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AVALIAÇÃO DO POTENCIAL ANTI-CÁRIE E ANTI-PLACA DE PRÓPOLIS DE *Apis mellifera* SELECIONADAS DE DUAS REGIÕES DO BRASIL

Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas, para obtenção do grau de DOUTOR em Odontologia, área de concentração em Biologia e Patologia Buco-Dental.

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Orientador: Prof. Dr. Jaime A. Cury

Comissão Examinadora:

Prof. Dr. Jaime Aparecido Cury

Prof. Dr. Yong Kun Park

Prof. Dr. José Domingos Fontana

Prof. Dr. Osmar Malaspina

Prof. Dr. Pedro Luiz Rosalen

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A Comissão Julgadora dos trabalhos de Defesa de Tese de DOUTORADO, em sessão pública realizada em 14 de Outubro de 1999, considerou o candidato HYUN KOO aprovado.

1. Prof. Dr. JAIME APARECIDO CURY

2. Prof. Dr. YONG KUN PARK

3. Prof. Dr. OSMAR MALASPINA

4. Prof. Dr. JOSÉ DOMINGOS FONTANA

5. Prof. Dr. PEDRO LUIZ ROSALEN

DEDICATÓRIA

À DEUS...

... por ter iluminado e abençoado o meu caminho
pela força nos momentos de desânimo
pela inspiração no trabalho

Aos meus pais...

...por tudo que fizeram por mim
por suportarem a minha ausência
pela compreensão, estímulo e amor

Aos meus queridos irmãos, Daniel e Simone...

...pelo companheirismo, pela alegria em
todos os momentos

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...obrigado pelo seu amor puro, honesto e verdadeiro
...obrigado pela sua paciência e compreensão

Por tudo isso e muito mais,

A minha sincera e eterna gratidão

SUMÁRIO

SUMÁRIO

RESUMO / ABSTRACT

RESUMO

O objetivo do presente estudo foi avaliar o potencial anti-cárie e anti-placa da própolis de *Apis mellifera* coletada de duas regiões do Brasil. O extrato etanólico da própolis (EEP) foi preparado a partir de amostras de própolis bruta da região Sudeste e Sul. Os EEPs foram previamente selecionadas através de dois parâmetros: 1) análise da composição química através da cromatografia em camada delgada de alta eficiência e cromatografia líquida de alta eficiência, ambas em fase reversa, 2) análise *in vitro* da atividade antimicrobiana e inibição da glucosiltransferase bruta (Gtf). Duas amostras - uma de Minas Gerais (EEP MG) e outra do Rio Grande do Sul (EEP RS) - foram analisadas por: 1) estudos *in vitro*: a) atividade antimicrobiana sobre alguns patógenos bucais através do método de difusão em ágar, b) concentração inibitória mínima -CIM e concentração bactericida mínima-CBM do EEP contra os estreptococos do grupo mutans, c) inibição da aderência de *Streptococcus mutans* e *S. sobrinus* sobre a superfície de vidro, d) inibição da síntese de glucano insolúvel em água-GIA, e) inibição da atividade das Gtfs em solução e aderidas sobre a superfície de hidroxiapatita, f) inibição da aderência de *S. mutans* e *S. sobrinus* sobre a superfície de hidroxiapatita; 2) estudo em animais utilizando o modelo de cárie experimental em ratos desalivados; 3) estudo *in vivo* através da inibição da formação de placa dental. Ambas as amostras de própolis (EEP MG e RS) demonstraram atividade inibitória significativa ($p<0,05$) em todos os parâmetro *in vitro* testados, quando comparadas com o controle (etanol 80%, v/v). Entretanto, os resultados do EEP RS foram significantemente melhores do que os obtidos pelo EEP MG ($p<0,05$). No estudo em animais, apenas o EEP RS demonstrou redução do índice da cárie de superfície lisa e de sulco, e os dados foram estatisticamente diferentes do grupo controle ($p<0,05$). O enxaguatório bucal contendo EEP RS reduziu significantemente a formação de placa dental *in vivo* (44,7%), quando comparado com o placebo ($p<0,05$). Em conclusão, a própolis demonstrou propriedades anti-cárie e anti-placa. Entretanto, o seu efeito biológico é variável dependendo da origem geográfica das amostras de própolis devido à diferenças da composição química desta substância natural.

ABSTRACT

The aim of the present study was to evaluate the anti-caries and anti-plaque potential of *Apis mellifera* propolis collected from two regions of Brazil. The ethanolic extract of propolis (EEP) was prepared from crude propolis samples collected in Southeastern Brazil and Southern Brazil. The EEPs were screened and selected by two parameters: 1) chemical composition analysis by reversed-phase High Performance Thin Layer Chromatography and reversed-phase High Performance Liquid Chromatography; 2) *In vitro* antimicrobial activity and inhibition of crude glucosyltransferase (Gtf) enzyme assays. Two samples - one from Minas Gerais state (EEP MG) and another from Rio Grande do Sul state (EEP RS) - were analyzed by: 1) *in vitro* studies - a) Antimicrobial activity against some oral pathogens by agar diffusion method, b) determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of EEP against mutans streptococci, c) inhibition of adherence of *Streptococcus mutans* and *S. sobrinus* to glass surface, d) inhibition of water insoluble glucan (WIG) synthesis, e) Inhibition of activity of purified Gtfs in solution and adsorbed on the surface of hydroxyapatite, f) Inhibition of adherence of *S. mutans* and *S. sobrinus* on the surface of hydroxyapatite; 2) animal study performed by experimental dental caries model using desalivated rats; 3) *in vivo* study conducted to evaluate dental plaque inhibition. Both propolis samples (EEP MG and RS) showed significant inhibitory activities on all *in vitro* parameters tested when compared to control (80 % aqueous ethanol, v/v) ($p<0.05$). However, EEP RS demonstrated significantly better results than EEP MG ($p<0.05$). In animal study, only EEP RS reduced both smooth-surface and sulcal caries and the data were statistically different to the control group ($p<0.05$). The mouthrinse formulation containing EEP RS significantly reduced dental plaque formation on smooth-surface *in vivo* (44,7%) when compared to placebo ($p<0.05$). In conclusion, propolis showed anti-caries and anti-plaque properties. However its biological effects is variable depending on geographic origin of propolis samples due to differences in the chemical composition of this natural substance.

ARTIGOS ORIGINAIS

A tese de Doutoramento foi baseado nos seguintes artigos, os quais estão no item **Anexos**.

I. KOO, H.; GOMES, B.P.F.A.; ROSALEN, P.L.; AMBROSANO, G.M.B.; PARK, Y.K.; CURY, J.A. *In vitro* antimicrobial activity of propolis and *Arnica montana* against oral pathogens. **Arch. Oral Biol.** 1999, *in press*.

II. KOO, H.; ROSALEN, P.L.; CURY, J.A.; AMBROSANO, G.M.B.; IKEGAKI, M; PARK, Y.K. Effect of A*pis mellifera* propolis from different geographical origins on mutans streptococci. **FEMS Microbiol. Letters**, submitted.

III. KOO, H.; VACCA SMITH, A.M.; BOWEN, W.H.; ROSALEN, P.L.; CURY, J.A.; PARK, Y.K. Effects of A*pis mellifera* propolis on the activities of streptococcal glucosyltransferases in solution and adsorbed onto saliva-coated hydroxyapatite. **Arch. Oral Biol.**, submitted.

IV. KOO, H.; VACCA SMITH, A.M.; BOWEN, W.H.; ROSALEN, P.L.; CURY, J.A.; AMBROSANO, G.M.B.; PARK, Y.K. Effects of quercetin, 3, 3', 4', 5, 7 – pentahydroxyflavone, on the activities of streptococcal glucosyltransferases in solution and adsorbed onto saliva-coated hydroxyapatite. **J. Nat. Prod.**, submitted.

V. KOO, H.; ROSALEN, P.L.; CURY, J.A.; IKEGAKI, M; PARK, Y.K.; SATTLER, A. Effect of *Apis mellifera* propolis from two Brazilian regions on caries development in desalivated rats. **Caries Res.**, 33: 393-400, 1999.

Os dados preliminares para o desenvolvimento desta tese foram publicados no artigo:

PARK, Y.K; KOO, H.; ABREU, J.A.S.; IKEGAKI, M.; CURY, J.A.; ROSALEN, P.L. Antimicrobial activity of propolis on oral microorganisms. **Current Microbiol., 36**: 24-28, 1998.

1. INTRODUÇÃO

A cárie dental e a doença periodontal são os principais indicativos que determinam o estado fisiopatológico da cavidade bucal de um ser humano (AL IAFI & ABABNEH, 1995).

Um dos fatores etiológicos mais importantes das doenças cárie e gengivite são os microrganismos de origem bacteriana que formam um biofilme patogênico que se adere sobre a superfície dental, de modo a produzir ácidos e produtos citotóxicos que levam, respectivamente, a desmineralização do esmalte dental e/ou inflamação gengival (MARSH, 1994). Este biofilme é genericamente conhecido como placa dental. A placa dental que se forma sobre a superfície do dente apresenta uma composição bacteriana e bioquímica variável dependendo de fatores intrínsecos e extrínsecos, podendo mudar de modo a tornar este biofilme patogênico (MARSH, 1992; 1994). Os primeiros elementos que se aderem sobre a superfície dental, após por exemplo uma profilaxia, são as proteínas salivares e glicoproteínas de modo a formar a película adquirida (HAY & MORENO, 1993). As bactérias interagem com esta película através de uma série de mecanismos específicos, dentre elas a interação tipo lectina, envolvendo as adesinas localizadas na superfície bacteriana e os receptores da película (GIBBONS, 1984; GIBBONS, 1996; JENKINSON & LAMONT, 1997). Subseqüentemente, outras bactérias da mesma ou de espécies diferentes se aderem não apenas a película como também às bactérias pré-existentes (KOLENBRANDER et al., 1990). Diversos estudos têm demonstrado que os primeiros colonizadores durante a formação da placa dental inicial são bactérias do grupo dos estreptococos, particularmente *Streptococcus sanguis, S. mitis* e *S. oralis* (CARLSSON et al., 1970; GIBBONS & VAN HOUTE, 1975; STAAT & PEYTON, 1984; NYVAD & KILIAN, 1990); além da presença de *Actinomyces spp; Neisseria spp; Haemophilus spp* (LILJEMARK et al., 1986; NYVAD & KILIAN, 1987). Uma vez desenvolvida, a composição desta placa se mantém relativamente estável e não apresenta patogenicidade (MARSH, 1992; 1994). Dentro deste contexto, esta situação é chamado placa dental-saúde (MARSH, 1992). Assim, fatores que levam ao desequilíbrio desta comunidade microbiana favorecendo o crescimento de bactérias odonto ou periodontopatogênicas vão direcionar para o surgimento de uma placa dental-doença (MARSH, 1992). Assim, este biofilme dependendo do desequilíbrio ecológico bacteriano poderá ser patogênico para as estruturas duras (esmalte, dentina) ou moles (periodonto) da cavidade bucal.

Um dos fatores de desequilíbrio fundamental para o aparecimento de uma placa dental cariogênica, é a dieta rica e freqüente de carboidratos fermentáveis (Figura 1). Esta dieta promove um aumento da proporção de estreptococos do grupo mutans e lactobacilos, com concomitante queda de níveis de *S. oralis, S. sanguis* e *S. mitis* (DE STOPPELAAR et al., 1970; DENNIS et al., 1975; STAAT et al., 1975; MINAH et al., 1985).

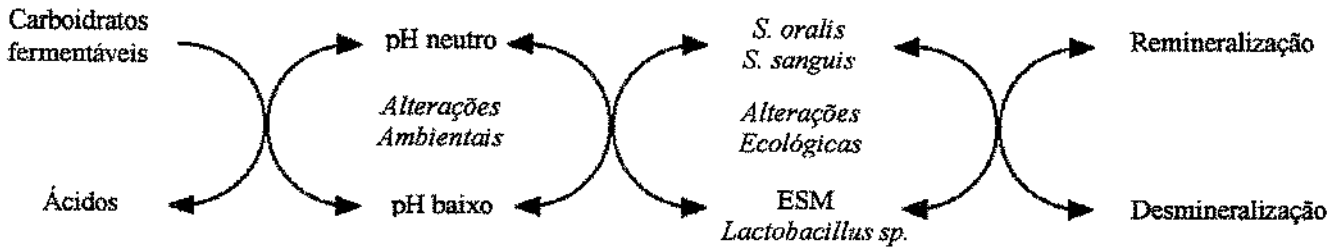

Figura 1. O diagrama mostra o processo dinâmico da formação de uma placa-doença relacionada à cárie dental. ESM = estreptococos do grupo mutans (diagrama adaptado de MARSH, 1994).

Por outro lado, quando as bactérias se acumulam na superfície dental na ausência de açúcar (Figura 2) ocorre uma alteração da composição bacteriana de uma microbiota estreptococos dominante (SLOTS, 1977) para um número cada vez maior de *Actinomyces spp.* e um aumento progressivo de capnófilos e bactérias anaeróbias estritas, como *Fusobacterium nucleatum, Prevotella spp,* entre outros (SAVITT & SOCRANSKI, 1984; MOORE et al., 1987). Com acúmulo contínuo da placa na região subgengival, inicia-se um aumento do número de anaeróbios produtores de pigmentos negros, como *Prevotella intermedia, Prevotella nigrescens, Porphyromonas gingivalis,* caracterizando-se a formação da placa dental relacionada à doença periodontal (MARCOTTE & LAVOIES, 1998; SOCRANSKI et al., 1998). Outras bactérias gram negativas que têm sido isolados em doença periodontal são *Actinobacillus actinomycetemcomitans, Bacteroides forsythus* e *Campylobacter rectus* (SLOTS et al., 1986; DZINK et al., 1988; CHRISTERSSON et al., 1989; MOORE et al., 1991; SOCRANSKI et al., 1998).

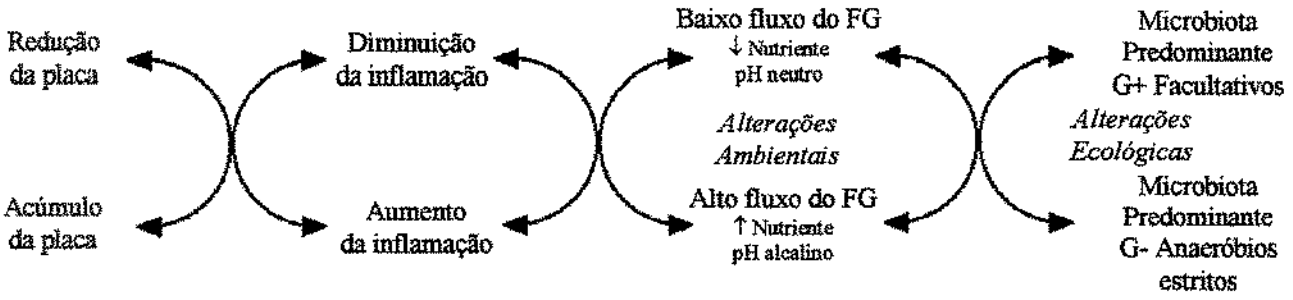

Figura 2. O diagrama mostra o processo dinâmico da formação de uma placa-doença relacionada à doença periodontal. FG = fluido gengival; G+ / G- = gram positivo / negativo (diagrama adaptado de MARSH, 1994).

Os diagramas das figuras 1 e 2 ilustram os fatores ambientais responsáveis pelo desequilíbrio ecológico bacteriano e conseqüente desenvolvimento da placa dental-doença. Entretanto, um dos fatores críticos para o desenvolvimento inicial e formação de uma placa dental mais cariogênica é a dieta rica em sacarose. Os estreptococos do grupo mutans apresentam algumas vantagens ecológicas quando da presença da sacarose no meio bucal que permitem a sua aderência e colonização da superfície lisa do esmalte dental, e assim, o seu posterior acúmulo. Os estreptococos do grupo mutans, além de serem acidogênicos e acidúricos, não só fermentam a sacarose como a partir desta sintetizam polissacarídeos extracelulares (DE STOPPELAAR et al. 1971; GIBBONS & VAN HOUTE, 1975; HAMADA & SLADE, 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) (RÖLLA et al., 1983; HAMADA & SLADE, 1980; LOESCHE, 1986). Entre os glucanos destacam-se os insolúveis em água os quais têm sido considerados como os principais fatores para a aderência e o acúmulo dos estreptococos cariogênicos sobre a superfície do esmalte dental tanto em experimentos com animais quanto em humanos (KRASSE, 1965; FROSTELL et al., 1967; HAMADA & SLADE, 1980; RÖLLA et al., 1983; TANZER et al., 1985). Em acréscimo, tem sido demonstrado que estes glucanos aumentam a porosidade (DIBDIN & SHELLIS, 1988; VAN HOUTE, 1994) bem como causam mudanças na composição inorgânica da matriz da placa (CURY et al., 1997) tornando-a ainda mais cariogênica. A figura 3 ilustra a dinâmica do efeito da sacarose na formação da placa cariogênica.

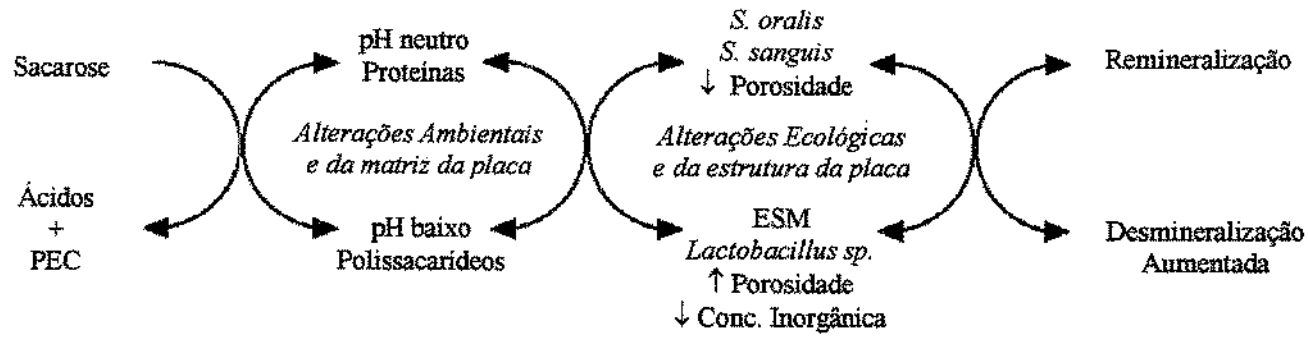

Figura 3. O diagrama mostra o papel da sacarose na formação da placa cariogênica. PEC = polissacarídeos extracelulares; ESM = estreptococos do grupo mutans (diagrama adaptado de MARSH, 1994).

As Gtfs são secretadas principalmente pelo *Streptococcus mutans*, e pelo menos 3 Gtfs estão bem caracterizadas tanto bioquimicamente como a nível molecular: 1) Gtf B - codificado pelo gene *gtfB*, que sintetiza glucanos insolúveis em água (GIA) tendo ligações glicosídicas principais α (1→3); 2) Gtf C - codificado pelo gene *gtfC*, que sintetiza uma mistura de glucanos insolúveis e solúveis (GSA), este último apresentando ligações glicosídicas principais α (1→6); e 3) Gtf D - codificado pelo gene *gtfD*, que sintetiza basicamente glucanos solúveis (LOESCHE, 1986; HANADA & KURAMITSU, 1989). A figura 4 ilustra a estrutura química destes 2 tipos de glucanos.

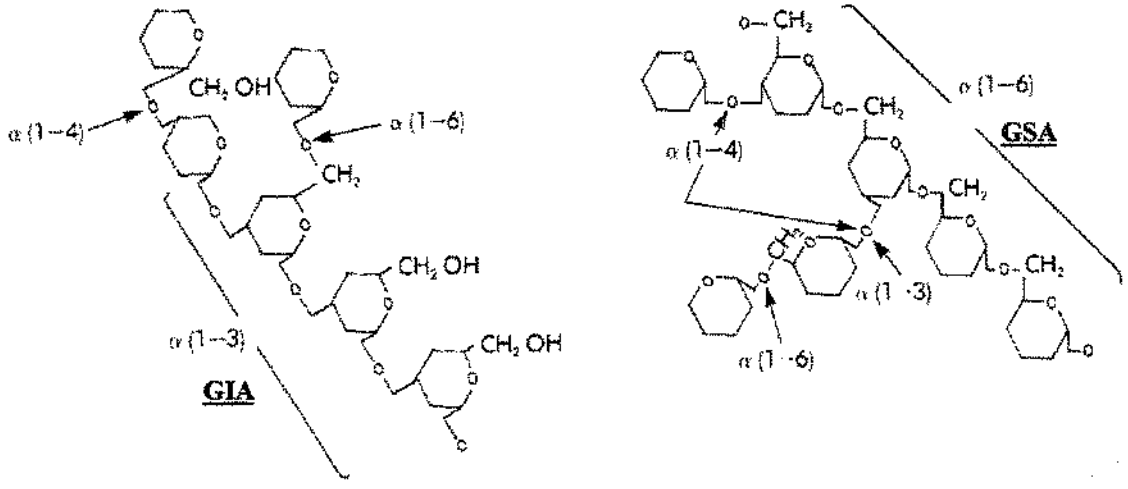

Figura 4. Estrutura química dos glucanos solúveis (GSA) e insolúveis em água (GIA).

Estas Gtfs estão presentes tanto na saliva humana como incorporadas a película adquirida que se forma sobre a superfície do esmalte dental (RÖLLA et al., 1983; SCHEIE et al., 1987). Em acréscimo, têm sido demonstrado que as Gtfs aderidas sobre uma película experimental apresentam propriedades físicas e cinéticas diferentes das mesmas enzimas em solução, expressando um aumento da atividade enzimática (SCHILLING & BOWEN, 1988; VENKITARAMAN et al., 1995). Assim, quantidades significantes de glucanos são formados sobre a película mediando a aderência seletiva dos estreptococos bucais, incluindo *S. mutans* e *S. sobrinus*, diretamente sobre a superfície dental (SCHILLING & BOWEN, 1992). Esta aderência sacarose-dependente que permite a colonização e o acúmulo destes estreptococos cariogênicos tem sido considerada um fator crítico no desenvolvimento da placa dental cariogênica (Figura 5).

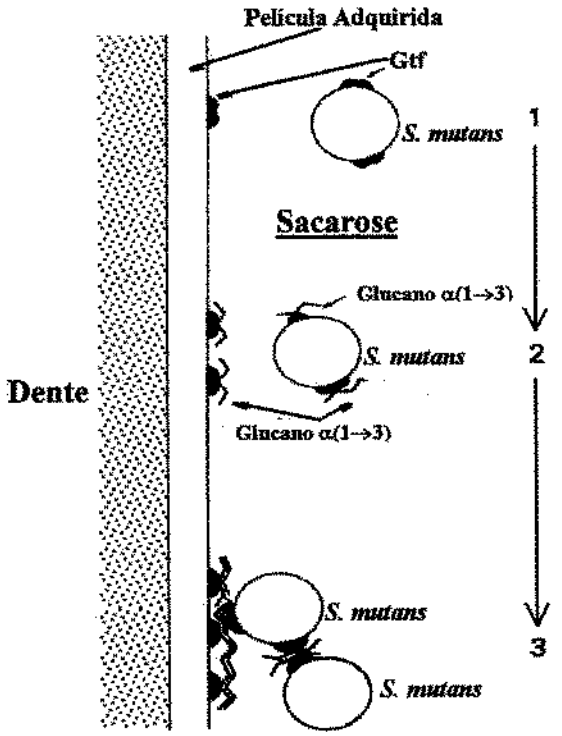

Figura 5. Um possível mecanismo da aderência sacarose-dependente na colonização bacteriana da superfície lisa dental. 1)Gtf é aderida na superfície do esmalte dental (na película adquirida) 2) Dieta rica e freqüente de sacarose causa a síntese de glucanos na superfície da película pelas Gtfs aderidas e também na superfície da parede bacteriana. 3) Com a formação de glucanos com ligação α (1→3) ocorre uma forte adesão entre as bactérias e o dente devido a interação entre as cadeias rígidas deste glucano (Adaptado de RÖLLA et al., 1983).

Deste modo, uma das estratégias visando o controle da formação de uma placa dental cariogênica tem sido a tentativa de inibir a atividade das Gtfs, principalmente as aderidas a superfície da película e que formam glucanos insolúveis. Assim, um agente que aliasse eficientemente propriedades antimicrobiana com inibição das Gtfs seria extremamente desejável para a prevenção da cárie dental.

Nas últimas décadas têm sido observado mundialmente um crescente uso de produtos naturais para prevenção de doenças bucais (WOLINSKY & SOTE, 1984; WU-YUAN et al., 1990; HARPER et al., 1990; ISRAELSON, 1991; IKENO et al., 1991; OTAKE et al., 1991; MORAN et al., 1991; OOSHIMA et al., 1993). Diversos relatos na literatura têm demonstrado que estes produtos, principalmente plantas medicinais, apresentam atividade antibacteriana sobre os patógenos bucais, entre elas o *S. mutans*, e propriedades anti-placa (SOUTHARD et al., 1984; WOLINSKY & SOTE, 1984; MOSADOMI, 1987; SAKANAKA et al., 1989; OSAWA et al., 1990; SAEKI et al., 1993; OOSHIMA et al., 1994; SHAPIRO et al., 1994; WOLINSKY et al., 1996; CAI & WU, 1996).

Assim a descoberta de novos produtos naturais e de seus compostos com atividade antibacteriana e/ou anti-Gtf seria muito importante, com a finalidade de obter um meio de controlar a formação de uma placa dental cariogênica de modo efetivo. Porém, para avaliar a efetividade de um produto são necessárias análises progressivas começando com estudos laboratoriais *in vitro*, passando por modelos de estudo *in vivo* e culminando com os estudos clínicos longitudinais (TEN CATE & MARSH, 1994).

Entre os vários produtos naturais, a própolis tem se destacado devido a vários relatos quanto aos seus efeitos farmacológicos de interesse médico, dentre eles antibacteriana, antioxidante, anti-inflamatória, cicatrizante, anestésica e anti-tumoral (GHISALBERTI, 1979; BANKOVA et al., 1989, MARCUCCI, 1995).

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), sendo posteriormente modificado pelas abelhas através da adição de secreções próprias (GHISALBERTI, 1979). A própolis é utilizada pelas abelhas como um meio de proteção da colmeia selando as frestas e embalsamando pequenos insetos mortos pelas abelhas evitando assim a decomposição (GHISALBERTI, 1979).

Vários artigos científicos têm sido publicados relatando a atividade antimicrobiana do extrato etanólico da própolis (EEP) sobre um amplo espectro de microrganismos patogênicos (LINDENFELSER, 1967; METZNER et al., 1979; GRANGE & DAVEY, 1990; BONHEVI et al., 1994, BANKOVA et al., 1995; KUJUMGIEV et al., 1999). Entretanto, pouca atenção tem sido dada aos patógenos bucais, tendo sido publicados apenas 4 artigos nos últimos 10 anos (IKENO et al., 1991; STEINBERG et al., 1996; MURRAY et al., 1997; PARK et al., 1998). Assim, IKENO et al. (1991) e PARK et al. (1998) demonstraram que o EEP apresentou atividade antibacteriana contra *S. mutans* e inibição da glicosiltransferase bruta quando em solução. Em acréscimo, IKENO et al. (1991) demonstraram que a cárie dental foi reduzida significantemente em animais que ingeriram água contendo própolis a 1%. Entretanto, neste experimento não foi levado em consideração o controle da freqüência de ingestão da dieta cariogênica. Segundo STEINBERG et al. (1996) a própolis apresentou atividade antibacteriana *in vitro* contra o *S. mutans* e reduziu significantemente a contagem desta na saliva de voluntários que bochecharam previamente uma solução de própolis. Em contraste, MURRAY et al. (1997) demonstraram que embora um enxaguatório bucal de própolis reduzisse o índice de placa, determinado pelo método de SILNESS & LÖE (1964), não houve diferença estatística em relação ao controle. Esta discrepância de resultados pode estar relacionada com a origem geográfica da própolis.

Entre os compostos ativos da própolis, os fenólicos e principalmente flavonóides, têm sido considerado suas substâncias biológicas ativas (GHISALBERTI, 1979; BANKOVA et al., 1982; GRANGE & DAVEY 1990, BONHEVI et al., 1994). Entretanto, a composição química da própolis é variável, quantitativamente e qualitativamente, dependendo da biodiversidade de cada região visitada pelas abelhas (KÖNIG, 1985; GREENAWAY et al., 1990; GARCÍA-VIGUEIRA et al., 1992; BANKOVA et al., 1992; TOMAS-BARBERAN et al., 1993; BONHEVI & COLL, 1994). Assim, considerando a grande biodiversidade do Brasil, uma extensa análise da própolis de *Apis mellifera* foi realizado englobando as regiões Sudeste, Sul, Centro Oeste e alguns estados do Nordeste. Aproximadamente 400 amostras brutas foram obtidas entre 1995-1998 pelos pesquisadores da Faculdade de Engenharia de Alimentos - FEA - UNICAMP, Laboratório de Bioquímica de Alimentos* para uma seleção através de análises químicas e biológicas. O resultado das análises por cromatografia em camada delgada de alta eficiência (CCDAE) e cromatografia líquida de alta eficiência (CLAE), ambas em fase reversa, demonstraram que a

composição química era extremamente variável (parcialmente publicados, PARK et al., 1997; KOO & PARK, 1997; PARK et al. 1998; PARK & IKEGAKI, 1998). Deste modo, os EEPs foram classificados em pelo menos 8 tipos diferentes de acordo com o perfil cromatográfico. Os diferentes tipos de EEPs e os seus perfis cromatográficos por CCDAE estão ilustrados na figura 6.

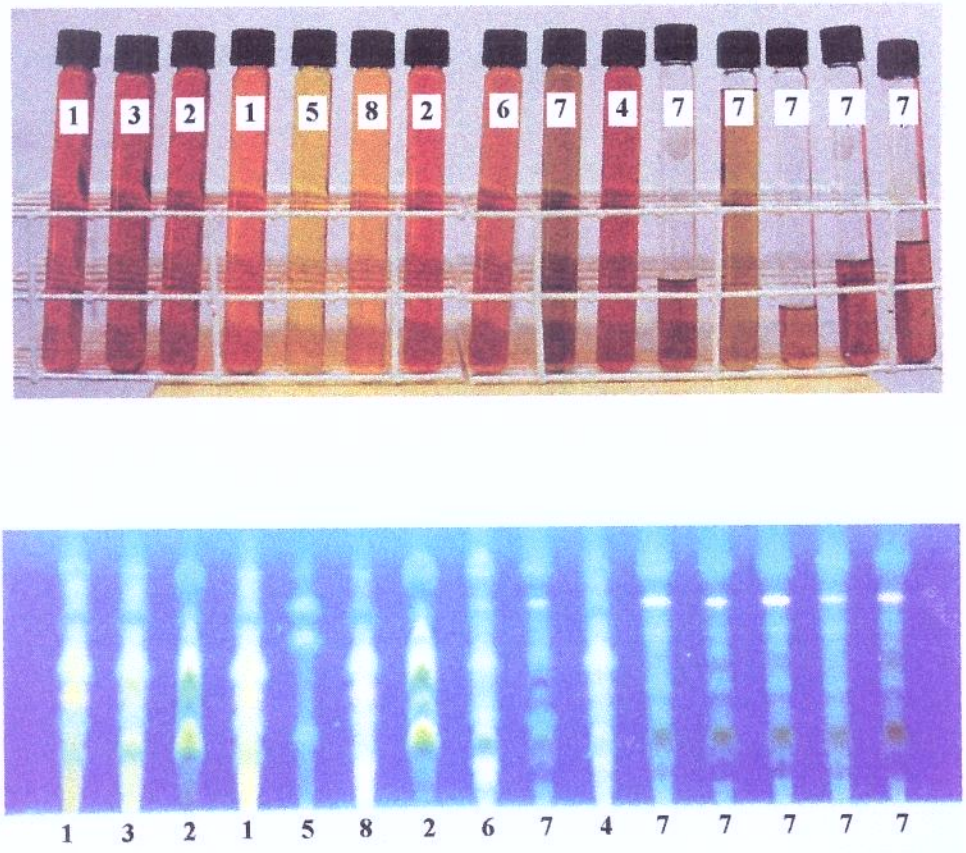

Figura 6. Extratos Etanólicos da Própolis e os perfis cromatográficos por Cromatografia em Camada Delgada de Alta Eficiência (CCDAE) em fase reversa. Os números eqüivalem aos diferentes tipos de própolis (classificação 1 a 8), de acordo com PARK et al. (1997, 1998) e PARK & IKEGAKI (1998).

