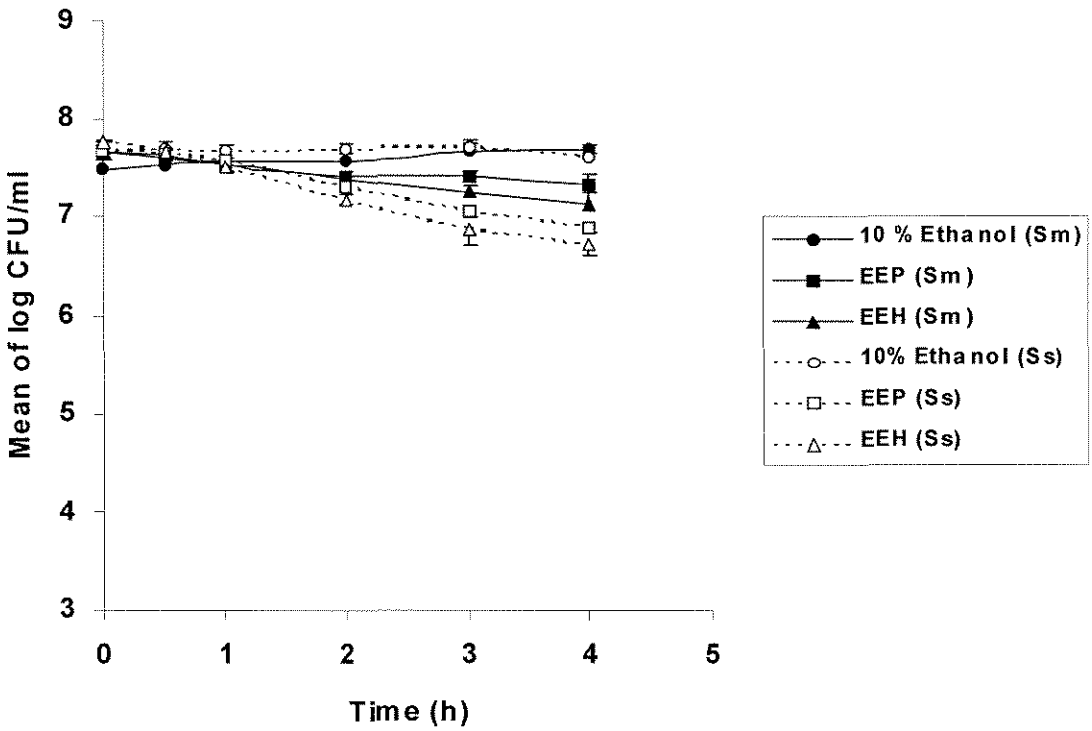


Figure 2 – Time-kill curves for *S. mutans* UA159 (Sm) and *S. sobrinus* 6715 (Ss) by crude ethanolic extract of propolis type-6 (EEP) and its hexane fraction (EEH). The final concentration of the test extracts was 800 μg/ml.

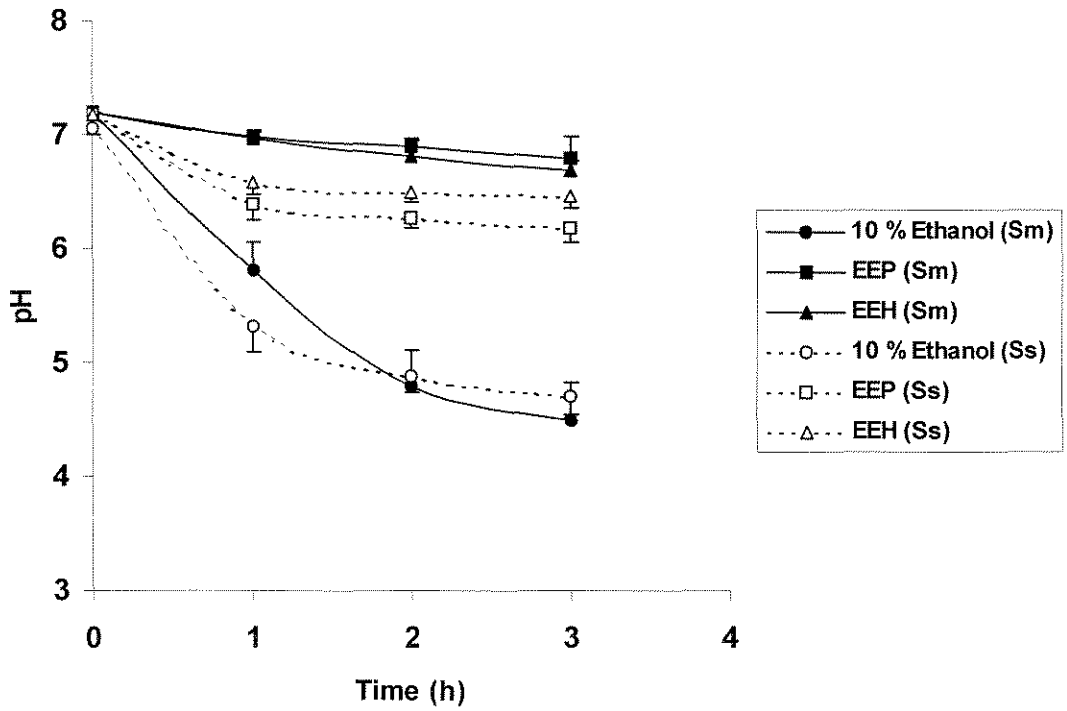


Figure 3 – Acidogenicity of intact *S. mutans* UA159 (Sm) and *S. sobrinus* 6715 (Ss) biofilms treated with crude ethanolic extract of propolis type-6 (EEP) and its hexane fraction (EEH). The final concentration of the test extracts was 800 μg/ml.

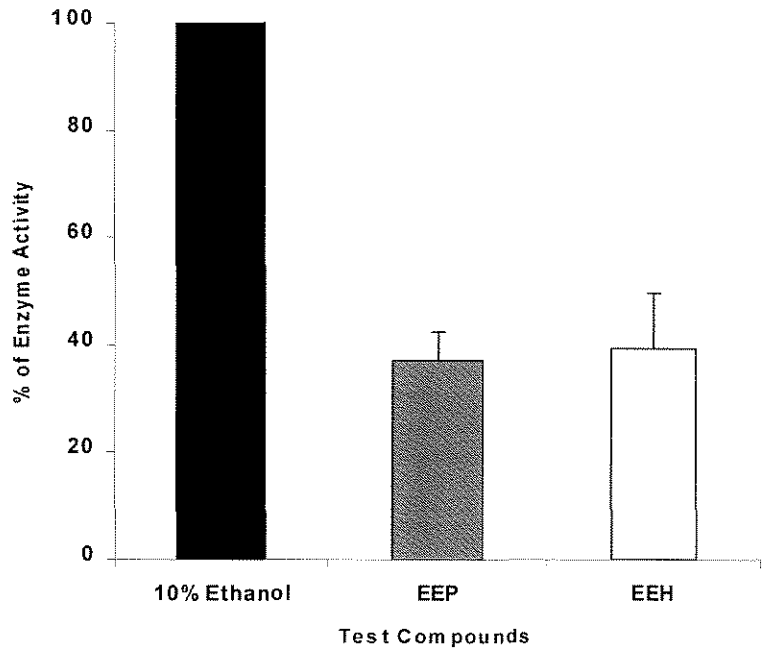


Figure 4 – Influence of crude ethanolic extract of propolis type-6 (EEP) and its hexane fraction (EEH) on F-ATPase activity. The final concentration of the test extracts was 800 μg/ml. n=3

In the animal study, the rats remained in apparent good health during the 5 weeks of experiment. During this time, the animals gained weight, and it was not statistically different among the groups. Furthermore, the percentage of *S. sobrinus* 6715 recovered from the jaws of the animals treated with EEP and EEH was lower than those treated with control, however the differences did not reach statistical significance ($p>0.05$).

