UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

Mitsue Fujimaki Hayacibara

Avaliação do potencial anti-cárie e anti-placa de frações isoladas de Própolis de *Apis mellifera* selecionadas de duas regiões do Brasil

Tese apresentada ao Programa de Pós-Graduação em Odontologia da Faculdade de Odontologia de Piracicaba – UNICAMP, como requisito para a obtenção do título de Doutor em Odontologia, área de concentração Cariologia.

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SUMÁRIO

RESUMO

Estudos recentes têm mostrado resultados promissores em relação ao efeito anti-placa e anti-cárie de extratos etanólicos brutos da própolis de *Apis mellifera* de algumas regiões do Brasil, particularmente do Rio Grande do Sul e de Minas Gerais, classificadas como tipo-3 e tipo-12, respectivamente. Entretanto, não estão bem estabelecidas quais substâncias ativas são responsáveis por estes efeitos. Desta maneira, o objetivo deste estudo foi avaliar o potencial anti-placa e anti-cárie das frações isoladas da própolis do tipo-3 e tipo-12, identificando a fração de maior atividade. Os extratos etanólicos das própolis (EEP) tipo-3 e tipo-12 foram fracionados utilizando os seguintes solventes em um gradiente crescente de polaridade: hexano, clorofórmio, acetato de etila e etanol. Os extratos foram avaliados por: 1) estudos *in vitro* para determinar: a) atividade antimicrobiana sobre *S. mutans* Ingbritt 1600 e *S. sobrinus* 6715 através do método de difusão em ágar, b) concentração inibitória mínima (CIM), c) concentração bactericida mínima (CBM), d) inibição da atividade das glucosiltransferases (Gtfs) B e C em solução e aderidas à superfície de hidroxiapatita e e) inibição da viabilidade do biofilme de *Streptococcus mutans* UA 159; 2) estudo em animais utilizando o modelo de cárie experimental em ratos. As frações hexano demonstraram os melhores resultados nos teste antimicrobianos, inibiram efetivamente as enzimas Gtfs em solução e adsorvidas à superfície da hidroxiapatita e também proporcionaram a inibição do biofilme de *S. mutans* quando comparadas ao controle ($p < 0.05$). Embora estas frações não tenham sido mais efetivas que a própolis bruta na redução de cárie, estas inibiram significantemente a microbiota total e microrganismos cariogênicos em ratos. Assim, pode-se concluir que as substâncias anti-placa e anti-cárie das própolis estudadas estão relacionadas com compostos ativos de caráter apolar.

ABSTRACT

Recent studies have shown promising results regarding the anti-plaque and anti-caries effect of crude ethanolic extracts of propolis from Southern (propolis type-3) and Southeastern (propolis type-12) regions of Brazil. However, it is not well established which active substances are responsible for these effects. Thus, the aim of this study was to evaluate the anti-plaque and anti-caries effects of isolated fractions of propolis (types-3 and 12), in order to identify the most active fraction. The ethanolic extracts of propolis (EEPs) type-3 and type-12 were fractionated using solvents in a chemical gradient of polarity: hexane, chloroform, ethyl acetate, and ethanol. The extracts were analyzed by: 1) *in vitro* studies to determine: a) antimicrobial activity against *Streptococcus mutans* Ingbritt 1600 and *Streptococcus sobrinus* 6715 by agar diffusion method; (b) minimum inhibitory concentration (MIC); (c) minimum bactericidal concentration (MBC), (d) inhibition of activity of purified glucosyltransferase (Gtfs) B and C in solution and adsorbed on the surface of hydroxyapatite, e) inhibition of the viability of *Streptococcus mutans* UA 159 biofilm; 2) animal study performed using an experimental dental caries model in rats. The hexane fraction showed better results for antibacterial activity, inhibited effectively Gtfs in solution and adsorbed on the hydroxyapatite surface and also demonstrated inhibition of *S. mutans* biofilm compared to the control ($p < 0.05$). Although these fractions were not more effective on caries reduction than the crude propolis, they showed higher antibacterial effect reducing the total viable microflora and the cariogenic microorganisms in rats. Thus, it was concluded that the anti-plaque and anti-caries substances of propolis are related to the active non-polar compounds.

I – INTRODUÇÃO

A cárie dental é uma doença de alta prevalência na cavidade bucal, constituindo ainda um problema de saúde pública, principalmente nos países em desenvolvimento (MARSH & MARTIN[29], 1992).

Atualmente está bem estabelecido que um dos fatores etiológicos mais importantes desta doença são os microrganismos de origem bacteriana, que formam um biofilme patogênico que se adere sobre a superfície dental, de modo a produzir ácidos que levam à desmineralização do esmalte (MARSH[27], 1994). Este biofilme é genericamente conhecido como placa dental. A placa dental apresenta uma composição microbiana e bioquímica variável dependendo de fatores intrínsecos e extrínsecos, podendo mudar de modo a tornar este biofilme patogênico (MARSH [28,27], 1992, 1994). Assim, fatores que levam ao desequilíbrio da comunidade microbiana de modo a favorecer o crescimento de bactérias odontopatogênicas vão direcionar para o surgimento de uma placa dental relacionada a cárie dental (MARSH[28], 1992).

Um dos fatores de desequilíbrio fundamental para o aparecimento de uma placa dental cariogênica é a dieta rica e freqüente de carboidratos fermentáveis, principalmente a sacarose. Esta dieta promove um aumento na proporção de microrganismos que apresentam vantagens ecológicas na presença da sacarose no meio bucal, como por exemplo, os estreptococos do grupo mutans. Estes, além de serem acidogênicos e acidúricos, sintetizam polissacarídeos extracelulares a partir da sacarose (GIBBONS & VAN HOUTE[12], 1975; HAMADA & SLADE[15], 1980). Esta síntese é feita por enzimas chamadas genericamente de glicosiltransferases, podendo ser sintetizados glucanos pela ação das glucosiltransferases (Gtf) e frutanos pela atividade das frutosiltransferases (Ftf) (HAMADA & SLADE[15], 1980; LOESCHE[25], 1986). Os glucanos, principalmente os insolúveis em água, facilitam a aderência e acúmulo dos estreptococos cariogênicos sobre a superfície lisa do esmalte dental, tanto em experimentos com animais, quanto em humanos (KRASSE[24], 1965; FROSTELL *et al.*[9], 1967; HAMADA & SLADE[15], 1980; TANZER *et al.*[35], 1985). Atualmente, 3 Gtfs distintas secretadas pelo *S. mutans* estão bem caracterizadas tanto bioquimicamente como em nível molecular: 1) Gtf B - codificada pelo gene *gtfB*, que sintetiza glucanos insolúveis em água tendo ligações glicosídicas predominantes α (1$\rightarrow$3);

2) Gtf C - codificada pelo gene *gtfC*, que sintetiza uma mistura de glucanos insolúveis e solúveis, este último apresentando ligações glicosídicas predominantes α (1$\rightarrow$6); e 3) GtfD - codificada pelo gene *gtfD*, que sintetiza basicamente glucanos solúveis (LOESCHE[25], 1986; HANADA & KURAMITSU[16], 1989). Em acréscimo, tem sido demonstrado que estes glucanos aumentam a porosidade (DIBDIN & SHELLIS[8], 1988; VAN HOUTE[37], 1994) bem como contribuem para mudanças na composição inorgânica da matriz da placa (CURY *et al.*[7], 1997) tornando-a ainda mais cariogênica. Assim, estreptococos do grupo mutans e glucanos são considerados fatores críticos para desenvolvimento do biofilme dental cariogênico.

Deste modo, uma das estratégias visando o controle da formação de uma placa dental patogênica relacionada à doença cárie tem sido a tentativa de inibir: 1) o crescimento dos estreptococos do grupo mutans; 2) a atividade das Gtfs, principalmente aquelas responsáveis pela síntese de glucanos insolúveis (Gtf B e C). Um agente que aliasse eficientemente propriedades antimicrobianas com inibição das Gtfs seria desejável para utilização no controle da cárie dental.

Nas últimas décadas, tem sido observado mundialmente um crescente uso de produtos naturais devido as suas diversas propriedades biológicas. Dentre estes, a própolis tem se destacado devido a seus efeitos farmacológicos de interesse médico, tais como antimicrobiano, anti-inflamatório, cicatrizante, anestésico, antioxidante e anti-tumoral (GHISALBERTI[11], 1979; BANKOVA *et al.*[3], 1989; MARCUCCI[26], 1995). Em relação às patologias bucais, em particular à cárie dental, os estudos são ainda recentes, tendo sido iniciados na década de 90 (IKENO *et al.*[17], 1991).