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* Departamento de Ciências de Alimentos, FEA / UNICAMP, coordenado pelo Prof. Dr. Yong K. Park.

Consequentemente as suas propriedades biológicas *in vitro*, tais como atividade antibacteriana, anti-inflamatória, anti-oxidante entre outros, foram nitidamente variáveis (PARK et al., 1997; PARK et al., 1998; PARK & IKEGAKI, 1998). Dentre os diferentes tipos de própolis, duas se destacaram nesta seleção inicial, sendo uma da Região Sudeste (equivalente a classificação 7) e a outra da região Sul (equivalente a classificação 2). Estas amostras apresentaram os melhores resultados *in vitro* quanto a inibição do crescimento do *S. mutans* e da glicosiltransferase bruta, sendo assim selecionadas para as análises do presente trabalho.

Considerando o potencial farmacológico da própolis, a sua variabilidade da composição química e a escassez de dados na literatura com relação as suas propriedades antibacterianas e anti-Gtf, o objetivo geral do presente trabalho foi determinar por meio de estudos *in vitro*, em animais e *in vivo* o potencial anti-cárie e anti-placa do extrato etanólico da própolis proveniente de duas regiões do Brasil.

2. OBJETIVOS

Os objetivos do presente trabalho foram avaliar o potencial anti-cárie e anti-placa da própolis de *Apis mellifera* provenientes de 2 regiões do Brasil através das seguintes análises:

1) Atividade antimicrobiana *in vitro* contra os patógenos bucais;

2) Determinação da Concentração Inibitória Mínima (CIM) e Concentração Bactericida Mínima (CBM) dos extratos etanólicos da própolis (EEP) contra os estreptococos do grupo mutans;

3) Inibição da aderência celular de *S. mutans* e *S. sobrinus* sobre a superfície do vidro;

4) Inibição da síntese de glucanos insolúveis em água (GIA);

5) Inibição da atividade das glucosiltransferases B, C, D e G, avaliadas em solução ou aderidas a superfície de hidroxiapatita;

6) Inibição da aderência de *S. mutans* e *S. sobrinus* a superfície da hidroxiapatita;

7) Efeito da própolis sobre o desenvolvimento de cárie dental em ratos;

8) Efeito da própolis sobre a formação da placa dental *in vivo*.

MATERIAIS E MÉTODOS

3. MATERIAIS E MÉTODOS

3.1. Obtenção do Extrato Etanólico da Própolis (EEP)

Duas amostras de própolis bruta de *Apis mellifera* foram coletadas nas cidades de Santa Luzia – Minas Gerais (MG) e Porto Alegre – Rio Grande do Sul (RS) após prévia seleção (PARK et al., 1997; PARK et al., 1998). A própolis foi de-hidratada e triturada, e o seu extrato etanólico preparado na concentração de 10% (p/v) em etanol 80% (v/v) de acordo com a metodologia descrita por KOO & PARK (1997) e PARK et al. (1997).

3.2. Atividade Antimicrobiana *in vitro*

3.2.1. Microrganismos

Os microrganismos utilizados nesta análise foram divididos em 3 grupos: 1) aeróbios - *Staphylococcus aureus* ATCC 25923, *Candida albicans* F72, *Candida albicans* NTCC 3736, *Enterococcus faecalis* ATCC 29212; 2) Facultativos - *Streptococcus mutans* Ingbritt 1600, *Streptococcus mutans* OMZ-175, *Streptococcus sobrinus* 6715, *Streptococcus cricetus* HS-6, *Streptococcus sanguis* ATCC 10556, *Actinomyces naeslundii* ATCC 12104, *Actinomyces naeslundii* W1053, *Actinomyces viscosus* OMZ 105; 3) Anaeróbios estritos - *Porphyromonas gingivalis, Porphyromonas endodontalis, Prevotella denticola* (anaeróbios estritos). Os três últimos microrganismos foram isolados clinicamente. Os anaeróbios facultativos foram gentilmente doados pelo Center for Oral Biology, University of Rochester, NY, USA.

3.2.2. Determinação da atividade antimicrobiana pelo método de difusão em ágar

Para esta análise foram utilizados os seguintes meios de cultura em ágar: Fastidious Anaerobe (Lab. M, Burry, UK) com 5% de sangue de carneiro desfibrinado – Fasc, Brain Heart Infusion – BHI (Difco, MA, USA) e Mueller Hinton – MH (Difco, MA, USA). Os microrganismos foram inoculados no meio por "pour plate", com exceção do anaeróbios estritos.

Culturas ativas de anaeróbios e anaeróbios facultativos foram cultivados em placas BHI ágar por 18-24 h, a 37 °C (10% de CO_2 para os facultativos). Os anaeróbios estritos foram cultivados em placas Fasc ágar pré-reduzidos por 24-48 h, a 37°C em câmara de anaerobiose. Após o crescimento bacteriano, as colônias individuais foram removidas com auxílio de um alça de platina e suspendidas em uma solução estéril de NaCl 0,89%. Após a homogeneização com auxílio de um vortex, a suspensão bacteriana foi ajustada para atingir 0,5 da escala de Mc Farland. Uma alíquota de 400 µL de cada suspensão bacteriana foi homogeneizada com 40 mL de BHI agar a 45 °C, sendo distribuída sobre uma camada base pré-existente de MH ágar. Para os anaeróbios, a suspensão bacteriana foi preparada como descrito acima, porém turbidez da suspensão foi equivalente a 1,0 da escala de Mc Farland. Com auxílio de uma zaragatoa estéril, a suspensão bacteriana foi inoculada sobre placas Fasc ágar pré-reduzidas. Os procedimentos do inóculo foram apropriados para providenciar um crescimento semi-confluente dos microrganismos testados.

Seis cilindros de aço-inoxidável estéril 8.0 x 10 mm (diâmetro interno de 6 mm) foram colocados sobre cada placa de ágar inoculado. O volume de 40 µL do EEP e o seu controle negativo – etanol 80% (v/v) foi colocado nos cilindros. As placas foram mantidas por 2 h na temperatura ambiente para permitir a difusão dos extratos através do ágar. Após estes procedimentos, as placas foram incubadas a 37°C nas seguintes condições: aeróbios – 24h, facultativos – 24/48 h e 10% CO_2, e anaeróbios estritos – 7 dias em uma câmara de anaerobiose controlada (Don Whitley Scientific, Bradford, UK) em uma atmosfera de 5-10% H_2, 10% CO_2 e 80-85% N_2. Zonas de inibição do crescimento bacteriano ao redor do cilindro contendo os EEPs foram medidos após o tempo de incubação. Seis (6) replicatas foram feitas para cada microrganismo.

3.2.3. Determinação da concentração inibitória mínima (CIM) e concentração bactericida mínima (CBM) dos EEPs contra os estreptococos do grupo mutans.

As bactérias utilizadas nesta análise foram: *Streptococcus mutans* Ingbritt 1600, *Streptococcus sobrinus* 6715, *Streptococcus cricetus* HS-6. Os procedimentos para a obtenção da suspensão bacteriana foram feitas de acordo com o item 3.2.2. Entretanto, a suspensão foi ajustada, espectrofotometricamente entre absorbâncias de 0,48 e 0,5 a 660 nm, para obter

0,5 - 1 x 10^9 ufc/mL. A suspensão bacteriana foi diluída na proporção de 1:1000 (v/v) com o meio líquido BHI estéril e 4960 µL foram transferidos para um tubo de ensaio estéril. Posteriormente, foram pipetados 40 µL do EEP (com a concentração variando de 6,2 a 1600 µg/mL com diluição seriada, razão 2) no tubo e homogeneizado com auxílio de um vortex em baixa velocidade. As culturas foram incubadas nas mesmas condições descritas no item 3.2.2 para os facultativos. O CIM foi considerado a menor concentração do EEP onde não houve crescimento bacteriano visível, ou seja uma diferença de leitura de absorbância menor que 0,05 a 660 nm entre as leituras feitas antes e depois da incubação dos microrganismos testados.

Para determinação do CBM, uma alíquota de 50 µL das suspensões utilizadas no teste do CIM foram inoculados em placas BHI ágar suplementado com 5% de sangue de carneiro desfibrinado estéril. O CBM foi considerado a menor concentração que causou 99,9% de morte celular, ou seja, sem crescimento bacteriano sobre o ágar.

3.3. Aderência de *S. mutans* e *S. sobrinus* sobre a Superfície do Vidro.

Para análise da aderência celular de *S. mutans* Ingbritt 1600 *e S. sobrinus* 6715 sobre a superfície de vidro, os microrganismos foram incubados a 37°C por 18 h em tubos de vidro com inclinação de 30°, como descrito por HAMADA & TORII (1978). Os procedimentos para o preparo da suspensão bacteriana foram os mesmos do item 3.2.2. A suspensão foi diluída na proporção 1:100 (v/v) com o meio líquido BHI contendo 1% de sacarose, e subseqüentemente 2480 µL do meio foram transferidos para um tubo de vidro. Após esta etapa, foram feitos os seguintes procedimentos: 1) 20 µL do EEP (com a concentração variando de 6,2 a 400 µg/mL, em diluição seriada, razão 2) foram pipetadas no interior dos tubos contendo o inóculo bacteriano, 2) A mistura foi homogeneizado com auxílio de um vortex em baixa velocidade, 3) Os tubos foram incubados a 37°C por 18 h em tubos de vidro com inclinação de 30°. Após a incubação, o meio de cultura e as bactérias não aderidas foram descartadas. As bactérias aderidas foram lavadas duas vezes com o tampão fosfato de potássio – KPB (0,05M, pH 6,8) contendo 0,02% de azida sódica. Em seguida, 5 mL de KPB foram adicionados ao tubo e as células foram ressuspensas extensivamente com ultrassom (250 W, com pulsos de 10s, intervalo de 5s, por 20 minutos, em ultrassonicador-banho Thorton). A quantificação das bactérias aderidas foi feita por

espectrofotometria através da leitura da absorbância a 550 nm – Abs. $_{550}$ (HAMADA & TORII, 1978). Seis (6) replicatas foram realizadas para cada concentração dos EEPs.

3.4. Síntese de Glucanos Insolúveis em Água (GIA) pela Glicosiltransferase bruta.

3.4.1. Preparo da glicosiltransferase extracelular bruta

A glicosiltransferase extracelular bruta de *S. sobrinus* 6715 foi preparada de acordo com a modificação do método descrito por SCHILLING & BOWEN (1988). A bactéria foi inicialmente reativada da cultura estoque (mantida a –20°C) e a sua cultura pura inoculada em 10 mL do meio de cultura LMW ("Low Molecular Weight", 2,5% triptona; 1,5% de extrato de levedura; 0,3% de glicose e 1,0% de sorbitol ultrafiltrados através de uma membrana com exclusão de 5,0 kd) suplementado de 10 μg/mL de estreptomicina e incubado a 37°C, 10% CO_2 por 18 h. Subseqüentemente, a cultura inicial foi transferida para 200 mL de LMW e novamente incubada nas condições descritas anteriormente. Após a incubação, as bactérias foram removidas por centrifugação a 12000xg, 5°C, 15 min. e o sobrenadante separado. Em seguida foi acrescentado o inibidor de protease, fenilmetilsulfonilfluoreto (PMSF, 1,0 mmol/L) e o conservante azida sódica-NaN_3 (0.02%, concentração final). O sobrenadante assim obtido foi tratado com sulfato de amônio a 55% de saturação e mantido sob agitação por 18 h a 5°C. Posteriormente, este sobrenadante tratado foi centrifugado (10000xg por 10 min.) e o precipitado foi suspendido em tampão KPB contendo PMSF e NaN_3, nas concentrações finais de 1,0 mmol/L e 0,02% respectivamente. O precipitado ressuspendido foi extensamente dialisado no mesmo tampão utilizando uma membrana com exclusão de 12,0-14,0 kd. O preparado dialisado foi utilizado como glicosiltransferase extracelular bruta.

3.4.2. Inibição pelo EEP da formação de glucanos insolúveis em água (GIA).

Para analisar a formação de GIA foi realizada uma reação contendo 1000 μL de sacarose 0,25M, 100 μL da glicosiltransferase bruta, 20 μL do EEP (concentração variando de 7,8 a 500 μg/mL de reação) num volume total de 2 mL de KPB contendo PMSF 1,0 mmol/L e NaN_3 0.02%. A mistura foi incubada a 37°C por 18 h em um ângulo de 30° Após a incubação, a

solução foi cuidadosamente removida e 1 mL de água destilada e deionizada estéril foi adicionada ao tubo para remoção do material não aderido. O procedimento de lavagem foi feita mais duas vezes e o material aderido foi utilizado para a determinação do GIA (TAKADA et al., 1985). O GIA foi disperso com auxílio de ultrasom (250W, pulso de 10 s e 5 s de intervalo, por 20 minutos, em ultrassonicador-banho Thorton) e coletado por centrifugação (8500xg, 5°C, 10 min.). O precipitado (GIA) foi extraído com 3 mL de NaOH 1N e o açúcar total quantificado pelo método fenol-sulfúrico (DUBOIS et al., 1956). Seis (6) replicatas foram realizadas para cada concentração dos EEPs.

3.5. Atividade das Glucosiltransferases (Gtf) B, C, D e G Purificadas

3.5.1. Microrganismos

Os microrganismos utilizados foram: 1) *Streptococcus milleri* KSB8, que contém o gene *gtfB*, para a produção de Gtf B; 2) *S. milleri* NH5, que contém o gene *gtfD*, para produção de Gtf D;3) *S. mutans* WHB410, onde os genes *gtfB, gtfD* e o *ftf* foram deletados, para produção de Gtf C; 4) *S. sanguis* 10904, para produção de Gtf G. As metodologias para a construção destas linhagens foram descritas detalhadamente por FUKUSHIMA et al. (1992) e WUNDER & BOWEN (1999).

3.5.2. Obtenção e purificação das glucosiltransferases

As bactérias foram inicialmente cultivadas em meio de cultura TEG (Tryptic soy broth, 3,0%; extrato de levedura, 0,5% e glicose, 10%). O meio TEG foi suplementado com antibióticos como descrito a seguir: eritromicina (10 μg/mL) para a cepas *S. milleri*, e eritromicina (10 μg/mL), kanamicina (500 μg/mL) e tetraciclina (5 μg/mL) para *S. mutans* WHB410.

As Gtf B ,D e G foram obtidas a partir do sobrenadante das culturas de *S. milleri* KSB8, *S. milleri* NH5 e *S. sanguis* 10904 de acordo com o método descrito por VENKITARAMAN et al. (1995).

Para a obtenção da Gtf C, foi realizado de acordo com a modificação da metodologia descrita por WUNDER & BOWEN (1999). A cultura inicial de *S. mutans* WHB 410 também foi inoculada em um saco de diálise (exclusão 50,0 kd) contendo 200 mL do meio de cultura Low Molecular Weight - LMW (triptona 2.5%, extrato de levedura 1.5%, glicose 0.3 %, frutose 0.1% e sorbitol 0.1%; SCHILLING & BOWEN, 1988), sendo esta imersa em 800 mL do mesmo meio. O meio de cultura LMW foi suplementado com os seguintes antibióticos: eritromicina (10 μg/mL), kanamicina (500 μg/mL) e tetraciclina (5 μg/mL). Após o crescimento bacteriano, as células foram obtidas por centrifugação a 5000xg, 4°C por 20 min e o sobrenadante foi descartado. As células foram ressuspendidas em 20 mL do tampão fosfato de potássio 50 mM (KPO_4) pH 7,5 e 0,01% de Triton X-100, sendo posteriormente sonicadas sob gelo (250W, 5 pulsos de 30s e 10s de intervalo, Braun-Sonic 1510). Após a sonicação, as células ressuspendidas foram centrifugadas e o sobrenadante separado e utilizado como fonte de Gtf C.

Todos os sobrenadantes obtidos como descritos anteriormente, foram purificados em colunas de hidroxiapatita 1x30 cm (MacroPrep ceramic Hydroxyapatite Type-I, 80 μm-particle size, BioRad, Hercules, CA) de acordo com VENKITARAMAN et al., 1995). As enzimas foram armazenadas a 4°C em tampão Adsorption buffer – AB (50 mM KCl, 1,0 mM KPO_4, 1,0 mM $CaCl_2$, 0,1 mM $MgCl_2$, pH 6,5) contendo 10% glicerol. As enzimas foram analisadas, quanto ao grau de purificação, através de SDS-PAGE e coloração por prata, como descrito anteriormente (VENKITARAMA et al., 1995). A quantificação protéica foi feita pelo método de absorbância a 280 nm como descrito por DEUTSCHER (1990).

A atividade da glucosiltransferase foi determinada pela incorporação do ^{14}C-glicose da sacarose marcada [^{14}C-(glucosil)-sacarose; NEN Research Products, Boston, MA] no glucano sintetizado. As atividades específicas das Gtfs foram: Gtf B – $1,24 \times 10^{-3}$ μmol de glicose incorporado no glucano/min/μg de proteína, Gtf D – $6,9 \times 10^{-4}$ μmol de glicose incorporado no glucano/min/μg de proteína e Gtf C $1,63 \times 10^{-4}$ μmol de glicose incorporado no glucano/min/μg de proteína. Uma unidade da enzima foi definida como a quantidade de enzima necessária para incorporar 1 μmol de glicose no glucano por 4 horas de reação. A Gtf utilizada em todas as análises foi equivalente a quantidade requerida para incorporar 1,0 - 1,5 μmol de glicose no glucano durante 4 horas de reação (1,0 - 1,5 unidades).

3.5.3. Atividade das Gtfs em solução

As Gtfs B, C e D foram misturadas com EEP (concentração variando de 0,078 a 5,0 mg/mL) e incubado com substrato de ^{14}C-(glucosil)-sacarose (200,0 mmol/L sacarose, 40 μmol/L dextrana 9000, 1,0 mmol/L PMSF, 0,02% de NaN_3 em tampão AB, pH 6,5). A concentração final de sacarose foi de 100 mmol/L (200 μL, volume final). Para o controle, a mesma reação foi feita onde etanol 80% foi adicionado em vez do EEP. A concentração final do etanol foi de 8%. As amostras foram incubadas a 37°C sob agitação por 4 h. Após a incubação, etanol (1,0 mL; -20°C) foi adicionado para a precipitação dos glucanos e armazenados por 18 h, 4 °C. O glucano formado foi determinado por cintilometria como descrito anteriormente (VENKITARAMAN et al., 1995). O flavonóide aglicona quercetina, 3, 3', 4', 5, 7 - pentahidroxiflavona (Sigma Chemical Co., St. Louis, MO, USA), identificado em ambos EEPs também foi testado nas concentrações variando de 3,9 a 250,0 μg/mL.

3.5.4. Atividade das Gtfs aderidas a superfície de hidroxiapatita (HA)

As esferas de hidroxiapatita (10 mg, tamanho 80 μm e área de superfície 0,24 m^2) foram tratadas com saliva humana clarificada (saliva total estimulada obtida de um único doador e centrifugada a 8500xg, 4°C, por 10min) para a formação da película experimental como detalhado por SCHILLING & BOWEN (1988) e VENKITARAMAN et al. (1995). Em seguida, a superfície de HA foi tratada com 300 μL do EEP (nas mesmas concentrações citadas no item 3.5.3) por 30 min, 37 °C. Para o controle, etanol 80% foi adicionado ao invés do EEP. A concentração final de etanol foi de 8%. As esferas de HA foram lavadas e expostas a 300 μL do substrato ^{14}C-(glucosil)-sacarose, sendo posteriormente incubadas por 4h, 37 °C. Após a incubação, etanol (1,0 mL, -20°C) foi adicionado em cada amostra e armazenado por 18h, 4 °C. Os glucanos foram quantificados como descrito no item 6.3. O flavonóide aglicona quercetina, 3, 3', 4', 5, 7 - pentahidroxiflavona (Sigma Chemical Co., St. Louis, MO, USA), identificado em ambos EEPs também foi testado nas concentrações variando de 3,9 a 250,0 μg/mL.

3.6. Aderência de *S. mutans* e *S. sobrinus* sobre Hidroxiapatita (HA)

A análise da aderência de *S. mutans* GS-5 e *S. sobrinus* 6715 marcadas com ^{3}H-timidina (NEN Research Products, Boston, MA) sobre o glucano formado por Gtf C incorporado na película experimental foi realizada de acordo com SCHILLING & BOWEN (1992). A Gtf C foi aderida na superfície de HAS e em seguida tratada com EEP (concentração variando de 0,078 a 2,5 mg/mL) como descrito no item 3.5.4. Após a incubação com o substrato ^{14}C-(glucosil)-sacarose, as esferas de HA foram lavadas e expostas as bactérias marcadas como descrito detalhadamente por SCHILLING & BOWEN (1992). As bactérias aderidas foram determinadas por cintilometria (SCHILLING & BOWEN, 1992).

3.7. Desenvolvimento de Cárie em Modelo Experimental com Ratos Desalivados

Trinta ratos fêmea Wistar SPF, 19 dias de idade, foram adquiridos do CEMIB/UNICAMP (Campinas, SP, Brasil). Os animais foram verificados com relação a presença de estreptococcus do grupo mutans indígenos de acordo com a metodologia descrita por BOWEN et al. (1988a). Os ratos foram infectados com *S. sobrinus* 6715 por 3 dias consecutivos (21°, 22° e 23° dia). Os animais foram alimentados com dieta 2000 (KEYES, 1968) e água estéril contendo 5% de sacarose *ad libitum* até o 25° dia para estabelecer a infecção com *S. sobrinus*. A infecção foi confirmada através do cultivo das bactérias bucais dos ratos sobre meio de cultura mitis salivarius contendo estreptomicina - MSS (Sigma). No 25° dia, os ratos foram desalivados através da ligadura dos ductos da parótida e remoção cirúrgica das glândulas sublingual e submandibular (BOWEN et al, 1988b). No dia seguinte, os ratos foram divididos randomicamente em 3 grupos com 10 animais cada, sendo colocados em gaiolas individuais da máquina de alimentação programada König-Höfer (KÖNIG et al., 1968). Em intervalos de 1 hora, começando a partir da 4 horas da tarde, 17 refeições consistindo de dieta 2000 (400 mg) foram fornecidas aos ratos. Os tratamentos começaram no 26° dia terminando no 48° dia, onde foram aplicados 100 µL do EEP ou etanol 80% sobre os molares dos ratos (25 µL sobre os molares de cada quadrante) duas vezes ao dia. Os grupos foram divididos em: Grupo 1 – etanol 80 % (grupo controle), Grupo 2 – EEP de MG e Grupo 3 - EEP do RS.

Durante todo o período experimental, todos os animais foram pesados semanalmente e a aparência física avaliadas diariamente. O número da dieta consumida também foi anotado diariamente. No final do experimento (48° dia), os ratos foram mortos e decapitados 60-90 min após a última refeição. Todas as mandíbulas esquerdas foram assepticamente removidas, dissecadas, colocadas em solução de NaCl 0,89% e sonicadas em banho de ultra-som (250 W, 10 s de pulso e intervalos de 5s, por 20 minutos, em ultrassonicador-banho Thorton). A suspensão obtida foi diluída (1:5, 1:100 e 1:000, v/v) e inoculada em placas de MSS e ágar sangue (5% de sangue desfribrinado de carneiro) para recuperação de *S. sobrinus* 6715 e microbiota total respectivamente, as quais foram incubadas a 37 °C, 48h e 10% CO_2. As placas de ágar sangue foram incubadas 24h adicionais em atmosfera normal a 37°C. Posteriormente a microbiota foi contada e estabelecida em ufc/mL. Posteriormente, os dentes foram preparados para o índice de cáries de acordo com o método modificado de Keyes por Larson (LARSON, 1981). A determinação do índice foi cego através da codificação das mandíbulas e foi feito por um examinador experiente.

3.8. Formação de Placa Dental *in vivo*.

3.8.1. Enxaguatório Bucal

Foram preparadas duas formulações de enxaguatório bucal: 1) Experimental - Extrato etanólico da própolis RS (EEP RS) 20% (p/v, em etanol 80%, v/v); propilenoglicol; água destilada e deionizada. A concentração final da própolis foi 5% (p/p) e de etanol 20% (p/p), 2) Placebo, os mesmos componentes acima com exceção do EEP RS, que foi substituído por etanol 80% (v/v). A concentração final do etanol foi 20% (p/p). Os enxaguatórios bucais foram armazenadas em frasco âmbar, iguais e codificados de tal modo que nem os voluntários, nem os examinadores tinham conhecimento da sua composição.

3.8.2. Voluntários

Seis voluntários adultos (idade entre 20-38 anos; média 26,8 ± 7,8), de ambos os sexos (3 do sexo feminino e 3 do sexo masculino) e saudáveis participaram espontaneamente desta

pesquisa. Os voluntários receberam um documento informativo sobre os objetivos e as informações sobre a pesquisa. Um termo de consentimento esclarecido foi assinado pelos voluntários (Anexo 2) autorizando a realização do experimento após a explanação sobre os riscos e benefícios da pesquisa (Resolução n° 196, de 10 de outubro de 1996, do Conselho Nacional de Saúde).

3.8.3. Delineamento experimental

O delineamento experimental foi do tipo cruzado, duplamente cego, em duas etapas de três dias, durante os quais toda a higiene bucal foi paralisada. Em cada etapa experimental 3 voluntários utilizavam duas vezes por dia o enxaguatório bucal experimental, e a outra metade utilizava placebo. Após uma semana de "wash-out", para eliminação de qualquer efeito residual, foi feito o segundo cruzamento. Durante as etapas, os voluntários de ambos os grupos bochecharam 5 vezes ao dia uma solução de açúcar (sacarose 20%, p/v) para estimular a formação de placa dental. No início de cada período experimental os voluntários foram submetidos a profilaxia com ultra-som. Sete dias antes do período experimental e no intervalo entre os mesmos, os voluntários escovaram os dentes com dentifrício sem agentes antimicrobianos.

3.8.4. Placa dental

O índice de placa dental foi avaliada de acordo com Silness & Löe (1964), sendo assinalados os valores de 0 a 3 para as superfícies disto-vestibular, vestibular, mésio-vestibular e palatina/lingual sendo: 0 = ausência de placa; 1 = placa detectada com auxílio de sonda exploradora; 2 = placa visível; 3 = acúmulo de placa. Os índices foram determinados por um único examinador experiente.

3.9. Análises Estatísticas

As análises estatísticas de cada experimento estão descritas detalhadamente nos artigos colocados nos anexos.

4. RESULTADOS

4.1. Atividade Antimicrobiana do EEP

Os resultados das análises da atividade antimicrobiana da própolis estão demonstrados nas tabelas 1 e 2. Ambos os extratos foram capazes de inibir significantemente o crescimento de todos os grupos de microrganismos testados formando zonas de inibição de crescimento entre 0,83 a 9,50 mm ($p<0,05$) (Tabela 1). O grupo dos *Actinomyces spp*, principalmente o *Actinomyces viscosus*, demonstraram as maiores zonas inibitórias (entre 7,86 e 9,50 mm), enquanto que o crescimento das cepas de *Candida albicans* foram fracamente inibidas (zonas de inibição entre 0,83 e 2,33 mm). *Streptococcus sanguis* e os estreptococcus do grupo mutans também foram significantemente inibidas pela própolis. Vale destacar que a própolis apresentou atividade antibacteriana sobre os anaeróbio estritos, principalmente *Porphyromonas gingivalis*.

Tabela 1. Médias das zonas de inibição de crescimento dos microrganismos pelo extrato etanólico da própolis (EEP).

Microrganismos	Tratamentos		
	EEP de MG	EEP do RS	Etanol 80%
Staphylococcus aureus ATCC 25923	2,33 a	2,76 a	0,00 b*
Enterococcus faecalis ATCC 29212	2,17 a	2,25 a	0,00 b
Candida albicans NTCC 3736	0,83 a	2,33 b	0,00 c*
Candida albicans F72	0,83 a	1,95 b	0,00 c*
Streptococcus mutans OMZ 175	2,00 a	2,75 b	0,00 c*
Streptococcus mutans Ingbritt 1600	2,17 a	2,80 b	0,00 c*
Streptococcus cricetus HS-6	1,83 a	2,91 b	0,00 c*
Streptococcus sobrinus 6715	2,25 a	2,95 b	0,00 c
Streptococcus sanguis ATCC 10556	4,42 a	4,35 a	0,00 b*
Actinomyces naeslundii ATCC 12104	9,25 a	8,26 b	0,00 c*
Actinomyces naeslundii W1053	8,17 a	7,86 a	0,00 b*
Actinomyces viscosus OMZ-105	9,50 a	9,00 b	0,00 c*
Porphyromonas gingivalis	3,42 a	3,30 a	0,00 b*
Porphyromonas endodontalis	2,25 a	2,05 a	0,00 b*
Prevotella denticola	2,58 a	2,58 a	0,00 b*

* Inibição por contato direto. Médias (n=6) seguidas de mesma letra, na horizontal, não diferem estatisticamente entre si , $p>0,05$, teste não paramétrico Kruskal-Wallis por comparações múltiplas.

De modo geral, podemos observar que a própolis do RS obteve maiores zonas de inibição de crescimento de *Candida albicans* e estreptococcus do grupo mutans que o EEP de MG ($p<0,05$). Por outro lado, o EEP MG apresentou melhores resultados para o grupo dos *Actinomyces spp.*

Os dados do CIM e CBM dos EEPs para o *S. mutans* Ingbritt 1600, *S. sobrinus* 6715 e *S. cricetus* HS-6 estão apresentados na tabela 2.

Tabela 2. Valores de CIM e CBM dos EEPs para os estreptococos do grupo mutans.

Microrganismos	CIM*		CBM*	
	EEP de MG	EEP do RS	EEP de MG	EEP do RS
Streptococcus mutans Ingbritt 1600	200 - 400	50-100	800 - 1600	200 - 400
Streptococcus sobrinus 6715	200 - 400	25-50	800 - 1600	100 - 200
Streptococcus cricetus HS-6	200 - 400	25-50	800 - 1600	100 - 200

* Os resultados estão representados em μg de própolis/mL de reação.

A amostra RS demonstrou nitidamente uma maior atividade antibacteriana sobre os estreptococos testados do que o MG. O valor de CIM do EEP RS foi de 25 - 50 μg/mL para *S. sobrinus* e *S. cricetus* e 50 - 100 μg/mL para *S. mutans*, enquanto que a amostra MG apresentou valores entre 200 - 400 μg/mL para todas as cepas testadas. A concentração bactericida foi de 4 - 8 vezes maiores que os valores de CIM.

4.2. Inibição pelo EEP da Aderência de *S. mutans* e *S. sobrinus* sobre a Superfície do Vidro

Ambos os EEPs reduziram a aderência de *S. mutans* Ingbritt 1600 e *S. sobrinus* 6715 sobre a superfície de vidro demonstrando inibição significante em todas as concentrações testadas quando comparada com o controle (etanol 80%, v/v) ($p<0,05$), como ilustrado na figura 7. Entretanto, o EEP RS foi mais efetivo apresentando maior inibição da aderência celular que o EEP MG nas concentrações entre 25-200 μg/mL para o *S. mutans* e 12,5-200 μg/mL para o *S. sobrinus* ($p<0,05$).