The smooth-surface and sulcal caries scores are shown in Table 2 and 3, respectively. The 5% EEH was the only treatment that was able to affect, under a high

cariogenic challenge, both the incidence and severity of smooth and sulcal surface caries, when compared to the control group ($p<0.05$).

Table 2 – Effects of EEP (ethanolic extract of crude propolis type 6) and EEH (ethanolic extract of hexane fraction) on total caries and caries severity in SMOOTH-SURFACE: means (SD)

Groups	Total	Ds	Dm	Dx
EEP	39.82 (13.01) [a]	17.81 (12.17) [a]	5.18 (6.99) [a]	1.00 (1.84) [ab]
EEH	35.18 (11.79) [a]	15.64 (11.63) [a]	6.00 (9.41) [a]	0.18 (0.41) [a]
Ethanol 80%	51.33 (14.74) [b]	27.17 (13.48) [a]	12.17 (11.11) [a]	2.67 (2.61) [b]

Values followed by the same superscripts are not significantly different from each other ($p<0.05$). ANOVA, comparison for all pairs using Tukey-Kramer HSD. Ds, Dm and Dx represent the severity of dental caries.

Table 3 – Effects of EEP (ethanolic extract of crude propolis type 6) and EEH (ethanolic extract of hexane fraction) on total caries and caries severity in SULCAL caries lesions: means (SD)

Groups	Total	Ds	Dm	Dx
EEP	37.81 (2.44) [ab]	24.00 (6.34) [a]	12.36 (5.26) [a]	1.45 (2.51) [ab]
EEH	34.64 (4.46) [a]	20.09 (7.67) [a]	10.73 (6.51) [a]	0.36 (0.81) [a]
Ethanol 80%	40.50 (2.65) [b]	26.00 (3.81) [a]	15.25 (5.99) [a]	1.92 (1.44) [b]

Values followed by the same superscripts are not significantly different from each other ($p<0.05$). ANOVA, comparison for all pairs using Tukey-Kramer HSD.

For the incidence of smooth-surface, the animals treated with the non-polar hexane fraction (EEH) presented lower scores, followed by the animals treated with the crude propolis type 6 (EEP); both EEH and EEP showed statistically significant difference when compared to the control group ($p<0.05$). A similar pattern was observed in terms of severity of smooth surface caries; the EEH group showed the lowest scores, differing statistically from the control group at Dx level, which represents the most severe score. However, the EEP did not show difference neither when compared to the EEH nor to the control group.

For the sulcal caries, EEH also showed the lowest scores, showing statistically significant difference compared to the control group, but not to the EEP group. Again, the EEP group did not show statistical difference when compared to either EEH or control group ($p>0.05$).

DISCUSSION

Natural products, such as propolis, are receiving increased attention due to their diverse range of biological properties, providing sources for discovery of novel and effective bioactive compounds.[36]

Propolis extracts have been recognized for their wide range of pharmacological activities, including prevention of oral diseases,[3,5,6,9,11] and flavonoids and some terpenoids have been widely cited as their main bioactive compounds.[3,13,37] However, we recently found a new and biologically active variety of propolis, which was completely devoid of any of the cited bioactive compounds. The present study attempted to elucidate the putative active compounds and the mechanisms of action of this novel propolis *in vitro*, and to examine its influence on caries development *in vivo*.

The chemical analyses of propolis type 6 revealed that fatty acids are the main constituents, especially oleic (37.19%) and Palmitic (23.06%) acids and this information is relevant once these constituents can be also found in a high relative percentage on its bioactive non-polar hexane fraction, mainly oleic (43.91%) and palmitic (26.59%), also in addition to linoleic and stearic acids; these compounds could be related to the biological effects of propolis type-6.

Indeed, several fatty acids have shown anti-caries properties *in vivo* when incorporated in the rats diet,[38–42] suggesting that these compounds may represent a virtually

non-toxic and non-allergenic means of controlling the acidogenic organisms associated with dental caries.[43,44] According to Hayes and Berkovitz,[41] fatty acids act as anionic surfactants and have antibacterial and anti-fungal properties at low pH values; in addition, they can be selective against Gram-positive organisms.[45] The potential biological target of fatty acids is the bacterial membrane, which could disrupt the integrity and function of the membrane.[43] Furthermore, the fatty acid may form micelles surrounding each bacterium, which would prevent adherence or cell metabolism related to acid production;[43] the polar carboxyl end of the fatty acids may bind to the salivary pellicle or the bacterial wall, leaving the non-polar ends free and preventing the bacterium operative for the fatty acids that decrease the amount of plaque accumulation.[46] However, most of the previous studies have been conducted against microorganisms in suspension (planktonic state). It is well known that microorganisms growing in biofilms are more resistant to antimicrobial agents than their planktonic counterparts;[47,48] oral pathogens, including mutans streptococci, form biofilms in the oral cavity.

In the present study, the propolis extracts were devoid of any major killing activity against biofilms of mutans streptococci at a concentration of 800 μg/ml, despite showing major killing activity against the same bacteria in suspensions (MIC values between 25-100 μg/ml against planktonic cells of *S. mutans* and *S. sobrinus*).[9] The lack of the killing activities of the propolis extracts was further confirmed in our *in vivo* study, where none of the treated animals showed significant reduction in the percentage of *S. sobrinus* infection. These findings clearly show the relevance of using biofilm models to determine the antibacterial activity of test agents. Nevertheless, propolis extracts, especially EEH at 5% (w/v) were able to reduce the caries incidence on rats under a high cariogenic challenge, by

mechanisms other that affecting the viability of the organisms, such as (1) reduction of the acid production by both *S. mutans* UA159 and *S. sobrinus* 6715 biofilms, and (2) inhibition of the proton-translocating F-ATPAse activities. One of the mechanisms which mutans streptococci have developed to alleviate the influences of acidification is to up-regulate proton-translocating F-ATPase (H^+-ATPase) activity in response to low pH.[26] This membrane-associated enzyme pumps protons (H^+) out of the cell in association with ATP hydrolysis to maintain intracellular more alkaline than extracellular pH. The high acid tolerance of these microorganisms plays a critical role in their virulence to dental caries. By inhibiting the activity of ATPase, propolis type-6 could have affected the acid tolerance of mutans streptococci. In addition, the disruption of the intracellular pH would also inhibit the pH-sensitive glycolitic enzymes, thereby reducing the ability of the microorganisms to produce acids. These results are in accordance with Sheu and Freeze[49] and Iwami *et al.*,[50] whom previously described that there are many reports on possible mechanisms of the antibacterial activity of fatty acids, such as the inhibition of ATP regeneration, and the maintenance of a pH gradient across the cell membrane of bacteria. Furthermore, propolis type-6 also inhibited the glucans synthesis by GTFs in solution and adsorbed onto a saliva-coated hydroxyapatite, which was shown by Duarte *et al.*;[9] glucans are responsible for the adherence, accumulation of oral bacteria to the tooth surface as well as providing bulk and structural integrity to the biofilms. By disrupting these essential virulence factors of mutans streptococci, propolis type-6 was able to exert cariostatic properties in rats without affecting the viability of the oral flora population; we are currently exploring the molecular mechanisms involved in the inhibitory activities observed here.

In summary, fatty acids such as oleic, palmitic, linoleic and stearic acids, are the putative active compounds in the propolis type 6 and its non-polar bioactive hexane

fraction. It is likely that these compounds are influencing some of the critical virulence factors associated with the pathogenesis of dental caries, including acid production, F-ATPase and GTFs activities.

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Capítulo 2

Bioactive fractions of a novel Brazilian propolis on *Streptococcus mutans* glucosyltransferases and biofilm development.

S. Duarte[a]; P.L. Rosalen[a]; J.A. Cury[a]; V.L.G. Rehder[b]; A. Sartoratto[b]; M. Ikegaki[c]; B.D. Schobel[d]; W.H. Bowen[d]; H. Koo[d]*.

[a] Department of Physiological Science, Dentistry School of Piracicaba, State University of Campinas (UNICAMP), Piracicaba, SP, Brazil.