A própolis (PRO – em defesa de; POLIS – cidade) é o nome genérico dado para uma resina coletada pelas abelhas da espécie *Apis mellifera* de diversas partes da planta (como broto e botões florais), que posteriormente é modificada pelas abelhas através da adição de secreções próprias e funciona como meio de proteção da colméia. (GHISALBERTI[11], 1979). Por outro lado, a composição química da própolis é muito complexa e variada. Os compostos fenólicos, particularmente flavonóides e derivados do ácido cinâmico, bem como alguns diterpenos têm sido associados com as propriedades biológicas da própolis (GHISALBERTI[11], 1979; BANKOVA *et al.*[2], 1982; GRANGE & DAVEY[13], 1990; BONHEVI & COLL[6], 1994, AGA *et al.*[1], 1994; BANKOVA *et al.*[4],

1996). Porém, a composição química da própolis é bastante variável dependendo da biodiversidade de cada região visitada pelas abelhas (KÖNIG[18], 1985; GREENAWAY *et al.*[14], 1990; GARCÍA-VIGUEIRA *et al.*[10], 1992; BANKOVA *et al.*[5], 1992; TOMAS-BARBERAN *et al.*[36], 1993; BONHEVI & COLL[6], 1994).

Num extenso estudo da própolis brasileira, onde foram coletadas aproximadamente 600 amostras provenientes das regiões Sudeste, Sul, Centro Oeste e alguns estados do Nordeste, a composição dos flavonóides foi extremamente variável (PARK *et al.*[30], 1997; KOO & PARK[20], 1997; PARK & IKEGAKI[31], 1998). Conseqüentemente, as suas propriedades biológicas *in vitro* também mostraram-se nitidamente distintas (PARK *et al.*[30], 1997; PARK *et al.*[32], 1998; PARK & IKEGAKI[31], 1998). Dentre as própolis analisadas, duas amostras quimicamente distintas classificadas de acordo com Park *et al.* [33](2000) como tipo-3 (amostra representativa da região Sul do Brasil - RS) e tipo-12 (região Sudeste – MG) se destacaram por apresentarem *in vitro* uma alta atividade antibacteriana sobre os estreptococos do grupo mutans (IKENO *et al.*[17], 1991; STEINBERG *et al.*[34], 1996; PARK *et al.*[32], 1998; KOO *et al.*[19], 2000) e inibição da atividade da glucosiltransferase (IKENO *et al.*[17], 1991; PARK *et al.*[32], 1998, KOO *et al.*[23], 2000). Posteriormente, estas amostras demonstraram redução de cárie dental utilizando um modelo experimental animal de alto desafio cariogênico (KOO *et al.*[21], 1999), bem como na redução da formação de placa supragengival quando utilizada topicamente na forma de bochechos (KOO et *al.*[24], 2002).

Considerando a complexidade na sua composição química, pouco se conhece dos princípios ativos da própolis. Tendo em vista que os extratos brutos da própolis tipo-3 e tipo-12 demonstraram efeitos anti-placa e anti-cárie promissores, a análise de frações isoladas destes extratos é o passo inicial para a determinação do(s) composto(s) ativo(s) desta substância natural.

Assim, o objetivo deste trabalho foi avaliar em qual(is) fração(ões) do extrato da própolis de *Apis mellifera* do tipo-3 e do tipo-12 estão presentes os princípios ativos que apresentam propriedades anti-cárie e anti-placa.

II – PROPOSIÇÃO

Propomos neste estudo avaliar o potencial anti-placa e anti-cárie de frações isoladas das própolis tipo-3 e tipo-12 através de:

➘ 1-modelos *in vitro* para determinar:

1.1- suscetibilidade de *Streptococcus mutans* Ingbritt 1600 e *Streptococcus sobrinus* 6715 pela técnica de difusão em ágar;

1.2- concentração mínima inibitória (CIM) e a concentração bactericida mínima (CBM) contra *S. mutans* Ingbritt 1600 e *S. sobrinus* 6715;

1.3- inibição da aderência de *S. mutans* Ingbritt 1600 e *S. sobrinus* 6715 ao vidro;

1.4- inibição da atividade das glucosiltransferases (Gtfs) B e C em solução e adsorvidas à superfície de hidroxiapatita;

1.5- inibição da viabilidade do biofilme de *S. mutans* UA 159;

➘ 2-modelo animal para avaliar:

2.1-efeito da própolis e suas frações hexano sobre o desenvolvimento de cárie dental em ratos.

III – CAPÍTULOS

Artigo 1

"Effects of isolated chemical fractions of Brazilian propolis on mutans streptococci, glucosyltransferase activity and caries development in rats". Este artigo foi submetido à publicação na Biological & Pharmacological Bulletin.

Artigo 2

"Effect of Brazilian propolis type-3 and its hexane fraction on *S. mutans* biofilm and on glucosyltransferase activity." Este artigo será submetido à publicação na revista FEMS Microbiol.

Artigo 1

Effects of isolated chemical fractions of Brazilian propolis on mutans streptococci, glucosyltransferase activity and caries development in rats

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Summary

Considering that the crude ethanolic extract of Brazilian propolis type-3 and type-12 has shown anti-caries effect, the aim of this study was to evaluate the effects of their isolated chemical fractions on the growth and adherence of mutans streptococci, on the ability to inhibit glucosyltransferase enzymes (GTFs) and on caries reduction in rats. The ethanolic extracts of propolis (EEPs) were serially fractionated in the solvents hexane, chloroform, ethyl acetate, and ethanol. The ability of the 4 fractions and EEP to inhibit the growth of *Streptococcus mutans* Ingbritt 1600 and *Streptococcus sobrinus* 6715 were tested by the following *in vitro* assays: (1) the agar diffusion method; (2) determination of minimum inhibitory concentration (MIC) and (3) minimum bactericidal concentration (MBC). The inhibitory effect of the extracts on GTFs B and C activities in solution was also determined. For the caries study, 60 Wistar rats were infected with *Streptococcus sobrinus* 6715 and submitted to the following five groups of treatments : (1) EEP type-3, (2) hexane fraction type-3, (3) EEP type-12, (4) hexane fraction type-12 and (5) 80% ethanol (control). The hexane fraction from both types of propolis showed higher antibacterial activity than the crude propolis. GTFs were effectively inhibited by all fractions. The hexane fractions of propolis were not more effective on caries reduction than the crude extract, but they showed higher antibacterial effect reducing the total viable microflora and the cariogenic microorganisms of the rats mouth. The data suggest that the active anti-caries substances of the propolis evaluated are mostly non-polar compounds.

Keywords: propolis; glucosyltransferase; antimicrobial activity; mutans streptococci; caries

Introduction

In the last decades, it has been observed worldwide an increased use of natural products for pharmacological purposes[1)], including propolis. Propolis is a resinous hive product collected by *Apis mellifera* bees from tree buds and mixed with secreted beeswax.[2)] Many pharmacological activities are attributed to the ethanolic extract of propolis, such as antimicrobial, cytostatic, anti-inflammatory properties.[1,3)] The chemical composition of propolis is complex; flavonoids and (hydroxyl) cinnamic acid derivatives have been considered the primary biological active compounds in propolis.[3)] Also, the composition of propolis is highly variable and depends on their geographical origin.[2,4,5)] Twelve chemically distinct types of Brazilian propolis have been characterized to the present date.[6)]

Recent studies have shown anti-caries potencial of propolis from Southern (type-3) and from Southeastern (type-12) regions of Brazil.[7-11)] Furthermore, propolis in drinking water[1)] or applied topically reduced the incidence of dental caries in rats.[8)] Two mechanisms of action are related to the anti-caries effect of propolis: antimicrobial properties against cariogenic bacteria and the effect on the glucosyltransferase enzymes (GTFs) activity.[9)] GTFs are of central importance in adhesive interactions by *S. mutans* and it is essential in the expression of virulence by these microorganisms.[12-15)] The glucans synthesized by GTFs not only promote the accumulation of cariogenic streptococci on the tooth surface, but also contribute significantly to the bulk of dental plaque.[16,17)] *S. mutans* produces at least 3 GTFs: GTF B, which synthesizes mostly insoluble α1,3-linked glucan; GTF C, which synthesizes insoluble and soluble α1,6-linked glucan, and GTF D, which synthesizes soluble glucan. Among them, GTF B and C appear to be the most important GTFs related to dental caries.[15)]

Thus, the fractionation of propolis is the first step toward identifying the active compound(s) of this natural product. The objective of the present study was to evaluate the effects of isolated fractions of propolis type-3 and type-12 on bacterial growth, activity of glucosyltransferase enzymes (GTFs) in solution and caries development in rats.