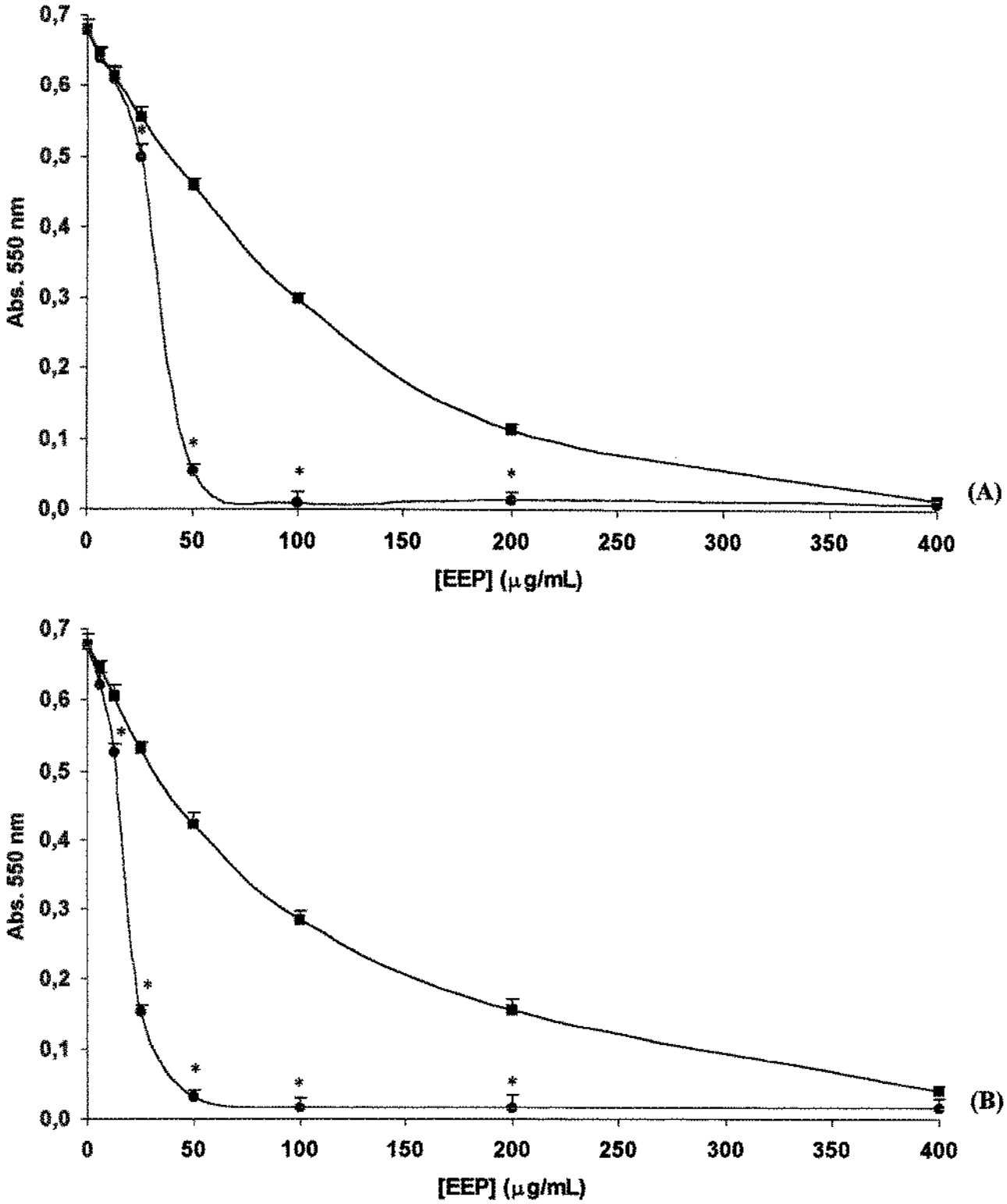

Figura 7. Efeito do extrato etanólico da própolis (EEP) sobre a aderência de *Streptococcus mutans* Ingbritt 1600 (A) e *S. sobrinus* 6715 (B). Símbolos: —●— EEP RS (Região Sul); —■— EEP MG (Região Sudeste).[1] Os valores apresentados são a média de 6 replicatas ± D.P. [2] Os valores seguido por um asterisco (*) entre os tratamentos, em cada concentração, são significantemente diferente entre si, teste F, $p<0,05$. Os valores obtidos por ambos os EEPs diferiram estatisticamente do controle em todas as concentrações analisadas, teste Dunnet, $p<0,05$.

A porcentagem de inibição para ambas as cepas pelo EEP RS foi em torno de 90% nas concentrações entre 50 e 400 μg/mL, enquanto que o EEP MG inibiu nesta mesma intensidade apenas na concentração de 400 μg/mL de reação.

4.3. Inibição pelo EEP da Síntese de GIA

Os dados da análise da inibição de síntese de glucanos insolúveis em água (GIA) pelos EEPs estão ilustrados na figura 8.

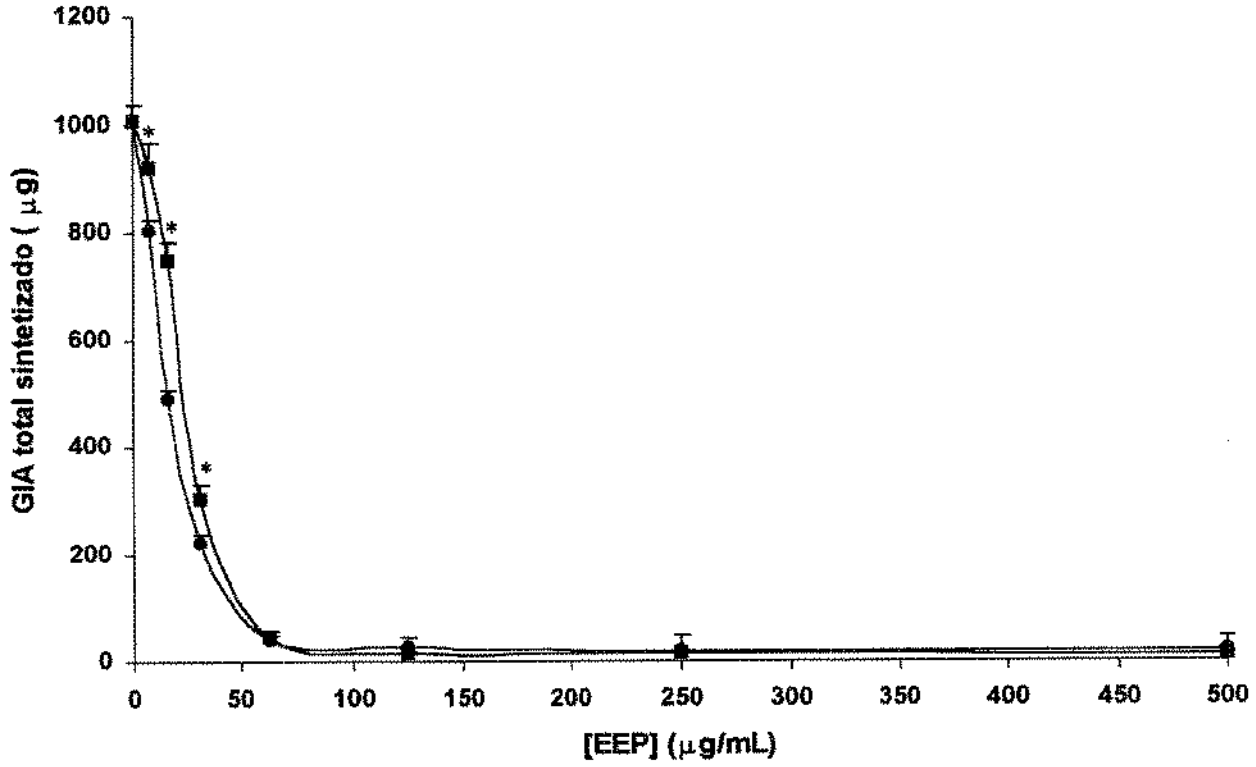

Figura 8. Efeito do extrato etanólico da própolis (EEP) sobre a síntese de glucanos insolúveis em água (GIA) pela glicosiltransferase. Símbolos: —●— EEP RS (Região Sul); —■— EEP MG (Região Sudeste).[1] Os valores apresentados são a média de 6 replicatas ± D.P. [2] Os valores seguido por um asterisco (*) entre os tratamentos, em cada concentração, são significantemente diferente entre si, teste F, $p<0,05$. Os valores obtidos por ambos os EEPs diferiram estatisticamente do controle em todas as concentrações analisadas, teste Dunnet, $p<0,05$.

A própolis demonstrou ser um potente inibidor da síntese GIA. Os EEPs inibiriam a síntese de GIA quase na sua totalidade nas concentrações entre 62,5 e 500 μg/mL. O EEP RS demonstrou uma inibição significantemente maior nas concentrações entre 7,8 e 31,3 μg/mL quando comparado com EEP de MG ($p<0,05$). Os EEPs inibiram significantemente a síntese de GIA em todas as concentrações testadas quando comparados com o controle ($p<0,05$).

4.4. Inibição da Atividade das Gtfs Purificadas

4.4.1. Inibição pelo EEP das Gtfs purificadas, em solução e aderidas a superfície de HA

Os dados da inibição das Gtfs purificadas estão ilustradas nas figuras 9 a 12. Os EEPs demonstraram ser potentes inibidores das Gtfs. A atividade enzimática das Gtfs B, D e G foram reduzidas entre 85-95% nas concentrações de EEP entre 1,25 e 5,0 mg/mL. A atividade do Gtf C foi reduzido neste mesmo nível quando a concentração foi entre 2,50 e 5,0 mg/mL. Entretanto, o efeito inibitório foi variável dependendo do EEP e a Gtf testada. O EEP RS inibiu mais eficientemente as atividades das Gtfs B e C do que o EEP MG, como mostrado nas figuras 9 e 10. As diferenças de inibição foram estatisticamente significantes nas concentrações entre 0,078 a 0,625 mg/mL para o Gtf B, e 0,078 a 1,25 mg/mL para o Gtf C. Por outro lado ambos os EEPs foram igualmente efetivas para inibir as atividades das Gtf D e G. As Gtfs D e G foram as enzimas mais susceptíveis para serem inibidas pelos EEPs em solução, demonstrando mais de 70% e 80% de inibição quando a concentração foi de 0,312 mg/mL.

Os resultados do efeito da própolis sobre as mesmas enzimas aderidas a uma película experimental formada sobre a superfície de hidroxiapatita (HA) demonstraram que o grau de inibição foi menor que os obtidos em solução, principalmente a Gtf G (Figuras 9 a 12). Novamente o efeito inibitório foi variável dependendo do EEP e da Gtf analisada. EEP RS foi mais eficiente na inibição das Gtf B e C aderidas na superfície de HAS do que o EEP MG, na qual foi estatisticamente significante nas concentrações entre 0,078 e 0,312 mg/mL ($p<0,05$). Em constraste, o EEP MG inibiu as Gtf D e G aderidas de modo mais efetivo que o EEP RS. O EEP MG demonstrou maior inibição da Gtf D que o EEP RS nas concentrações de 0,625 a 2,5 mg/mL,

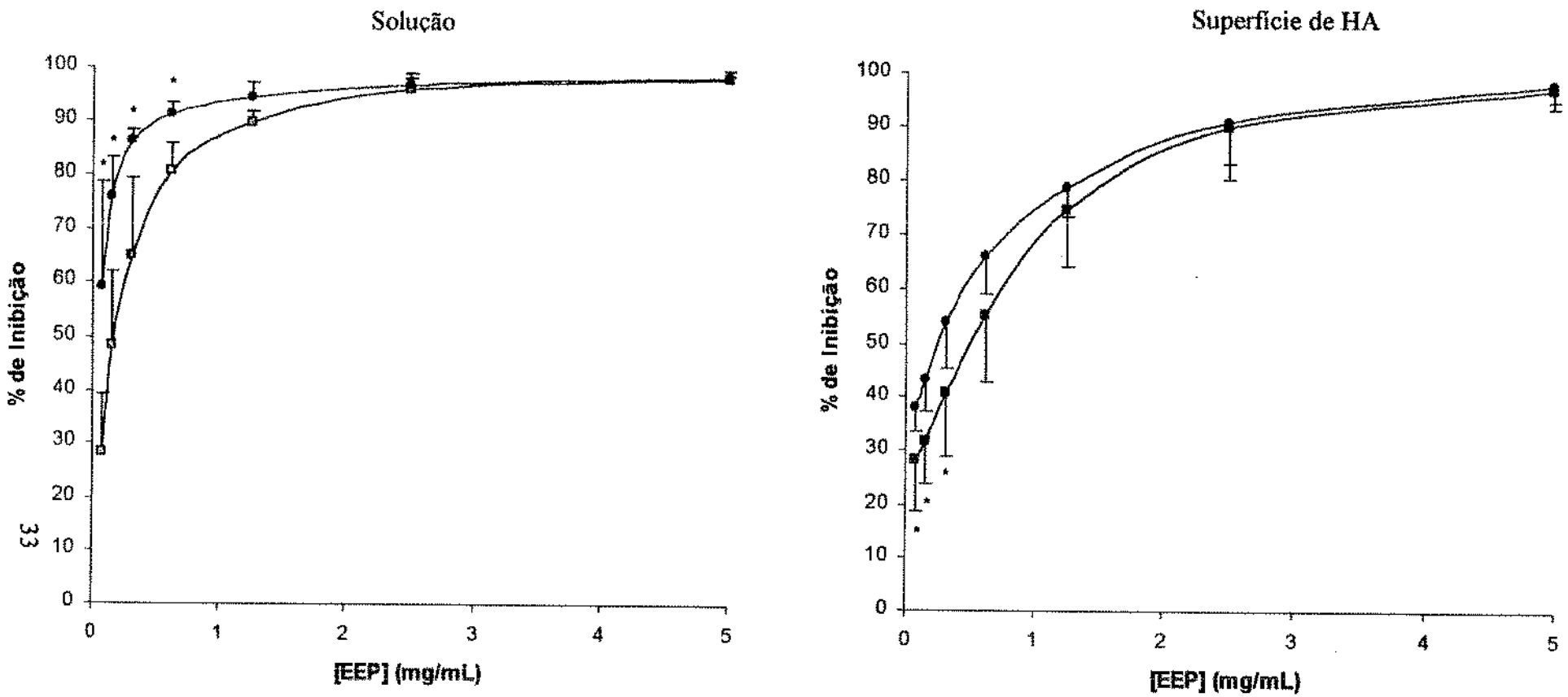

Figura 9. Efeito de extrato etanólico da própolis (EEP) sobre a atividade da glucosiltransferase B (Gtf B) em solução e aderida a superfície de HA. Símbolos:—●— EEP RS (Região Sul);—■—EEP MG (Região Sudeste).

[1] A porcentagem de inibição foi calculada considerando o controle como a atividade máxima da Gtf. O controle foi o etanol 80% (v/v).

[2] Os valores apresentados são a média de 6 replicatas ± D.P.

[3] Os valores seguido por um asterisco (*) entre os tratamentos, em cada concentração, são significantemente diferente entre si, t de Student, $p<0,05$.

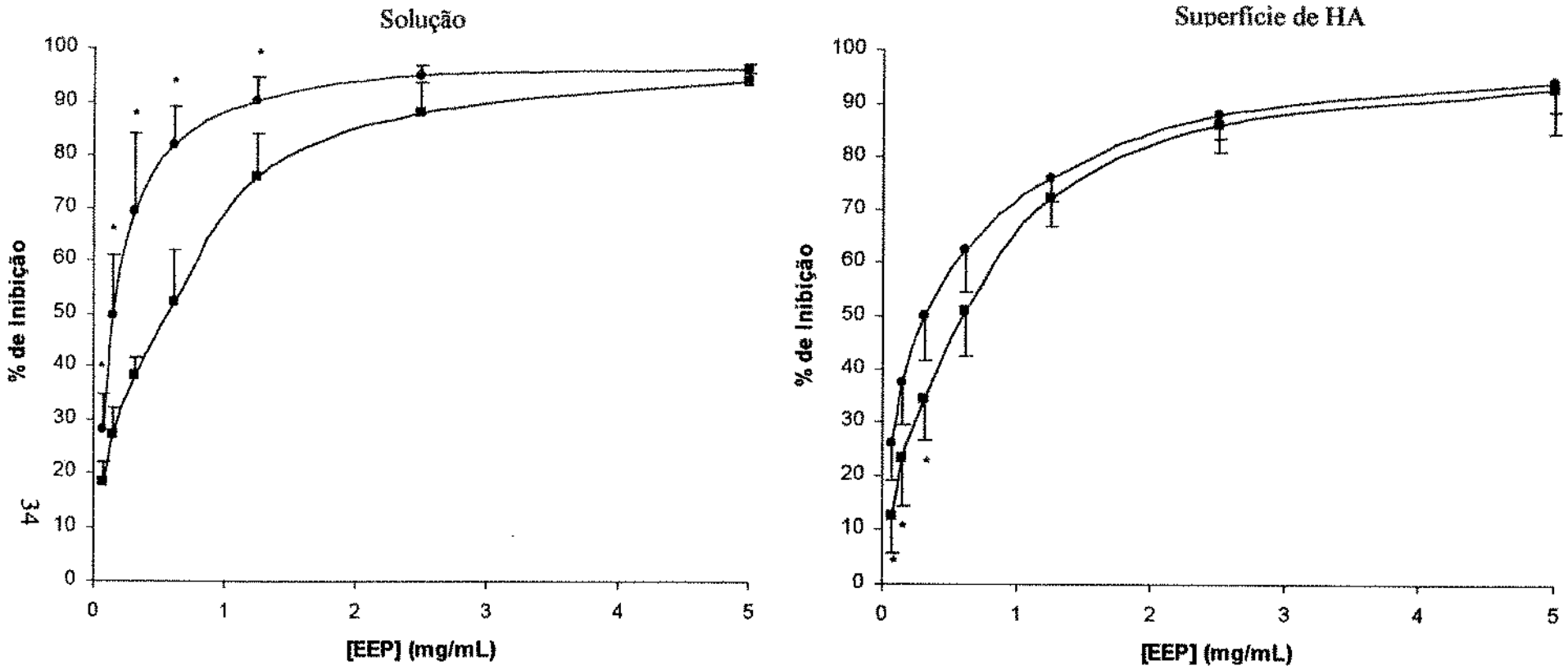

Figura 10. Efeito de extrato etanólico da própolis (EEP) sobre a atividade da glucosiltransferase C (Gtf C) em solução e aderida a superfície de HA. Símbolos: —●— EEP RS (Região Sul); —■— EEP MG (Região Sudeste).

[1] A porcentagem de inibição foi calculada considerando o controle como a atividade máxima da Gtf. O controle foi o etanol 80% (v/v).

[2] Os valores apresentados são a média de 6 replicatas ± D.P.

[3] Os valores seguido por um asterisco (*) entre os tratamentos, em cada concentração, são significantemente diferente entre si, t de Student, $p<0,05$.

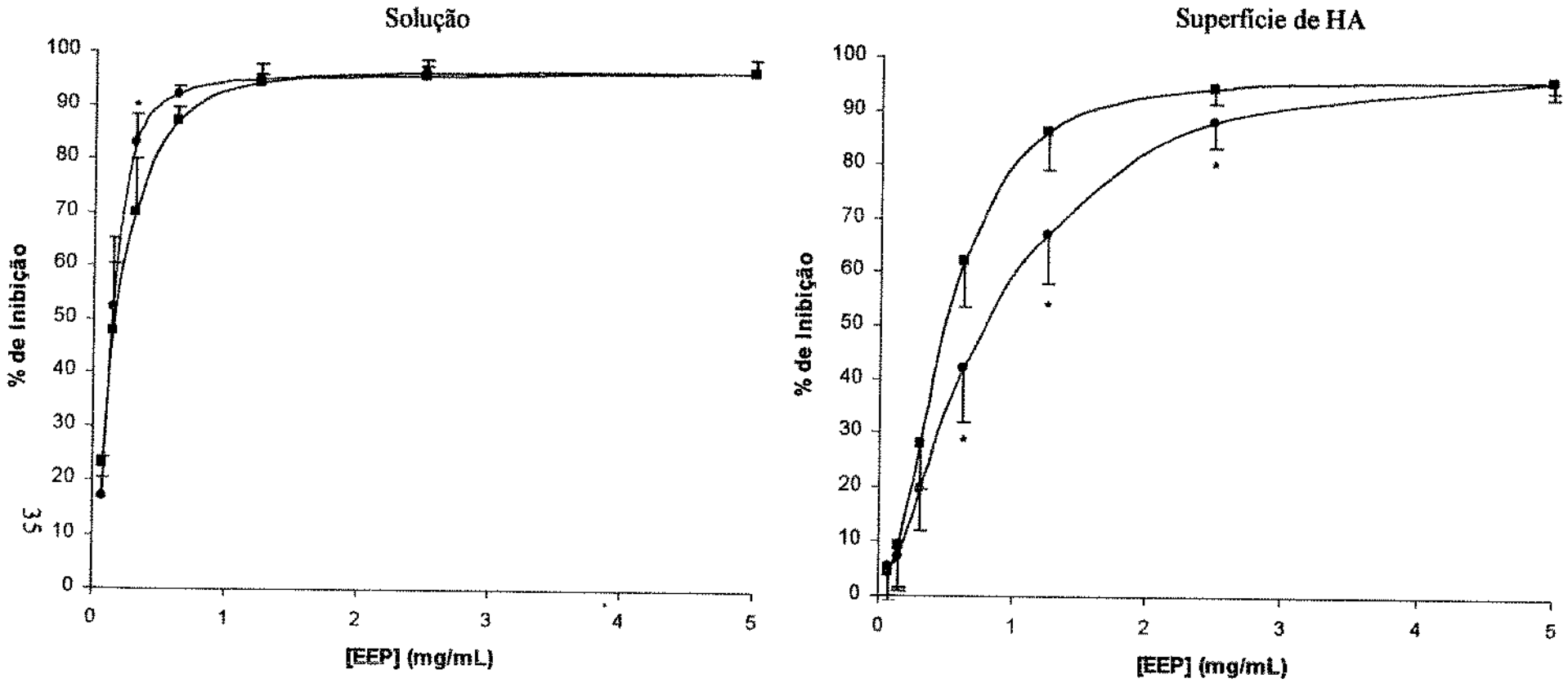

Figura 11. Efeito de extrato etanólico da própolis (EEP) sobre a atividade da glucosiltransferase D (Gtf D) em solução e aderida a superfícies de HA. Símbolos: —●— EEP RS (Região Sul); —■— EEP MG (Região Sudeste).

[1] A porcentagem de inibição foi calculada considerando o controle como a atividade máxima da Gtf. O controle foi o etanol 80% (v/v).

[2] Os valores apresentados são a média de 6 replicatas ± D.P.

[3] Os valores seguido por um asterisco (*) entre os tratamentos, em cada concentração, são significantemente diferente entre si, t de Student, $p<0,05$.

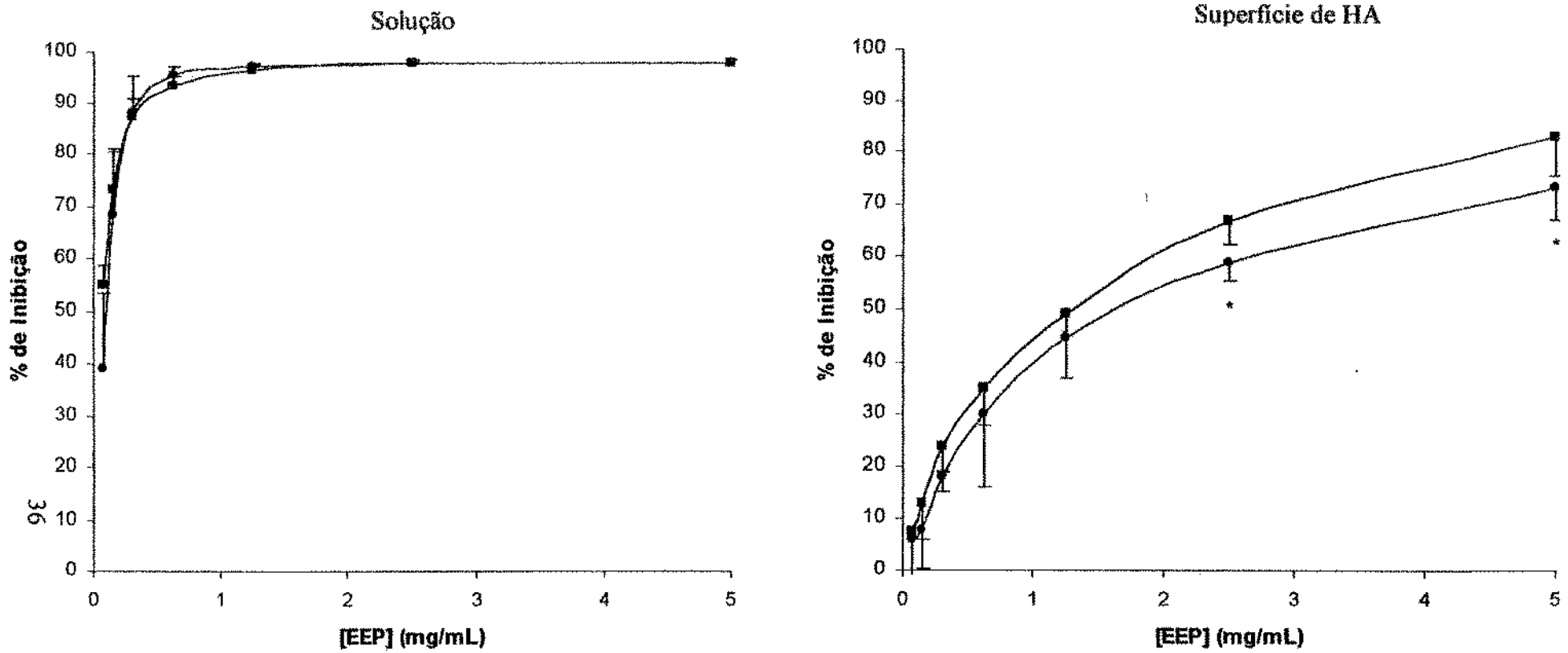

Figura 12. Efeito de extrato etanólico da própolis (EEP) sobre a atividade da glucosiltransferase G (Gtf G) em solução e aderida a superfície de HA. Símbolos: —●— EEP RS (Região Sul); —■—EEP MG (Região Sudeste).

[1] A porcentagem de inibição foi calculada considerando o controle como a atividade máxima da Gtf. O controle foi o etanol 80% (v/v).

[2] Os valores apresentados são a média de 6 replicatas ± D.P.

[3] Os valores seguido por um asterisco (*) entre os tratamentos, em cada concentração, são significantemente diferente entre si, t de Student, $p<0,05$.

e da Gtf G nas concentrações entre 2,5 e 5,0 mg/mL, como ilustrado nas figuras 11 e 12. Vale ressaltar que ambos EEPs não reduziram a atividade da Gtf G aderida na mesma intensidade observada quando a mesma enzima estava em solução. Por exemplo, na concentração de 5,0 mg/mL a inibição foi apenas de 73 ± 6 e 83 ± 7% para EEP RS e MG respectivamente. Em solução ambos EEPs inibiram em torno de 95% nas concentrações entre 0,625 e 5,0 mg/mL. De modo geral, apesar da diminuição da eficácia da própolis, ambos os EEPs foram capazes de inibir em mais de 90% a atividade de todas as Gtfs em superfície na concentração de 5,0 mg/mL, com exceção da Gtf G.

4.4.2. Inibição pela quercetina das Gtfs purificadas, em solução e aderidas a superfície de HA

A quercetina reduziu a atividade de todas as Gtf em solução de uma maneira dose-dependente (r^2 de 0.910 a 0.999), como ilustrado na figura 13. Entretanto, o grau inibição da atividade enzimática foi variável dependendo da Gtf testada. As Gtf B e C foram inibidas 20-60% quando a concentração de quercetina foi entre 62.5 to 250 μg/mL, demostrando curvas de inibição semelhante entre estas Gtfs. Por outro lado, Gtf D e G demonstraram um perfil inibitório diferente daqueles obtidos para Gtf B e C. A inibição de Gtf D foi de 35-50%, enquanto que a atividade de Gtf G foi reduzido mais de 90% nas concentrações entre 62,5 to 250 μg/mL. Quercetina inibiu fortemente a atividade de Gtf G em solução, onde 40% a 60% de inibição foi observada entre 7,8 and 15,6 μg/mL.

O perfil inibitório das Gtfs aderidas pela quercetina foram diferentes das mesmas em solução como demonstrado na figura 13. Dentre as Gtfs testadas, apenas Gtf B e C foram moderadamente inibidas pela quercetina (20-40% de inibição para Gtf B e 15-25% para Gtf C nas concentrações entre 62,5-250 μg/mL). As atividades das Gtf D e Gtf G não foram reduzidas pela quercetina mesmo na concentração mais alta (250 μg/mL). Assim, embora quercetina fosse um potente inibidor de Gtf G em solução o mesmo composto não foi capaz de inibir esta enzima quando aderida na película experimental. De modo geral, todas as Gtfs aderidas sobre HAS mostraram um aumento da resistência para inibição pela quercetina, particularmente Gtf D e G.

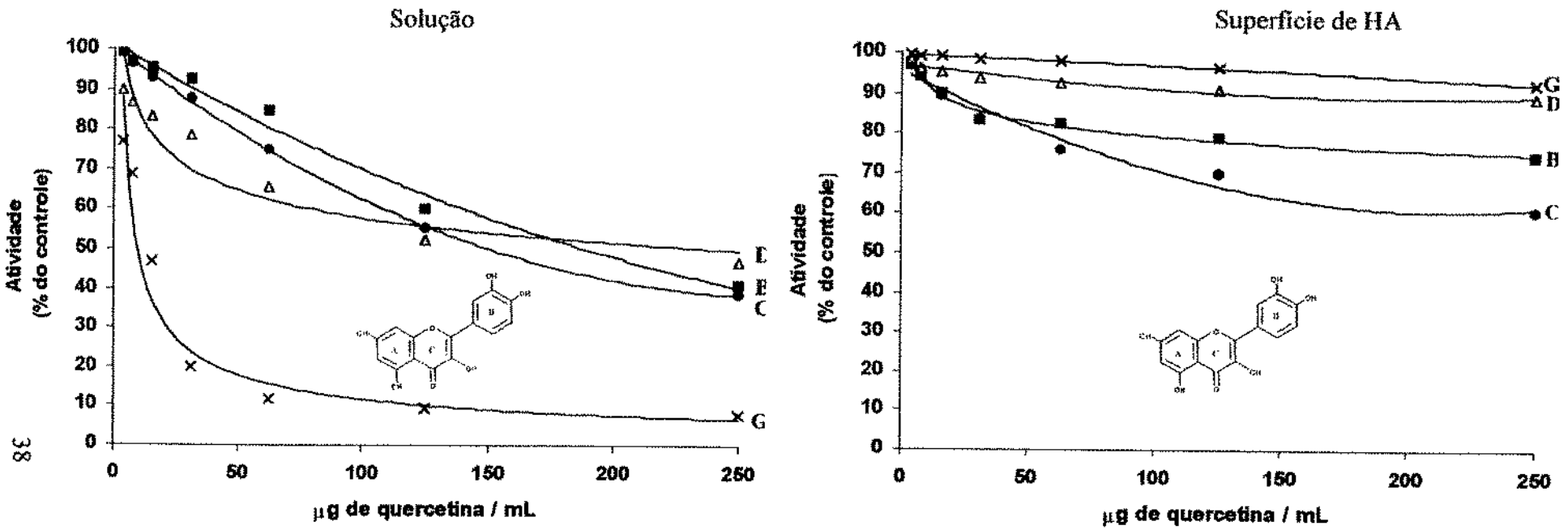

Figura 13. Efeito da quercetina sobre a atividade das glucosiltransferases (Gtfs) em solução e aderidas a superfície de HA.

[1] A porcentagem da atividade da Gtf foi calculado considerando o controle (etanol 95%) como atividade enzimática máxima.

[2] Os valores mostrados são médias de 6 replicatas.

[3] Equação das curvas das Gtfs em solução: Gtf B: $y = 0,0009x^2 - 0,4671x + 100,61$ / $r^2 = 0,9993$; Gtf C: $y = 0,0005x^2 - 0,3576x + 101,32$ / $r^2 = 0,9879$; Gtf D: $y = 123,23x^{-0,1643}$ / $r^2 = 0,9108$; Gtf G: $y = 205,84x^{-0,6253}$ / $r^2 = 0,9533$

[4] Equação das curvas das Gtfs aderidas a superfície de HAS: Gtf B: $y = 106,48x^{-0,0642}$ / $r^2 = 0,9831$; Gtf C: $y = 119x^{-0,113}$ / $r^2 = 0,9606$; Gtf D: $y = 0,0002x^2 - 0,0802x + 97,154$ / $r^2 = 0,909$; Gtf G: $y = -7E\text{-}05x^2 - 0,0166x + 99,368$ / $r^2 = 0,9963$

4.5. Inibição pelo EEP da Aderência de *S. mutans* e *S. sobrinus* sobre HA

A figura 14 ilustra a inibição da aderência de *S. mutans* GS-5 e *S. sobrinus* 6715 sobre o glucano formado pelo Gtf C. Os EEPs inibiram a aderência de ambas as cepas, sendo que o *S. mutans* foi mais afetado que o *S. sobrinus*. Embora a amostra RS demonstrasse melhor perfil de inibição em ambas as cepas, esta apenas diferiu do EEP MG nas concentrações de 1,25 mg/mL, para o *S. mutans* e 1,25 - 2,5 mg/mL, para o *S. sobrinus*.