[b] Pluridisciplinar Center of Chemical, Biological and Agricultural Researches, CPQBA/ UNICAMP, Paulinia, SP, Brazil.

[c] School of Pharmacy and Dentistry of Alfenas; Alfenas, MG, Brazil

[d] Center for Oral Biology and Eastman Department of Dentistry, University of Rochester Medical Center, Rochester, NY, USA.

* Hyun Koo
University of Rochester Medical Center, Center for Oral Biology
601 Elmwood Avenue Box 611,
14642, Rochester, NY, USA
Tel.: +1(585) 275-1433 / FAX +1(585) 473-2679
e-mail: hyun_koo@urmc.rochester.edu

SUMMARY

A novel Brazilian propolis, classified as type 6, devoid of any of the compounds commonly found in this natural substance, such as flavonoids, displayed inhibitory activities against mutans streptococci. The purpose of this study was to analyze the chemical composition and the influence of two bioactive non-polar fractions of propolis type 6 on glucosyltransferases (GTFs) activity, and on *Streptococcus mutans* biofilm accumulation and polysaccharide composition. The chemical composition of the bioactive fractions of propolis Hfr-2 and Hfr-3 was examined by gas chromatography / mass spectrometry (GC/MS) technique. The Hfr-2 and Hfr-3 (final concentration ranging from 6.25 to 500 μg/ml) were tested on GTF B and GTF C both in solution (GTF-sol) and adsorbed onto saliva-coated hydroxyapatite (GTF-surf). *S. mutans* UA159 biofilms were formed on saliva-coated hydroxyapatite discs in batch culture for 5 days. The biofilms were treated for 1 min twice/day with either Hfr-2 or Hfr-3 at 500 μg/ml during the biofilm formation. After the treatments, the dry weight, viability, and polysaccharide composition per biofilm were determined. Ethanol at 25% (v/v) was used as vehicle-control. The chemical analysis showed that fatty acids, such as oleic (Hfr-2 = 41.45%; Hfr-3 = 16.15 %) and palmitic (Hfr-2 = 17.62 %; Hfr-3 = 8.67 %) acids are the major constituents in the bioactive fractions. Both fractions inhibited the activity of GTFs B and C in solution (52-86% of inhibition) and on surface (22-40% of inhibition) at concentrations as low as 500 μg/ml. The dry weight of the biofilms treated with Hfr-2 and Hfr-3 were, respectively, 51.3 (±12.8)% and 15.63 (±11.6)% less than those treated with vehicle-control. Furthermore, biofilms treated with Hfr-2 resulted in statistically lower amounts of insoluble and intracellular polysaccharides when compared to the control ($p<0.05$). Our findings suggest that the fatty acids present in the Hfr-2 and Hfr-3 fractions may be responsible for the inhibition of GTFs activity and disruption of *S. mutans* biofilm polysaccharides matrix, which play a critical role in the expression of virulence of dental biofilm.

INTRODUCTION

Dental caries formation is ascribed to the adherence of specific oral bacteria on tooth surface to form dental biofilm and subsequent production of acids from sugars, resulting in the demineralization of tooth enamel. From this biofilm, mutans streptococci have been considered as the primary cariogenic bacteria, mainly because of their acidogenic and aciduric characteristics, and the ability to synthesize water-soluble and water-insoluble glucans from sucrose, catalyzed by glucosyltransferases (GTFs).[1–3] Among these virulence factors, glucans synthesized by GTFs play a critical role in the development and establishment of pathogenic dental biofilms related to caries. Glucans not only promote the adherence and accumulation of cariogenic streptococci on the tooth surface, but also contribute to the bulk, cariogenicity and structural integrity of the dental biofilm.[4–7]

Glucan production is an essential virulence factor of *S. mutans* associated with the pathogenesis of dental caries.[8] It is known that *Streptococcus mutans* produces at least three GTFs: GTF B, which synthesizes mostly insoluble α1,3-linked glucans; GTF C, which synthesizes a mixture of insoluble and soluble α1,6-linked glucans; and GTF D, which synthesizes soluble glucans.[8] Among these biofilm-building enzymes, GTFs B and C appear to be the most important GTFs related to dental caries.[8] These observations suggest several avenues for chemotherapeutic intervention other than attempting to eliminate *S. mutans*; these include reducing or elimination of glucan production by the GTFs, and reducing the ability of organisms to produce and tolerate acid.

Propolis is a natural non-toxic resinous product collected by *Apis mellifera* bees from various plant sources that has several pharmacological activities, including prevention of oral diseases, such as dental caries. In addition, it is known that its chemical composition

and pharmacological activities are dependent on the biodiversity of the region that it is collected.[9–12]

At this moment, the major biological active substances in propolis have been considered to be (poly)phenolic compounds, such as flavonoid aglycones[11,13–16] and derivatives of cinnamic acids and diterpenoids.[17,18] Recently, a novel type of Brazilian propolis, classified as type-6 (from northeastern Brazil, Atlantic Forest, Bahia state), in which none of the commonly found phenolics were detected, showed remarkable antimicrobial activities against oral pathogens including mutans streptococci.[12,19] Furthermore, it was able to inhibit the activity of GTF enzymes both in solution and on hydroxyapatite surface.[12] Considering the unusual chemical profile and the *in vitro* biological activities against mutans streptococci of this novel propolis, the purpose of this investigation was to identify the putative active compounds through chemical fractionation and analysis of the isolated fractions on the activity of GTFs and on development of *S. mutans* biofilms.

MATERIAL AND METHODS

Test Compounds

Crude samples of *A. mellifera* bee propolis were obtained from Atlantic forest region of Bahia state, northeastern Brazil, and were classified as type 6.[20] The EEP (ethanolic extract of crude propolis type 6) was subjected to a chemical fractionation, as described by DUARTE *et al.*[12] The hexane fraction was further fractionated by dried column chromatography (cellulose 2 X 30 cm) using Si gel 60 (Merck) and chloroform:ethyl acetate (70:30, v/v). The columns were cut in five parts and extracted using chloroform yielding the following fractions: Hfr-1, Hfr-2, Hfr-3, Hfr-4 and Hfr-5,

which were screened by standard methods of determination of minimum inhibitory and bactericidal concentration, and cell adherence inhibition using *S. mutans* planktonic cells as detailed in DUARTE *et al.*[12] Hfr-2 and Hfr-3 were the most effective fractions, and were selected for the present study (data not shown).

Gas Chromatography – Mass Spectrometry (CG-MS)

The Hfr-2 and Hfr-3 were analyzed by Gas Chromatography coupled to a Mass Seletive Detector (GC-MS). For best results, before the GC-MS analysis, the extracts were methylated using diazomethane solution. The analyses were obtained in gas chromatography Hewlett-Packard 5890 Series II (Palo Alto, CA, USA) equipment, with mass selective detector HP-5971 in the electron impact ionization mode (70eV), injector *split/splitless*, capillary column J & W Scientific DB-5 (25 m x 0,2 mm x 0,33 μm). Temperatures: injector = 250°C, column = 60°C, 3°C/min, 240°C (7 min), detector = 300°C. Carrier gas (He) = 1,0 ml/min. The GC-MS peaks were identified by comparison with data from literature[21] and the profiles from the Wiley 138 and Nist 98 libraries.

GTF B and C Assays

The GTF B and C enzymes were obtained from culture supernatants and purified to near homogeneity by hydroxyapatite column chromatography as described by VENKITARAMAN *et al.*[22] and WUNDER and BOWEN.[23] GTF activity was measured by the incorporation of [^{14}C]glucose from labeled sucrose (NEN Research Products, Boston, Mass., USA) into glucans.[19,22] The GTF enzyme added to each sample for all assays was equivalent to the amount required to incorporate 1 to 1.5 μmol of glucose over the 4 h reaction.