Material and Methods

Propolis samples and fractionation

Crude samples of *Apis mellifera* propolis were obtained from two different regions of Brazil: Southern and Southeastern, classified as type-3 and type-12, respectively.[6] The ethanolic extract of propolis (EEP) at 20% (w/v) in aqueous ethanol (80% v/v) was prepared as detailed elsewhere.[5] A portion of the EEP was used to make a chemical fractionation, according to a polarity gradient. First, the EEP was fractioned with hexane to make the first fraction, following chloroform, ethyl acetate and ethanol, resulting in 4 fractions. Each fraction was monitored by paper chromatography and developed by UV light (λ=254 and 366 nm).[18] The ethanolic extracts of these fractions (10%, w/v) in ethanol (80%, v/v) were tested.

Susceptibility testing

The antimicrobial activity was determined by agar diffusion method, and by determining the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) in accordance with the National Committee for Clinical Laboratory Standards (NCCLS) guidelines and KOO et al.[10] The bacterial strains used were: *S. mutans* Ingbritt 1600 and *S. sobrinus* 6715. For the agar diffusion method, the inoculum procedures were appropriate to provide a semiconfluent growth of the microorganisms tested (1-2 x 10^8 CFU/ml) onto a brain heart infusion agar plate. Six sterilized steel cylinders of 8.0 x 10.0 mm (inside diameter 6 mm) were placed onto the inoculated agar plates. The test extracts (400 μg/ml) or control (80% ethanol, v/v) (40 μl) were applied inside the cylinders. The plates were incubated at 37 ^oC, for 24 h, in a 10% CO_2 incubator.[18] The zones of inhibition of microbial growth around the cylinder containing the extracts were measured. Three different experiments in triplicate were made for each microorganism. For the MIC determination, the starting inoculum was 5×10^5 CFU/ml, and the concentration of test extracts ranged from 12.5 to 1600 μg/ml (for EEPs) and 6.25 to 800 μg/ml (for propolis fractions). In order to determine MBC, an aliquot (50 μl) of all incubated tubes with higher concentrations than MIC was subcultured on BHI agar supplemented with 5% of defibrinated sheep blood with a Spiral plater (Whittley Automatic Spiral Plater®). MBC was defined as the lowest concentration that allows no visible growth

on the agar.[10] Three different experiments were made for each concentration of the extracts tested.

Assays of activity of GTFs in solution

The bacterial strains used for the production of GTFs were: 1) *Streptococcus milleri* KSB8, which harbors the *gtfB* gene from *S. mutans* GS-5 (for GTF B production) and 2) *S. mutans* WHB 410, whose genes for GTF B, D and fructosyltransferase were deleted (for GTF C). The cultures were stored at –80 ^{o}C in brain heart infusion (BHI) or tryptic soy broth (TSB) containing 20% glycerol. The *S. milleri* constructs were a kind gift from Dr. Howard K. Kuramitsu (SUNY, Buffalo, NY, USA). The GTF B and C enzymes were obtained from culture supernatants and purified to near homogeneity by hydroxyapatite column chromatography.[19,20] GTF activity was measured by the incorporation of [^{14}C]glucose from labeled sucrose (NEN Research Products, Boston, Mass., USA) into glucans.[9,19] The GTF enzyme added to each sample for all assays was equivalent to the amount required to incorporate 1 μmol of glucose over the 4 h reaction. GTF B or C was mixed with a twofold dilution series of the EEP or the fractions (concentration ranging from 50.0 to 400.0 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 (200 μl final volume). For the control, the same reaction was done with ethanol (final concentration of 80%, v/v) replacing the test extracts. The samples were incubated at 37 ^{o}C with rocking for 4 h. After incubation, ice-cold ethanol (1.0 ml) was added and the samples were stored for 18 h at 4 ^{o}C for precipitation of glucans. Radiolabeled glucan was determined by scintillation counting.[9,19]

Caries study

The animal experiment was performed as described previously by KOO et al., 2002.[21] Sixty pups of Wistar female rats, aged 19 days, mutans-free, were purchased from CEMIB-UNICAMP (Brazil). The pups were infected on two successive days with an

actively growing culture of *Streptococcus sobrinus* 6715 and checked to confirm the heavy infection. Pups aged 24 days were randomly divided into five groups and their teeth treated topically using a camel hair brush for 30 s, twice daily as follows: (1) propolis type-3; (2) hexane fraction type-3; (3) propolis type-12; (4) hexane fraction type-12 and (5) ethanol 80% (v/v). The concentration of EEPs and hexane fractions tested was 2.5 %. Each group of 12 animals was provided with diet 2000[22], containing 56% of sucrose, *ad libitum*. The experiments proceeded for 5 weeks, at the end of which the animals were killed by CO_2 asphyxiation. The lower left jaw was aseptically dissecated, suspended in 5.0 ml of sterile saline solution and sonicated (three 10 s-pulses at 5 s-intervals, at 30 W (Sonic & Materials Inc. Vibracell®). The suspension was plated on mitis salivarius agar plus streptomycin to estimate *S. sobrinus* populations and on blood agar to determine total cultivable flora. The jaws were defleshed and the teeth prepared for caries scoring. The smooth and sulcal surfaces caries and their severity (Ds, dentin exposed; Dm, ¾ of the dentin affected) were evaluated by means of the Larson modification of the Keyes' system.[23]

The data were subjected to ANOVA in the Tukey-Kramer Honest Standard Deviation (HSD) test for all pairs using JMP version 3.1, software for statistical visualization.[25] The level of significance was 5%.

Results

In vitro study

The means of bacterial growth inhibition zones by tested extracts are shown in Table 1. All extracts produced inhibitory zones against all the microorganisms tested, except the ethanol fraction that did not display any antibacterial activity. In general, extracts from propolis type-3 produced slightly higher means of inhibitory zones than propolis type-12. The control (80% aqueous ethanol, v/v) did not form an inhibitory zone with any of the strains tested.

The MIC, MBC and the values at which the bacterial adherence was inhibited are shown in Table 2. The MIC values of EEPs and their hexane and chloroform fractions from both types of propolis ranged from 25 to 400 μg/ml. In contrast, ethyl acetate and ethanol fractions did not show inhibition at the concentrations tested in this study. The potency order against *S. mutans* and *S. sobrinus* was: hexane fraction > EEP > chloroform fraction,

except for hexane fraction type-3 that did not differ from EEP and chloroform fraction. The MBC values from propolis type-3 were 2 to 8 times higher than the MIC values. Among the extracts from propolis type-12, the hexane fraction was the only one that displayed bactericidal effect at the concentrations tested in this study. *S. sobrinus* appears to be more susceptible to propolis type-3 than *S. mutans* but no difference was observed with propolis type-12.

The effects of the propolis on the activity of GTF B and C are shown in Figs.1, 2, 3 and 4. The EEPs extracts and their 4 fractions reduced the activity of GTF B and C in solution as follows: 13 to 50 % inhibition for propolis type-3 and 9 to 75% inhibition for propolis type-12 at a concentration of 100 μg/ml.

Caries study

The rats remained in apparent good health during the 5-week experiment. Weight gains were not significantly different among the treatment groups ($p > 0.05$). Table 3 shows the effects of the treatments on the number of total viable microorganisms, *S. sobrinus* 6715 and percentage of *S. sobrinus* recovered after 5-week treatment. The percentage of *S. sobrinus* recovered from the jaws of the rats was calculated from the total cultivable flora and the *S. sobrinus* population. The groups treated with hexane fractions types-3 and 12 displayed significant lower levels of total viable microorganisms ($p < 0.05$). In contrast, EEP from type-3 and type-12 did not affect the number of total bacteria, showing comparable numbers to control group. In addition, the effect of hexane fractions of both types on *S. sobrinus* population was higher than the control group ($p < 0.05$). Although the fractions have affected the number of microorganisms, they did not affect the proportion of *S. sobrinus* in all groups.

The effects of the treatments on the incidence and severity of smooth-surface and sulcal caries are shown in Table 4. Animals treated with propolis type-3 developed fewer lesions than did the control group ($p < 0.05$), approximately 2 times less caries than the control group and no statistical difference was noted when compared among other treatments ($p > 0.05$). Significant differences were observed in the severity of lesions on smooth surfaces. All treatments showed similar inhibition (Ds and Dm) and differed from

the control except for the hexane fraction of propolis type-12. Statistically significant differences in sulcal caries score and its severity were not observed for any treatment compared to the control.