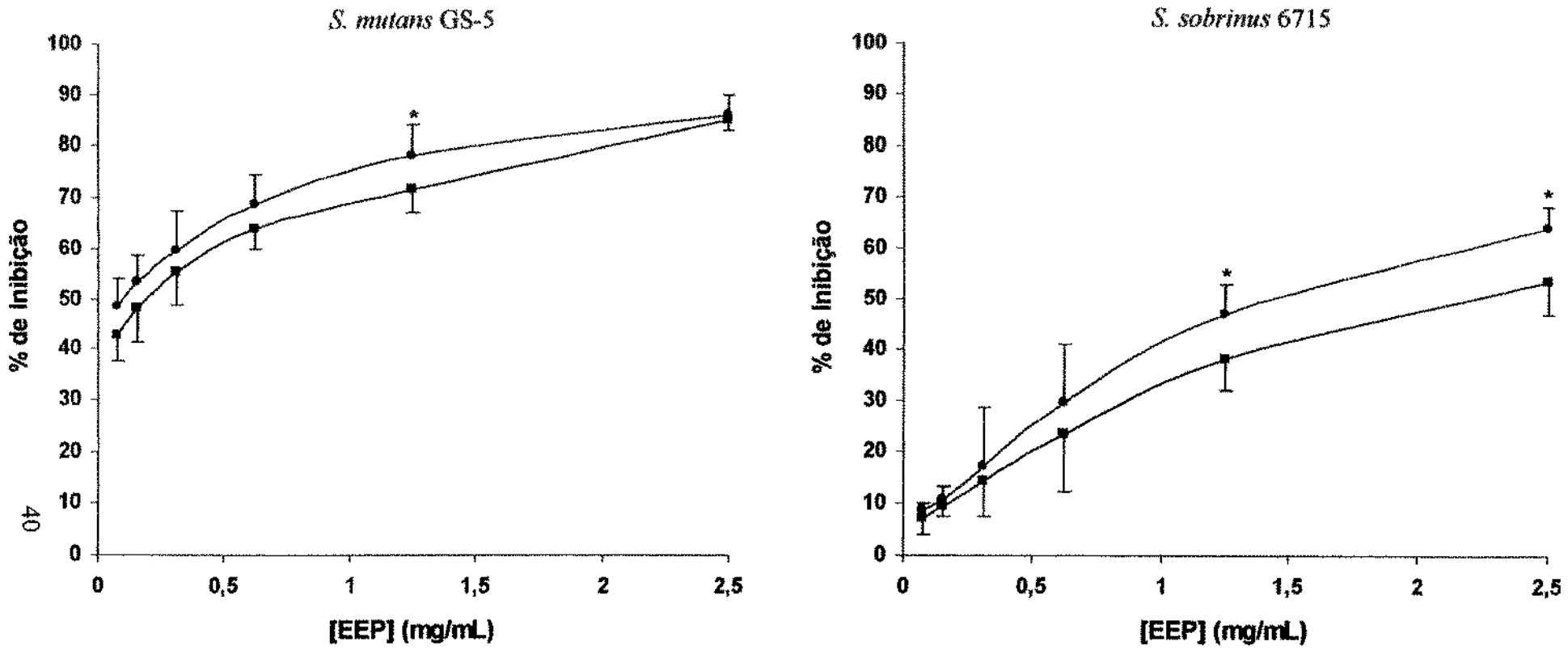

Figura 14. Efeito de extrato etanólico da própolis sobre a aderência de *S. mutans* GS-5 e *S. sobrinus* 6715 sobre o glucano formado por Gtf C aderida a superfície de HA. Símbolos: —●— EEP RS (Região Sul); —■— EEP MG (Região Sudeste).

[1] As bactérias foram marcadas com ^{3}H-timidina.

[2] A porcentagem de inibição foi calculada considerando o controle como máximo de aderência das bactérias estudadas. O controle foi o etanol 80% (v/v).

[3] Os valores apresentados são a média de 6 replicatas ± D.P.

[4] Os valores seguido por um asterisco (*) entre os tratamentos, em cada concentração, são significantemente diferente entre si, t de Student, $p<0,05$.

4.6. Efeito do EEP sobre o Desenvolvimento de Cárie em Ratos Desalivados

Os pesos ganhos e o número de refeições consumidas pelos animais não diferiram estatisticamente entre os grupos experimentais. Na tabela 3 estão apresentados a porcentagem de infecção por *S. sobrinus* 6715. Embora os grupos tratados com própolis apresentassem uma menor porcentagem de infecção por *S. sobrinus*, esta não diferiu estatisticamente do controle (p=0,232). Os resultados do índice de cáries estão demonstrados nas tabelas 4 e 5.

Tabela 3. Efeito do extrato etanólico da própolis (EEP) sobre a porcentagem de *Streptococcus sobrinus* 6715: média (D.P.).

Tratamentos	Porcentagem (%) de infecção por *S. sobrinus**
Etanol 80% (controle)	17,57 (12,68)
EEP RS (Região Sul)	10,49 (8,21)
EEP MG (Região Sudeste)	11,30 (7,48)

* Não houve diferença estatisticamente significante entre os tratamentos, p=0.232, ANOVA, comparação de todos os pares através de Tukey-Kramer HSD.

Tabela 4. Efeito do EEP sobre o desenvolvimento de cáries (superfície lisa e sulco) em ratos desalivados: média (D.P.) escore de Keyes.

Tratamentos	Superfície lisa	Sulco
Etanol 80% (controle)	88,10 (12,07) [a]	53,10 (2,96) [a]
EEP MG	75,22 (10,84) [ca]	49,56 (3,91) [ca]
EEP RS	65,60 (13,81) [bc]	47,10 (2,85) [bc]

Tratamentos, cujas médias são seguidas de mesma letra (na vertical), não diferem estatisticamente entre si, p<0,01, ANOVA, Tukey-Kramer HSD, comparação de todos pares.

Na tabela 4 estão demonstrados os índices de cárie da superfície lisa e de sulco. Os animais tratados com EEP RS apresentaram os menores índices para ambos os tipos de cárie e foram estatisticamente diferente do grupo controle. Entretanto, os animais tratados com EEP MG não sofreram redução significativa da cárie de superfície lisa e de sulco quando comparado com controle. A severidade das lesões da superfície lisa e de sulco estão demonstradas na tabela 5.

Tabela 5. Efeito do EEP na severidade de cáries (superfície lisa e sulco) em ratos desalivados: média (D.P.), índice de Keyes.

Tratamentos	Severidade da superfície lisa		Severidade do sulco	
	Ds	Dm	Ds	Dm
Etanol 80% (controle)	84,20 (12,50) [a]	11,00 (5,10) [a]	43,40 (2,95) [a]	14,90 (4,58) [a]
EEP MG	73,44 (10,03) [ca]	8,22 (4,71) [a]	39,78 (4,06) [b]	8,44 (3,78) [b]
EEP RS	61,60 (13,00) [bc]	3,20 (1,62) [b]	38,60 (2,22) [b]	4,80 (1,81) [b]

Tratamentos, cujas médias são seguidas de mesma letra (na vertical), não diferem estatisticamente entre si, $p<0,01$, ANOVA, Tukey-Kramer HSD, comparação de todos pares.

Assim como foi observado para a cárie de superfície lisa, apenas o EEP RS demonstrou menor severidade de cárie (Ds e Dm) do que os animais tratados no grupo controle. A severidade da cárie de sulco (Ds e Dm) foram significantemente menores nos animais tratados com ambos os EEPs quando comparado com o grupo controle. Deste modo, apenas os animais tratados com o EEP RS demonstraram redução da cárie de superfície lisa e de sulco, bem como menor severidade da cárie de superfície lisa.

4.7. Efeito de um Enxaguatório Bucal contendo Própolis sobre a Formação de Placa Dental *in vivo*

A tabela 6 mostra o índice de placa dos voluntários após os tratamentos com os enxaguatórios bucais. Os resultados demonstraram que houve uma redução de formação de placa dental da ordem de 44,7% pelo enxaguatório experimental (contendo própolis RS a 5%) quando

comparado com o placebo, sendo que esta redução foi estatisticamente significante ($p< 0,05$). A figura 15 ilustra os dados da tabela 6. Foi observado que o enxaguatório com própolis reduziu os índices de placa 2 e 3 ao mesmo tempo que aumentou a porcentagem de superfícies dentais livres de placa dental (índice 0).

Tabela 6. Índice de placa dental (I.P.) após os tratamentos. Média (desvio-padrão).

Tratamentos	I.P.	Diferença
Placebo	1,41 (0,14)	-
Experimental	0,78 (0,17)	44,7%*

* Diferença entre os tratamentos foi estatisticamente significante pelo teste não-paramétrico de Wilcoxon, $p< 0,05$.

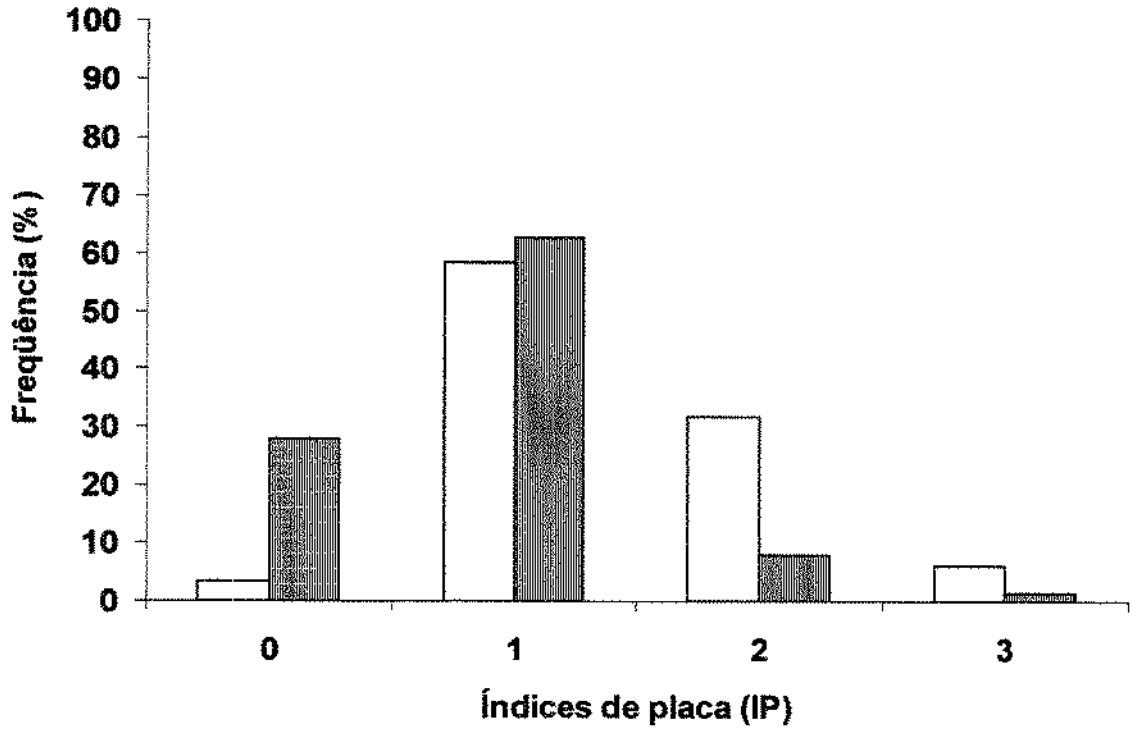

Figura 15. Freqüência relativa dos índices de placa - IP (0, 1, 2, 3) em função dos tratamentos. Símbolos: ☐ Placebo; ▥ Experimental.

DISCUSSÃO

5. DISCUSSÃO

Dentre os produtos naturais, a própolis de *Apis mellifera* tem tido grande destaque na literatura pelas suas propriedades biológicas, principalmente antimicrobiana, anti-inflamatória e anti-tumoral (GHISALBERTI, 1979; BANKOVA et al., 1989; MARCUCCI, 1995). Paralelamente, também houve um crescente interesse na identificação dos compostos ativos da própolis, onde têm sido isolado basicamente compostos fenólicos, principalmente flavonóides agliconas. Os flavonóides têm sido associado com as propriedades farmacológicas da própolis (GHISALBERTI, 1979; BANKOVA et al., 1982; GRANGE & DAVEY, 1990; BONHEVI et al., 1994), embora outros compostos fenólicos estejam também envolvidos, como os derivados do ácido cinâmico e seus ésteres (IKENO et al., 1991; AGA et al., 1994; TAZAWA et al., 1998), diterpenos (BANKOVA et al., 1996), entre outros. Entretanto a composição deste compostos biologicamente ativos é bastante variável dependendo da região de origem da própolis (KÖNIG, 1985; GREENAWAY et al., 1990; GARCÍA-VIGUEIRA et al., 1992; BANKOVA et al., 1992; TOMAS-BARBERAN et al., 1993; BONHEVI & COLL, 1994). As análises dos EEPs por CCDAE e CLAE demonstraram uma nítida diferença no perfil cromatográfico entre as amostras de MG e RS (PARK et al., 1997; 1998; PARK & IKEGAKI, 1998). Apigenina, ramnetina, crisina, galangina e tectocrisina foram identificados apenas no EEP do RS. Por outro lado, EEP de MG apresentou isoramnetina quer não foi encontrada no EEP do RS. Em acréscimo, em ambos EEPs foram identificados a quercetina e o ácido 4-hidroxicinâmico (ácido *p*-coumárico). A análise quantitativa demonstrou que a própolis do RS apresentava maiores concentrações de pinocembrina, crisina, acacetina e galangina, sendo que a própolis de MG demonstrou maior concentração de kaempferol e kaempferide. Deste modo, a composição dos flavonóides agliconas foi qualitativamente e quantitativamente diferente entre as amostras da região Sudeste e Sul, provavelmente devido a diferença da biodiversidade entre as 2 regiões.

Os EEPs demonstraram que foram capazes de inibir significantemente todos os grupos de microrganismos testados, inclusive os anaeróbios estritos periodontopatogênicos, como *P. gingivalis*, verificados através do método de difusão em ágar. Os estreptococos do grupo mutans e *S. aureus* também demonstraram formação de zonas de inibição de crescimento, confirmando os resultados dos estudo prévios (LINDENFELSER, 1967; GRANGE & DAVEY, 1990; IKENO et al., 1991; BONHEVI, 1994; BANKOVA et al., 1995; STEINBERG et al., 1996; PARK et al.,

1998; KUJUMGIEV et al., 1999). O tamanho da zona de inibição variou dependendo das diferentes cepas testadas, embora estes dados não signifiquem obrigatoriamente que um microrganismo é mais susceptível que o outro. Os dados obtidos com a determinação do CIM e CBM confirmaram os resultados obtidos da determinação das zonas inibitórias de crescimento, onde o EEP RS demonstrou atividade tanto bacteriostática como bactericida superior quando comparado com o EEP MG. Alguns flavonóides encontrados na própolis são considerados agentes antimicrobianos como pinocembrina, galangina, sakuranetina e pinobanksina (VILLANUEVA et al., 1969; VILLANUEVA et al., 1970; METZNER et al., 1979; DIMOV et al., 1992). Em acréscimo, kaempferol e miricetina, isolado de *Syzygium aromaticum*, apresentaram forte atividade antimicrobiana sobre *P. gingivalis* e *P. intermedia*, entretanto foi observado apenas um efeito moderado sobre o crescimento de *S. mutans* e *A. viscosus* (CAI & WU, 1996). Dentre os flavonóides citados, galangina, pinocembrina, kaempferol e sakuranetina foram identificados nas amostras analisadas. Entretanto, galangina foi encontrado apenas no EEP de RS e a concentração de pinocembrina foi 9 vezes maior nesta própolis do que aquela obtida em MG. Por outro lado, a concentração de kaempferol foi 4 vezes maior no EEP MG quando comparado com EEP RS. Esta variabilidade quantitativa e qualitativa dos flavonóides antimicrobianos poderia ser um dos fatores que sugeririam as diferenças das atividades antibacterianas sobre os patógenos bucais pelos EEPs, particularmente os estreptococos do grupo mutans. Entretanto, nenhuma conclusão pode ser definida uma vez que outros flavonóides e compostos fenólicos podem estar envolvidos. Por exemplo, recentes relatos na literatura têm demonstrado que os derivados de ácido cinâmico, como ácido 3,5-diprenil-4-hidroxicinâmico (AGA et al.,1994; TAZAWA et al., 1998), bem como alguns derivados de diterpenos (BANKOVA et al., 1996) encontrados na própolis brasileira apresentam atividade antimicrobiana.

O mecanismo de ação antimicrobiana da própolis é complexo e ainda não está completamente elucidado. De acordo com BONHEVI et al. (1994) e AMOROS et al. (1992), a atividade inibitória da própolis sobre o crescimento microbiano está mais relacionado com o efeito sinérgico dos flavonóides (e outros fenólicos) do que compostos individuais. Esta observação está em acordo com os resultados obtidos por TAKAISIKIKUNI & SCHILCHER (1994), onde os autores relataram que a atividade antibacteriana sobre *S. agalactiae* era complexa envolvendo uma série de mecanismos como: formação multicelular de pseudo

estreptococos; desorganização do citoplasma, da membrana citoplasmática, e da parede celular; bacteriólise parcial; e inibição da síntese protéica. Assim, os autores concluíram que não era possível fazer uma analogia com nenhum dos antibióticos clássicos conhecidos. Dentro deste contexto, o efeito sinérgico dos compostos ativos da própolis EEP RS foi mais efetivo do que aqueles de MG para inibir o crescimento de *S. mutans* e *S. sobrinus*.

A aderência celular de *S. mutans* e *S. sobrinus* na presença de sacarose foi efetivamente inibida por ambos EEPs. Embora a atividade antibacteriana sobre estas cepas explicassem em parte a inibição da aderência sobre a superfície de vidro, a síntese *de novo* de glucanos a partir da sacarose pelas Gtfs tem um papel fundamental para mediar esta aderência sacarose-dependente. A síntese de GIA foi fortemente inibida pela própolis, mesmo em baixas concentrações. A GIA tem sido considerado o principal glucano responsável pela colonização e acúmulo de *S. mutans* e *S. sobrinus* na superfície lisa do esmalte dental (HAMADA & SLADE, 1980, RÖLLA et al., 1983; LOESCHE, 1986). Este dado indica que os EEPs estão inibindo em solução a atividade das Gtfs responsáveis pela síntese deste glucanos insolúveis. Deste modo, o EEP RS que demonstrou maior efetividade tanto da atividade antibacteriana como da inibição da síntese de GIA, também apresentou melhores resultados da inibição da aderência celular sobre a superfície de vidro.

Os dados preliminares obtidos *in vitro* sugeriram que o EEP pode ter um efeito biológico *in vivo* na prevenção da formação da placa dental cariogênica, uma vez que houve inibição do crescimento dos estreptococos do grupo mutans, da aderência celular de *S. mutans* e *S. sobrinus* e da síntese de GIA. Dentre estes dados, vale destacar o potente efeito inibitório dos EEPs na síntese da GIA pela glicosiltransferase bruta. Entretanto, os dados obtidos foram feito com um preparado enzimático bruto e analisando apenas o que ocorre em solução. Porém, dentro do contexto atual, as enzimas aderidas na película desempenham um papel primordial na aderência de *S. mutans* e *S. sobrinus*, e consequentemente, formação e acúmulo de placa dental cariogênica. Em acréscimo, as enzimas aderidas demonstraram maior resistência aos principais inibidores enzimáticos sintéticos conhecidos (VACCA SMITH & BOWEN, 1997; WUNDER & BOWEN, 1999). Assim, a simples inibição da síntese de GIA em solução não significa que isto necessariamente irá ocorre com as enzimas aderidas na película.

Assim, com intuito de analisar mais detalhadamente a ação da própolis sobre a formação dos glucanos pela glucosiltransferase, as Gtfs individuais e purificadas foram testadas quanto a susceptibilidade aos EEPs quando estas estavam em solução e aderidas a superfície de

hidroxiapatita (HA). Os resultados obtidos demonstraram que os EEPs das duas regiões reduziram efetivamente a atividade de todas as Gtfs testadas em solução e aderidas sobre uma película experimental formada sobre HA. Entretanto, as duas amostras de EEP mostraram diferente padrão de inibição da atividade das Gtfs, além de ser evidente que ambos EEPs afetaram diferentemente as Gtfs quando estas estavam em solução e na superfície de HA. Em geral, o EEP RS inibiu as atividades das Gtfs B e C mais eficientemente que o EEP de MG. O EEP MG, porém, demonstrou maior efetividade sobre as Gtfs D e G aderidas sobre a superfície de HA.

As Gtfs B e C são as glucosiltransferases mais importantes relacionadas a cárie dental (YAMASHITA et al., 1993). O glucano produzido por estas enzimas aumentaram a aderência de *S. mutans* e *S. sobrinus* sobre uma película experimental formada sobre a hidroxiapatita (VENKITARAMAN et al., 1995). Particularmente, a inibição da Gtf C é de extrema importância uma vez que é a Gtf predominante da superfície apatítica, demonstrando maior afinidade sobre HA dentre todas as Gtfs (VACCA-SMITH et al., 1996; VACCA SMITH & BOWEN, 1998). A maioria dos inibidores de Gtfs (incluindo as formulações comerciais), analisados através do mesmo modelo experimental do presente estudo, não mostraram redução da atividade da Gtf C (VACCA SMITH & BOWEN, 1997; WUNDER & BOWEN, 1999). A própolis inibiu esta enzima antes (ou seja em solução) ou depois de aderir a superfície da película, reduzindo assim eficientemente a atividade da Gtf C. Este nível de inibição da Gtf C não foi observada mesmo quando 3 diferentes classes de inibidores enzimáticos sintéticos foram testados utilizando o mesmo modelo experimental realizado neste estudo (WUNDER & BOWEN, 1999).

A função das Gtfs D e G na formação da placa dental ainda não está completamente elucidada, entretanto têm sido considerado que os glucanos sintetizados por estas enzimas poderiam servir como "primer" para outras Gtfs, como a B e C (KOGA et al., 1983; Keevil et al., 1984; GILMORE et al., 1993; VENKITARAMAN et al., 1995).

Em acréscimo, foi observado que o efeito inibitório do EEP na atividade da Gtf diminui da solução para superfície. Isto se deve pela mudança conformacional que as enzimas sofrem quando imobilizadas em superfícies sólidas (MANJON et al., 1984; STEVENATO et al., 1989), podendo levar a alterações das suas propriedades físicas e cinéticas. Este fenômeno tem sido observado para as Gtfs (SCHILLING & BOWEN, 1988; VENKITARAMAN et al., 1995).

Assim, esta nova conformação estrutural das Gtfs tornaram-nas mais resistentes ao EEP, particularmente Gtf G.

Os possíveis compostos biologicamente ativos da própolis que estariam modulando a inibição das Gtfs ainda são desconhecidos. Entretanto, têm sido amplamente relatado que flavonóides são potentes inibidores enzimáticos (HAVSTEEN, 1983; MIDDDLETON & KANDASWAMI, 1994; HOLLMAN, 1997). Dentre elas, flavonas e flavonols, os quais possuem ligação dupla entre os carbonos C2-C3, têm sido considerados os principais flavonóides relacionados com a inibição enzimática (WHEELER & BERRY, 1986; FERRIOLA et al., 1989; MERLOS et al., 1991; EATON et al., 1996). Dentre elas, quercetina, galangina, kaempferol, kaempferide (flavonols); e crisina, apigenina e acacetina (flavonas) foram identificadas nos EEPs analisados neste estudo. Este flavonóides podem estar envolvidas na inibição das Gtfs pela própolis. Entretanto, o único relato na literatura avaliando o efeito de flavonóides sobre as Gtfs foi o trabalho realizado por IIO et al. (1984). Os autores demonstraram que a quercetina inibiu a atividade da Gtf em solução, entretanto foi utilizado um preparado enzimático bruto e sem levar em consideração as Gtfs aderidas.

Neste estudo a quercetina demonstrou inibição de todas as Gtfs em solução em concentrações de μM, podendo ser um dos compostos ativos da própolis para a inibição da Gtf G em solução. A própolis inibiu em torno de 95% a atividade de Gtf G em solução na concentração 5,0 mg/mL. Nesta mesma concentração, a quantidade de quercetina é entre 10 e 20 μg/mL. A Gtf G foi inibida pela quercetina 40-80 % nas concentrações entre 7,8 e 31,3 μg/mL em solução. Entretanto, a sua contribuição para a inibição das Gtf B, C e D em solução foi mínima, além de não ter efeito sobre todas as Gtfs aderidas nas concentrações entre 7,8 e 31,3 μg/mL. É evidente que a presença de quercetina não explica o potente efeito inibitório da própolis sobre as Gtfs.

A eficiente inibição da Gtf C aderida na película experimental, e consequentemente na síntese de glucano, refletiu na aderência dos *S. mutans* GS-5 e *S. sobrinus* 6715 sobre a superfície de HA. Ambas as cepas expressam GBPs - "glucan binding proteins" que apresentam uma alta afinidade por glucanos, intermediando assim a aderência destas bactérias com estes polissacarídeos (RUSSEL, 1979; McCABE & HAMELIK, 1978; MOOSER & WONG, 1988). Em acréscimo, os *S. mutans* também expressam adesinas que permitem uma aderência específica com a película adquirida numa interação do tipo lectina (CLARK & GIBBONS, 1977; GIBBONS, 1984; JENKINSON & LAMONT, 1997). Entretanto, os dados neste estudo não

permitem concluir se a própolis interagiu ou não com os GBPs e/ou as adesinas do *S. mutans*. Outro ponto importante a ser elucidado em estudos futuros é avaliar um possível efeito da própolis na estrutura dos glucanos sintetizados pelas Gtfs aderidas a HA. Assim, apenas podemos sugerir que um dos fatores responsáveis pela diminuição da aderência celular de *S. mutans* e *S. sobrinus* a superfície de HA seja a inibição da formação de glucanos sobre a película pela Gtf C.

O modelo experimental de cárie dental em ratos utilizados neste estudo promoveu um severo desafio cariogênico pela desalivação dos ratos e uma alimentação com dieta cariogênica contendo 56% de sacarose (dieta 2000). Além disso, a freqüência de alimentação da dieta 2000 pelos ratos foi controlada, uma vez que é reconhecido que o nível de cáries está diretamente relacionada com a freqüência de ingestão da sacarose (GUGGENHEIM & REGOLATI, 1972; NEWBRUN & FROSTELL, 1978; BOWEN et al., 1980). Como não houve diferença estatisticamente significante entre o número de refeições consumidas pelos animais entre os grupos de tratamentos, pode se considerar que os animais foram submetidos ao mesmo desafio cariogênico. Esta é uma variável crítica nos modelos de alimentação da dieta *ad libitum*. Embora ambas as amostras demonstrassem redução da percentagem de infecção por *S. sobrinus*, não houve diferença estatisticamente significante em relação ao controle. Os resultados deste experimento demonstraram que apenas o EEP do RS foi capaz de reduzir a cárie de superfície lisa, além de diminuir a severidade destas, principalmente em nível Dm (dentina moderada, ou seja, dentina exposta) com 74,5% de redução quando comparado com controle. A cárie de sulco foi reduzida apenas pelo EEP do RS, embora ambos reduzissem igualmente a severidade desta cárie. Vale salientar que em todos os parâmetros avaliados o EEP de MG apresentou uma tendência de redução de cárie dental. Estes dados estão em acordo com aqueles obtidos *in vitro*, onde a própolis do RS apresentou os melhores resultados nos parâmetros avaliados, particularmente inibição das Gtfs B e C aderidas na película experimental. Ressalta-se que este é um modelo onde os animais são submetidos a um desafio cariogênico extremamente severo, e que provavelmente em condições de menor severidade o efeito da própolis poderia ser ainda mais evidente.

Assim, as amostras de própolis demonstraram em estudos *in vitro* e em animais que apresentam propriedades anti-placa e anti-cárie. Porém, o seu efeito variou dependendo da origem da própolis. A amostra RS apresentou resultados significantemente superiores que o MG, embora ambos demonstrassem potencial biológico nos parâmetros avaliados. Deste modo, o EEP

RS foi selecionado para o estudo *in vivo*, analisando o efeito de um enxaguatório bucal contendo esta amostra de própolis sobre a formação da placa estimulada por sacarose.

A redução de placa observada foi da ordem de 44,7% mesmo em condições de alto desafio induzido pelo bochecho com sacarose a 20%. Assim os dados comprovam a eficácia demonstrada *in vitro* e em animais pela amostra RS. Estes resultados são promissores uma vez que o enxaguatório bucal foi baseado num preparado bruto da própolis. A identificação dos compostos ativos seria fundamental para determinar o(s) composto(s) responsáveis pelas propriedades anti-placa.

Os resultados deste estudo demonstraram que ambas as amostras de própolis possuem grande potencial na redução da cárie e placa dental, embora haja a necessidade de estudos *in situ* e clínicos longitudinais tanto em termos de formação da placa como cárie dental para uma conclusão definitiva. Um dos possíveis mecanismos anti-cárie e anti-placa da própolis seria a eficiente inibição da atividade das Gtfs B e, principalmente, C. A ação antibacteriana da própolis também pode estar envolvida, embora não tenha sido constatado o seu efeito no estudo em animais. Entretanto o EEP RS apresentou resultados significantemente superiores que o EEP MG. Esta diferença pode ser explicado pela variabilidade qualitativa e quantitativa da composição química entre as duas amostras de própolis, como descrito anteriormente (PARK et al., 1997; PARK et al., 1998; PARK & IKEGAKI, 1998). Dentro deste contexto, a grande variabilidade da própolis brasileira é ao mesmo tempo interessante, do ponto de vista científico, e preocupante, para a padronização desta substância natural e o conseqüente uso terapêutico. Devemos lembrar que as amostras RS e MG foram previamente selecionadas de 400 amostras de própolis subdivididas em 8 grupos diferentes, onde muitas amostras demonstraram pouca ou nenhuma atividade biológica *in vitro* que justificassem as suas analises posteriores tanto *in vitro* como *in vivo*. Deste modo, fica evidente alguns pontos importantes: 1 - Necessidade de estudos clínicos longitudinais; 2 - Necessidade de analisar os mecanismos de ação da própolis; 3 - Isolamento e identificação do(s) composto(s) ativo(s) da própolis; 4 - Necessidade de estudos para elucidar a variabilidade da composição química da própolis; 5 - A utilização da própolis deve ser estritamente controlada devido a sua grande variabilidade química.

CONCLUSÕES

6. CONCLUSÕES

Tendo em vista que as amostras de própolis demonstraram: 1) *in vitro* - a) atividade antimicrobiana sobre os patógenos bucais; b) inibição da aderência celular de *S. mutans* e *S. sobrinus* sobre a superfície de vidro; c) inibição da síntese dos glucanos insolúveis em água (GIA); d) inibição da atividade das Gtfs B, C, D e G em solução e aderidas na superfície da hidroxiapatita; e) inibição da aderência de *S. mutans* e *S. sobrinus* sobre o glucano formado por Gtf C; 2) em animais - redução da cárie dental em ratos desalivados; 3) *in vivo* - redução do índice de placa por enxaguatório bucal contendo própolis RS, conclui-se que:

1 - A própolis apresenta potencial anti-cárie e anti-placa, porém o seu efeito biológico variou entre as amostras provenientes de duas regiões do Brasil. O extrato etanólico da própolis - EEP RS demonstrou resultados significantemente superiores quando comparado com o EEP MG.

2 - A própolis apresenta componentes biologicamente ativos mediando o efeito anti-placa e anti-cárie observada neste estudo. A quercetina, 3, 3', 4', 5, 7 - pentahidroxiflavona, demonstrou ser um dos compostos ativos na inibição da Gtf G em solução. É extremamente desejável o isolamento e a identificação destes compostos bioativos.

3 - As diferenças das atividades biológicas nos parâmetros avaliados pode ser atribuída a diferença qualitativa e quantitativa da composição química entre os EEPs como descrito anteriormente (PARK et al., 1997; PARK et al., 1998; PARK & IKEGAKI, 1998).