For solution assays, GTF B or C was mixed with the Hfr-2 and Hfr-3 (at 6.25, 12.5 or 25 μg/ml) and incubated with ^{14}C-(glucosyl)-sucrose substrate (0.2 μCi/ml; 200.0 mmol/l sucrose, 40 μmol/l dextran 9000, 0.02% sodium azide in adsorption buffer, pH 6.5) to a final concentration of 100 mmol/l sucrose (300 μl final volume). For the control, the same reaction was done with the solvent, ethanol (final concentration of 25%, v/v), replacing the test extracts. The technique was followed as described by VENKITARAMAN *et al.*[22] radiolabeled glucans was determined by scintillation counting.

For surface assays, the GTFs were adsorbed onto hydroxyapatite beads (Macro-Prep Ceramic Hydroxyapatite Type I, 80 μm, Bio-Rad®) coated with clarified whole saliva (sHA), as described by VENKITARAMAN *et al.*[22] Following adsorption of the enzyme, the beads exposed to the test fractions (at 100, 250 or 500 μg/ml) or control (25%, v/v of ethanol) for 30 min. Then, the beads were exposed to ^{14}C-(glucosyl)-sucrose substrate (100.0 mmol/l sucrose, final concentration). The radiolabeled glucan formed was collected and quantified by scintillation counting.[19,22] All of the solution and surface assays were done in triplicate in at least three different experiments.

Biofilm Formation and Treatments

Biofilms of *S. mutans* UA159 were formed on saliva-coated hydroxyapatite discs placed in a vertical position (HAP ceramic – Calcium Hydroxyapatite, 0.5" diameter ceramic – Clarkson Calcium Phosphates, Williamsport, PA, USA) in batch cultures using microplate, modified from KOO *et al.*[16] *S. mutans* UA159 was selected because it is a proven virulent cariogenic pathogen and was the strain selected for genomic sequencing.[24] Cells of *S. mutans* were grown in ultrafiltered (10 kDa molecular weight cut-off membrane; Amicon) tryptone-yeast extract broth with addition of 30 mM sucrose at 37°C and 5% of

CO_2.[16] The culture medium was replaced daily; biofilms were grown for 30 h to allow initial biofilm formation. At this point (30 h old), the biofilms were treated twice daily until the fifth day of the experimental period with one of the following: (1) 500 μg/ml of Hfr-2; (2) 500 μg/ml of Hfr-3; and (3) vehicle-control (25%, v/v of ethanol). The biofilms were exposed to the treatments for 1 min; double-dip rinsed in sterile 0.89% NaCl solution; and transferred to fresh culture medium, until the fourth day. Then, the biofilms were incubated an additional 18 h and harvested until the fifth day of the experimental period. Each biofilm was exposure to the respective treatment seven times. The biofilm assays were performed in duplicate in at least three different experiments.

Biofilm analyses

At the end of the experimental period, the biofilms were dip-washed three times in sterile 0.89% NaCl solution to remove loosely adherent material. The biofilms were placed in 0.89% NaCl solution, and they were subjected to 10 min sonication by an Aquasonic model 150HT (VWR®) sonicating bath. The resulted biofilm suspension was used for dry weight (biomass), bacterial viability (Colony Forming Units – CFU / ml) and insoluble, soluble and intracellular polysaccharide analysis as detailed elsewhere.[16,25,26]

Statistical Analysis

The data were analyzed using ANOVA, and the F-test was used to test any difference between the groups. When significant differences were detected, all pair comparison was made between all the groups using Tukey-Kramer HSD method to adjust for multiple comparisons. Statistical software JMP version 3.1 [SAS institute (1989), Cary, NC, USA] was used to perform the analysis. The significant level was 5%.

RESULTS

Chemical analysis

The GC-MS chromatograms of Hfr-2 and Hfr-3 are shown in Figure 1. The relative percentage of the identified compounds in Hfr-2 and Hfr-3 is presented in Table 1. The chemical analyses of Hfr-2 and Hfr-3, which were derived from hexane fraction of propolis type-6, indicates that these fractions have a high relative percentage of fatty acids, mainly oleic (Hfr-2 = 41.5%; Hfr-3 = 16.2%) and Palmitic (Hfr-2 = 17.6%; Hfr-3 = 8.7%) acids. Furthermore, among the compounds, it is possible to find other fatty acids, as linoleic, behenic (docosanoic), and tetracosanoic acids. Flavonoids were not found in these fractions.

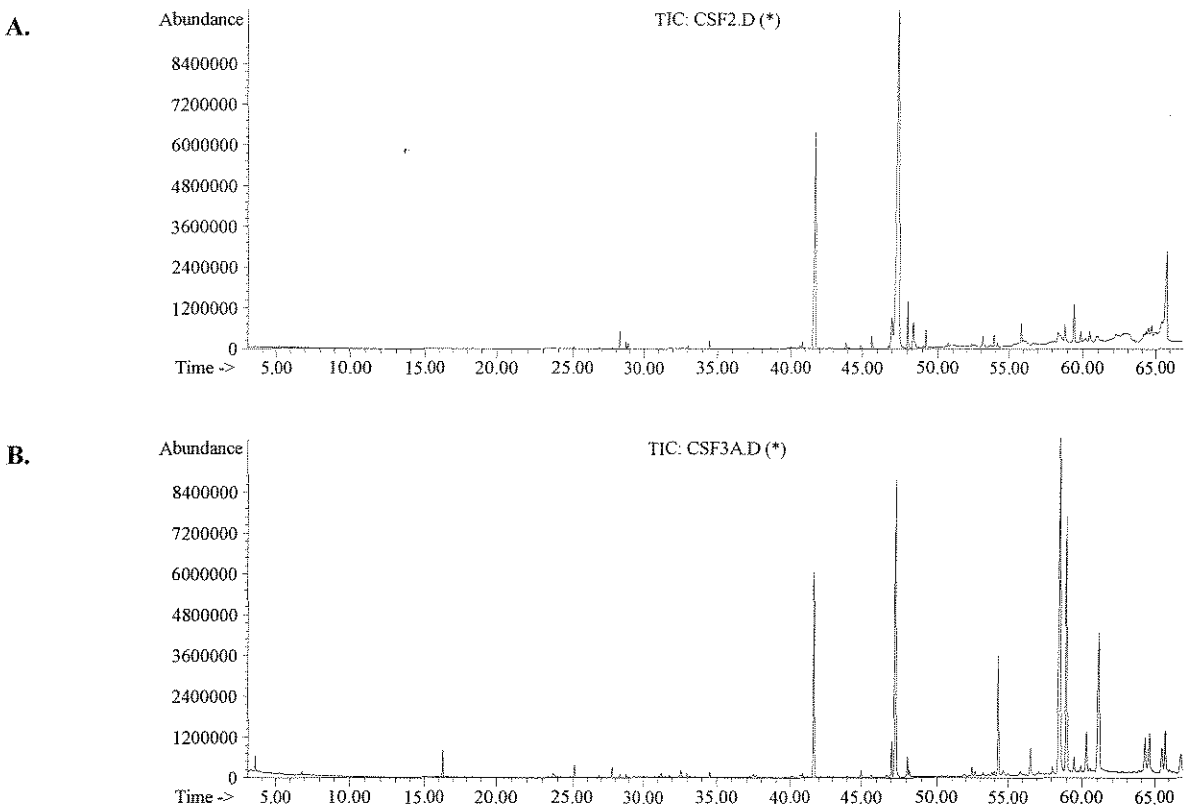

Figure 1. A. GC-MS of the Hfr-2 sub-fraction from Hexane fraction, propolis type-6. **B.** GC-MS of the Hfr-3 sub-fraction from Hexane fraction, propolis type-6.

Table 1. Chemical composition of sub-fractions Hfr-2 and Hfr-3 by GC/MS.