Discussion

Dental caries development involves a series of events in a biofilm where bacterial interactions with diet occur on a tooth surface. There is a general consensus that the frequent consumption of carbohydrates, mainly sucrose, can result in the emergence of cariogenic microorganisms, such as mutans streptococci. These microorganisms have been implicated as a primary etiological agent of dental caries in animal and humans.[25-27)]

According to the results of this study (Table 1 and 2) propolis type-3 and type-12 displayed antimicrobial activity and is in agreement with previous *in vitro* studies.[7,9)] Regarding the fractions, lower polarity ones showed antimicrobial effect (Table 1 and 2). This effect was initially seen in the agar diffusion assay through the measurement of the inhibition zones produced by the extracts. It is noteworthy that agar diffusion method is limited in predicting efficacy when comparing different antimicrobial substances since the inhibition zone may also depend on the diffusion capacity of the agents. The fractions, extracted according to the solvents polarity, may have distinct diffusibility. Therefore, more specific assays should be conducted in order to compare antimicrobial efficacy among the different fractions, such as MIC and MBC determinations.

The determination of MIC demonstrated that EEPs and fractions from propolis type-3 and type-12 presents significant activity against mutans streptococci growth (Table 2), showing MIC values between 12.5 to 50 μg/ml (type-3) and between 25 to 400 μg/ml (type-12); and MBC values ranging from 50 to 400 μg/ml (type-3) and from 200 to 400 μg/ml (type-12). Higher polarity fractions (ethyl acetate and ethanol) from both types of propolis were devoid of antibacterial activity. The hexane fractions were clearly more active than EEPs, suggesting that the putative compounds are mostly apolar.

With regard to the effect on GTF enzymes, the data showed that propolis type-3 and type-12 and their fractions effectively reduced the activities of GTFs B and C in solution.

These are preliminary results as it is well known that it is desirable to determine the effects of potential inhibitors of GTFs on the surface-adsorbed enzymes.[18)]

The findings are in accordance with Duarte et al.[18)], who found that the biological activities of propolis type-6 are associated with its nonpolar components; type-6 is a flavonoid-free propolis. Since particularly the hexane fraction obtained from both types of propolis showed potent and multiple inhibitory effects, it should be the fraction of choice for further studies for identification of novel bioactive compounds for controlling biofilm formation and accumulation.

The results of caries showed that the topical application of hexane fractions significantly affected the number of *S. sobrinus* and total viable microorganisms after 5-week treatment. This result is consistent with our *in vitro* data showing MBC values for hexane fraction type-3 4 times more potent than for EEP. Also, MBC values for the hexane fraction type-12 was between 200-400 μg/ml whereas the EEP was not able to kill the microorganism at 1600 μg/ml. The percentage of *S. sobrinus* was lower in the treated groups, but did not differ statistically from the control group. This result is in accordance with previous report.[8)] The recovered microorganisms from the jaw of each animal represent mainly the bacteria attached to the teeth, or present in the dental biofilm of the rats. Thus, the lower number of bacteria present could also be a result of the inhibition of glucosyltransferase enzymes (GTFs), that are of central importance in adhesive interaction by *S. mutans*.[12-15)]

Although a significant reduction of the number of *S. sobrinus* was found when the animals were treated with the hexane fractions, the caries inhibition was not consistent. The incidence of smooth-surface caries and severity in rats was similar among treatments, showing numerically less smooth-surface caries than the control. Following the same pattern of result, the severity of smooth-surface caries was significantly lower in all treatments, when compared to control, except for hexane fraction of propolis type-12 and did not differ among treatments. These data suggest that the reduction in number of smooth-surface caries lesions by the treatments was probably more related to the inhibition of glucosyltransferase activity rather than antimicrobial action. It is likely that the antimicrobial effect seen was masked by other mechanism.

The composition of propolis type-3 and propolis type-12 are distinct and complex. Regarding propolis type-3, *p*-coumaric, pinobanksin and some flavonoids (quercetin, kaempferol, apigenin, pinicembrin, chrysin, acacetin, galangin) were identified.[7)] In contrast, only a few flavonoids in propolis type-12 were identified, in addition to *p*-coumaric, cinnamic acid derivatives and terpenes.[7,28)] Despite the chemical composition differences, both types of propolis displayed significant antimicrobial activity likely due to phenolic compounds, hydroxycinnamic acid derivatives and some terpenoids which are present in both types of propolis; these compounds are related to the antimicrobial activity of propolis.[1,29,30)]

Although the hexanic fraction was not more effective than the crude extract on caries reduction, its higher antibacterial effect can be important, mainly when associated with other anticaries agents, for example, fluoride. Fluoride is the most effective anticaries agent known to date and it interferes physicochemically with caries development by reducing demineralization and enhancing remineralization of dental enamel.[31)] Thus, substances that act on virulence factors and/or metabolism of cariogenic bacteria may enhance the anticariogenic effect of fluoride. Therefore, we suggest further studies evaluating the combination of fluoride and hexane fraction in order to achieve enhanced benefits for caries control.

In conclusion**,** the data suggest that the active anti-caries substances of the propolis evaluated are mostly of non-polar nature.

Acknowledgments

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Table 1. Means (SD; n=3) of inhibition zone values (mm) of ethanolic extract of propolis type-3 and type-12 and their fractions against *S. mutans* and *S. sobrinus*.

Treatments	*Propolis type-3*		*Propolis type-12*	
	S. mutans Ingbritt 1600	*S. sobrinus* 6715	*S. mutans* Ingbritt 1600	*S. sobrinus* 6715
Crude propolis (EEP)	2.45 ± 0.44	2.47 ± 0.42	1.31 ± 0.27	0.92 ± 0.30
Hexane fraction	1.68 ± 0.60	1.35 ± 0.35	1.21 ± 0.45	1.03 ± 0.25
Chloroform fraction	2.87 ± 0.52	2.35 ± 0.32	1.18 ± 0.17	1.23 ± 0.21
Ethyl acetate fraction	2.93 ± 0.38	2.55 ± 0.57	1.02 ± 0.21	1.16 ± 0.29
Ethanol fraction	0.0	0.0	0.0	0.0
80% Ethanol (negative control)	0.0	0.0	0.0	0.0

Table 2. Values of Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of ethanolic extracts of propolis type-3 and type-12 and their fractions against mutans streptococci.

Treatments	*Propolis type-3*				*Propolis type-12*			
	S. mutans Ingbritt 1600		*S. sobrinus* 6715		*S. mutans* Ingbritt 1600		*S. sobrinus* 6715	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Crude propolis (EEP)	25-50	200-400	25-50	100-200	200-400	-	200-400	-
Hexane fraction	25-50	50-100	12.5-25	25-50	25-50	200-400	25-50	200-400
Chloroform fraction	25-50	200-400	25-50	200-400	200-400	-	200-400	-
Ethyl acetate fraction	-	-	-	-	-	-	-	-
Ethanol fraction	-	-	-	-	-	-	-	-

The values are expressed in a range of concentration in μg/ml. The MIC and MBC values are between the concentrations above.

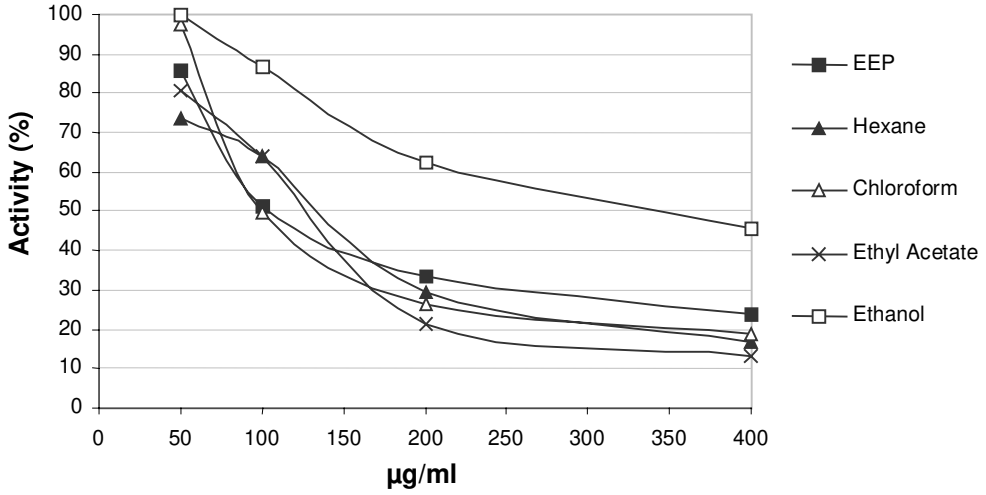

Figure 1. Effect of ethanolic extract of propolis (EEP) type-3 and its fractions on the activities of glucosyltransferases (GTF B) in solution. For the control, ethanol (80%, final concentration) was used. The percentage of GTF activity was calculated considering the control as maximum enzymatic activity.