4 - Os resultados obtidos sugerem que um dos mecanismos principais para a redução de cárie dental em ratos e da placa dental em humanos é a inibição eficiente das Gtfs B e C em solução e, principalmente aderidas a película experimental formada sobre a hidroxiapatita.

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ANEXOS

Anexo 1 - Artigos Publicados / Aceitos e Submetidos

In vitro *antimicrobial activity of propolis and* Arnica montana *against oral pathogens*

H.Koo[1], B.P.F.A. Gomes[1], P.L. Rosalen[1], G.M.B. Ambrosano[1], Y.K. Park[2], J.A. Cury[1]

School of Dentistry of Piracicaba[1] and School of Food Engineering[2],
State University of Campinas, Piracicaba , SP, Brazil.

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Key words: propolis, *Arnica montana,* microorganism, dental plaque

Corresponding author, to whom requests for reprints should be addressed:

Dr. Brenda P.F.A. Gomes
Faculdade de Odontologia de Piracicaba - FOP-UNICAMP
Av. Limeira, 901, Areião,
Piracicaba -S.P.
Brazil
Caixa Postal 52. CEP 13414-900.
tel: #55-19-430-5215
fax: #55-19-430-5218
e-mail: bpgomes@fop.unicamp.br

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Dr D. B. Ferguson
University of Manchester
School of Biological Sciences
G. 38 Stopford Building
Oxford Road
Manchester M13 9PT

Your manuscript entitled "In vitro antimicrobial activity of propolis and ..."

has been accepted for publication and was forwarded to the publisher on 13.8.99

A reprint order form will be sent with the galley proof.

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Editor in Chief

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Summary

Arnica and propolis have been used for thousands of years in folk medicine for several purposes. They have been shown to possess several biological activities such as antiinflammatory, antifungal, antiviral and tissue regenerative activities, among others. Although the antibacterial activity of propolis has already been demonstrated for different bacteria, very few studies were done on bacteria of clinical relevance in dentistry. Also, the antimicrobial activity of *Arnica* has not been extensively investigated. Therefore the aim of this study was to evaluate *in vitro* the antimicrobial activity (AMA), inhibition of adherence (Adh) of mutans streptococci and inhibition of formation of water insoluble glucan (WIG) by *Arnica* and propolis extracts. *Arnica montana* (10%, w/v) and propolis (10%, w/v) extracts from Minas Gerais State were compared with their (-) controls. Fifteen microorganisms were used as follows: Candida albicans - NTCC 3736, F72; Staphylococcus aureus - ATCC 25923; Enterococcus faecalis - ATCC 29212; Streptococcus sobrinus 6715; Streptococcus sanguis - ATCC 10556; Streptococcus cricetus - HS-6; Streptococcus mutans - Ingbritt 1600; Streptococcus mutans – OMZ 175; Actinomyces naeslundii – ATCC 12104, W 1053; Actinomyces viscosus OMZ 105; Porphyromonas gingivalis; Porphyromonas endodontalis and Prevotella denticola (the last 3 were clinical isolates). AMA was determined by the agar diffusion method and the zones of growth inhibition were measured. To assess the cell adherence on a glass surface, the organisms were grown for 18 h at 37°C in test tubes at a 30° angle. To assay WIG formation, a mixture of crude glucosyltransferase and 0.125 M sucrose was incubated for 18 h at 37°C in test tubes at a 30° angle. *Arnica* and propolis extracts (20 µL) were added to these tubes to evaluate the % of inhibition of cell Adh and WIG formation. The propolis extract significantly inhibited all microorganisms tested ($p<0.05$), showing the highest inhibitory zone for Actinomyces spp. The *Arnica* extract did not demonstrate significant AMA. The cell adherence and WIG formation were almost completely inhibited by the propolis extract at a final concentration of 400 µg/mL and 500 µg/mL, respectively. Nevertheless, the *Arnica* extract showed a slight inhibition of Adh of the growing cells (19% for Strep. mutans and 15% for Strep. sobrinus) and of WIG formation (29%) at these same concentrations. In conclusion, the propolis extract showed *in vitro* antibacterial activity, inhibition of cell adherence and inhibition of water insoluble glucan formation, while the *Arnica* extract demonstrated only slight activities for these three parameters.

1. Introduction

Dental plaque is a film of microorganisms found on the dental surface which plays an important role in the development of dental caries and periodontal disease (Marsh, 1992). Mutans streptococci can colonise the tooth surface and initiate plaque formation by their ability to synthesise extracellular polysaccharides from sucrose, mainly water-insoluble glucan, using glucosyltransferase enzyme (Gibbons and Van Houte, 1975; Hamada and Slade, 1980; Tanzer et al. 1985). This sucrose-dependent adherence and accumulation of cariogenic streptococci are critical processes in the development of pathogenic dental plaque related to caries. The further accumulation of dental plaque around the gingival margin and subgingival region may lead to a shift in the microbial composition of plaque from a streptococcus-dominated microflora to a higher level of Actinomyces spp. and an increased number of capnophilic and obligatory anaerobic bacteria, such as Porphyromonas gingivalis (Marsh, 1994). These microorganisms seem to be involved in root caries and periodontal disease, respectively (Schüpbach et al., 1995; Slots and Rams, 1992). Therefore, antimicrobial agents against these oral pathogens could play an important role in the prevention of dental caries and periodontal disease, particularly those that can affect dental plaque formation.

Natural products have been used for thousands of years in folk medicine for several purposes. Among them, propolis has attracted increased interest due to its antimicrobial activity against a wide range of pathogenic microorganisms. Propolis is a resinous hive product collected by *Apis mellifera* bees from tree buds and mixed with secreted beeswax. It is known that the ethanolic extract of propolis (EEP) shows some pharmacological activities, such as anti-inflammatory, anaesthetic and cytostatic properties, besides antimicrobial activity (Ghisalberti, 1979; Bankova et al., 1989; Grange and Davey, 1990). It was observed previously that the EEP showed antibacterial activity against Streptococcus mutans (Ikeno et al., 1991; Steinberg et al., 1996; Park et al., 1998). However, little is know about the antimicrobial activity of propolis against others oral pathogens and, mainly, its effects on dental plaque formation *in vitro*. Another natural product that has many applications in phytotherapy is *Arnica montana*, a perennial herbaceous plant with creeping roots whose extracts possess anti-inflammatory and cytotoxic properties (Woerdenbag et al., 1994; Lyss et al., 1997). The antimicrobial activity of *Arnica montana* has not been thoroughly investigated.

Considering that only few studies have been reported on the *in vitro* effect of propolis and *Arnica montana* extracts against oral pathogens and dental plaque formation, the aim of the present investigation was to evaluate *in vitro* the antimicrobial activity of these extracts on some oral microorganisms, especially their effects on adherence of mutans streptococci and inhibition of water insoluble glucan formation.

2. Materials and methods

2.1. Microorganisms

The following fifteen microorganisms were used in this study: Staphylococcus aureus ATCC 25923, Enterococcus faecalis ATCC 29212, Candida albicans NTCC 3736, Candida albicans F72, Streptococcus sanguis ATCC 10556, Strep. mutans Ingbritt 1600, Strep. mutans OMZ-175, Strep. sobrinus 6715, Strep. cricetus HS-6, Actinomyces naeslundii ATCC 12104, Act. naeslundii W1053, Act. viscosus OMZ 105, Porphyromonas gingivalis, Porph. endodontalis and Prevotella denticola. The last three microorganisms and C. albicans F72 were isolated from clinical trials. The facultative anaerobes were kindly donated by the Center for Oral Biology, University of Rochester, NY, USA.

2.2. Ethanolic extracts of propolis and Arnica montana

Ethanolic extracts of propolis and *Arnica montana* were used in this study. The ethanolic extract of propolis (10%, w/v) from Minas Gerais state - south-eastern Brazil, was prepared as described previously (Koo and Park, 1997; Park et al., 1997) and the *Arnica* extract (10%, w/v) was obtained from the flowerheads of *Arnica* and prepared according to the Brazilian Pharmacopoeia (Farmacopéia Homeopática Brasileira, 1977).

2.3. *Antimicrobial activity assay*

The antimicrobial activity was determined by agar diffusion using Fastidious Anaerobe Agar (Lab. M, Burry, UK) plus 5% sheep-blood - FAAsb, Brain Heart Infusion - BHI (Difco, MA, USA) and Mueller Hinton - MH (Difco, MA, USA) agars. The microorganisms were seeded by pour plate, except the anaerobes.

Isolated 18-24h colonies of aerobes and facultatives grown on BHI agar were suspended in sterile 0.85% NaCl solution. The cell suspension was adjusted spectrophotometrically to match the turbidity of a McFarland 0.5 scale. A 400 μL aliquot of each test-microorganism suspension was mixed with 40 mL BHI agar at 45°C, and poured onto a previously set layer of MH agar (140 x 25 mm plate). For the anaerobes, isolated colonies were suspended to reach 1.0 on the McFarland scale. Sterile swabs were dipped into the bacterial suspension and inoculated onto pre-reduced FAAsb agar plates. The inoculum procedures were appropriate to provide a semi-confluent growth of the tested microorganisms.

Six sterilised (6) stainless steel cylinders of 8.0 x 10 mm (inside diameter 6mm) were placed onto each inoculated agar plate. The test extracts or control (80% aqueous ethanol, v/v) (40 μL) were applied inside the cylinders. The plates were kept for 2 hours at room temperature to allow the diffusion of the agents through the agar. Afterwards, the plates were incubated at 37°C in an appropriate gaseous condition and for an appropriate period of time: aerobes - 24 h, facultatives - 24 -48 h in a CO_2 incubator (10% CO_2), and anaerobes in the anaerobic work station (Don Whitley Scientific, Bradford, UK) in an atmosphere of 5-10% H_2, 10% CO_2 and 80-85% N_2 for 7 days. Zones of inhibition of microbial growth around the cylinder containing the extracts were measured and recorded after the incubation time. The inhibitory zone was considered the shortest distance (mm) from the outside margin of the cylinder to the initial point of the microbial growth. Six (6) replicates were made for each microorganism.

2.4. *Inhibition of adherence of growing cells to a glass surface*

To assess the adherence of growing cells of *S. mutans* Ingbritt 1600 and *S. sobrinus* 6715 to a glass surface, organisms were grown at 37 °C, at an angle of 30° for 18 h in test tubes, as

described by Hamada and Torii (1978). Individual 18-24 h colonies from BHI agar plates were suspended in 5.0 mL of sterile 0.85% NaCl, and the suspension adjusted to 0.5 on the McFarland scale. An aliquot of the suspension was mixed with BHI broth (1:100 dilution, v/v) containing 1% sucrose, and then 2.48 mL were transferred to a test tube. Subsequently, 20 µL of a two-fold dilution series of the test extracts (concentrations ranging from 6.2 to 400 µg/mL reaction) and their control (80% ethanol, v/v) was inoculated, gently stirred and then incubated. After incubation, the adhered cells were washed and suspended using the procedures outlined by Hamada and Torii (1978). The amount of adherent cells was measured at 550 nm - $O.D._{550}$ (Hamada and Torii, 1978). Six (6) replicates were made for each concentration of the test extracts.

2.5. Preparation of crude extracellular glucosyltransferase (Gtase)

Streptococcus sobrinus 6715 was grown in a medium containing 2.5% tryptone, 1.5% yeast extract, 0.3% glucose and 1.0% sorbitol which was ultrafiltered through a 5.0-kd molecular weight cut-off membrane (Low molecular weight medium - LMW) as described by Schilling and Bowen (1988).

Actively growing Strep. sobrinus 6715 was incubated in 200 mL of LMW at 37 °C, 10% CO_2, 18 hours. After incubation, the cells were removed by refrigerated centrifugation at 12,000x g and the protease inhibitor, phenylmethylsulphonyl fluoride (PMSF) at a final concentration of 1.0 mmol/L, and the preservative sodium azide-NaN_3 (at a final concentration of 0.02 %) were added to the culture-supernatant fluid. None of the reagents has any adverse effects on enzyme activity or stability (Wunder and Bowen, unpublished data). The pH of the culture supernatant fluid was adjusted to 6.8 by the addition of 2M KOH. The culture-supernatant fluid was treated with ammonium sulphate at 55% saturation and then centrifuged (Chludzinski et al., 1974). The precipitate was resuspended in potassium phosphate buffer (KPB), pH 6.8, containing PMSF (1.0 mmol/L) and NaN_3 (0.02%), and extensively dialysed against the same buffer using a 12.0~14.0-kd molecular weight cut-off membrane. The dialysed preparation was used as crude extracellular glucosyltransferase (Gtase).

2.6. *Inhibition of water insoluble glucan formation*

To assay the formation of water insoluble glucan (WIG), it was used a reaction mixture consisting of 1000 µL of 0.25 M of sucrose, 100 µL of crude Gtase and 20 µL of a two-fold dilution series of test extracts (concentrations ranging from 7.8 to 500 µg/mL of reaction) in a total volume of 2 mL of KPB containing NaN_3 and PMSF to a final concentration of 0.02% and 1.0 mmol/L, respectively. For the control, the same reaction was done which 80% ethanol (v/v) was added instead of test extracts. The mixture was incubated at 37°C for 18 h at an angle of 30°. After incubation, the fluid was carefully removed and the tube content was washed with sterile water as described by Takada et al. (1985). The WIG was determined by the phenol-sulphuric method (Dubois et al., 1956). Six (6) replicates were made for each concentration of the test extracts.

2.7. *Statistical analysis*

An exploratory data analysis was performed to determine the most appropriate statistical test. The data from zones of inhibition of growth of each microorganism related to the treatments were compared by means of nonparametric Kruskal-Wallis and multiple comparison tests. The values obtained for inhibition of cell adherence and inhibition of water-insoluble glucan formation were analysed by ANOVA considering a factorial 2x7 (treatments x concentration) and an additional treatment (control). The Dunnett test was applied for comparison between each treatment concentration and the respective control. The level of significance for all statistical tests was $p<0.05$.

3. Results

3.1. Antimicrobial activity

The means of the zones of microbial growth inhibition by propolis and *Arnica montana* extracts are shown in Table 1. It was found that only the ethanolic extract of propolis (EEP) showed inhibitory zones against all tested microorganisms ($p<0.05$), demonstrating strong inhibition of Actinomyces spp. growth, mainly Act. viscosus. Streptococcus sanguis and the mutans group of streptococci were also significantly inhibited by propolis. However, propolis extract showed only a slight inhibitory zone against Candida albicans species. It is important to mention that the EEP also affected the growth of anaerobes, mainly Porphyromonas gingivalis. On the other hand, the ethanolic extract of *Arnica montana* (EEA) demonstrated only a slight activity, in which 12 from 15 strains presented no zone of growth inhibition. The EEA showed only a slight inhibitory action on Porph. gingivalis and Act. naeslundii (ATCC 12104 and W1053) growth, in which the values of the inhibitory zones were not statistically different from control ($p>0.05$). The control (80% aqueous ethanol, v/v) did not demonstrate the formation of inhibitory zone for any tested microorganisms, although it had shown inhibition in direct contact with some of them.

3.2. Inhibition of adherence of Strep. mutans and Strep. sobrinus to a glass surface

Figure 1 shows the effects of EEP and EEA on adherence of growing cells of Strep. mutans and Strep. sobrinus. The *in vitro* adherence inhibition of these microorganisms was evident when these cells were grown in broth containing sucrose and different concentrations of EEP. The rate of inhibition was 98% for Strep. mutans and 90% for Strep. sobrinus, at a concentration of 400 μg/mL of reaction. Furthermore, even at a concentration of 50 and 100 μg/mL, the rate of inhibition for both microorganisms was about 35 and 55% respectively. However, EEA demonstrated only a slight inhibition of Strep. mutans (19%) and Strep. sobrinus (15%) adherence at a concentration of 400 μg/mL. In addition, the *Arnica* extract showed a significant inhibition only at concentration of 400 and 200 μg/mL ($p<0.05$), while EEP inhibited

the adherence of both microorganisms at all tested concentrations ($p<0.05$) when compared with its control (80% ethanol).

3.3. Inhibition of water insoluble glucan (WIG) synthesis.

EEP clearly inhibited the synthesis of WIG by crude Gtase. As shown in Fig. 2, the synthesis of WIG was almost completely abolished at concentrations between 125 and 500 μg / mL of reaction. EEP also showed marked inhibition at low concentrations, e.g. 31.3 μg / mL of reaction (> 80% of inhibition). The EEA only demonstrated significant inhibition of WIG synthesis at the concentration of 250 and 500 μg /mL of reaction ($p<0.05$). In addition, only a slight reduction was observed at these concentrations (18% and 29%, respectively).

4. Discussion

Natural products have been used for medical purposes throughout the world for thousand of years in folk medicine. Many of them have demonstrated pharmacological properties, such as antimicrobial, anti-inflammatory and cytostatic properties, among others (Wu-yuan et al., 1990).

The main etiological factor of dental caries and periodontal disease is dental plaque accumulation. Therefore, it is necessary to search for natural products which have antiplaque properties and antimicrobial activity against oral pathogens. Propolis has been extensively studied for its biological properties, mainly antimicrobial activity (Lindenfelser, 1967; Metzner et al., 1979; Grange and Davey, 1990; Bonhevi et al., 1994). Although the antimicrobial activity of EEP has already been reported against various pathogenic microorganisms, few studies have been conducted against oral pathogens (Ikeno et al., 1991; Steinberg et al., 1996; Park et al., 1998). *Arnica montana* seems to have anti-inflammatory and cytotoxic properties (Woerdenbag et al., 1994; Lyss et al., 1997), but little is known about its antimicrobial property. Since mutans streptococci are involved in caries and dental plaque formation, they were chosen as the main test microorganisms in this study. Actinomyces spp. and three anaerobic bacteria, such as Porphyromonas gingivalis, were also selected due to their relation to root caries and periodontal disease, respectively. In addition, the antimicrobial activity against Staphylococcus aureus, Candida albicans and Enterococcus faecalis, which are important in other oral diseases, was also tested.

The findings of antimicrobial assay showed that only EEP has significant activity against microbial growth *in vitro* when compared to its control (80% ethanol, v/v). EEP showed inhibitory zone for every group of tested microorganisms, including the periodontopathogenic anaerobes. The mutans streptococci and Staph. aureus also demonstrated significant growth inhibition, confirming previously results (Ikeno et al., 1991; Steinberg et al., 1996; Park et al., 1998; Lindenfelser, 1967; Grange and Davey, 1990; Bonhevi et al., 1994). The size of the zones of growth inhibition was variable depending on the strain tested, although it does not necessarily mean that one microorganism is more susceptible than another. In addition, some strains, e.g. Candida albicans, showed very small inhibitory zones. The biological relevance of these zones of inhibition needs to be evaluated using appropriate *in vivo* models. The present data suggest that propolis presents bioactive compounds which possess antimicrobial activity *in vitro*.

The main biologically active compounds in propolis are polyphenolic compounds, mainly flavonoids (Ghisalberti, 1979; Bankova et al., 1989, 1992). Some flavonoids in propolis are considered to be antimicrobial agents, such as pinocembrin, galangin and sakuranetin (Villanueva et al., 1964; Villanueva et al., 1970; Metzner et al., 1979; Ghisalberti, 1979). In addition, Osawa et al. (1992) reported that kaempferol had antimicrobial activity against Strep. mutans and Act. viscosus. Recently, Cai and Wu (1996) showed that kaempferol, isolated from *Syzygium aromaticum*, also inhibited the growth of Porph. gingivalis and Prev. intermedia. In previous works (Park et al., 1997, 1998) it was reported the composition of flavonoid aglycones in Brazilian propolis, in which galangin, pinocembrin, kaempferol and sakuranetin were identified. In addition, other phenolic compounds could be involved. Ikeno et al. (1991) related that cinnamic acid, identified from Chinese and Japanese propolis, demonstrated antimicrobial activity (Strep. mutans). Aga et al. (1994) showed that some hydroxycinnamic derivatives, e.g. 3,5-diprenyl-4-hydroxycinnamic acid, isolated and identified in Brazilian propolis showed antimicrobial activity against Bacillus cereus, Enterobacter aerogenes, Arthroderma benhamiae. The mechanism of antimicrobial action of propolis seems to be complex and it is not completely understood. According to Amoros et al. (1992) and Bonhevi et al. (1994), the activity of propolis against microorganisms is more related to the synergistic effect of flavonoids (and other phenolics) than to the individual compounds. These findings are in agreement with Takaisikikuni and Schilcher (1994), who observed that the antibacterial action against Strep. agalactiae was complex, involving several mechanisms such as formation of pseudo-multicellular streptococci; disorganisation of the cytoplasm, the cytoplasmatic membrane, and the cell wall; partial bacteriolysis; and inhibition of protein synthesis. These authors concluded that a simple analogy cannot be made to the mode of action of any classic antibiotics.

On the other hand, the EEA did not demonstrate significant antimicrobial activity when compared to its control (80% ethanol), although a slight inhibition was noted against Porph. gingivalis and Act. naeslundii (ATCC 12104 and W 1053). The pharmacological effect of *Arnica montana* preparations has been attributed mainly to sesquiterpene lactones (Woerdenbag et al. 1994). Although these compounds demonstrated cytotoxic activity, in this study they seemed to have no effect on the growth of oral pathogens.

EEP showed *in vitro* inhibition of adherence of growing Strep. mutans and Strep. sobrinus cells to a glass surface at all tested concentrations. Furthermore, EEP demonstrated to be a potent

inhibitor of WIG synthesis. Again, EEA presented only slight activity on these two parameters. The glucosyltransferase-catalysed synthesis of WIG from dietary sucrose is known to enhance the pathogenic potential of dental plaque by promoting the adherence and accumulation of large numbers of Strep. mutans and Strep. sobrinus on the teeth of animals and humans (Frostell et al., 1967; Hamada and Slade, 1980). It is well known that Strep. sobrinus produces at least two distinguish types of glucosyltransferase (Gtase): an enzyme (Gtase-I) that produces water insoluble glucan, and another (Gtase-S) that produces water-soluble glucan. Apparently propolis strongly inhibited the Gtase-I activity of Strep. sobrinus. Studies are in progress to determine the effect of propolis on activity of individual and purified glucosyltransferases.

Therefore, only propolis demonstrated effectiveness to reduce both cell adherence and WIG formation by crude glucosyltransferase *in vitro*, which are critical factors in dental plaque formation. Iio et al. (1984) demonstrated that quercetin and chrysin inhibited *in vitro* the glucosyltransferase activity and glucan formation. Quercetin and chrysin was also found in significant amounts in Brazilian propolis (Park et al., 1997; 1998). Cai and Wu (1996) related that some flavonoids, e.g. kaempferol, inhibited glucosyltransferase activity and adherence of Strep. mutans cells to a glass surface. In addition, cinnamic acid demonstrated inhibitory activity of crude glucosyltransferase (Ikeno et al., 1991).

The present investigation demonstrated that *Arnica montana* extract has no or little antimicrobial activity, cell adherence of Strep. mutans and Strep. sobrinus, and WIG synthesis. On the other hand, EEP was effective not only in inhibiting the growth of oral pathogens, but also in reducing dental plaque formation *in vitro*. Probably, the bioactive flavonoids, e.g. galangin, pinocembrin, kaempferol, sakuranetin and quercetin, and other phenolic compounds, e.g. cinnamic acid and its derivatives, present in propolis are involved in its biological activity against oral pathogens. Nevertheless, its phenolic composition is qualitatively and quantitatively variable depending on the region and season of propolis collection (König, 1985; Greenaway et al., 1991; Bankova et al., 1992; Park et al., 1997). It is clear that it is highly desirable to identify and isolate the active compounds of propolis responsible for the inhibitory effects.

In conclusion, the present study suggest that propolis can prevent dental caries and periodontal disease, since it demonstrated significant antimicrobial activity against the microoganisms involved in such diseases and, especially, inhibition of dental plaque formation *in vitro*. However, *Arnica montana* extract showed only slight effects on these *in vitro* parameters,

although it has proved to have a strong anti-inflammatory and cytotoxic activity. Researches are in progress to evaluate these biological effects of propolis using *in vivo* models.

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Table 1. Mean area of the zones of microbial growth inhibition in mm (n=6) provided by the ethanolic extracts of propolis and *Arnica montana*.

Microorganisms	Treatments		
	Propolis	*Arnica montana*	Control (80% ethanol)
Staphylococcus aureus ATCC 25923	2.33 a	0.00 b*	0.00 b*
Enterococcus faecalis ATCC 29212	2.17 a	0.00 b	0.00 b
Candida albicans NTCC 3736	0.83 a	0.00 b*	0.00 b*
Candida albicans F72	0.83 a	0.00 b*	0.00 b*
Streptococcus mutans OMZ 175	2.00 a	0.00 b*	0.00 b*
Streptococcus mutans Ingbritt 1600	2.17 a	0.00 b*	0.00 b*
Streptococcus cricetus HS-6	1.83 a	0.00 b*	0.00 b*
Streptococcus sobrinus 6715	2.25 a	0.00 b	0.00 b
Streptococcus sanguis ATCC 10556	4.42 a	0.00 b*	0.00 b*
Actinomyces naeslundii ATCC 12104	9.25 a	0.33 b	0.00 b*
Actinomyces naeslundii W1053	8.17 a	0.67 b	0.00 b*
Actinomyces viscosus OMZ 105	9.50 a	0.00 b*	0.00 b*
Porphyromonas gingivalis	3.42 a	0.48 b	0.00 b*
Porphyromonas endodontalis	2.25 a	0.00 b*	0.00 b*
Prevotella denticola	2.58 a	0.00 b*	0.00 b*

* Direct contact inhibition only.

The inhibitory zone was considered the shortest distance (mm) from the outside margin of the cylinder to the initial point of the microbial growth.

Means followed by the same letters on a line are not significantly different from each other, $p>0.05$, nonparametric multiple comparison tests.

Fig. 1. Effect of ethanolic extracts of propolis (-♦-) and *Arnica montana* (-Δ-) on the adherence of growing *S. mutans* Ingbritt 1600 (A) and *S. sobrinus* 6715 (B) cells. Means ($O.D._{550}$, n=6) marked by different letters between treatments, in each concentration, differ significantly from each other, $p<0.05$, F test.

Fig. 2. Inhibition of water insoluble glucan (WIG) synthesis by ethanolic extracts of propolis (-♦-) and *Arnica montana* (-Δ-). Means (n=6) marked by different letters between treatments, in each concentration, differ significantly from each other, $p<0.05$, F test.

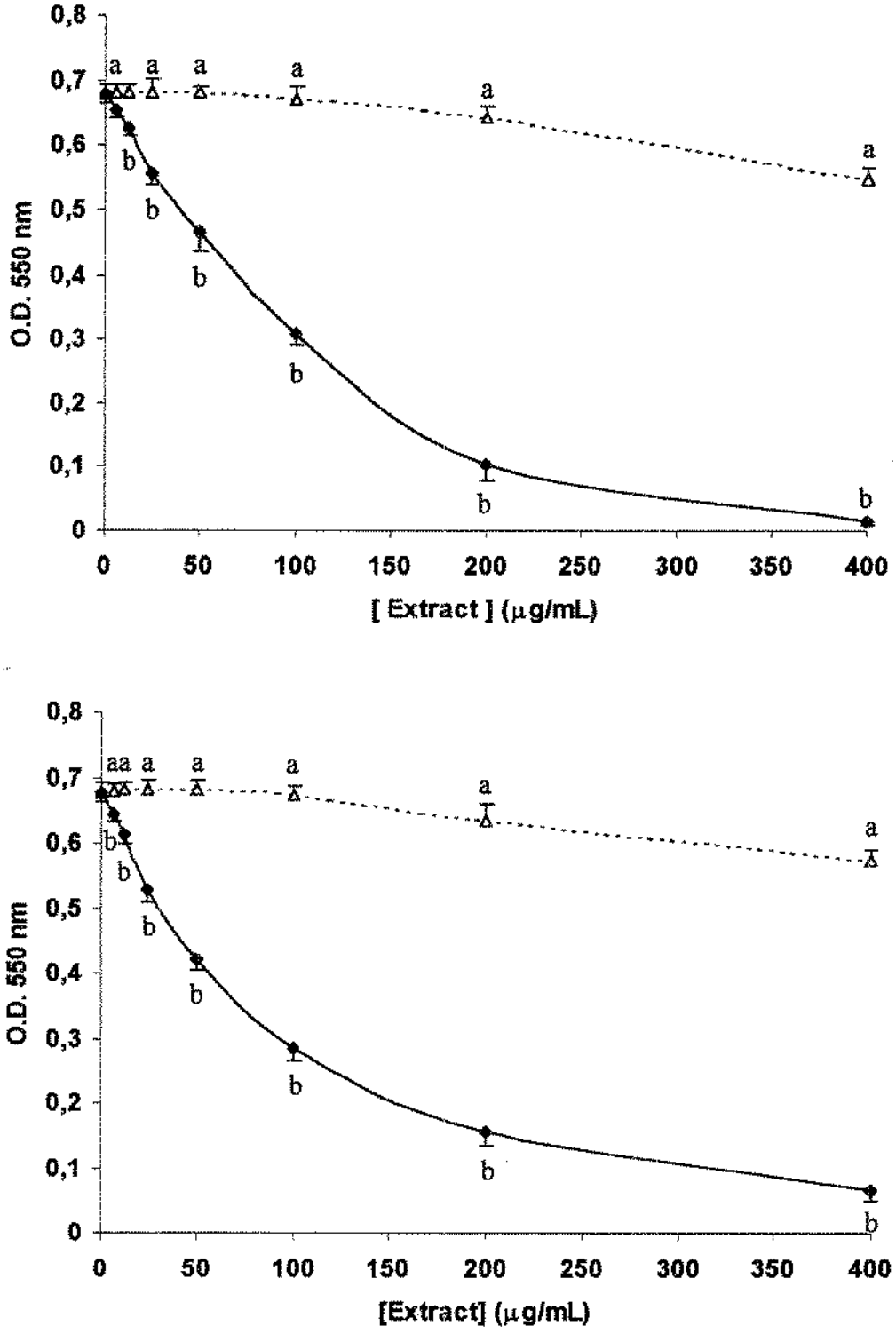
O.D. 550 nm
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
0
50
100
150
200
250
300
350
400
[Extract] (µg/mL)
[Extract] (µg/mL)

Figure 1

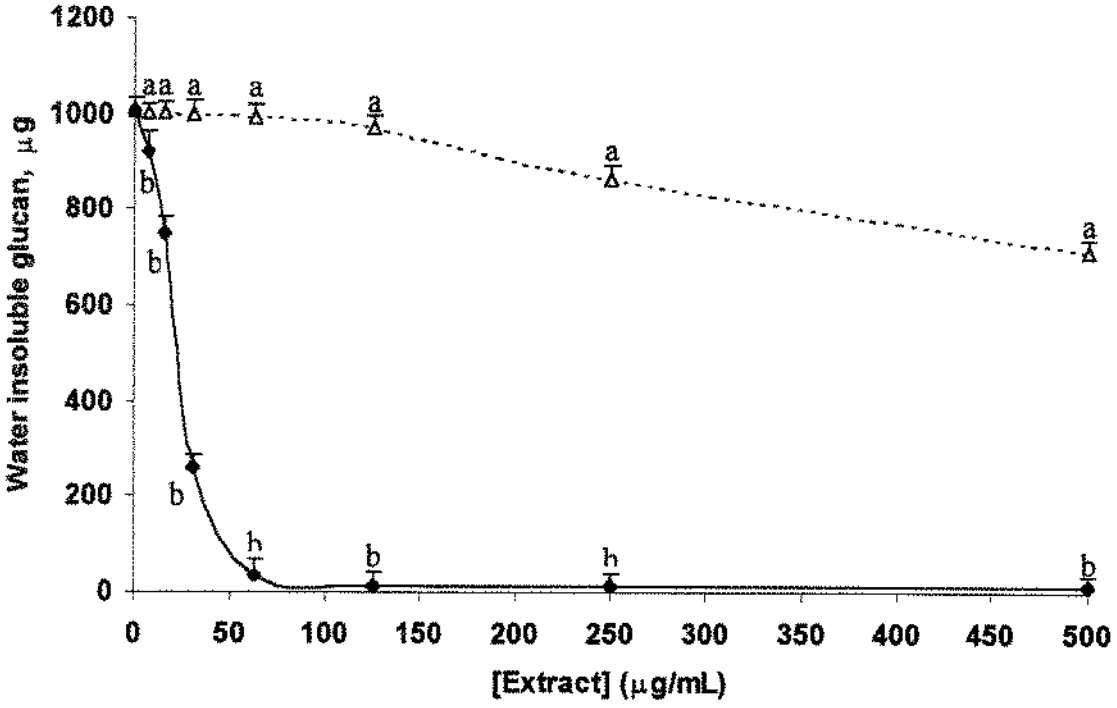
Water insoluble glucan, µg
1200
1000
800
600
400
200
0
0
50
100
150
200
250
300
350
400
450
500
[Extract] (µg/mL)

Figure 2

EFFECT OF *Apis mellifera* PROPOLIS FROM DIFFERENT GEOGRAPHICAL ORIGINS ON MUTANS STREPTOCOCCI

Hyun Koo [a], Pedro Luiz Rosalen [a], Jaime Aparecido Cury [a,*], Gláucia M. B. Ambrosano[a], Masaharu Ikegaki [b], Yong Kun Park [b]

[a] Department of Physiological Sciences, Faculty of Dentistry of Piracicaba, State University of Campinas, Caixa Postal 52, Piracicaba, 13414-900, SP, Brazil

[b] Department of Food Science, College of Food Engineering, State University of Campinas, Caixa Postal 6177, Campinas, 13081-970, SP, Brazil.