Time (min)	Identification	Relative %	
		Hfr-2	Hfr-3
17,47	Benzenepropanoic acid, methyl ester	-	0.86
43,15	Palmitic acid, methyl ester	17.62	8.67
48,56	Linoleic acid, methyl ester	2.57	1.43
48,87	Oleic acid, methyl ester	41.45	16.15
49,59	Stearic acid, methyl ester	-	22.82
59,01	Unknown	-	13.71
61,08	Behenic acid, methyl ester	1.98	0.72
62,01	1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	0.63	0.32
65,86	Tetracosanoic acid, methyl ester	10.66	2.69

Palmitic acid, methyl ester = hexadecanoic acid, methyl ester = methyl palmitate; Linoleic acid, methyl ester = (Z,Z)-9,12- octadecadienoic acid, methyl ester = methyl linoleate; Oleic acid, methyl ester = (Z)-9-octadecnoic acid, methyl ester = methyl oleate; Stearic acid, methyl ester = octadecanoic acid, methyl ester = methyl stearate; Behenic acid, methyl ester = docosanoic, methyl ester = methyl docosanoate; Tetracosanoic acid, methyl ester = lignoceric acid, methyl ester = methyl tetrasosanoate.

GTF B and C assays

The effect of Hfr-2 and Hfr-3 on the activity of GTF B and C are shown in Table 2. Both Hfr-2 and Hfr-3 reduced the activity of all enzymes tested in solution (52-86% inhibition) at concentrations as low as 25 μg/ml. Both fractions reduced the activity of GTFs on the surface of saliva coated hydroxyapatite beads, although their effects was not as potent as that observed when the same enzymes were in solution phase.

Table 2. Average (SD) of the percentage of glucosyltransferases (GTF B and C) activity inhibition in SOLUTION and on SURFACE by **Hfr-2** and **Hfr-3** sub-fraction from Hexane fraction, propolis type-6.

	Concentration	GTF B		GTF C	
		Hfr-2	Hfr-3	Hfr-2	Hfr-3
SOLUTION	6.25 µg/ml	52.45 (5.25)	80.17 (3.53)	20.00 (5.22)	68.27 (2.47)
	12.5 µg/ml	72.47 (2.85)	86.11 (4.73)	53.09 (10.18)	77.79 (0.45)
	25.0 µg/ml	82.43 (8.87)	82.74 (1.05)	63.20 (1.02)	83.19 (0.63)
SURFACE	100 µg/ml	25.66 (12.81)	21.83 (15.17)	32.82 (19.27)	22.66 (3.93)
	250 µg/ml	30.22 (7.35)	30.22 (12.92)	48.57 (14.30)	27.47 (6.54)
	500 µg/ml	39.73 (14.48)	32.82 (19.20)	48.07 (5.26)	41.52 (14.43)

The GTF activity of the control group was considered the most. n=3

Effects of Hfr-2 and Hfr-3 on biofilm accumulation and composition

The population of viable cells recovered from biofilms, and their biomass and polysaccharides composition are shown in Table 3. *S. mutans* UA159 biofilms treated with the test agents showed slightly lower numbers of recoverable viable cells compared with the vehicle-control (Table 3). However, none of them appeared to be bactericidal for *S. mutans* UA159 in the biofilms.

Table 3. Average (SD) of units forming colonies (UFC) /biofilm, dry weight (DW), extracelluar Insoluble (INS) and soluble (SOL) polyssacharides, and Intracellular (IPS) polysaccharides in the biofilms after 7 treatments.

Treatments	Log UFC / Biofilm	DW (mg)	INS (µg/biofilm)	SOL (µg/biofilm)	IPS (µg/biofilm)
Hfr-2 500 µg/ml	8.85 (0.67) [a]	3.4 (0.6) [a]	1538.3 (97.5) [a]	502.2 (104.1) [a]	50.6 (26.0) [a]
Hfr-3 500 µg/ml	8.62 (0.17) [a]	6.1 (1.1) [b]	2694.1 (139.1) [b]	632.0 (142.9) [a]	255.5 (103.5) [ab]
Vehicle – control	9.07 (0.51) [a]	7.4 (1.1) [b]	3899.1 (1836.6) [b]	366.3 (346.6) [a]	314.9 (119.8) [b]

Values followed by the same superscripts are not significantly different from each other ($p<0.05$). ANOVA, comparison for all pairs using Tukey-Kramer HSD. n=3

The biofilms treated with Hfr-2 at 500 μg/ml showed 54.1% less biomass (dry weight), than those treated with vehicle control. The total amount of insoluble polysaccharides (INS) was also affected by Hfr-2 and Hfr-3 treatments showing respectively 60.5% and 30.9 % less INS than vehicle control, but only the Hfr-2 was statistically different. The total amount of water-soluble glucans (SOL) in the biofilms was unaffected by the test agents compared with the vehicle control. In addition, biofilms treated with Hfr-2 displayed significantly less intracellular iodophilic polysaccharides (IPS) than those treated with vehicle control ($p<0.05$).

DISCUSSION

Numerous drugs have been tested for their effect on dental biofilm and the most common of them contain antibacterial agents.[27] Although effective, such antibacterial applications have several undesirable side effects.[28] Thus, manipulation of the oral bacteria ecology by altering bacterial virulence factors in biofilm, without affecting their viability represents a novel targeting approach.

The data of the present study show that Hfr-2 and Hfr-3 remarkably reduced the activities of GTF B and C when these enzymes were both in solution and adsorbed to a saliva-coated hydroxyapatite. This inhibition is relevant since GTF plays a critical virulence in *S. mutans* biofilms, which allows the glucans production from sucrose by their cooperative action, permitting the microorganisms to adhere firmly to the tooth surface.[5,8,29]

Among GTFs produced by mutans streptococci, GTF B and C appear to be the most important GTFs related to dental caries,[8] since both produce insoluble α1,3-linked glucans. In addition, even if Hfr-2 and Hfr-3 inhibited GTFs adsorbed to a saliva-coated hydroxyapatite surface less than in solution, this influence can be very significant, once the

surface-adsorbed GTF enzymes display an increased resistance to the most common anti-plaques agents, including commercially available mouthrinses.[23,30] Our findings are in agreement with DUARTE, *et al.*[12] that also showed that hexane fraction from propolis type 6 was able to reduce the GTFs B and C activity even on surface, and at this time we found that the Hfr-2 and Hfr-3 could be responsible for that.

This GTF activity inhibition could be related to the chemical composition found in the bioactive fractions of propolis type 6, in which we can find mainly fatty acids, such as oleic, palmitic, linoleic, behenic (docosanoic), and tetracosanoic acids. This founds are in accordance with OSAWA *et al.*,[31] that presented fatty acids, like oleic and linoleic, among others, as possible active compounds related to the cariostatic activity of cacao bean husk, showing anti-glucosyltransferase and antimicrobial activities, and also inhibited experimental dental caries in rats infected with mutans streptococci.

Therefore, we studied the effect of Hfr-2 and Hfr-3, which are rich in fatty acids, on *S. mutans* UA159 biofilm treated twice daily in an attempt to mimic likely exposure in humans.[16] Although our mono-species biofilm model does not mimic the complex microbial community found in dental plaque, it is more advantageous when examining specific actions of test agents on *S. mutans* physiology, especially on the glucan-mediated processes involved in the biofilm development, which appears to be essential for expression of virulence by *S. mutans*. In addition, it is established that the biofilm model is more resistant than planktonic cells,[32] and even using a biofilm model, the treatments with Hfr-2 at 500 μg/ml were able to reduce significantly the biomass (dry weight) and the total amount of insoluble polysaccharides (INS), when compared to the control. The treatment with Hfr-3 at 500 μg/ml also reduced the amount of INS, but it was not statistically

different. The reduction of INS production could be related to the GTF B and C inhibition, already shown. In addition, this sub-fraction did not show antibacterial activity in the biofilm model.