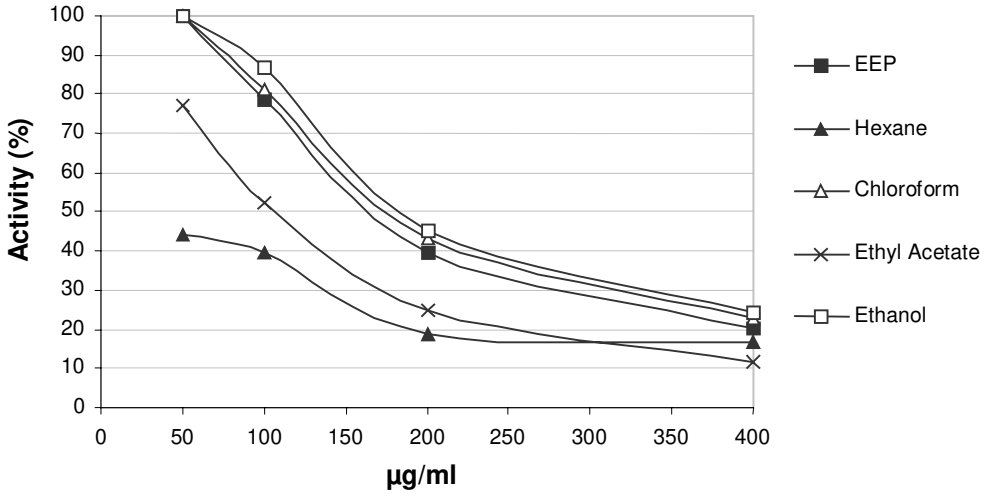

Figure 2. Effect of ethanolic extract of propolis (EEP) type-12 and its fractions on the activities of GTF B in solution. For the control, ethanol (80%, final concentration) was used. The percentage of GTF activity was calculated considering the control as maximum enzymatic activity.

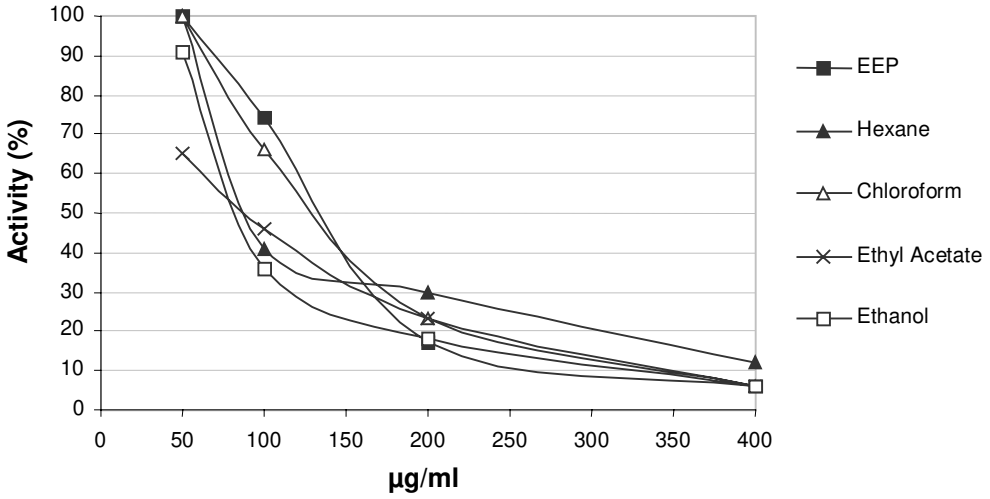

Figure 3. Effect of ethanolic extract of propolis (EEP) type-3 and its fractions on the activities of GTF C in solution. For the control, ethanol (80%, final concentration) was used. The percentage of GTF activity was calculated considering the control as maximum enzymatic activity.

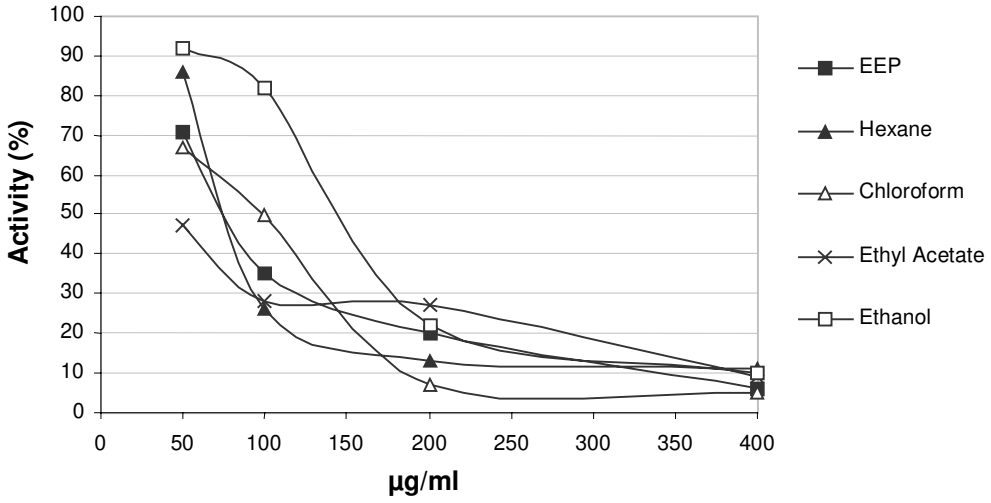

Figure 4. Effect of ethanolic extract of propolis (EEP) type-12 and its fractions on the activities of GTF C in solution. For the control, ethanol (80%, final concentration) was used. The percentage of GTF activity was calculated considering the control as maximum enzymatic activity.

Table 3. Means (SD; n=12) of total microorganisms, *S. sobrinus* 6715 and percentage of *S. sobrinus* recovered after 5-week experiment.

Treatments	Total cultivable population (x 10^7)	*S. sobrinus* (x 10^7)	% of *S. sobrinus*
Propolis type-3	1.5 (1.7) [a]	1.0 (1.4) [a,b]	58.1 (20.8) [a]
Hexane fraction type-3	0.1 (0.1) [b]	0.1 (0.1) [c]	72.3 (24.0) [a]
Propolis type-12	2.1 (2.5) [a]	1.2 (1.0) [a]	66.0 (21.1) [a]
Hexane fraction type-12	0.3 (0.5) [b]	0.2 (0.2) [b,c]	57.0 (25.7) [a]
Ethanol 80% (Control)	1.9 (1.9) [a]	1.5 (1.6) [a]	79.0 (24.6) [a]

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. (SAS, 1995).

Table 4. Effects of propolis types 3 and 12 and their hexane fractions on dental caries development in rats: means (SE), Keyes' scores.

Treatments	Total Smooth-Surface	Smooth-surface Severity		Total Sulcal	Sulcal Severity	
		Ds	D_m		Ds	D_m
Propolis type-3	10.4 (2.0) [b]	1.9 (1.0) [b]	0.3 (0.3) [b]	33.7 (2.4) [a]	11.9 (1.7) [a]	n.d. [b]
Hexane fraction type-3	13.6 (2.3) [a,b]	0.7 (0.4) [b]	0.1 (0.1) [b]	31.1 (1.1) [a]	7.4 (1.8) [a]	0.2 (0.2) [a,b]
Propolis type-12	13.8 (2.3) [a,b]	2.5 (1.5) [b]	n.d. [b]	30.0 (1.9) [a]	8.0 (1.0) [a]	0.5 (0.3) [a,b]
Hexane fraction type-12	19.4 (2.7) [a,b]	4.6 (0.9) [a,b]	0.6 (0.6) [a,b]	30.9 (1.4) [a]	11.6 (3.0) [a]	1.4 (0.2) [a]
Ethanol 80% (Control)	22.0 (3.8) [a]	8.3 (2.1) [a]	3.0 (1.1) [a]	35.4 (2.3) [a]	11.3 (1.9) [a]	1.3 (0.6) [a,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. (SAS, 1995).
n.d., not detectable

Artigo 2

Effect of Brazilian propolis type-3 and its hexane fraction on *S. mutans* biofilm and on glucosyltransferase activity

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ABSTRACT

Recent studies have shown that propolis from Southern region of Brazil (propolis type-3) exhibits cariostatic effects; preliminary data on the fractionation of this propolis has shown that hexane fraction displayed the best inhibitory activities. The aim of this study was to evaluate the inhibitory effects of propolis type-3 and its hexane fraction on *Streptococcus mutans* UA 159 biofilm viability and on the activity of glucosyltransferase (GTF) B (synthesis of insoluble glucan) and C (insoluble/soluble glucan) in solution and adsorbed onto saliva-coated hydroxiapatite (sHA). In addition, the chemical composition of the hexane fraction was determined by GC-MS. The EEP and its hexane fraction reduced the activity of the enzymes tested in solution (81.4 to 93.2 % inhibition) and on a surface (25.2 to 38.3% inhibition) at a concentration of 0.4 mg/mL. Both tested substances significantly inhibited *S. mutans* biofilm compared to the negative control after 1 hour of experiment ($p<0.05$). The composition analysis of hexane fraction by GC-MS showed the presence of active compounds already identified in the EEP. These data suggest that the hexane fraction type-3 probably carry most of the active components found in this type of propolis.