Keywords: propolis, flavonoid, mutans streptococci, glucan, antibacterial, plaque

[*] Full address of corresponding author, to whom requests for reprints should be addressed:

Jaime Aparecido Cury
Av. Limeira, 901, Caixa Postal 52
13414-900, Piracicaba, SP, Brazil
Tel.: #55-19-430-5302 / Fax: #55-19-430-5218
e-mail: jcury@fop.unicamp.br

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ABSTRACT

The effect of propolis from different regions of Brazil on mutans streptococci growth, cell adherence and water insoluble glucan (WIG) synthesis was evaluated. Ethanolic extract of propolis (EEP) from Northeastern (PE), Central (MT), Southeastern (MG) and Southern Brazil (RS-P and RS-B) were prepared and analyzed by reversed-phase HPLC. For the antibacterial activity assays, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of EEPs against *Streptococcus mutans, S. sobrinus* and *S. cricetus* were determined. Cell adherence of *S. mutans* and *S. sobrinus* to a glass surface was measured spectrophotometrically at 550 nm. WIG synthesized by glucosyltransferase (Gtf) from sucrose was extracted and quantified by phenol-sulfuric method. Flavonoid aglycones were detected only in samples MG and RS-P, which showed significant inhibitory effects when compared to control ($p<0.05$). In general, EEP RS-P demonstrated better biological activities than others. The MIC values of EEP RS-P were 50 - 100 μg ml^{-1}, for *S. mutans;* and 25-50 μg ml^{-1}, for *S. sobrinus* e *S. cricetus*. For EEP MG, the MIC values were 200-400 μg ml^{-1} for all tested strains. The bactericidal concentration of EEP RS-P and MG was 4-8 times the MIC values of the respective samples. The other samples did not demonstrate antibacterial activity (MIC > 1600 μg ml^{-1}). The adherence of *S. mutans* and *S. sobrinus* cells was markedly inhibited by RS-P (95% at concentrations between 50-400 μg ml^{-1}) and MG (95% at 400 μg ml^{-1}) samples. The EEPs MG and RS-P demonstrated to be potent inhibitors of WIG synthesis showing more than 95% inhibition between 125 and 500 μg ml^{-1}. In contrast, the other samples showed only negligible effects on cell adherence and WIG synthesis. The data show that the geographical origin of propolis may have influence on its biological effect against mutans streptococci due to the variability of the chemical composition of this natural substance.

1. INTRODUCTION

Bacterial adherence on tooth surface is fundamental for dental plaque formation and development of oral diseases. Mutans streptococci can colonize the tooth surface and initiate plaque formation by their ability to synthesize water-insoluble glucan (WIG) from sucrose using glucosyltransferase (Gtf) enzymes [1, 2]. This sucrose-dependent adherence and accumulation of mutans streptococci are critical processes in the development of pathogenic dental plaque related to caries [1, 2]. Therefore, antimicrobial substances against these bacteria or those that can affect the synthesis of WIG should be considered for prevention of dental plaque and caries.

Propolis, a resinous *Apis mellifera* bees product [3], has been studied due to its antimicrobial activity against a wide range of pathogenic microorganisms [4,5]. However, the evaluations made against mutans streptococci [6,7] have not estimated the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the ethanolic extract of this natural substance. Although there are some evidences that ethanolic extract of propolis (EEP) inhibited *in vitro* the activity of crude Gtf [6,7], little is known about its effect on WIG synthesis. Furthermore, the active substances, e.g. polyphenolic compounds (mainly flavonoid aglycones) [3,4,5], of propolis from different geographical origin can not be the same because its composition depends on the regional plant ecology [8,9].

Considering the evaluations made and that composition of propolis can affect their biological activity, the aim of the present study was to evaluate *in vitro* the activity of this natural substance from different regions of Brazil against mutans streptococci growth, cell adherence and water insoluble glucan synthesis.

2. MATERIALS AND METHODS

2.1. Propolis samples

Crude propolis samples collected by *Apis mellifera* were obtained from the cities: Palmares, Pernambuco State – PE (Northeastern Brazil); Cárceres, Mato Grosso State – MT (Central Brazil); Santa Luzia, Minas Gerais State – MG (Southeastern Brazil); Porto Alegre and Bagé, Rio Grande do Sul State – RS (Southern Brazil). EEP at 10% (w/v) in aqueous ethanol (80%, v/v) were prepared according to Koo and Park (10).

2.2. Reversed Phase High Performance Liquid Chromatography (HPLC) of EEP.

The analysis of flavonoids was performed by reversed phase HPLC with a chromatograph equipped with YMC PACK ODS-A column and diode array detector (SPD-M10A, Shimadzu Co.) as described elsewhere (7). The authentic standards of flavonoids (Extrasynthese A.A. Co., Genay-Sedex, France) used in this assay were: quercetin, kaempferol, apigenin, pinocembrin, chrysin, acacetin, galangin and kaempferide. The *p*-coumaric acid (4-hydroxycinnamic acid) was also used in this assay.

2.3. Microorganisms

The microorganisms used in this study were *Streptococcus mutans* Ingbritt 1600, *S. sobrinus* 6715 and *S. cricetus* HS-6. The cultures were stored at –80°C in Brain Heart Infusion (BHI) broth containing 20% glycerol. These microorganisms were kindly donated by the Center for Oral Biology, University of Rochester, NY, USA.

2.4. Determination of MIC and MBC of EEP

Isolated 18-24 h colonies of the mutans streptococci strains were suspended in sterile 0.89% NaCl solution [11]. The cell suspension was properly inoculated in BHI broth containing a two-fold dilution series of the EEP (concentrations ranging from 6.2 to 1600 μg ml^{-1} reaction) or the control (80% ethanol, v/v) to achieve an assay concentration of 1-2 x 10^5 cfu ml^{-1} [11]. The tubes were incubated in 10% CO_2, 37 °C, for 24 h. After incubation, bacterial growth was assayed by measurement of absorbance at 660 nm. MIC was defined as the lowest concentration of EEP that had restricted growth to a level <0.05 at 660 nm (no visible growth). For the determination of MBC, an aliquot (50 μL) of all incubated test tubes was subcultured on BHI agar supplemented with 0.5% of desfibrinated sheep blood. MBC was defined the lowest concentration that enables no growth on the agar (99.9 % kill).

2.5. Inhibition of adherence of growing cells to a glass surface

To assess the adherence of growing cells of *S. mutans* Ingbritt 1600 and *S. sobrinus* 6715 to a glass surface, organisms were grown at 37 °C, at an angle of 30° for 18 h in test tubes, as described by Hamada and Torii [16]. These microorganisms were grown in BHI broth plus 1% sucrose containing EEP (concentrations ranging from 6.2 to 400 μg ml^{-1} reaction) or control (80% ethanol, v/v). After incubation, the adhered cells were washed and suspended using the procedures outlined by Hamada and Torii [16]. The amount of adherent cells was measured at 550 nm - $O.D._{550}$ [16].

2.6. Inhibition of water insoluble glucan (WIG) synthesis

The glucosyltransferase (Gtf) was prepared according to methods outlined by Chludzinski et al. [12] and Schilling and Bowen [13]. The WIG synthesis was determined as described previously [14]. Briefly, a reaction mixture consisting of 1000 μL of 0.25 M of sucrose, 100 μL of Gtf and 20 μ L of a two-fold dilution series of EEP (concentrations ranging from 7.8 to 500 μg ml^{-1} of reaction) or control (80% ethanol, v/v) in a total volume of 2.0 ml of potassium phosphate buffer (KPB) was incubated at 37°C for 18 h at an angle of 30°. After incubation, the fluid was removed and the tube content was washed with sterile water as described by Takada et al. [14]. The WIG was extracted with NaOH 1N and quantified by phenol-sulphuric method [15].

2.7. Statistical analysis

Six replicates were made for each concentration of the tested extracts for all assays. An exploratory data analysis was performed to determine the most appropriate statistical test. The values obtained for inhibition of cell adherence and inhibition of WIG formation were analyzed by ANOVA considering a factorial 5 x 7 (treatments x concentration) and an additional treatment (control). The Dunnett test was applied for comparison between each concentration of the treatment, and the respective control. The level of significance for all statistical tests was $p<0.05$.

3. RESULTS AND DISCUSSION

The biological activity of propolis is related to its chemical composition, which varies according to regional plant ecology [8,9,]. Considering the high biodiversity of Brazil, the present study tested the hypothesis that propolis collected from different geographical regions, during the same period of the year, could present variable biological effects on mutans streptococci.

Table 1 shows some of the physico-chemical characteristics of propolis from different geographical regions of Brazil. The color varied from black to dark-green color and the texture from soft and sticky to hard and brittle. The composition of flavonoid aglycones was also remarkably variable among the propolis samples. The flavonoid aglycones identified in the EEP RS-P were quercetin, kaempferol, apigenin, pinocembrim, chrysin, acacetin and galangin. For EEP MG the detected flavonoids were quercetin, kaempferol, pinocembrim and kaempferide. On the other hand, neither flavonoids nor *p*-coumaric acid was found in EEPs PE, MT and RS-B, although some unidentified peaks were detected in the EEP RS-B (data not shown). The variability of flavonoid contents is probably due to the difference of plant ecology of particular region of propolis collection. Further isolation, purification and identification of flavonoids and other phenolic compounds are in progress through mass spectrometry and GC-MS analysis.

The MIC and MBC values of EEP are shown in Table 2. Among the propolis samples, the EEP RS showed the strongest inhibitory activity on the growth of mutans streptococci strains. The range of MIC of EEP RS-P was 50 - 100 μg ml^{-1}, for *S. mutans*, and 25 - 50 μg ml^{-1} *for S. sobrinus* and *S.cricetus*. The EEP MG also showed antibacterial activity in which MIC values of 200 - 400 μg ml^{-1} were achieved. As expected, the bactericidal concentration of EEP was higher (4 - 8 times) than MIC values. The MBC values of EEP RS-P were 200 - 400 μg ml^{-1} for *S. mutans* and 100 - 200 μg ml^{-1} for *S. sobrinus* and *S. cricetus*, whereas the MBC values of EEP MG were 800 - 1600 μg ml^{-1} for all tested strains. However, no appreciable activity was observed when EEPs PE, MT and RS-B were tested against the mutans streptococci strains (> 1600 μg ml^{-1}). Some flavonoids in propolis are considered to be antimicrobial agents, such as pinocembrin, galangin, sakuranetin, pinobanksin [3]. Kaempferol and myricetin showed strong antimicrobial activity against *Porphyromonas gingivalis* and *Prevotella intermedia*; and moderate effect on *S. mutans*, *Actinomyces viscosus* growth [17].

The mechanism of antimicrobial action of propolis seems to be complex and it is not completely understood. According to Bonhevi et al. [5] and Amoros et al. [18], the activity of propolis against microorganisms is more related to the synergistic effect of flavonoids (and other phenolics) than to

individual compounds. This hypothesis is in agreement with Takaisikikuni and Schilcher [19], who observed that the antibacterial action against *Streptococcus agalactiae* was complex involving several mechanisms such as formation of pseudo-multicellular streptococci; disorganization of the cytoplasm, the cytoplasmatic membrane, and the cell wall; partial bacteriolysis; and inhibition of protein synthesis. These authors concluded that a simple analogy cannot be made to the mode of action of any classic antibiotics. In addition, Mirzoeva et al [20] showed that propolis uncoupled the energy tranducing cytoplasmic membrane and inhibited bacterial motility of some gram-positive and gram-negative strains.

Figure 1 illustrates the effect of EEPs on adherence to glass by cells of *S. mutans* Ingbritt 1600 and *S. sobrinus* 6715. The EEP RS-P and MG markedly inhibited the adherence of both strains, showing significant inhibition at all concentrations when compared to control (80% ethanol) ($p<0.05$). EEP RS-P showed better effect than EEP MG at concentrations between 25 - 200 μg ml^{-1} for *S. mutans* and between 12.5 - 200 μg ml^{-1} for *S. sobrinus* ($p<0.05$). The degree of inhibition for both strains by EEP RS-P was more than 95% at concentrations between 50 – 400 μg ml^{-1}, whereas EEP MG inhibited at this same degree only at 400 μg ml^{-1}. The EEP RS-B showed slight effects in which 15% inhibition of cell adherence was achieved at concentration of 400 μg ml^{-1} for both strains. The other samples, PE and MT, showed negligible effects, where no significant inhibition was noted when compared to control ($p>0.05$).

The inhibition of WIG synthesis by EEPs is shown in Figure 2. The EEP MG and RS-P proved to be potent inhibitors of WIG synthesis *in vitro*. At concentrations between 125 and 500 μg ml^{-1}, the WIG synthesis was almost completely abolished. The EEP RS-P showed higher inhibition at concentrations of 31.3, 15.6, 7.8 μg ml^{-1} than EEP MG ($p<0.05$). Both samples of propolis significantly inhibited the WIG synthesis at all tested concentrations when compared to their control ($p<0.05$). EEP RS-B reduced weakly the WIG formation only at concentration of 500 μg ml^{-1} (10% inhibition). In contrast, the EEPs PE and MT did not show any significant inhibitory activity ($p>0.05$). The glucosyltransferase-catalysed synthesis of WIG from dietary sucrose is known to enhance the pathogenic potential of dental plaque by promoting the adherence and accumulation of large numbers of *S. mutans* and *S. sobrinus* on the teeth of animals and humans [1, 2]. It is well known that mutans streptococci produce at least two distinguished types of glucosyltransferase (Gtf): an enzyme (Gtf-I) that produces water insoluble glucan, and another (Gtf-S) that produces water-soluble glucan [1, 2]. Apparently EEP RS-P and MG showed themselves to be a potent inhibitor of

the Gtf-I activity of *S. sobrinus*. Studies are in progress to determine the effect of propolis on activity of individual and purified Gtfs.

Both cell adherence and WIG synthesis are critical factors in dental plaque formation. Therefore, these EEP samples demonstrated *in vitro* that they could participate in the reduction of dental plaque formation and further accumulation. Some flavonoids detected in these samples, e.g. quercetin, chrysin and kaempferol, are known to inhibit *in vitro* the Gtf activity and glucan formation [17, 21]. Kaempferol also inhibited the adherence of *S. mutans* cells to a glass surface [17].

The findings of the present study showed that the composition of flavonoid aglycones and the biological activities of propolis on all tested *in vitro* parameters were highly variable. Only samples RS-P and MG, where flavonoids compounds were detected, showed antimicrobial activity, inhibition of bacterial adherence, and especially to be potent inhibitors of WIG synthesis. In addition, EEP RS-P demonstrated that it is more effective than EEP MG.

Further investigation on the chemical characterization of these EEP samples should be carried out before any definitive conclusion regarding to possible bioactive compounds, due to other flavonoids and phenolics could be involved in their biological activities. Aga et al. [22] reported that some hydroxycinnamic acid derivative of propolis extracts, e.g. 3,5-diprenyl-4-hydroxycinnamic acid, showed antimicrobial activity against *Bacillus cereus*, *Enterobacter aerogenes*, and *Arthroderma benhamiae*. Although few bioactive flavonoids were detected in propolis from MG, recent studies demonstrated that propolis from this region presents various (hydroxy) cinnamic acid derivative, such as 3,5-diprenyl-4-hydroxycinnamic acid [22, 23]. In the present study, 4-hydroxycinnamic acid (*p*-coumaric acid) was identified in EEP MG and RS-P. It is clear that it is highly desirable to identify and isolate the active compounds of propolis.

In conclusion, the biological effects of propolis on mutans streptococci were variable depending on the geographical origin of propolis due to differences on their chemical composition. The composition of propolis is related to plant ecology of each region visited by bees [8,9].

Researches are in progress to elucidate the biological significance of these *in vitro* findings by using appropriate *in vivo* model. The use of propolis to prevent dental caries and plaque formation is worthy of exploration.

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Table 1. Some physico-chemical aspects of *Apis mellifera* propolis from different geographical origin.

Sample of propolis	Origin	Physical characteristics	Flavonoid aglycones identified
PE	Complex flora, Palmares, state of Pernambuco (Northeastern Brazil, located between 5° and 10° south latitude)	Brown color, pliable and soft	-
MT	Complex flora, Cárceres, state of Mato Grosso (Central Brazil, located between 15° and 20° south latitude)	Black color, very soft and extremely sticky.	-
MG	Complex flora, Santa Luzia, state of Minas Gerais (Southeastern Brazil, located between 20° and 25° south latitude)	Dark-green color, hard and brittle	Quercetin, kaempferol, pinocembrin, kaempferide, *p*-coumaric acid (4-hydroxycinnamic acid)
RS-P	Complex flora, Porto Alegre, state of Rio Grande do Sul (Southern Brazil, located at 30° south latitude)	Reddish-brown color, pliable and soft.	Quercetin, kaempferol, apigenin, pinocembrin, chrysin, acacetin, galangin, *p*-coumaric acid (4-hydroxycinnamic acid)
RS-B	Complex flora, Bagé, state of Rio Grande do Sul (Southern Brazil, near the border of Uruguay, located between 30° and 35° south latitude)	Dark-brown color, hard and brittle	No identified peaks

Table 2. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of EEP against the mutans streptococci strains.

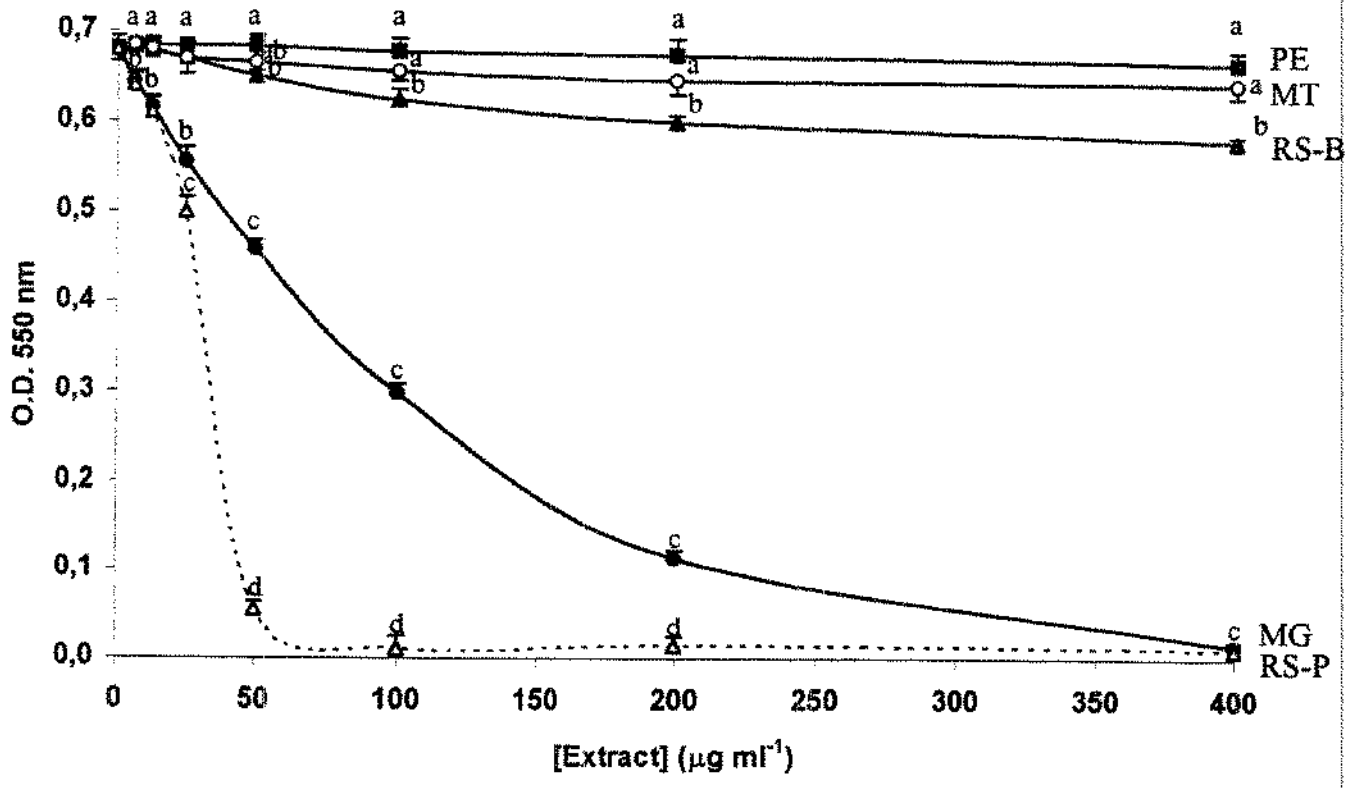
Propolis samples	Microorganisms					
	Streptococcus mutans Ingbritt 1600		*Streptococcus sobrinus* 6715		*Streptococcus cricetus* HS-6	
	MIC	MBC	MIC	MBC	MIC	MBC
PE	> 1600	> 1600	> 1600	> 1600	> 1600	> 1600
MT	> 1600	> 1600	> 1600	> 1600	> 1600	> 1600
MG	200-400	800-1600	200-400	800-1600	200-400	800-1600
RS-P	50-100	200-400	25-50	100-200	25-50	100-200
RS-B	> 1600	> 1600	> 1600	> 1600	> 1600	> 1600

* The MIC and MBC values are expressed in μg ml^{-1}. The concentrations of EEP ranged from 6.2 to 1600 μg ml^{-1}.

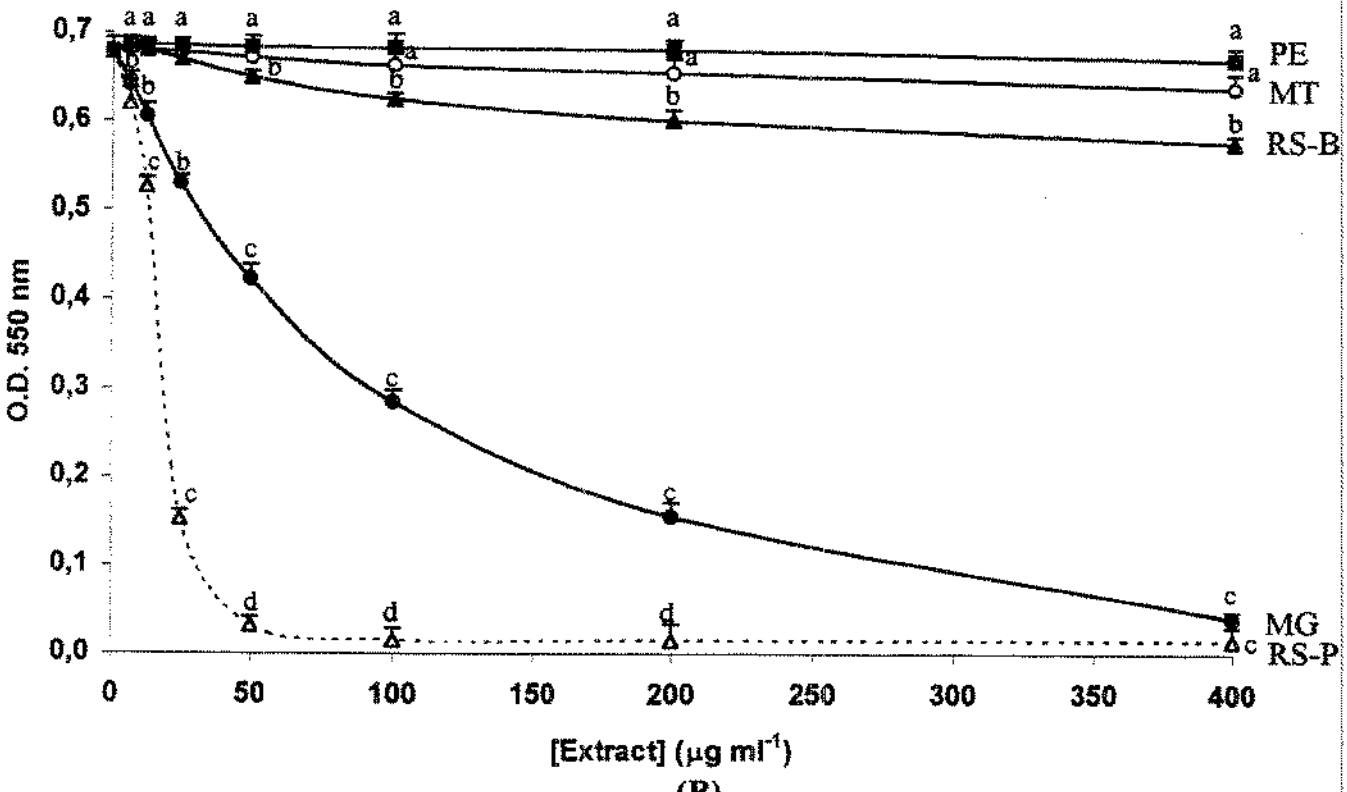
**Six replicates were made for each concentration of the tested extracts for all assays.

Fig. 1. Effect of ethanolic extract of propolis (EEP) on adherence of growing cells of *Streptococcus mutans* Ingbritt 1600 (A) and *S. sobrinus* 6715 (B). Inhibitory activities of EEP were measured at various concentrations. The amount of adherent cells was measured at 550 nm - O. D. $_{550}$. Means (O.D. $_{550}$, n=6) marked by different letters between treatments, in each concentration, differ significantly from each other, $p<0.05$, F test.

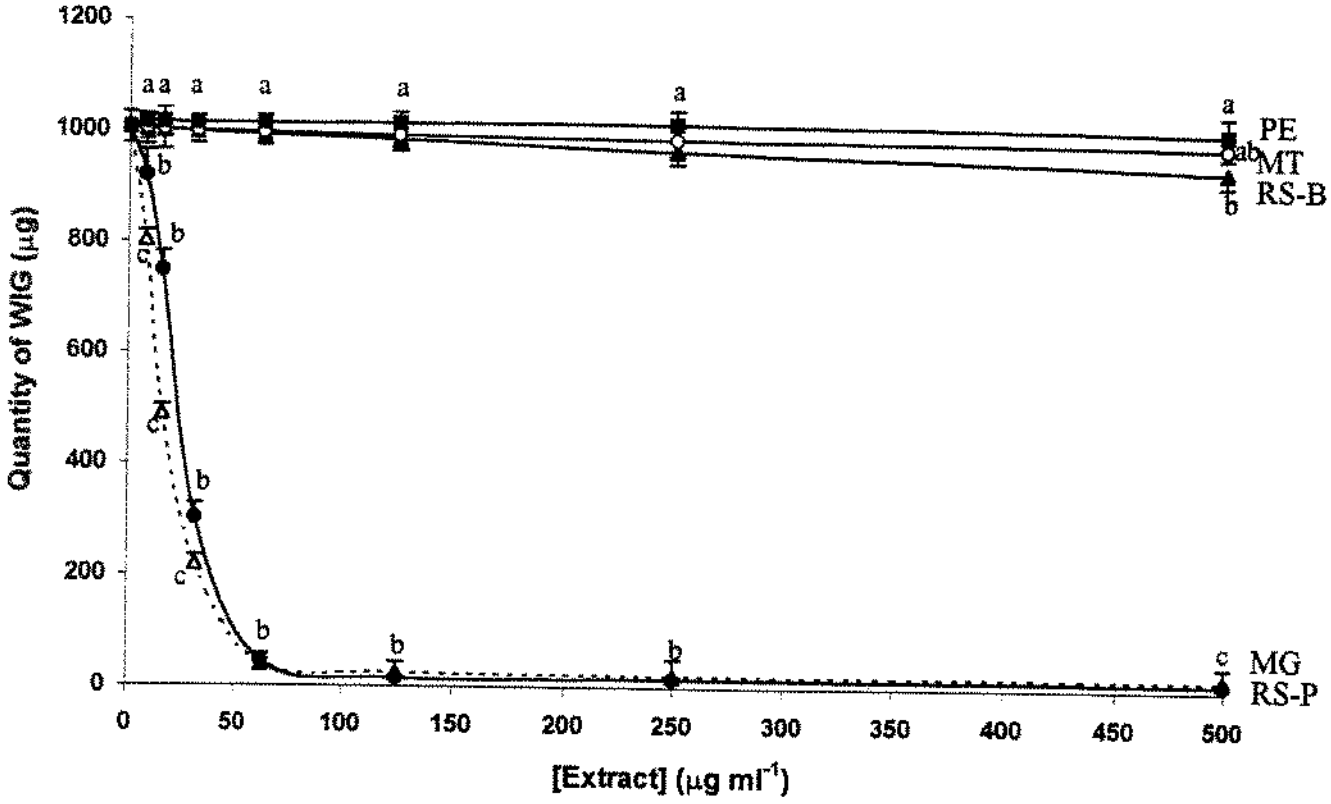
Fig. 2. Effect of ethanolic extract of propolis (EEP) on water insoluble glucan synthesis (WIG) by glucosyltransferase (Gtf). Inhibitory activities of EEP were measured at various concentrations. The WIG was extracted with NaOH 1N and quantified by phenol-sulphuric method. Means (n=6) marked by different letters between treatments, in each concentration, differ significantly from each other, $p<0.05$, F test.


O.D. 550 nm
[Extract] (µg ml-1)
PE
MT
RS-B
MG
RS-P

(A)


O.D. 550 nm
[Extract] (µg ml-1)
PE
MT
RS-B
MG
RS-P

(B)


1200
1000
800
600
400
200
0
Quantity of WIG (µg)
0
50
100
150
200
250
300
350
400
450
500
[Extract] (µg ml-1)
PE
MT
RS-B
MG
RS-P

Artigo III

EFFECTS OF *Apis mellifera* PROPOLIS ON THE ACTIVITIES OF STREPTOCOCCAL GLUCOSYLTRANSFERASES IN SOLUTION AND ADSORBED ONTO SALIVA-COATED HYDROXYAPATITE

Hyun Koo[1]*, Anne M. Vacca Smith[2], William H. Bowen[2], Pedro L. Rosalen[1], Jaime A. Cury[1], Yong K. Park[3]

[1] Faculty of Dentistry of Piracicaba, State University of Campinas, Caixa Postal 52, Piracicaba, 13414-900, SP, Brazil

[2] Center for Oral Biology, University of Rochester, 601 Elmwood Avenue, Box 611, Rochester, 14642, NY, USA

[3] School of Food Engineering, State University of Campinas, Caixa Postal 6177, Campinas, 13081-970, SP, Brazil

Running Title: Effects of propolis on glucosyltransferases

Key Words: propolis, streptococci, glucosyltransferase, hydroxyapatite, glucan, plaque

Abbreviations: EEP, ethanolic extract of propolis; Gtf, glucosyltransferase; sHA, saliva-coated hydroxyapatite; PMSF, phenylmethylsulfonyl-fluoride; LMW, low molecular weight

* Full address of corresponding author, to whom requests for reprints should be addressed:
Hyun Koo
University of Rochester
Center for Oral Biology
601 Elmwood Ave. Box 611
Rochester, NY 14642
USA
Tel (716) 275-1433 / Fax (716) 473-2679
E-mail: hyunkoobr@yahoo.com

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ABSTRACT

Propolis, a resinous hive product collected by *Apis mellifera* bees, has been used for thousands of years in folk medicine. The ethanolic extract of propolis (EEP) has been shown to inhibit the activity of crude glucosyltransferase (Gtf) enzymes in solution. These enzymes synthesize glucans from sucrose, which are essential for pathogenic dental plaque formation. In the present study, the effects of propolis from two different regions of Brazil on the activity of separate, purified Gtf enzymes in solution and on the surface of saliva-coated hydroxyapatite (sHA) beads were evaluated. The EEP from Minas Gerais-MG (Southeastern Brazil) and Rio Grande do Sul-RS (Southern Brazil) were tested for their ability to inhibit the enzymes Gtf B (synthesis of insoluble glucan), Gtf C (insoluble/soluble glucan) and Gtf D (soluble glucan). An additional enzyme, Gtf G from *Streptococcus sanguis* (soluble glucan synthesis), was also analyzed. The EEP from both regions efficiently inhibited the activity of all Gtfs in solution (75 to 95%) and on surface of sHA beads (45 to 95%) at concentrations of 1.25 and 5.0 mg of propolis/mL. However, the two samples of propolis showed variable inhibitory effects. In general, EEP RS demonstrated significantly higher inhibitory activity on Gtf B and C activities (both solution and surface assays) than EEP MG at concentrations between 0.078 to 0.312 mg/mL ($p<0.05$). EEP MG on the other hand, exhibited greater inhibitory effect on the activities of surface Gtf D (at 0.625 and 1.25 mg/mL) and G (at 2.5 and 5.0 mg/mL) ($p<0.05$). The activities of Gtf D and G in solution were inhibited equally well by both EEPs. These data indicate that EEP is a potent inhibitor of Gtf enzymes in solution and adsorbed to an experimental pellicle. However, its effect on Gtf activity is variable depending on the region of collection of propolis samples.