The lack of antibacterial activity could be desirable since the treatments with Hfr-2 were able to reduce the biofilm accumulation without kill bacteria, and according to MARSH[33] treatments should attempt to control rather than eliminate the plaque microflora, since the resident plaque microflora, once established, remains relatively stable over time and reduces the chance of infection by acting as a barrier to colonization by exogenous (and often pathogenic) species, which is of benefit to the host.[34,35]

The treatments with Hfr-2 also reduced the amount of intracellular polysaccharide (IPS), which could be related to the acidification of the cell cytoplasm. DUARTE *et al.* (Submitted press) showed that Hexane fraction from propolis type 6, which contains mainly fatty acids in its chemical composition, reduced the proton translocation mediated by F-ATPase from *S. mutans* UA159. This enzyme is related to the ability of *S. mutans* to resist acidification, pumping protons out of the cell .[36–39] This movement of protons results in an internal pH more basic than that of the plaque environment, thereby protecting relatively acid-sensitive glycolytic enzymes.[40] Thus, the reduction of the F-ATPase activity would results in the acidification of the cell cytoplasm and, consequently, in the reduction of the intracellular polysaccharides production. Thus far, fatty acids have also been attributed to a decrease in ionization to produce an increase in their oil:water partition coefficient and presumably in their cell membrane:water partition coefficient,[41] which could also be related to the intracellular acidification.

Therefore, these results indicate that Hfr-2, a fraction from Hexane fraction of propolis type-6, may represent a novel source of natural bioactive compounds to prevention of dental caries, since it was able to reduce the biofilm accumulation without antibacterial activity, acting mainly on its virulence factors. In addition, the active compounds of propolis type 6 appear to be fatty acids.

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IV – DISCUSSÃO GERAL

Diversas drogas têm sido estudadas e utilizadas na prevenção de cárie dental por apresentarem inibição do biofilme bacteriano, sendo que a maioria delas contém agentes antimicrobianos (GILBERT *et al.*, 1997). Entretanto, embora efetivos, os agentes antimicrobianos têm sido considerados pelos seus efeitos adversos, como fator de seleção e/ou supressão de microrganismos resistentes e da microbiota oral (STEINBERG *et al.*; 1996).

Os resultados encontrados no nosso estudo demonstraram que o extrato etanólico da própolis do tipo 6 (EEP), bem como de sua fração hexano (EEH) embora não tenham demonstrado boa atividade antimicrobiana, foram capazes de reduzir o índice de cárie dental em animais submetidos a um desafio cariogênico, sendo que essa atividade pode estar relacionada à capacidade dessa própolis em inibir alguns fatores de virulência do biofilme dental bacteriano, como a atividade da enzima ATPase, uma das responsáveis pela sobrvivência dos *S. mutans* em meio ácido, e também a capacidade de inibir a atividade das enzimas glucosiltransferases (GTFs), como demonstrado por DUARTE *et al.* (2003).

Além disso, foram testadas duas sub-frações da fração hexano (Hfr-2 e Hfr-3), que foram pré-selecionadas por apresentarem os melhores resultados em estudos preliminares, sendo que a sub-fração Hfr-2 foi capaz de reduzir a atividade de GTFs B e C e, quando testada em modelo de acúmulo de biofilme de *S. mutans* UA159, embora não tenha apresentado atividade antimicrobiana, os tratamentos com Hfr-2 reduziram a quantidade de polissacarídeos insolúveis e intracelulares. Essa atividade em biofilme também pode estar relacionada a

inibição de seus fatores de virulência, como redução da atividades das GTFs B e C, o que pode ter resultado na redução da produção de polissacarídeos insolúveis, e redução da atividade da ATPase, causando uma acidificação do citoplasma, e conseqüentemente à inibição da produção de polissacarídeos intracelulares (IWAMY *et al.*, 1995).

Verificando a composição química desta própolis, observamos que tanto para o extrato bruto quanto para as frações biologicamente ativas, os constituintes que estão presentes nas maiores porcentagens relativas são ácidos graxos, entre eles ácido olêico, linolêico, palmítico e esteárico.

Os ácidos graxos têm sido reconhecidos na literatura pelas suas propriedades anticárie (ROSENBURY e KARCHAN, 1935; SCHWEIGERT *et al.*, 1946; SCHEMMEL *et al.*, 1978; HAYES e BERCOVITZ, 1979; OOSHIMA *et al.*, 2000), sendo que alguns autores relatam como possíveis mecanismos de ação desses compostos a capacidade de modificar a permeabilidade da membrana, o que faria com que a célula bacteriana perdesse constituintes citoplasmaticos (WILLIAMS *et al.*, 1982). Além disso, estes autores também discutem a possibilidade desses ácidos graxos formarem miscelas ao redor da célula, o que dificultaria sua aderência a outras superfícies, bem como o metabolismo celular (WILLIAMS *et al.*, 1982). Outro possível mecanismo de ação estaria relacionado à própria estrutura desses ácidos graxos, que são moléculas com uma das extremidades com características polares e a outra com características apolares. Desta forma, a extremidade polar se ligaria a membrana bacteriana e a extremidade que ficaria livre seria a que apresenta características apolares,

reduzindo então a aderência dessa bactéria a outras superfícies (LILJEMARK *et al.*, 1978).

Assim, os resultados desses trabalhos indicam que a própolis tipo 6 pode ser uma nova fonte de novos compostos naturais relacionados à prevenção de cárie dental. Em acréscimo, as análises químicas sugerem que esses compostos podem ser ácidos graxos, que talvez não apresentem atividade antimicrobiana, mas sim a capacidade de reduzir a virulência do biofilme dental bacteriano, entretanto investigações específicas devem ser conduzidas, a fim de se comprovar tal atividade.

V – CONCLUSÃO GERAL

Os resultados indicam que os efeitos biológicos observados na própolis tipo 6 podem ser atribuídos às altas concentrações de ácidos graxos encontradas nessa própolis, sendo que o seu efeito anticárie pode não estar relacionado à atividade antimicrobiana, mas à inibição de fatores de virulência do biofilme cariogênico, como a redução da produção de ácidos, a acidificação do pH intracelular, através da inibição da atividade da F-ATPase e a redução da produção de glucanos insolúveis através da inibição da atividade das GTFs B e C.

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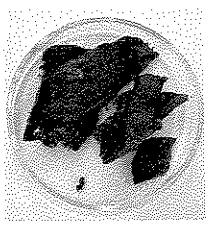
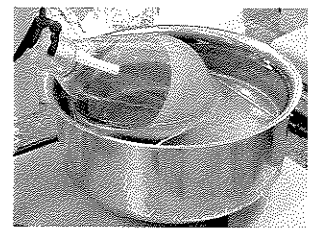
Apêndice

✓ Obtenção do extrato etanólico da própolis bruta (EEP), da fração hexano (EEH) e das sub-frações Hfr-2 e Hfr-3.

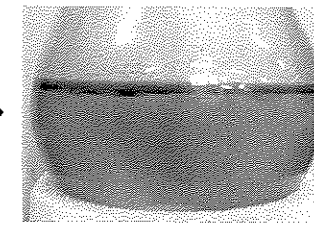


Propolis bruta

Extração com etanol 80%



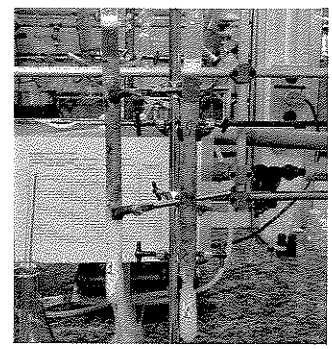
EEP concentrado em evaporador rotativo



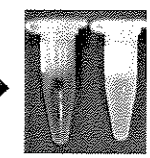
Fracionamento com Hexano

EEP e EEH

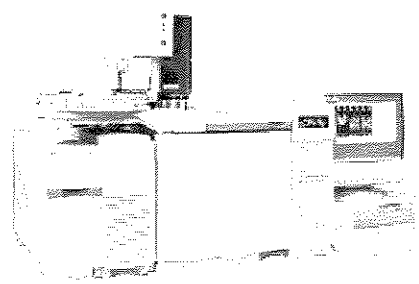
EEH



Fracionamento em coluna seca

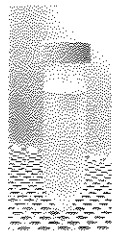


Hfr-2 e Hfr-3

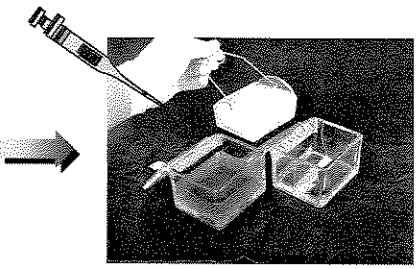


Cromatografia Gasosa – Espectrometria de Massa

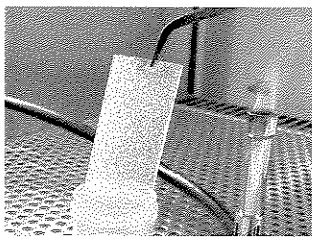
✓ **Biofilmes formados em laminas de vidro**