Keywords: propolis, glucosyltransferase, antimicrobial activity, mutans streptococci.

INTRODUCTION

Dental caries development involves a series of events in a biofilm termed dental plaque, where bacterial interactions with diet occur on a tooth surface. There is general consensus that the persistent consumption of carbohydrates, mainly sucrose, can result in the emergence of cariogenic microorganisms, such as *Streptococcus mutans* (Hamada and Slade, 1980; Burne, 1998). *S. mutans* possesses a variety of mechanisms, which facilitate its ability to colonize the tooth surfaces. Among them, the ability of *S. mutans* to synthesize extracellular polysaccharides, especially insoluble glucans from sucrose by the glucosyltransferases (GTFs) is of central importance in adhesive interaction by *S. mutans*. It is also essential in the expression of virulence by these microorganisms (Hamada and Slade, 1980; Tanzer, 1985, Schilling and Bowen, 1992, Yamashita et al., 1993). The glucans synthesized by GTFs not only promote the accumulation of cariogenic streptococci on the tooth surface, but also contribute significantly to the bulk of plaque; chemical analyses of dental plaque reveal that it is composed of up to 40% glucan (Critchley, 1969; Hortz et al., 1972). Therefore, inhibition of formation of cariogenic biofilm communities by affecting the virulence factors involved in this process is an attractive route in preventing dental caries and dental plaque formation. Clearly, glucosyltransferases should be an important target on the prevention of either the formation of dental plaque or dental caries.

Propolis is a resinous substance collected by *Apis mellifera* bees from tree exudates and secretions (Ghislberti, 1979). It is a non-toxic natural product and has been used as a folk medicine for centuries (Burdock, 1998). Propolis exhibits several biological activities such as antimicrobial, anti-inflammatory, anesthetic, and cytostatic properties. The chemical composition of propolis is complex and it is variable depending on the regional plant ecology (Burdock, 1998). Previous studies have shown that the chemical composition of Brazilian propolis is highly variable; twelve chemically distinct types of propolis have been characterized (Park et al., 2000). Among them, propolis type-3 (collected in Southern part of Brazil) showed remarkable inhibitory effects on the activity of GTFs and on the growth of mutans streptococci (Koo et al., 2000a,b). Furthermore, propolis type-3 significantly reduced the incidence of dental caries in rats (Koo et al., 1999) and the formation of supragingival plaque and its content of insoluble polysaccharide (Koo et al.,

2002a). However, the active compounds of this biologically active propolis are still unknown.

Recently, we attempted to fractionate propolis type-3 in order to identify the active fraction(s); preliminary data showed that the hexane fraction was the most active one. Therefore, the aim of the present study was to evaluate the effects of the hexane fraction of propolis type-3 on the viability of *S. mutans* biofilm and on GTF activity in solution and adsorbed onto saliva-coated hydroxiapatite. In addition, the chemical composition was determined by gas chromatography/mass spectrometry (GC/SM).

MATERIALS & METHODS

Propolis samples

Crude sample of *Apis mellifera* propolis, classified as type-3 according to Park et al. (2000) were obtained from the state of Rio Grande do Sul – RS (Southern Brazil). The ethanolic extract of propolis was prepared as described elsewhere (Koo and Park, 1997). Briefly, 500 g of crude propolis was extracted in 2 L ethanol 80% (v/v), 70 °C for 2 h. Then, the extract was centrifuged, the supernatant was filtrated and concentrated to remove all solvent, using a rotary evaporator. From this concentrated extract, the ethanolic extract (EEP) (w/v) in aqueous ethanol (80% v/v) was prepared to be used in the experiments.

Fractionation of propolis

A portion of the concentrated extract of propolis was used to make the hexane fraction by means of 6 L of hexane, at a temperature of 45 °C, for 12 h. The liquid phase of this extraction was concentrated to obtain the hexane fraction, which was monitored by paper chromatography and developed by UV light (λ=254 and 366 nm) (Duarte et al., 2003). The concentrated hexane fraction was diluted in aqueous ethanol (80% v/v) to be employed in all assays.

Gas Chromatography – Mass Spectrometry (GC-MS) Analysis of propolis

The analysis of the hexane fraction was performed after methylation of the sample as described by Markham et al. (1996). The methylated solution was analyzed by GC-MS using a 30 m x 0.25 mm x 0.25 µm HP-5 column installed in a GC HP-5890 instrument interfaced to a HP-5971 mass selective detector operated in scanning mode (m/z 40-500). GC-MS analysis was temperature programmed from 60 °C to 240 °C (7 min hold) at 3°C/min. The chemical compounds were tentatively identified by comparison with library mass spectra database (Wiley-138 and Nist-98).

Gtfs Activity in solution and adsorbed onto sHA beads assays

For the production of GTFs the microorganisms used were: *Streptococcus milleri* KSB8, which harbors the gtfB gene from *S. mutans* GS-5 (for Gtf B production); *S. mutans* WHB 410, which the genes for GTF B, D and fructosyltransferase were deleted (for Gtf C). The *S. milleri* constructs were kindly provided by Dr. Howard K. Kuramitsu (Suny, Buffalo, NY). The GTF B and C enzymes were obtained as described by Venkitaraman et al. (1995), and Wunder and Bowen (1999). GTF activity was measured by the incorporation of [^{14}C]glucose form labeled sucrose (NEN Research Products, Boston, Mass., USA) into glucans. For solution assay, Gtf B or C was mixed with a two fold dilution series of the propolis extract and the hexane fraction (concentration ranging from 100 to 400 µg/mL) and incubate with ^{14}C-(glucosyl)-sucrose substrate (0.2 µCi/mL; 200.0 mmmo/L sucrose, 40 µmol/L dextran 9000, 0.02% sodium azide in adsorption buffer, pH 6.5) to reach a final concentration of 100 mmol/L sucrose (300 µL final volume). For the control, the same reaction was carried out with ethanol (final concentration of 80%) replacing the tested extracts. The samples were incubated at 37°C with rocking for 4 h. After incubation, ice-cold ethanol (1.0 mL) was added and the samples were stored for 18 h at 4°C for precipitation of glucans. Radiolabeled glucans were determined by scintillation counting (Venkitaraman et al., 1995).

For surface assays, the GTFs were adsorbed to hydroxyapatite beads (HA) coated with clarified whole saliva. The sHA beads were exposed to sufficient enzyme to saturate the surface as determined experimentally. After allowing for adsorption of the enzyme, the beads were washed 3 times with adsorption buffer to remove the unadsorbed material and

exposed to 300 µL of the twofold dilution series of EEP (or control) in the same concentrations described above for 30 min. The beads were washed and exposed to 300µL ^{14}C-(glucosyl)-sucrose substrate (100.0 mmol/L sucrose, final concentration). The radiolabeled glucans formed was collected and quantified as described above.

Biofilm Killing assay

Streptococcus mutans UA 159 was used in order to produce mono-organism biofilm. Cells of *S. mutans* were grown in static cultures at 37° C in tryptone-yeast extract broth with addition of 1% (w/v) of an appropriate sugar. Biofilms of *S. mutans* were grown on glass microscope slides in tryptone-yeast-extract medium with 1% sucrose in 50 mL, conical tubes at 37 °C, as described previously by Curran et al., 1998; Ma et al. (1999). Each slide was transferred daily to a new tube containing fresh medium for 3 to 4 days before use. The immersed portions of the slides became completely covered by biofilm with a thickness of about 4 to 6 mm (Ma et al., 1999).