INTRODUCTION

Glucosyltransferase enzymes (Gtf, EC 2.4.1.5) produced by *Streptococcus mutans* are virulence factors associated with dental caries (De Stoppelaar et al., 1971; Tanzer et al., 1985; Yamashita et al., 1993). These enzymes catalyze the formation of soluble and insoluble α-linked glucans from sucrose and contribute significantly to the polysaccharide composition of dental plaque matrix (Rölla et al., 1983; Loesche, 1986). These glucans, mainly the insoluble, can enhance the pathogenic potential of dental plaque by promoting the accumulation of cariogenic streptococci on the teeth of humans and experimental animals (Krasse, 1965; Frostell et al., 1967; Hamada and Slade, 1980). *S. mutans* produces at least 3 Gtfs: Gtf B, which synthesizes produces a polymer of mostly insoluble α1,3-linked glucan; Gtf C, which synthesizes a mixture of insoluble α1,3-linked glucan and soluble α1,6-linked glucan; and GtfD which synthesizes an α 1,6-linked soluble glucan (Loesche, 1986; Aoki, 1986; Hanada and Kuramitsu, 1988; 1989). An additional enzyme, Gtf G, may also be involved in the development of dental plaque. Gtf G is synthesized by *S. sanguis*, an early colonizer of tooth surface, and catalyzes predominantly α1,6-linked soluble glucan (Carlsson et al., 1969; Beeley and Black, 1977). Among these enzymes, Gtf B and C have been considered the most important Gtfs related to dental caries (Yamashita et al., 1993).

The Gtfs released by *S. mutans* are present in the soluble fraction of whole human saliva and are, also, incorporated into salivary pellicle which forms on the teeth (Rölla et al., 1983; Scheie et al., 1987). Furthermore, the Gtfs incorporated into an experimental pellicle demonstrated different physical and kinetic properties when compared to the same enzymes in solution, expressing enhanced enzymatic activity (Schilling and Bowen, 1988). Thus, a large proportion of the glucans formed *in situ* is retained in the pellicle. This glucan can provide attachment sites for selective adherence and colonization by *S. mutans* and *S. sobrinus* onto the surface of the tooth and contribute to the formation and bulk of dental plaque (Schilling and Bowen, 1992; Vacca-Smith and Bowen, 1998). Therefore, inhibition of Gtfs activities, mainly those adsorbed to salivary pellicle, could play an important role in the prevention of pathogenic dental plaque formation related to caries.

Propolis is a resinous substance collected by *Apis mellifera* bees from various parts of plant (buds and bark lesions) and mixed with beeswax (Ghisalberti, 1979). Basically, propolis is

used by bees as glue to seal the opening of the hives and to embalm dead invading insects (Ghisalberti, 1979). Ethanolic extracts of propolis (EEP) exhibit some pharmacological activities, such as antimicrobial, anti-inflammatory, anaesthetic, and cytostatic properties (Ghisalberti, 1979; Bankova et al., 1989). Polyphenolic compounds, mainly flavonoid aglycones, are considered the biological active substances in propolis (Ghisalberti, 1979; Grange and Davey, 1990; Bonhevi et al., 1994). Earlier reports demonstrated that EEP inhibited the growth of *S. mutans* and *S. sobrinus* (Ikeno et al., 1991; Steinberg et al., 1996; Park et al., 1998), besides reducing dental caries in rats (Ikeno et al., 1991; Koo et al., 1999). In addition, EEP strongly inhibited the activity of crude preparations of Gtf in solution (Ikeno et al., 1991; Park et al., 1998). Nevertheless, the previous studies could not determine precisely which Gtf (or glucan) had been inhibited, since they used crude Gtf and an indirect method for Gtf activity assay; and more importantly, whether EEP is an effective inhibitor of pellicle-adsorbed enzyme. Considering the evaluations made and the pharmacological potential of propolis the aim of the present study was to determine the effect of propolis from two different Brazilian region on the activity of individual, purified Gtf enzymes both in solution and adsorbed onto saliva coated hydroxyapatite surface.

MATERIALS AND METHODS

Propolis Samples

Crude propolis samples collected by *Apis mellifera* were obtained from the cities: Santa Luzia, Minas Gerais State - MG (Southeastern Brazil, located between 20° and 25° south latitude) and Porto Alegre, Rio Grande do Sul State - RS (Southern Brazil, located between 30° and 35° south latitude). These two samples were selected from our previous screening (Park et al., 1998). The samples of propolis were dehydrated with a low-vacuum pump, and the ethanolic extracts of propolis - EEP (10% w/v, in 80% aqueous ethanol, v/v) were prepared as described by Koo and Park (1997).

Saliva

Whole saliva was collected on ice from one donor by chewing paraffin-film and was clarified by centrifugation (8500g, 4°C, 10 min.). Afterwards, the saliva was diluted 1:1 with adsorption buffer (50 mM KCl, 1.0 mM KPO_4, 1.0 mM $CaCl_2$, 0.1 mM $MgCl_2$, pH=6.5), supplemented with sodium azide (0.02%, final concentration) and the protease inhibitor phenylmethylsulfonyl-fluoride (PMSF, 1.0 mmol/L, final concentration) (Vacca-Smith et al., 1996a). Negligible levels of Gtf were detected in the whole saliva from this donor.

Hydroxyapatite Beads

Hydroxyapatite (HA) beads (10 mg) were used for surface assays (Bio-Rad Laboratories, Hercules, CA, USA). The surface area of the beads was 0.24m^2, and the particle size was 80 μm.

Bacterial Strains

The bacterial strains used in this experiment were: 1) *Streptococcus milleri*, which harbors the *gtfB* gene (for Gtf B production); 2) *S. milleri* NH5, which contain the *gtfD* gene (for Gtf D); *S. mutans* WHB 410, which the genes for Gtf B, D and fructosyltransferase were deleted (for Gtf

C) (Wunder and Bowen, 1999); 4) *S. sanguis* 10904 (for Gtf G). The *S. milleri* constructs were a kind gift from Dr. Howard K. Kuramitsu (SUNY, Buffalo, NY).

Purification of Gft Enzymes

All the purification procedures were carried out using buffers containing the protease inhibitor, PMSF (0.1 mM, final concentration) and NaN_3 (0.02%, final concentration) as preservative. Neither reagents had any adverse effects on enzyme activity or stability. The enzymes were stored at 4°C, in adsorption buffer supplemented with 10% glycerol. The purity of the enzyme preparations was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a Hoefer Mighty Small SE245 system (Hoefer Scientific Instruments, San Francisco, CA, USA) and silver staining as previously described (Venkitaraman et al., 1995). Protein concentration was determined by the 280-nm absorbance method outlined in Deutscher (1990).

The Gtf B and G enzyme was obtained and purified to near homogeneity from clarified culture supernatant of *S. milleri* KSB8 and *S. sanguis* 10904 using the method described by Venkitaraman et al. (1995) and Vacca Smith et al. (manuscript submitted).

The purification of Gtf D from *S. milleri* NH5 was performed using the procedures outlined by Venkitaraman et al. (1995).

Gtf C was obtained from culture of *S. mutans* WHB 410. Briefly, actively growing *S. mutans* WHB 410 was inoculated into dialysis tubing (molecular weight cutoff 50,000) containing 200 mL of Low Molecular Weight medium-LMW (2.5% tryptone, 1.5% yeast extract, 0.3 % glucose, 0.1% fructose and 0.1% sorbitol; Schilling and Bowen, 1988) supplemented with erythromycin-10 μg/mL, tetracycline-5 μg/mL, kanamycin-500 μg/mL and immersed into 800 mL of the same medium. After growth, the cells were pelleted by centrifugation at 5000g, 4°C, for 20 min and the supernatant fluid discarded. The cell pellet was resuspended in 20 mL of 50 mM KPO_4 pH 7.5 and 0.01% of Triton X-100 and sonicated on ice (five 30s bursts at 250 Watts and 10s intervals, Braun-Sonic 1510). After sonication, the resuspended cells were centrifuged at 5000g, 4°C, for 10 min. The supernatant was collected as the source of Gtf C. Gtf C was purified as previously described by Venkitaraman et al. (1995).

Glucosyltransferase activity was measured by the incorporation of ^{14}C - glucose from labelled sucrose (NEN Research Products, Boston, MA) into glucans. The specific activities of the Gtf enzymes were as follows: Gtf B, 1.24 x 10^{-3} μmol of glucose incorporated into glucan/min./ μg of protein; Gtf D, 6.90 x 10^{-4} μmol of glucose incorporated into glucan/min./ μg of protein; Gtf C, 1.63 x 10^{-4} μmol of glucose incorporated into glucan/min./ μg of protein; and Gtf G, 2.44 x 10^{-3} μmol of glucose incorporated into glucan/min./ μg of protein. One unit of enzyme was defined as the amount of enzyme needed to incorporate one μmol of glucose into glucan over a four-hour reaction period. The Gtf enzyme added to each sample for all assays was equivalent to the amount required to incorporate 1.0-1.5 μmol of glucose over the course of the reaction (1.0-1.5 units).

Activity of Gtf in solution

GtfB, C, D and G were mixed with a two-fold dilution series of the EEP (concentration ranging from 0.078 to 5.0 mg/mL) and incubated with ^{14}C-(glucosyl)-sucrose substrate (200.0 mmol/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 (200μL final volume). For the control, the same reaction was done in which 80 % aqueous ethanol was added instead of EEP. The final concentration of ethanol in all samples was 8%. The samples were incubated 37°C with rocking for 4 hrs. After incubation, ice cold ethanol (1.0 mL) was added and the samples were stored for 18 hrs, 4°C for precipitation of glucans. Radiolabeled glucan was determined by scintillation counting as described previously (Venkitaraman et al., 1995).

Activity of Gtf onto sHA beads

The Gtfs were adsorbed to hydroxylapatite beads coated with clarified whole saliva as detailed elsewhere (Schilling and Bowen, 1988; Venkitaraman et al., 1995). After allowing for adsorption of the enzyme, the beads were washed 3 times with absorption buffer to remove the unadsorbed material and exposed to 300 μL of the two-fold dilution series of EEP at the same concentrations described above for 30 minutes. For the control 80% aqueous ethanol was added instead of EEP. The final concentration of ethanol was 8%. The beads were washed and exposed

to 300 µL ^{14}C-(glucosyl)-sucrose substrate (100.0 mmol/L sucrose, final concentration). The samples were incubated with rocking for 4 hrs, 37 °C. After incubation, ice-cold ethanol was added to each vial, and the samples were stored for 18 hrs, 4°C. The radiolabeled glucan formed was collected and quantified as previously described (Venkitaraman et al., 1995).

Statistical Analysis

Each assay was performed in six replicates. The data were evaluated by Student's t test for each pair using software for statistical visualization, JMP version 2 (SAS Institute, Inc., 1989). The level of significance for all statistical analysis was $p<0.05$.

RESULTS

The two samples of propolis used in this experiment were selected from our previous survey of Southeastern and Southern Brazilian propolis, where we analyzed over 200 samples of this natural substance (Park et al., 1997; 1998a; 1998b). One representative sample from Southeastern Brazil (city of Santa Luzia, MG State) and another from Southern Brazil (city of Porto Alegre, RS State) were selected based on: 1) high performance thin layer chromatography and high performance liquid chromatography data, 2) *in vitro* antimicrobial action and inhibition of crude glucosyltransferase activity in solution. Both samples have demonstrated the highest inhibitory activities on *S. mutans* growth and crude Gtf activity.

The results of the effect of EEP on activity of Gtfs in solution are shown in Figs. 1, 2, 3 and 4. Both extracts greatly reduced the activity of all enzymes in solution. The enzymatic activity of Gtf B, D and G was reduced between 85-95% when the concentration of EEP was between 1.25 mg/mL - 5.0 mg/mL. The Gtf C activity was reduced at same degree when the concentration was between 2.5 mg/mL - 5.0 mg/mL. It is interesting to note that Gtf B and C were inhibited more by higher dilutions of EEP RS (Southern Brazil) than EEP from MG (Southeastern Brazil). As shown in Fig. 2, EEP RS demonstrated higher percentage (%) of inhibition of Gtf B than EEP MG at concentrations of 0.625, 0.312, 0.156, 0.078 mg/mL ($p<0.05$). EEP RS was more effective than EEP MG in inhibiting Gtf C at 1.25, 0.625, 0.312,

0.156, 0.078 mg/mL (Fig. 3) ($p<0.05$). In contrast, propolis from both regions inhibited Gtf D and G equally well. Gtf D and G were more susceptible to inhibition by EEP than other Gtfs. Even at low concentration, e.g. 0.312 mg/mL, the propolis samples were able to inhibit more than 70 and 80% of activity of Gtf D and G, respectively. EEP from both Brazilian regions demonstrated to be potent inhibitors of all purified Gtfs in solution.

The results regarding the effect of EEP on activity of Gtfs on surfaces (Figs. 1-4) demonstrated that both EEP inhibited the activity of all enzymes on surface of sHA beads, however to a lesser degree when compared with the data from solution assays. Propolis RS showed a better inhibition pattern on Gtf B and Gtf C activity than the EEP MG, demonstrating significant higher inhibitory activities at 0.078, 0.156, 0.312 mg/mL as shown in Figs. 1 and 2 ($p<0.05$). On the other hand, propolis from MG showed better effects on Gtf D and G activities than EEP RS. EEP MG was significantly more effective in inhibiting Gtf D at 2.5, 1.25 and 0.625, and Gtf G at 2.5 and 5.0 mg/mL, as demonstrated in Fig. 3 and 4 ($p<0.05$). The EEPs were not as effective inactivating sHA surface adsorbed Gtf G enzyme (Fig. 5) as they were inhibiting the same enzyme in solution. For example, at concentration of 5.0 mg/mL, the degree of inhibition was only 73±6 and 83± 7% for EEP RS and MG. In solution, Gtf G was inhibited 95% by both EEP at concentrations between 0.625 and 5.0 mg/mL. Despite the reduction of inhibitory effectiveness of EEP, both samples were able to inhibit more than 90% of all surface Gtf enzyme activity (except Gtf G) at concentration of 5.0 mg/mL.

DISCUSSION

Natural products have been used for medical purposes throughout the world for thousand of years in folk medicine. Many of them have demonstrated pharmacological properties, such as antimicrobial, anti-inflammatory and cytostatic properties, among others (Wu-Yuan et al., 1990). These products could be applied to prevent oral diseases.

Dental plaque is one of the main etiological factors of a common oral disease, dental caries. Much of the work done on identifying therapeutic agents to prevent tooth decay is aimed at disrupting the plaque matrix (Vacca-Smith and Bowen, 1997), 30-40% (dry wt) of which is polysaccharide (Critchley, 1969; Hotz et al., 1972). Indeed, most of the polysaccharide fraction

of plaque is synthesized chiefly by glucosyltransferases (Rolla et al., 1983; Loesche, 1986). The glucan produced by these enzymes play an essential role in the colonization and accumulation of cariogenic streptococci (Schilling and Bowen, 1992). Additionally, it has been demonstrated that a glucan-rich dental plaque matrix displays an increased porosity as well as changes in its inorganic composition becoming the plaque more cariogenic(Dibdin and Shellis, 1988; van Houte, 1994; Cury et al, 1997). Therefore, one of the strategies to control the dental plaque formation is to inhibit the activity of Gtfs enzymes. However, most of the work has focused mainly on enzymatic activity in solution, without regard to importance of enzyme adsorbed to the pellicle on the tooth surface. Because the development of dental caries can be attributed to events that occur at the tooth pellicle-plaque interface and enzimatically active Gtfs are presented adsorbed to the pellicle, it is fundamental to determine potential inhibitors to this adsorbed enzyme. At this point of view, surface adsorbed Gtfs gain in importance because these enzymes display increased resistance to the most common inhibitors agents, including currently available anti-plaque mouthrinses (Vacca-Smith and Bowen, 1997; Wunder and Bowen, 1999). Thus, it is highly desirable that potential inhibitors of Gtfs are as effective in inactivating pellicle adsorbed enzyme as they are in inhibiting the same enzymes in solution.

Among natural products, propolis has attracted increased interest due its biological properties (Ghisalberti, 1979; Bankova et al., 1989), and some studies have demonstrated that propolis inhibited *in vitro* the crude Gtf activity in solution (Ikeno et al., 1991; Park et al., 1998). The two distinct EEPs analyzed in the present study had showed the highest inhibitory activities on mutans streptococci growth and crude Gtf activity among all samples tested in our previous work (Park et al., 1998a). Recently, these two samples showed that were able to reduce dental caries of desalivated rats without affecting the microbial composition of dental plaque (Koo et al., 1999). Therefore, we hypothesized that one of the possible mechanisms of EEP to reduce smooth-surface caries were its activity on Gtf enzymes. However, all the work done to date have analyzed the inhibition of Gtf activity using crude preparations and solution assays. The present study is the first attempt to investigate the effect of EEP on the activity of separate, purified Gtfs in solution and adsorbed onto sHA surfaces.

The results obtained in the present study clearly demonstrated that EEP from two regions of Brazil reduced the activities of all individual Gtfs in solution and adsorbed onto sHA surface. The two samples of propolis demonstrated different pattern of inhibition of Gtf activity, and it is

evident that EEP from either regions act differently towards Gtf enzymes adsorbed onto sHA beads and in solution.

In general, ethanolic extract of propolis RS inhibited Gtf B and C activities more effectively than EEP MG; although the difference was less evident when these enzymes were adsorbed on a surface. Whereas EEP MG strongly inhibited the Gtf D and G activity demonstrating better effects than EEP RS when these enzymes were adsorbed onto sHA beads.

Among all enzymes tested in this study, Gtf B and C are the most important Gtfs related to dental caries (Yamashita et al., 1993). The glucan produced by these enzymes increased the adherence of *S. mutans* and *S. sobrinus* to an experimental pellicle (Venkitaraman et al., 1995). Particularly, the inhibition of Gtf C by EEP may be important, because this enzyme is the predominant Gtf associated with apatitic surfaces, demonstrating the highest affinity for sHA beads among all Gtfs (Vacca-Smith et al., 1996a; Vacca-Smith and Bowen, 1998). In addition, Gtfs of human whole saliva adsorbed onto HA beads have properties of Gtf C from *S. mutans* (Vacca-Smith et al., 1996a). Most of the known Gtf inhibitors tested so far, including the currently available mouthrinse, failed to inhibit Gtf C activity (Vacca-Smith and Bowen, 1997; Wunder and Bowen, 1999). Propolis strongly inhibited Gtf C irrespective of whether the enzyme is exposed before or after adsorption to a surface. This level of inhibition has not been observed before even when three different class of synthetic Gtf inhibitors were tested using the same model (Wunder and Bowen, 1999). The better inhibitory profile of Gtf B and, particularly, C activities by EEP RS could explain the results obtained in our recent animal study, where EEP RS was more effective to reduce smooth-surface dental caries than EEP MG when compared to control group (Koo et al., 1999).

Although the role of Gtf D in the mouth and in plaque formation remains unclear, the glucan synthesized by this enzyme may serve as a primer for other Gtfs enzymes, mainly those bounded to apatitic and bacterial surfaces (Vacca-Smith and Bowen, 1998). Glucosyltransferase G like Gtf D synthesizes primarily soluble glucan and its activity might catalyze sufficient glucan formation to stimulate the primer-dependent activity of the *S. mutans* Gtf enzymes (Keevil et al., 1984).

Additionally, the present data demonstrated that the inhibitory effect of EEP on Gtf activity was decreased from solution to surface assays. This observation may be related to conformational changes that occur when some enzymes are immobilized by solid surfaces

(Manjon et al., 1984; Stevanato et al., 1989). Furthermore, it is known that physical and kinetic properties of enzymes may be modified when they are adsorbed onto a surface (Tenovuo and Kurkijarvi, 1981; Demers and Wong, 1985) and this phenomena has been observed for Gtf enzymes (Schilling and Bowen, 1988). Therefore, this new conformational structure of Gtfs onto sHA surface may be less affected by EEP. For example, it is clear that both EEPs, potent inhibitors of Gtf G in solution, showed only moderate inhibitory effect when the same enzyme was adsorbed onto sHA surface. It seems that the new conformational structure of Gtf G on surface reduced, dramatically, the effect of EEP on this enzyme. The reason for these differences of enzymatic inhibition profile needs to be explored in further research.

The possible bioactive compound(s) of propolis that are modulating the Gtf inhibition are unknown and we are currently pursuing this issue. It has been widely cited that polyphenolic compounds, mainly flavonoids, are the main biologically active compounds of propolis (Ghisalberti, 1979; Bankova et al., 1989; 1992). Flavonoids are 2-phenyl-1,4-benzopyrone derivatives that are found throughout the plant kingdom (Havsteen, 1983). The high chemical reactivity of flavonoids is expressed in their binding affinity to biological polymers (Cody, 1988). A multitude of *in vitro* studies has shown that flavonoids can inhibit or activate a large variety of mammalian enzyme systems (Havsteen, 1983; Middleton and Kandaswami, 1994; Hollman 1997). Flavones and flavonols, which possess the C2-C3 double bond (Wheeler and Berry, 1986; Ferriola et al., 1989; Merlos et al., 1991; Eaton et al., 1996), has been considered the main flavonoids related to inhibition of enzymes. Among them, quercetin, galangin, kaempferol, kaempferide (flavonols) and chrysin, apigenin, acacetin (flavones) were identified in the EEPs tested in the present study (Park et al., 1997; 1998a; 1998b). These flavonoids may be involved in the Gtfs inhibition by propolis. Nevertheless, the effect of flavonoids on the activity of glucosyltransferases has not been extensively studied. So far, Iio et al. (1984) showed that quercetin and chrysin, inhibited *in vitro* the activity of crude preparation of glucosyltransferase and insoluble glucan formation. In addition, the composition of propolis is quantitatively and qualitatively variable, depending on the regional plant ecology and bees' variety (Konig, 1985; Greenaway et al., 1990; Bankova et al., 1992; Koo and Park, 1997). In our previous studies of Brazilian propolis we reported that the composition of flavonoid aglycones was qualitatively and quantitatively different between these two selected samples, EEP MG and RS (Park et al., 1997; 1998a; 1998b). Probably, the difference of the chemical composition between propolis RS and

MG can explain the different inhibitory effects on the activities of individual Gtfs in solution and adsorbed onto sHA surface.

In summary, the present findings demonstrated that EEP was exceptionally effective in inhibiting the Gtfs activities in solution and surface. Among them, two important Gtfs related to dental caries, B and C, were efficiently inhibited. However, the inhibitory effect was not the same between the two EEPs tested. EEP RS showed better inhibitory activities on Gtfs B and C, whereas EEP MG was more efficient on Gtf D and G activities. This variable effects may be related to the different chemical composition between these two samples as previously observed (Park et al., 1997; 1998a; 1998b). The inhibition of Gtf B and C, mainly the surface adsorbed enzymes, by propolis can be one of the mechanism to reduce smooth dental caries in rats (Koo et al, 1999). Therefore, the use of propolis to prevent dental plaque formation is worthy of exploration. It is clear that it is highly desirable to identify and isolate the active compounds of propolis.

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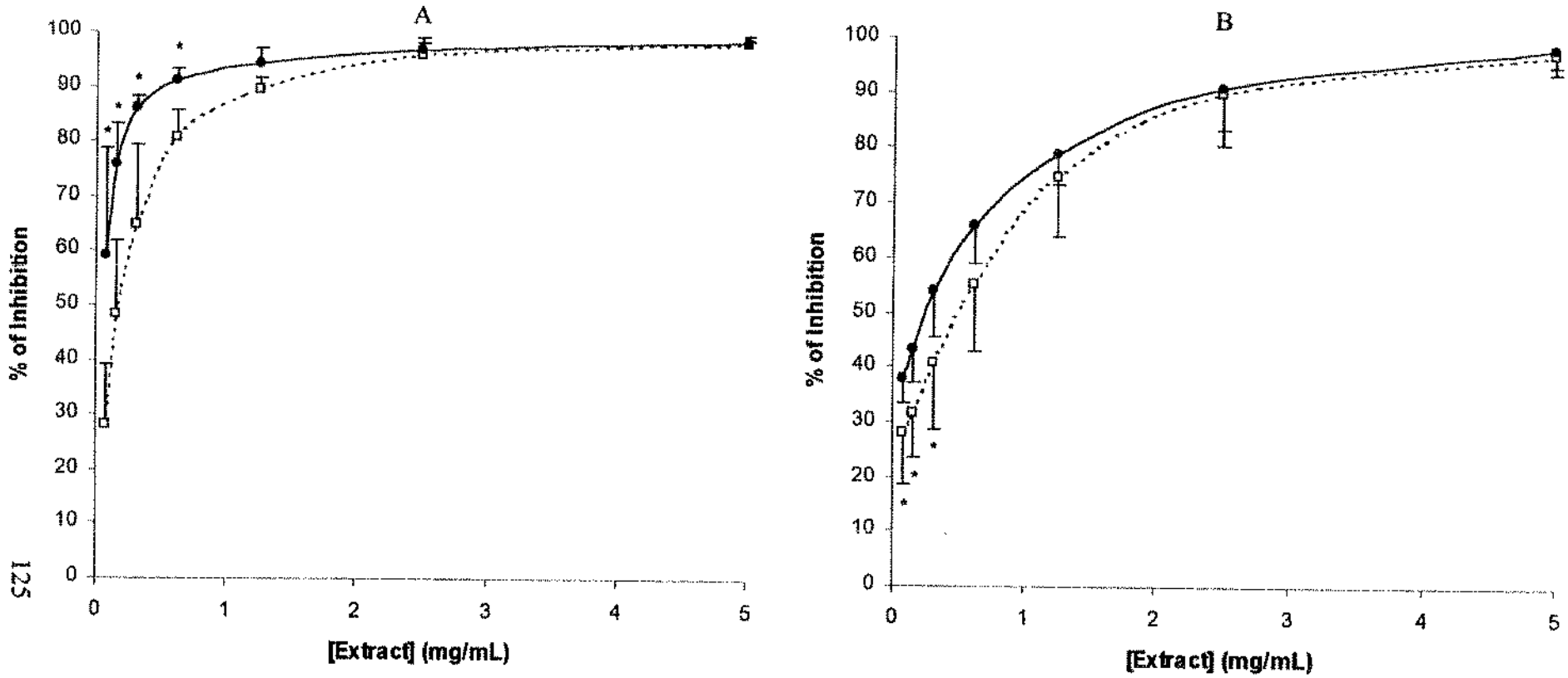

Fig. 1. Effect of Ethanolic Extracts of Propolis on the Activity of Glucosyltransferase B (Gtf B) in Solution (A) and Adsorbed onto sHA Surface (B). Symbols: —●— EEP RS (Southern Brazil); -□- EEP MG (Southeastern Brazil).

[1] The activity of Gtf in solution was determined by incubation in a reaction mixture containing 100 mmol/L ^{14}C-(glucosyl)-sucrose substrate (details in Material and Methods) and different concentrations of EEP. For the control, 80% aqueous ethanol was added instead of EEP.

[2] The activity of Gtf adsorbed onto sHA surface was determined by incubation in a reaction mixture containing 100 mmol/L ^{14}C-(glucosyl)-sucrose substrate (details in Material and Methods). The sHA beads were exposed to different concentrations of EEP after Gtf saturation on the sHA surfaces. For the control, 80% aqueous ethanol was added instead of EEP.

[3] The percentage of inhibition was calculated considering the control as maximum Gtf activity. The data shown are mean values for six replicates ± SD.

[4] The values followed by one asterisk (*) between treatments, in each concentration, are significantly different from each other at $p<0.05$ by Student's t test.

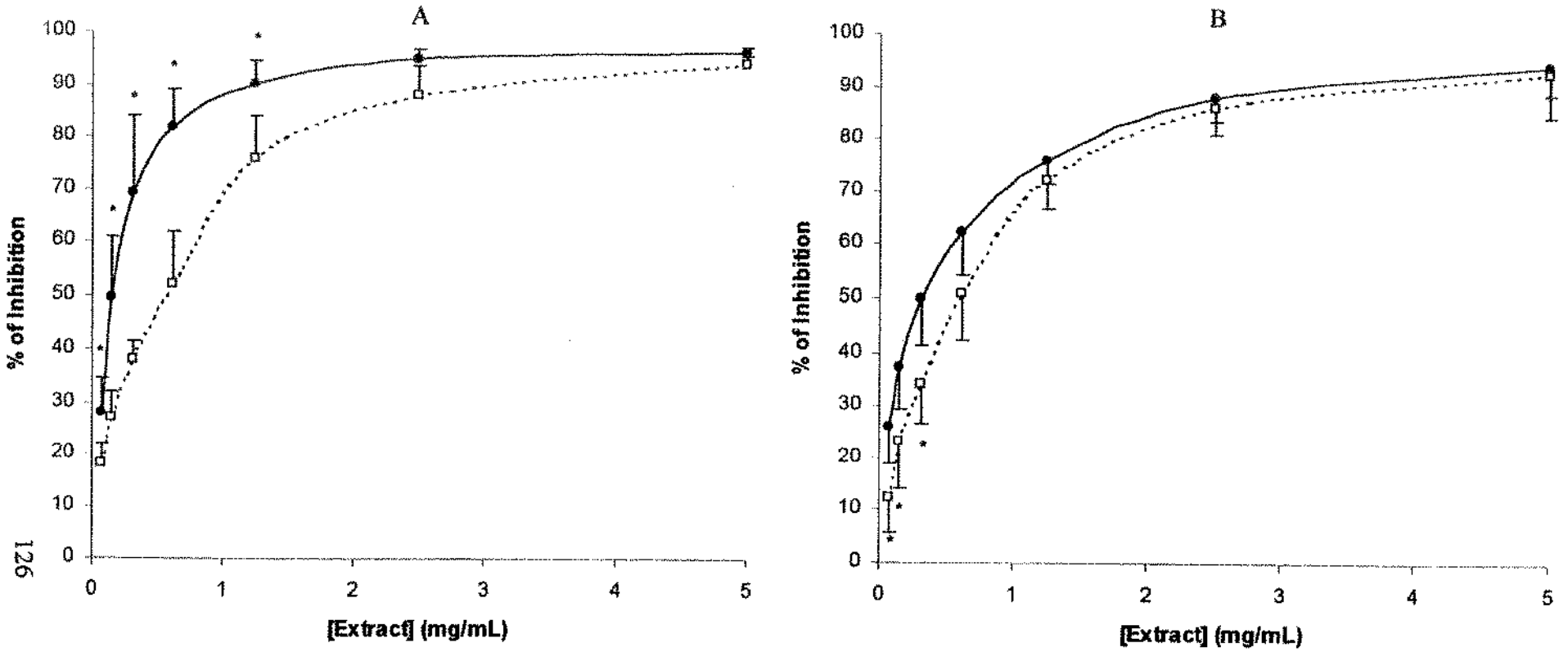

Fig. 2. Effect of Ethanolic Extracts of Propolis on the Activity of Glucosyltransferase C (Gtf C) in Solution (A) and Adsorbed onto sHA Surface (B). Symbols: —●— EEP RS (Southern Brazil); - -□- - EEP MG (Southeastern Brazil).