Inoculo – 18 h
S. mutans UA159
S. sobrinus 6715

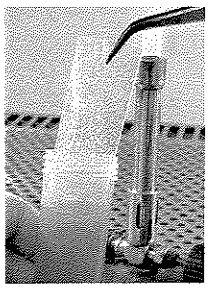


Incoulacao no 1º dia e troca do meio de cultura diariamente



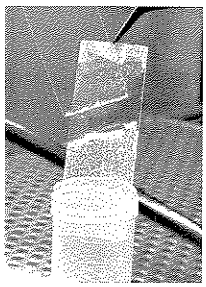
5º dia – biofilme maduro

➔ **Viabilidade**



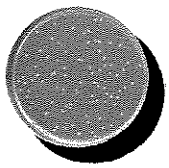
EEP, EEH (800 μg/mL) ou etanol 10%

0 – 4 h



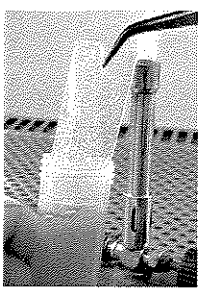
Raspagem dos biofilmes

Inoculacao

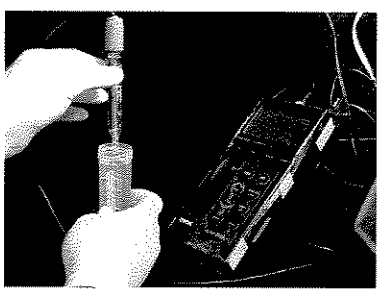


UFC/mL

➔ **Queda de pH**

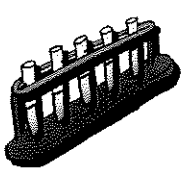


EEP, EEH (800 μg/mL) ou etanol 10%

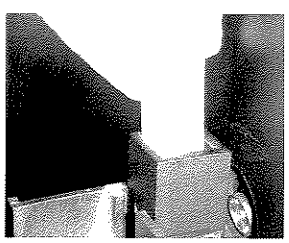


pH ajustado para 7,2
glicose 1%
leituras em 0, 1, 2 e 3 h

➔ **ATPase**

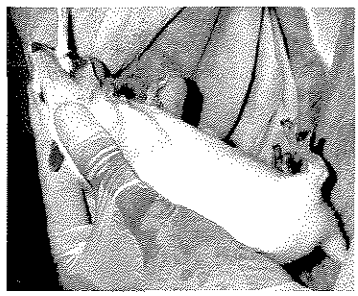


S. mutans UA159 permeabilizados
+
ATP
+
EEP, EEH (800 μg/mL) ou etanol 10%

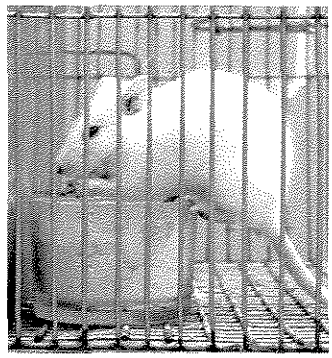


Leitura do Pi produzido

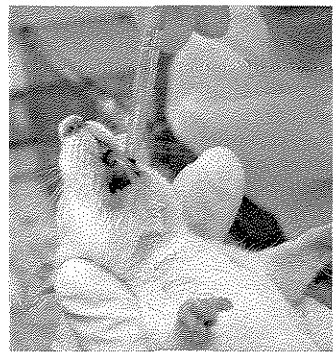
✓ **Desenvolvimento de carie experimental em ratos**



Ratas *Wistar spf* infectadas com *S. sobrinus* 6715

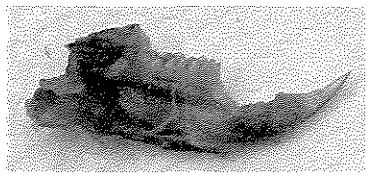


Dieta 2000 (56% sacarose)

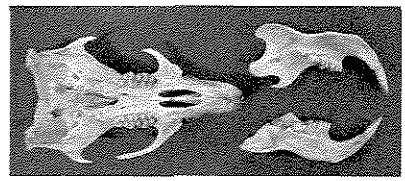


Tratamentos (2 X ao dia)

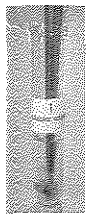
5 semanas



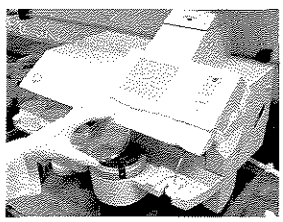
Mandibula esquerda



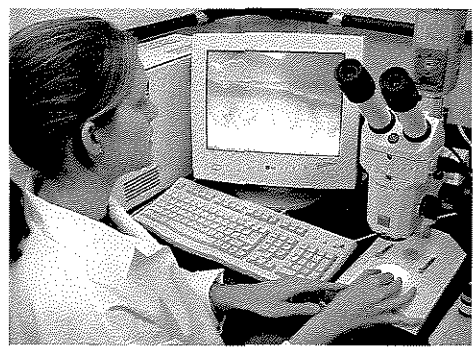
Mandibulas e Maxilas



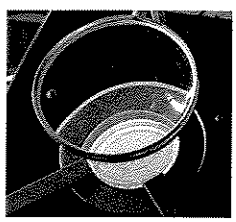
Sonicacao



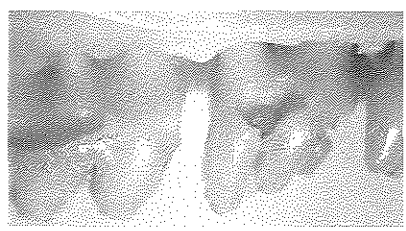
Inoculacao em MSS e agar sangue



Indice de carie (Keyes modificado por Larson, 1981)



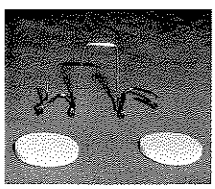
UFC/mL



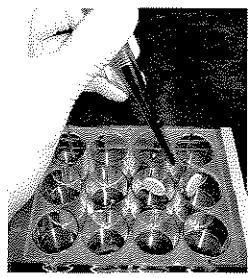
Carie de superficie lisa

Carie de sulco

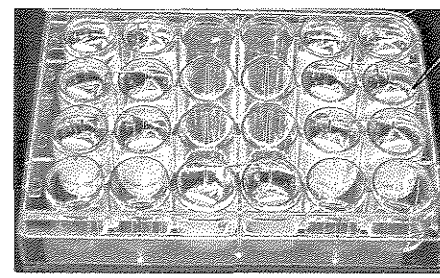
✓ **Biofilmes formados em superficie de hidroxiapatita**



Discos de HA



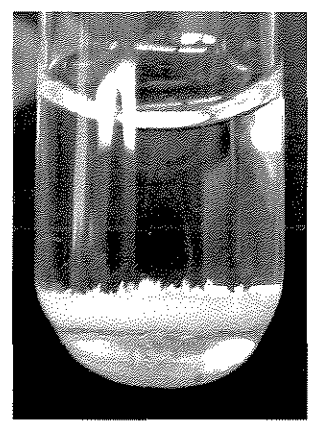
Saliva, 30 min, 37°C
– sob agitacao



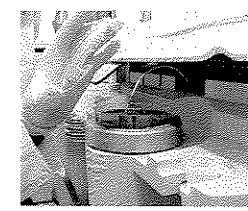
7 tratamentos – 1 min, 2 X ao dia
Hfr-2, Hfr-3 (500 μg/mL) ou etanol 25%