For killing assay, a five-day old biofilm was transferred to 1% peptone broth containing propolis or the hexane fraction at 400 µg/mL or ethanol 80% (v/v) as a control, and incubated at 37 °C, and the kinetics of killing were performed. At intervals, entire biofilms were dispersed and diluted with 1% peptone broth at pH 7.0. Biofilms were dispersed by first scraping them from glass slides with a spatula into 45 mL of peptone broth. The biofilm was sonicated on ice with a Branson Sonifier Cell Disruptor 200 at 60 w for 15 s. This treatment was sufficient to obtain suspensions with only single cells, which were used for plating and counting on tryptic soy agar (48 h, at 37° C). Logarithms of the surviving fraction by log N/N_0, where N_0 is the original colony forming units (or CFU)/mL, and N is the CFU/mL at sampling time vs. time were plotted in order to evaluate the killing. The same procedure was done with planktonic cells of *S. mutans* UA 159 and also with suspended cells from scraped biofilm.

Statistical Analysis

The results of biofilm killing were analyzed by a split-plot model analysis of variance (MANOVA) followed by Tukey test ($p < 0.05$).

RESULTS

The methylated extracts of the hexane fraction were analyzed by GC-MS. The total ion current (TIC) chromatograms of the samples are illustrated in Figure 1. The mass spectra for methylated compounds obtained by GC-MS tentatively identified the compounds by comparison with library programs.

The effects of the EEP and its hexane fraction on the activity of GTF B and C are shown in Table 1. They effectively reduced the activity of the enzymes tested in solution (82.2 to 93.2 % inhibition) and on a surface (25.2 to 38.3% inhibition) at a concentration of 0.4 mg/mL.

Figure 2 shows the killing of planktonic cells of *S. mutans* UA 159 by EEP and hexane fraction over time. Both tested substances significantly promoted *S. mutans* decrease compared to the negative control over time ($p<0.05$). Also, they were bactericidal for *S. mutans* cells in suspensions and killed all organisms in 3 hours. The killing of scraped biofilm cells of *S. mutans* UA 159 with EEP and its hexane fraction is shown in Figure 3. EEP and its hexane fraction acted equally and significantly against *S. mutans* during the studied period, compared to the control ($p<0.05$). In general, scraped cells showed similar pattern of results as planktonic cells, however, in a lesser extent of action. The extinction of bacterial cells was not found when scraped cells were treated, showing less susceptibility than planktonic cells. Figure 4 shows the killing of intact biofilms of *S. mutans* UA 159 with EEP and its fraction. EEP and its fractions are less effective in killing cells of *S. mutans* when forming biofilms. No statistically significant differences were observed in the first 30 minutes among the tested groups and control ($p<0.05$). In one hour both EEP and the hexane fraction differed from the control. On the second, third and forth hours, the difference between EEP and the hexane fraction was significant and they both also differed from the control. In general, the lowest values of inhibition were observed for intact biofilm, whereas the highest values were found in planktonic cells.

DISCUSSION

The chemical composition of Brazilian propolis is highly variable. Twelve chemically distinct types of propolis have been characterized (Park et al., 2000) and among

them, propolis type-3 has shown promising anti-caries effects (Koo et al., 1999, Koo et al., 2000a,b, Koo et al., 2002a). The chemical profile of propolis type-3 is very similar to those from European and North American temperate regions (Tomás-Barberán et al., 1993) and thus, the same type of propolis can be also found in other regions around the world. In the hexane fraction of propolis type-3 it was found pinobanksin, pinocembrin, pinobaksin 3-acetate, chrysin, and galangin, apigenin that are dominant flavonoids present in crude propolis type-3 (Park et al., 2002).

The data obtained in the present study showed that propolis type-3 effectively reduced the activities of GTFs B and C (Table 1). The inhibition of surface-adsorbed enzymes is particularly important because most of the currently commercially available compounds failed to affect surface GTF B and C significantly (Wunder and Bowen, 1999). These GTFs appear to be the most important enzymes related to dental caries (Yamashita et al., 1993). It has been suggested that flavonoids and other related compounds are the active compounds of propolis involved in the enzyme inhibition, e.g. flavonols and flavones. Apigenin, a present compound in the hexane fraction, has been shown to be the most effective glucosyltransferase inhibitor tested so far (Koo et al., 2002b). The exact mechanism by which apigenin inhibits glucosyltransferase activity is currently unknown; it appears that the presence of a C_2-C_2 double bond in the molecular structure of apigenin may provide a site for nucleophilic addition by side-chain of amino acids in glucosyltransferases, causing enzyme inhibition (Koo et al., 2002). In addition, apigenin has shown significant impact on the development and accumulation of biofilms by diminishing the synthesis of alkali-soluble glucans (Koo et al., 2003). Therefore, apigenin is an important compound that can contribute to the effects on GTFs activities.

It was found that EEP and the hexane fraction were bactericidal for cells of *S. mutans* in suspensions and can be used to kill all organisms in suspensions in 3 hours (Figure 2). However, EEP and the hexane fraction were less effective for killing cells of *S. mutans* in biofilms. The antimicrobial assays done focusing primarily on planktonic cells, or nonsessile bacteria, have been shown to be vulnerable to any kind of stress or extrinsic agent. Bacterial cells present in the mouth attach to different oral surfaces during colonization. Subsequently, further accumulation of large masses of bacteria and their products results in a sessile community known as biofilm or dental plaque (Gibbons and

Van Houte, 1975; Bradshaw and Marsh, 1999). One of the major advantages of the biofilm mode of life is that environmental stresses are moderated because of high population densities and because of diffusion barriers. Thus, cells in biofilms generally have more time to adapt to a stress applied to the film than if the same stress were applied to cells in suspension; thus, biofilms are more resistant to deleterious agents (Costerton et al., 1999). The data showed that *S. mutans* biofilms were more resistant to propolis and hexane fraction than cells in suspension (planktonic state), as reviewed by Lewis (2001).

The interference with bacterial attachment to the tooth surface may be one of the routes to achieve control of plaque accumulation (Addy et al., 1992). Further studies should elucidate the effects produced by chemical agents on oral bacteria and their interference in the overall interaction with the tooth surface that leads to the formation and accumulation of biofilm.

In conclusion, the results of the present study suggest that the hexane fraction may carry most of active components of propolis.

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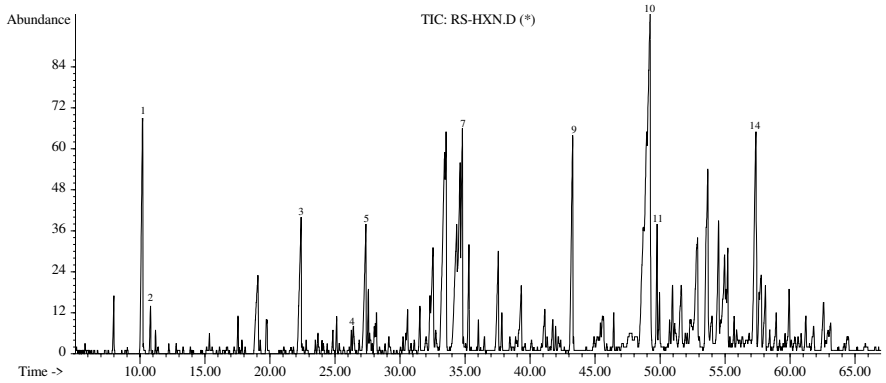

Figure 1 – GC-MS profiles of hexane fraction of propolis. 1-Coumaric acid; 2- Ferulic acid; 3- Pinobanksin; 4- Kaempferol; 5- Apigenin; 7- Pinocembrin; 9- Pinobanksin 3-acetate 10- Chrysin; 11- Galangin; 14- Tectochrysin.

Table 1 – Effect of EEP and its hexane fraction on the activities of streptococcal GTFs in solution and adsorbed onto an sHA surface*

% of activity inhibition				
	Gtf B		Gtf C	
Hexane fraction	Solution	Surface	Solution	Surface
100 μg/mL	50.4 ± 3.7	19.7 ± 5.5	18.2 ± 5.5	20.1 ± 6.1
200 μg/mL	73.5 ± 8.3	20.6 ± 3.6	80.2 ± 3.3	25.9 ± 5.5
400 μg/mL	81.4 ± 4.4	34.0 ± 7.8	93.2 ± 5.4	28.4 ± 5.7
EEP	Solution	Surface	Solution	Surface
100 μg/mL	36.3 ± 5.8	5.7 ± 2.3	49.0 ± 1.9	23.2 ± 1.6
200 μg/mL	70.8 ± 3.6	10.1 ± 3.1	66.2 ± 5.9	31.2 ± 2.3
400 μg/mL	82.2 ± 7.8	25.2 ± 2.3	88.2 ± 5.4	38.3 ± 4.8

*Percent inhibition was calculated by considering the control to have maximum GTF activity (100%)

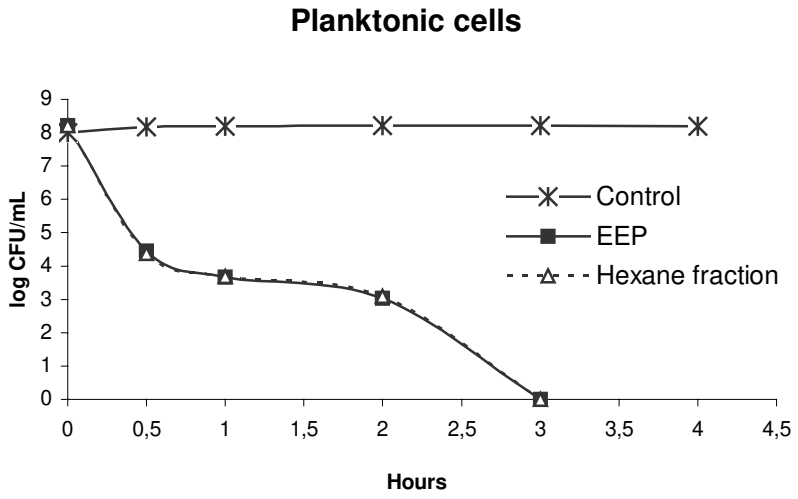

Figure 2 – Killing of planktonic cells (mean of log CFU/mL) of *S. mutans* UA 159 with EEP type-3 and its hexane fraction at 400 μg/mL.

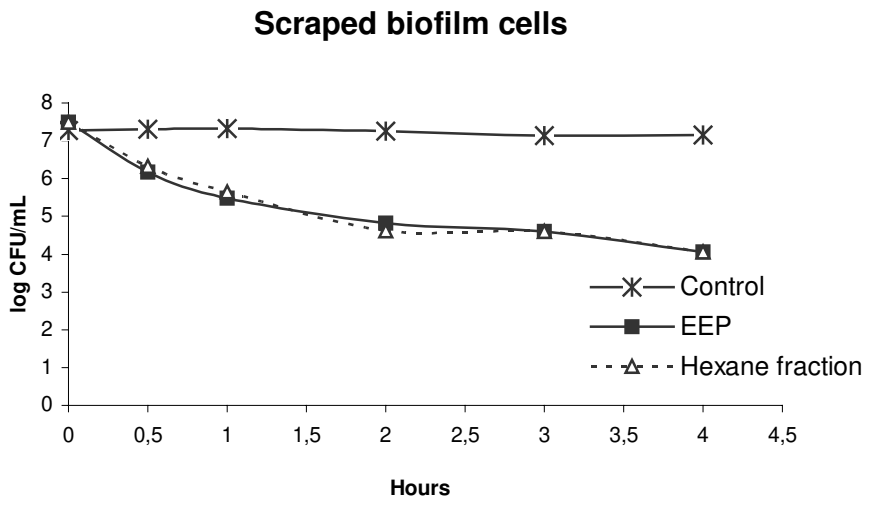

Figure 3 – Killing of scraped biofilm cells (mean of log CFU/mL) of *S. mutans* UA 159 with EEP type-3 and its hexane fraction at 400 μg/mL.

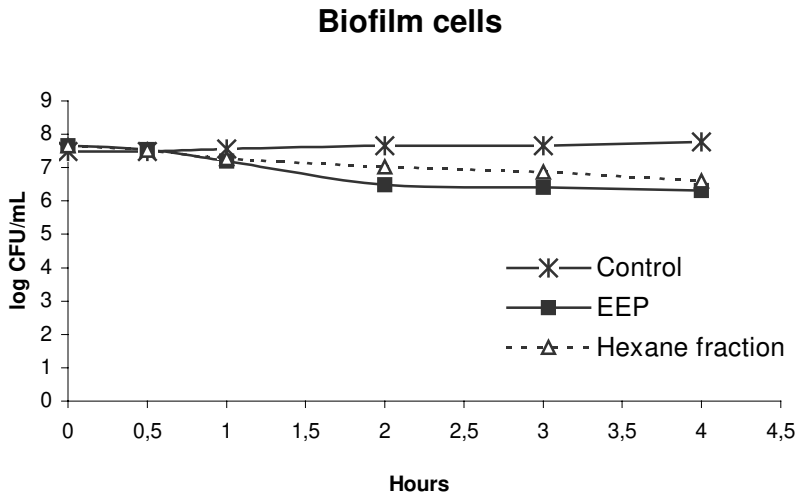

Figure 4 – Killing of intact biofilm cells (mean of log CFU/mL) of *S. mutans* UA 159 with EEP type-3 and its hexane fraction at 400 μg/mL.

IV- CONCLUSÕES

1- Os compostos ativos das própolis tipo-3 e tipo-12, apresentando atividade antimicrobiana e inibição da atividade das Gtfs, são predominantemente de caráter apolar.

2- A fração hexano tipo-3 inibiu efetivamente as enzimas adsorvidas à superfície da hidroxiapatita e também proporcionou a inibição da viabilidade do biofilme, sugerindo que grande parte dos compostos ativos da própolis apresentando esta atividade também são de natureza apolar.

3- As frações hexano das própolis tipo-3 e tipo-12 inibiram efetivamente o acúmulo de microrganismos cariogêncos em ratos, sugerindo também que os compostos apresentando esta atividade são apolares.

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ANEXO 1

Aprovação pelo Comitê de Ética em Pesquisa em Animais

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Instituto de Biologia
Universidade Estadual de Campinas
CEEA-IB-UNICAMP

CERTIFICADO

Certificamos que o Protocolo nº 109-1, sobre "Avaliação do potencial anti-cárie e anti-placa de frações isoladas de própolis de A. mellifera ...." sob a responsabilidade de Dr. Pedro 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 11/02/00. Este certificado expira em 11/02/01.

CERTIFICATE

We certify that the protocol nº 109-1, about "Evaluation of cariostatic and anti-plaque potential of isolated fractions of propolis from ...", is in agreement with the Ethical Principles in Animal Research adopted by Brazilian College of Animal Experimentation (COBEA) and was approved by the Institute of Biology/UNICAMP Ethical Committee for Animal Research (CEEA) in 11/02/00. Expiration date 11/02/01.

Campinas, 01 de fevereiro de 2000

A.R.Monteiro
Prof(a) Dr(a) Alba R. M. Souza Bruto
Presidente – CEEA/IB/UNICAMP

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ANEXO 2

Outros trabalhos em publicação ou publicados durante o período de doutorado:

1- Queiroz CS, Hayacibara MF, Tabchoury CP, Marcondes FK, Cury JA Relationship between stressful situations, salivary flow rate and oral volatile sulfur-containing compounds. ***Eur J Oral Sci***, 110 (5):337-340, 2002.

2- Hayacibara MF, Rosa OP, Koo H, Torres SA, Costa B, Cury JA. Effects of fluoride and aluminum from ionomeric materials on S. mutans biofilm. ***J Dent Res,*** 82 (4):267-271, 2003.

3- Duarte S, Koo H, Bowen WH, Hayacibara MF, Cury JA, Ikegaki M, Rosalen PL. Effect of a novel type of propolis and its chemical fractions on glucosyltransferases and on growth and adherence of mutans streptococci. ***Biol Pharm Bull,*** 26 (4):527-531, 2003.

4- Koo H, Hayacibara MF, Schobel BC, Cury JA, Rosalen PL, Park YK, Vacca-Smith, Bowen WH. Inhibition of *Streptococcus mutans* biofilms accumulation and polysaccharide production by apigenin and *tt*-farnesol. ***J Antimicrol Chem***, 52: 782-789, 2003.

5- Hayacibara MF, Ambrosano GMB, Cury JA. Simultaneous release of fluoride and aluminum from dental materials in various immersion media. ***Operative Dent,*** 29 (1): 16-22, 2004.

6- Hayacibara MF, Queiroz CS, Tabchoury CPM, Cury JA. Fluoride and aluminum in teas and tea-based beverages. ***Rev Saúde Pública,*** 38(1): 100-105, 2004.

7- Hayacibara MF, Paes Leme AF, Lima YBO, Gonçalves NCLAV, Queiroz CS, Gomes MJ, Kozlowski FC. Alkali-soluble fluoride deposition on enamel after professional application of topical fluoride *in vitro*. ***J Applied Oral Sci***, 2004. (aceito)

8- Hayacibara MF, Koo H, Vacca-Smith AM, Kopec LK, Scot-Anne K, Cury JA, Bowen WH. The influence of mutanase and dextranase on the production and structure of glucans synthesized by streptococcal glucosyltransferases. ***Glycobiology,*** (em submissão).