1º dia – inoculo cor
S. mutans UA159

Biofilmes de 5 dias



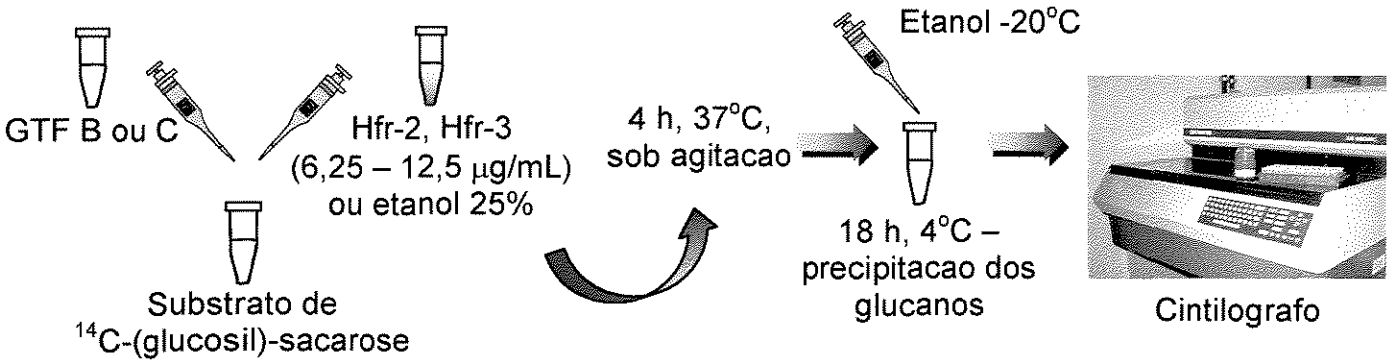
NaCl 0,89%



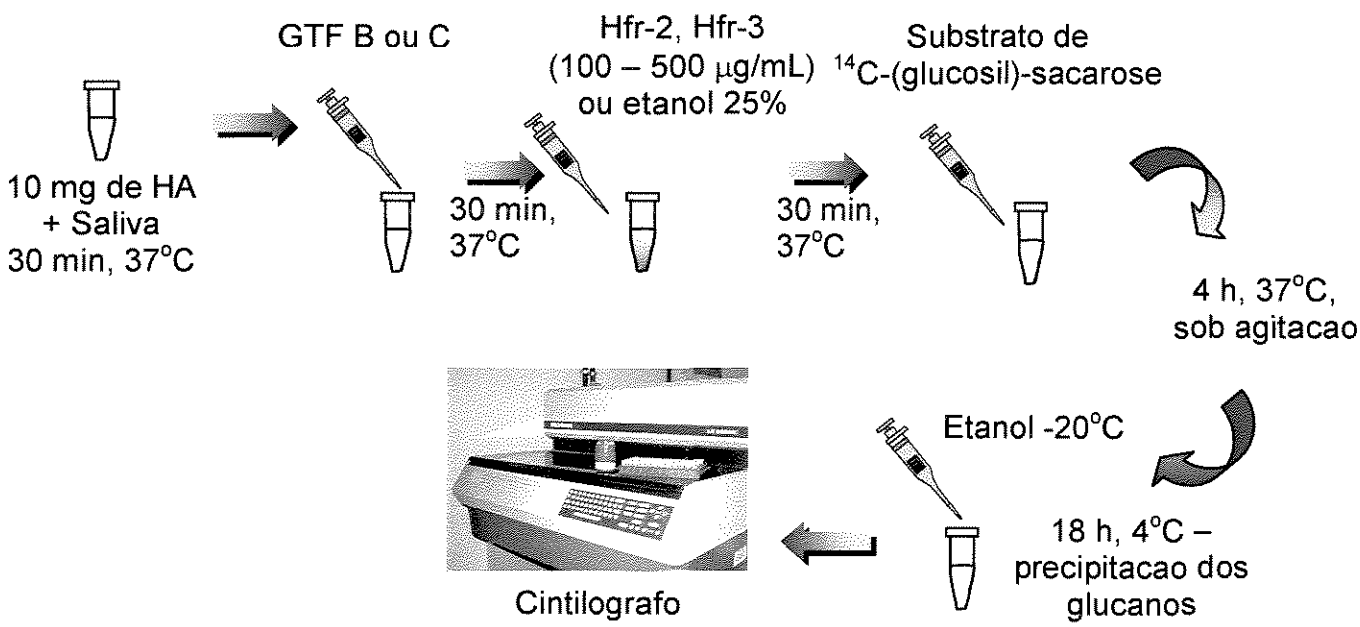
UFC/mL

Polissacarideos:
✓ Soluvel;
✓ Insoluvel;
✓ Intracelular

✓ **Atividade de GTF B e C em solucao**



✓ **Atividade de GTF B e C em superficie**



DELIBERAÇÃO CCPG – 001/98

Dispõe a respeito do formato das teses de Mestrado e de Doutorado aprovadas pela UNICAMP

Tendo em vista a possibilidade, segundo parecer PG Nº 1985/96, das teses de Mestrado e Doutorado terem um formato alternativo àquele já bem estabelecido, a CCPG resolve:

Artigo 1º - Todas as teses de mestrado e de doutorado da UNICAMP terão o seguinte formato padrão:

I) Capa com formato único, dando visibilidade ao nível (mestrado e doutorado), e à Universidade.
II) Primeira folha interna dando visibilidade ao nível (mestrado ou doutorado), à Universidade, à Unidade em foi defendida e à banca examinadora, ressaltando o nome do orientador e co-orientadores. No seu verso deve constar a ficha catalográfica.
III) Segunda folha interna onde conste o resumo em português e o Abstract em inglês.
IV) Introdução Geral.
V) Capítulo.
VI) Conclusão geral.
VII) Referências Bibliográficas.
VIII) Apêndices (se necessários).

Artigo 2º - A critério do orientador, 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.

Parágrafo único – Os veículos de divulgação deverão ser expressamente indicados.

Artigo 3º - A PRPG providenciará o projeto gráfico das capas bem como a impressão de um número de exemplares, da versão final da tese a ser homologada.

Artigo 4º - Fica revogada a resolução CCPG 17/97.

YAHOO! Mail

e: AOB@elsevier.com

ara: simonfop@yahoo.com

ssunto: Submission Confirmation for The influence of a novel propolis on mutans streptococci biofilms and caries development in rats.

ata: Mon, 13 Dec 2004 09:51:49 -0500

ear Mrs. Duarte,

he influence of a novel propolis on mutans streptococci biofilms and aries development in rats.

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On behalf of the Editors)
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Instituto de Biologia

UNICAMP

CEEA-IB-UNICAMP

Comissão de Ética na Experimentação Animal
CEEA-IB-UNICAMP

C E R T I F I C A D O

Certificamos que o Protocolo nº **512-1** sobre "**Avaliação das Atividades Anticárie e Antiplaca da Própolis Proveniente da Região de Mata Atlântica da Bahia (Brasil)**", sob a responsabilidade de **Prof. Dr. Pedro Luiz Rosalen** está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em reunião de **21 de Março de 2003**.

C E R T I F I C A T E

We certify that the protocol nº **512-1**, entitled "**Evaluation of Anti-Caries and Anti-Plaque Activities of Propolis from Atlantic Forest Regiona (Bahia State, Brazil)**", is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on **March 21, 2003**.

Campinas, 21 de Março de 2003.

Profa. Dra. Liana Verinaud
Presidente
CEEA/IB/UNICAMP

Fátima Alonso
Secretária Executiva
CEEA/IB/UNICAMP

UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE BIOLOGIA
CIDADE UNIVERSITÁRIA ZEFERINO VAZ
Caixa Postal 6109
CEP - 13083-970 - CAMPINAS - SP - BRASIL

TELEFONE: (19) 3788-6359
FAX 55 19 32893124