[1] The activity of Gtf in solution was determined by incubation in a reaction mixture containing 100 mmol/L ^{14}C-(glucosyl)-sucrose substrate (details in Material and Methods) and different concentrations of EEP. For the control, 80% aqueous ethanol was added instead of EEP.

[2] The activity of Gtf adsorbed onto sHA surface was determined by incubation in a reaction mixture containing 100 mmol/L ^{14}C-(glucosyl)-sucrose substrate (details in Material and Methods). The sHA beads were exposed to different concentrations of EEP after Gtf saturation on the sHA surfaces. For the control, 80% aqueous ethanol was added instead of EEP.

[3] The percentage of inhibition was calculated considering the control as maximum Gtf activity. The data shown are mean values for six replicates ± SD.

[4] The values followed by one asterisk (*) between treatments, in each concentration, are significantly different from each other at $p<0.05$ by Student's t test.

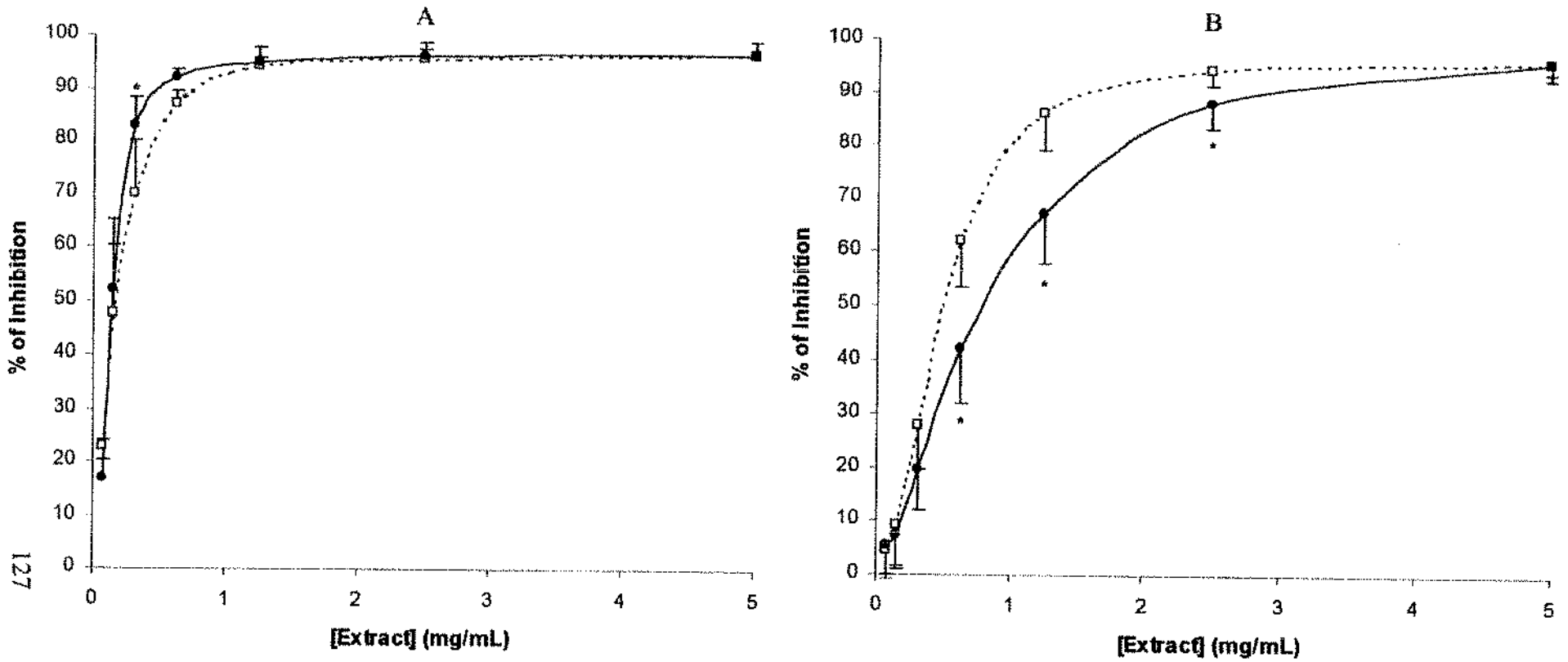

Fig. 3. Effect of Ethanolic Extracts of Propolis on the Activity of Glucosyltransferase D (Gtf D) in Solution (A) and Adsorbed onto sHA Surface (B). Symbols: —●— EEP RS (Southern Brazil); - -□- - EEP MG (Southeastern Brazil).

[1] The activity of Gtf in solution was determined by incubation in a reaction mixture containing 100 mmol/L ^{14}C-(glucosyl)-sucrose substrate (details in Material and Methods) and different concentrations of EEP. For the control, 80% aqueous ethanol was added instead of EEP.

[2] The activity of Gtf adsorbed onto sHA surface was determined by incubation in a reaction mixture containing 100 mmol/L ^{14}C-(glucosyl)-sucrose substrate (details in Material and Methods). The sHA beads were exposed to different concentrations of EEP after Gtf saturation on the sHA surfaces. For the control, 80% aqueous ethanol was added instead of EEP.

[3] The percentage of inhibition was calculated considering the control as maximum Gtf activity. The data shown are mean values for six replicates ± SD.

[4] The values followed by one asterisk (*) between treatments, in each concentration, are significantly different from each other at $p<0.05$ by Student's t test.

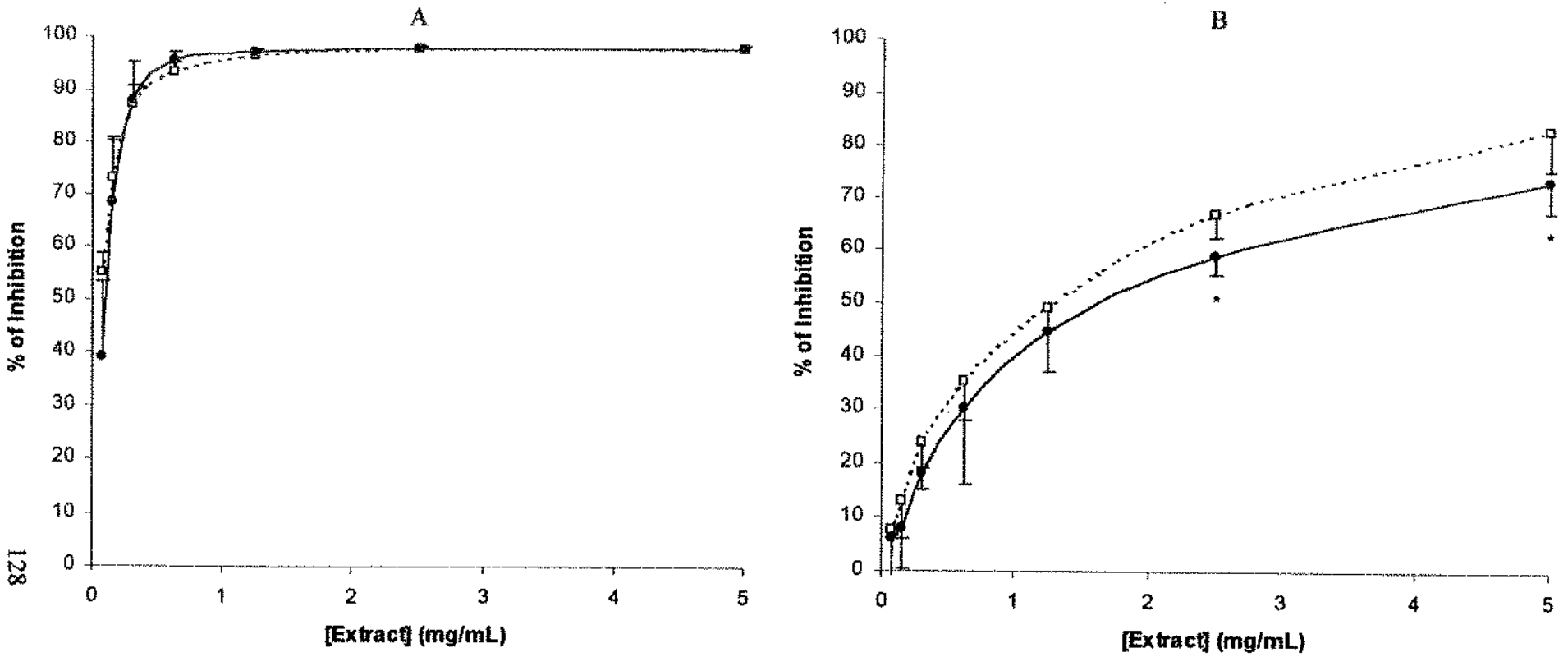

Fig. 4. Effect of Ethanolic Extracts of Propolis on the Activity of Glucosyltransferase G (Gtf G) in Solution (A) and Adsorbed onto sHA Surface (B). Symbols: —●— EEP RS (Southern Brazil); -□- EEP MG (Southeastern Brazil).

[1] The activity of Gtf in solution was determined by incubation in a reaction mixture containing 100 mmol/L ^{14}C-(glucosyl)-sucrose substrate (details in Material and Methods) and different concentrations of EEP. For the control, 80% aqueous ethanol was added instead of EEP.

[2] The activity of Gtf adsorbed onto sHA surface was determined by incubation in a reaction mixture containing 100 mmol/L ^{14}C-(glucosyl)-sucrose substrate (details in Material and Methods). The sHA beads were exposed to different concentrations of EEP after Gtf saturation on the sHA surfaces. For the control, 80% aqueous ethanol was added instead of EEP.

[3] The percentage of inhibition was calculated considering the control as maximum Gtf activity. The data shown are mean values for six replicates ± SD.

[4] The values followed by one asterisk (*) between treatments, in each concentration, are significantly different from each other at $p<0.05$ by Student's t test.

Artigo IV

EFFECTS OF QUERCETIN, 3, 3', 4', 5, 7 – PENTAHYDROXYFLAVONE, ON THE ACTIVITIES OF STREPTOCOCCAL GLUCOSYLTRANSFERASES IN SOLUTION AND ADSORBED ONTO SALIVA-COATED HYDROXYAPATITE

Hyun Koo[1]*, Anne M. Vacca Smith[2], William H. Bowen[2], Pedro L. Rosalen[1], Jaime A. Cury[1], Gláucia M. B. Ambrosano[1], Yong K. Park[3]

[1] Faculty of Dentistry of Piracicaba, State University of Campinas, Caixa Postal 52, Piracicaba, 13414-900, SP, Brazil

[2] Center for Oral Biology, University of Rochester, 601 Elmwood Avenue, Box 611, Rochester, 14642, NY, USA

[3] School of Food Engineering, State University of Campinas, Caixa Postal 6177, Campinas, 13081-970, SP, Brazil

Running Title: Effects of quercetin on glucosyltransferases

Key Words: flavonoids, quercetin, glucosyltransferase, hydroxyapatite, glucan, plaque

Abbreviations: Gtf, glucosyltransferase; sHA, saliva-coated hydroxyapatite; PMSF, phenylmethylsulfonyl-fluoride; LMW, low molecular weight

* Full address of corresponding author, to whom requests for reprints should be addressed:
Hyun Koo
University of Rochester
Center for Oral Biology
601 Elmwood Ave. Box 611
Rochester, NY 14642
USA
Tel (716) 275-1433 / Fax (716) 473-2679
E-mail: hyunkoobr@yahoo.com

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ABSTRACT

Quercetin, a benzo-γ-pyrone derivative, has been considered a potent inhibitor of various mammalian enzymes. In the present study, the effects of quercetin (3, 3', 4', 5, 7 – pentahydroxyflavone) on the activity of separate, purified glucosyltransferase (Gtf) enzymes in solution and adsorbed onto saliva-coated hydroxyapatite (sHA) beads were evaluated. Quercetin dissolved in 99.5% ethanol (v/v) (concentration ranging from 3.9 to 250.0 μg/mL, final concentration) was tested for its ability to inhibit the enzymes responsible for the synthesis of insoluble glucan (Gtf B), insoluble/soluble glucan (Gtf C) and soluble glucan (Gtf D) from *Streptococcus mutans*. Effects on Gtf G from *Streptococcus sanguis* (soluble glucan synthesis), was also determined. Quercetin inhibited the activity of all Gtfs in solution (50-60% for Gtf B, C and D; and 90% for Gtf G at concentration of 250 μg/mL), demonstrating that is a potent inhibitor of Gtf G (60-80% inhibition at concentrations between 15.6 and 31.3 μg/mL). The Gtf G was significantly more inhibited in all concentrations tested when compared to other Gtfs ($p<0.05$). However, the inhibitory action of quercetin on surface adsorbed Gtfs was not as effective as that on the same enzymes in solution. Among the Gtfs analyzed, only Gtf B and C were inhibited by quercetin (25 and 40% for Gtf B and C, at concentration of 250 μg/mL). The activities of surface adsorbed Gtf D and G were unaffected by quercetin, even at the highest concentration (250 μg/mL). The inhibitory effect of quercetin was different depending on the enzyme tested and its physical state. These data indicate that quercetin inhibited significantly the activity of Gtfs in solution at μM concentration, however its effect was greatly reduced when these enzymes were adsorbed onto sHA surface. Effects, if any, on structure of glucan remain to be elucidated.

INTRODUCTION

Glucosyltransferase enzymes (Gtf, EC 2.4.1.5) produced by *Streptococcus mutans* catalyze the formation of soluble and insoluble α-linked glucans from sucrose and contribute significantly to the polysaccharide composition of dental plaque matrix (Rölla et al., 1983; Loesche, 1986). These glucans synthesized by Gtfs can enhance the pathogenic potential of dental plaque by promoting the accumulation of cariogenic streptococci on the teeth of humans and experimental animals (Krasse, 1965; Frostell et al., 1967; Hamada and Slade, 1980). In addition, a glucan-rich dental plaque matrix displays an increased porosity (Dibdin and Shellis, 1988; van Houte, 1994) as well as changes in its inorganic composition (Cury et al, 1997). Three Gtfs have been identified from *S. mutans*: Gtf B, which synthesizes a polymer of mostly insoluble α1,3-linked glucan; Gtf C, which synthesizes a mixture of insoluble α1,3-linked glucan and soluble α1,6-linked glucan; and GtfD which synthesizes an α1,6-linked soluble glucan (Loesche, 1986; Aoki, 1986; Hanada and Kuramitsu, 1988; 1989). An additional enzyme, Gtf G, may also be involved in the development of dental plaque. Gtf G is synthesized by *S. sanguis,* an early colonizer of tooth surface, and catalyzes predominantly α1,6-linked soluble glucan (Carlsson et al., 1969; Beeley and Black, 1977). Gtfs are present in the soluble fraction of whole human saliva and, also, incorporated into salivary pellicle formed *in vivo* and *in vitro* (Rölla et al., 1983; Scheie et al., 1987; Schilling and Bowen, 1988). In addition, the Gtfs adsorbed to an experimental pellicle express enhanced activity producing large amount of glucans *in situ* when exposed to sucrose (Schilling and Bowen, 1988). This glucan provides attachment sites for *S. mutans* and *S. sobrinus* onto the surface of the tooth and contributes to the formation and bulk of dental plaque (Schilling and Bowen, 1992; Vacca-Smith and Bowen, 1998). Therefore, inhibition of the function of these important plaque building enzymes, could play an important role in the prevention of formation and accumulation of dental plaque, and the expression of virulence by mutans streptococci.

Quercetin is a benzo-γ-pyrone derivative and belongs to flavonoid group, a class of naturally occurring phenolic compound ubiquitously distributed in the plant kingdom. Flavonoid compounds have also been found in propolis, a resinous substance collected by *Apis mellifera* bees (Bankova et al., 1982; Bonhevi et al., 1994; Park et al., 1998). This class of phenolic compounds have been reported to possess widespread biological activities, especially toward

enzymes (Havsteen, 1983, Middleton and Kandaswami, 1994; Hollman 1997). Inhibition of a wide range of enzymes has been reported such as, phosphorylase kinase, lipoxygenases, phospholipases A_2, protein kinases, ATPases, reverse transcriptase, P-form phenolsulfotransferases, α-glucosidases, among others (Kyriakidis et al., 1986; Wheeler and Berry, 1986; Fawzy et al., 1988; Ferriola et al., 1989; Hirano et al., 1989; Ono et al., 1990; Middleton and Kandaswami, 1994; Eaton et al., 1996; Watanabe et al., 1997). Among flavonoids, quercetin is the most studied enzyme inhibitor. It is therefore, surprising that such large and important class of natural compounds have failed to attract researchers to test on glucosyltransferase enzymes. So far, Iio et al. (1984) showed that quercetin inhibited *in vitro* the activity of crude preparation of glucosyltransferase in solution. Nevertheless, the authors could not determine precisely which Gtf (or glucan) had been inhibited, since they used crude Gtf and an indirect method for Gtf activity assay; and more importantly, whether EEP is an effective inhibitor of pellicle-adsorbed enzyme. Considering the pharmacological potential of quercetin as enzyme inhibitor, the aim of the present study was to determine the effect of quercetin on the activity of individual and purified Gtf enzymes both in solution and adsorbed onto saliva coated hydroxyapatite (sHA) surface.

MATERIALS AND METHODS

Quercetin

Quercetin was purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in 99.5% ethanol (5.0 mg of quercetin/mL) just prior to carrying out the assay. The chemical structure of this flavonoid compound is shown in Fig. 1.

Saliva

Whole saliva was collected on ice from one donor by chewing paraffin-film and was clarified by centrifugation (8500g, 4 °C, 10 min.). Afterwards, the saliva was diluted 1:1 with adsorption buffer (50 mM KCl, 1.0 mM KPO_4, 1.0 mM $CaCl_2$, 0.1 mM $MgCl_2$, pH=6.5), supplemented with sodium azide (0.02%, final concentration) and the protease inhibitor

phenylmethylsulfonyl-fluoride (PMSF, 1.0 mmol/L, final concentration) (Vacca-Smith et al., 1996a). Negligible levels of Gtf were detected in the whole saliva from this donor.

Hydroxyapatite Beads

Hydroxyapatite (HA) beads (10 mg) were used for surface assays (Bio-Rad Laboratories, Hercules, CA, USA). The surface area of the beads was $0.24m^2$, and the particle size was 80 μm.

Bacterial Strains

The bacterial strains used in this experiment were: 1) *Streptococcus milleri*, which harbors the *gtfB* gene (for Gtf B production); 2) *S. milleri* NH5, which contain the *gtfD* gene (for Gtf D); *S. mutans* WHB 410, which the genes for Gtf B, D and fructosyltransferase were deleted (for Gtf C) (Wunder and Bowen, 1999); 4) *S. sanguis* 10904 (for Gtf G). The *S. milleri* constructs were a kind gift from Dr. Howard K. Kuramitsu (SUNY, Buffalo, NY).

Purification of Gft Enzymes

All the purification procedures were carried out using buffers containing the protease inhibitor, PMSF (0.1 mM, final concentration) and NaN_3 (0.02%, final concentration) as preservative. Neither reagents had any adverse effects on enzyme activity or stability. The enzymes were stored at 4°C, in adsorption buffer supplemented with 10% glycerol. The purity of the enzyme preparations was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a Hoefer Mighty Small SE245 system (Hoefer Scientific Instruments, San Francisco, CA, USA) and silver staining as previously described (Venkitaraman et al., 1995). Protein concentration was determined by the 280-nm absorbance method outlined in Deutscher (1990).

The Gtf B and G enzyme was obtained and purified to near homogeneity from clarified culture supernatant of *S. milleri* KSB8 and *S. sanguis* 10904 using the method described by Venkitaraman et al. (1995) and Vacca Smith et al. (manuscript submitted).

The purification of Gtf D from *S. milleri* NH5 was performed using the procedures outlined by Wunder and Bowen (1999).

Gtf C was obtained from culture of *S. mutans* WHB 410. Briefly, actively growing *S. mutans* WHB 410 was inoculated into dialysis tubing (molecular weight cutoff 50,000) containing 200 mL of Low Molecular Weight medium-LMW (2.5% tryptone, 1.5% yeast extract, 0.3 % glucose, 0.1% fructose and 0.1% sorbitol; Schilling and Bowen, 1988) supplemented with erythromycin-10 μg/mL, tetracycline-5 μg/mL, kanamycin-500 μg/mL and immersed into 800 mL of the same medium. After growth, the cells were pelleted by centrifugation at 5000g, 4°C, for 20 min and the supernatant fluid discarded. The cell pellet was resuspended in 20 mL of 50 mM KPO_4 pH 7.5 and 0.01% of Triton X-100 and sonicated on ice (five 30s bursts at 250 Watts and 10s intervals, Braun-Sonic 1510). After sonication, the resuspended cells were centrifuged at 5000g, 4°C, for 10 min. The supernatant was collected as the source of Gtf C. Gtf C was purified as previously described by Venkitaraman et al. (1995).

Glucosyltransferase activity was measured by the incorporation of ^{14}C - glucose from labelled sucrose (NEN Research Products, Boston, MA) into glucans. The specific activities of the Gtf enzymes were as follows: Gtf B, 1.24×10^{-3} μmol of glucose incorporated into glucan/min./ μg of protein; Gtf D, 6.90×10^{-4} μmol of glucose incorporated into glucan/min./ μg of protein; Gtf C, 1.63×10^{-4} μmol of glucose incorporated into glucan/min./ μg of protein; and Gtf G, 2.44×10^{-3} μmol of glucose incorporated into glucan/min./ μg of protein. One unit of enzyme was defined as the amount of enzyme needed to incorporate one μmol of glucose into glucan over a four-hour reaction period. The Gtf enzyme added to each sample for all assays was equivalent to the amount required to incorporate 1.0-1.5 μmol of glucose over the course of the reaction (1.0-1.5 units).

Activity of Gtf in solution

Gtf B, C, D and G were mixed with a two-fold dilution series of quercetin (concentration ranging from 3.9 to 250.0 μg/mL) and incubated with ^{14}C-(glucosyl)-sucrose substrate (200.0 mmol/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 (200 μL final volume). For the control, the same reaction was done in which 99.5% ethanol was added instead of quercetin. The final

concentration of ethanol in all samples was 9.95 %. The samples were incubated 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, 4°C for precipitation of glucans. Radiolabeled glucan was determined by scintillation counting as described previously (Venkitaraman et al., 1995).

Activity of Gtf adsorbed onto sHA beads

The Gtfs were adsorbed to hydroxylapatite beads coated with clarified whole saliva as detailed elsewhere (Schilling and Bowen, 1988; Venkitaraman et al., 1995). After allowing for adsorption of the enzyme, the beads were washed 3 times with absorption buffer to remove the unadsorbed material and exposed to 300 μL of the two-fold dilution series of quercetin at the same concentrations used in the solution assay for 30 minutes. For the control 99.5% ethanol was added instead of quercetin, again the final concentration of ethanol was 9.95%. The beads were washed and exposed to 300 μL ^{14}C-(glucosyl)-sucrose substrate (100.0 mmol/L sucrose, final concentration). The samples were incubated with rocking for 4 h, 37 °C. After incubation, ice-cold ethanol was added to each vial, and the samples were stored for 18 h, 4°C. The radiolabeled glucan formed was collected and quantified as previously described (Venkitaraman et al., 1995).

Statistical Analysis

Six replicates were performed for each assay. A one-way layout experimental design was used in 4x2x7 factorial scheme (enzyme x state X dose). An analysis of variance was run and qualitative treatments were compared using Tukey test at level of 5% of significance ($p<0.05$). A polynomial regression was applied in order to evaluate dose effects.

RESULTS

The results of the effect of quercetin on activity of Gtfs in solution are shown in Fig. 2. Quercetin reduced the activity of all enzymes in solution in a dose-dependant manner (r^2 values ranging from 0.909 to 0.999). However, the pattern of enzymatic inhibition was different among

the Gtfs tested. The enzymatic activity of Gtf B and C was reduced 20-60% when the concentration of quercetin was between 62.5 to 250 μg/mL, showing similar inhibition pattern. In contrast, Gtf D and G demonstrated different profile of enzymatic inhibition to those obtained for Gtf B and C. The inhibition of Gtf D was 35-50%, whereas the activity of Gtf G was reduced more than 90% at concentrations between 62.5 to 250 μg/mL. As shown in Fig. 2, quercetin strongly inhibited the activity of Gtf G in solution, demonstrating 40% to 60% inhibition at concentrations between 7.8 and 15.6 μg/mL. Quercetin demonstrated to be a potent inhibitor of Gtf G activity in solution.

The results regarding the effect of quercetin on activity of Gtfs on sHA surfaces are illustrated in Fig. 3. The inhibition profile of Gtf enzymes changed when compared to the solution phase assays. Among the four Gtfs analyzed only Gtf B and C were inhibited by quercetin. Neither Gtf D nor Gtf G was inhibited by quercetin, even at the highest concentration (250 μg/mL). On the other hand, quercetin reduced 15-25% of the activity of Gtf B and 20 – 40% of the activity of Gtf C at concentrations between 62.5 – 250 μg/mL. Thus, quercetin was not as effective inactivating sHA surface adsorbed Gtfs enzymes as it was inhibiting the same enzyme in solution, mainly for Gtf D and G. It is interesting to note that although Gtf G in solution was strongly inhibited by quercetin, even at low concentrations, the same compound failed to inhibit this enzyme when it was adsorbed onto sHA surface. On the other hand, quercetin maintained a similar pattern of inhibition of Gtf B and C independently if the enzymes were in solution or on surface. In general, all Gtfs adsorbed to sHA showed a greater resistance to inactivation by quercetin, particularly Gtf D and G.

DISCUSSION

The presence of flavonoids is widespread among plants and plant products (e.g. fermented and processed foods) (Formica and Regelson, 1995). Consequently, flavonoids are unavoidably consumed daily with an estimated total consumption of 1g/day (for US diet), although this value is quite variable depending on the region and season (Künhau, 1976; Pierpoint, 1986; Bravo, 1998). Among them, quercetin is one of the most consumed (25 – 50 mg/day/person) (Brown, 1980; US Department of Health and Human Services, 1992) and its concentration is high in some

food stuffs, as fruit juices (5 – 13mg/L), red wine (4-16 mg/L), tea (10-25 mg/L), onion (284-486 mg/kg), kale (110 mg/kg), broccoli (30mg/kg), apple (21-72 mg/kg) (Formica and Regelson, 1995). In addition, quercetin could be also found in propolis (20 – 130 mg/100mg), a natural bee product commonly used in folk medicine for various purposes (Bankova et al, 1982; Bonhevi et al, 1994; Park et al., 1997; 1998).

Flavonoids are recognized as a class of natural products of high pharmacology potency and many studies have been reported demonstrating various biological properties, e.g. antimicrobial, antioxidant, anti-inflammatory, anti-tumoral, among others (Havsteen, 1983; Hollman and Katan, 1998). One explanation for such variety of pharmacological activities is their high chemical reactivity expressed by their affinity to biological polymers and heavy metal ions as well as in their ability to catalyze electron transport and to scavenge free radicals (Havsteen, 1983). Most of the analyses with flavonoids were carried out using different mammalian enzymatic systems (Havsteen, 1983; Middleton and Kandaswami, 1994). Quercetin is the most studied flavonoid and it has been demonstrated that quercetin is a potent inhibitor of several enzymes, besides demonstrating anti-tumoral and antioxidant activities (Kyriakidis et al., 1986, Formica and Regelson, 1995). This class of natural compounds, a common component of diet, could be an important ally in the prevention of oral diseases.

Recently, we demonstrated that ethanolic extracts of Brazilian propolis strongly reduced the activities of purified Gtfs in solution and adsorbed onto sHA (manuscript submitted). Several flavonoid aglycones were identified and quantified in Brazilian propolis collected by *Apis mellifera* bees (Park et al, 1997; 1998). Quercetin was found in significant amounts in the ethanolic extract of propolis (0.9 – 3.9 mg/g). Considering that quercetin is a potent enzyme inhibitor, we considered the hypothesis that quercetin could be one of the active compounds of propolis in inhibiting the separate and purified Gtfs. The present study is the first attempt to investigate the effect of quercetin on the activity of separate, purified Gtfs in solution and adsorbed onto sHA surface.

The results presented in this study demonstrated that quercetin was able, at μM concentration, to reduce the activity of all Gtfs in solution, however it is evident that quercetin acts differently towards these enzymes. Quercetin was a potent inhibitor of Gtf G and was moderately inhibitory to Gtfs B, C and D activities. It is interesting to note that Gtf B and C had susceptibility profiles more alike to each other than to those obtained for Gtf D and G, which may

be explained by the greater similarity between Gtf B and C at the amino-acid level (Shiroza et al., 1987; Ueda et al., 1988). In our previous study, the ethanolic extracts of propolis reduced more than 90% of the enzymatic activity of Gtf G in solution at a concentration of 5mg/mL using roughly the same model of analysis (unpublished data). At this same concentration of propolis, the amount of quercetin is between 10 to 20 μg/mL. As shown in Fig. 2, the Gtf G was inhibited 40 to 80% when the concentration was between 7.8 to 31.3 μg/mL. The Gtf D was inhibited between 15 to 20% at these same concentrations, whereas the activity of Gtf B and C were not affected. Therefore quercetin could be one of the active compounds of propolis to inhibit Gtf G, and at a lesser degree Gtf D, when these enzymes are in solution, but its presence clearly does not explain completely the inhibition of Gtf by propolis.

In contrast, surface adsorbed Gtfs displayed increase resistance to inhibition by quercetin. The activities of Gtfs B and C were reduced mainly at higher concentrations, e.g. 125 and 250 μg/mL. Whereas Gtf D and G were practically unaffected by quercetin in all concentrations tested. This observation may be related to conformational changes that occur when some enzymes are immobilized by solid surfaces (Manjon et al., 1984; Stevanato et al., 1989). Furthermore, it is known that physical and kinetic properties of enzymes may be modified when they are adsorbed onto a surface (Tenovuo and Kurkijarvi, 1981; Demers and Wong, 1985) and this phenomena has been observed for Gtf enzymes (Schilling and Bowen, 1988). Therefore, this new conformational structure of Gtfs onto sHA surface may be less affected by quercetin. It is evident that the greatest differences in susceptibility profiles of the Gtfs to quercetin were observed for Gtf D and, mainly, G. Quercetin a potent inhibitor of Gtf G in solution showed negligible effect when the same enzyme was adsorbed on surface. On the other hand, the same was not observed for Gtf B and C. The inhibition curve was very similar between the solution and surface assays, although the effectiveness of quercetin was slightly reduced when these enzymes were adsorbed to sHA. Based on the present findings, quercetin contributes slightly to the inhibition of surface Gtfs by ethanolic extracts of propolis, where the activities of Gtf B and C were reduced 5 to 15 % at concentrations between 7.8 to 31.3 μg/mL.

Quercetin was as effective in reducing pellicle-adsorbed Gtfs B and C as it was inhibiting the same enzymes in solution. The inhibition of these enzymes may have significant influence on dental plaque formation, because the glucan produced by these enzymes increases the adherence of *S. mutans* and *S. sobrinus* to experimental pellicles (Venkitaraman et al., 1995). In addition,

Gtf B and C have been considered the most important Gtfs related to dental caries (Yamashita et al., 1993). Particularly, the inhibition of Gtf C by quercetin is important, because this enzyme is the predominant Gtf associated with apatitic surfaces, demonstrating the highest affinity for sHA beads among all Gtfs (Vacca-Smith et al., 1996a; Vacca-Smith and Bowen, 1998). In addition, Gtfs of human whole saliva adsorbed onto HA beads have properties of Gtf C from *S. mutans* (Vacca-Smith et al., 1996a). Most of the known Gtf inhibitors, including currently available commercial anti-plaque agents, failed to inhibit Gtf C activity (Vacca-Smith and Bowen, 1997, Wunder and Bowen, 1999). Quercetin was able to inhibit more than 40% the activity of Gtf C irrespective of whether the enzyme is exposed before or after adsorption to a surface at concentration of 250 μg/mL.

The mechanism of inhibition of enzymes by flavonoids is complex and it is not completely understood. Flavonoids belong to polyphenolic compounds group and they are well known to complex with proteins through hydrogen and covalent bonds causing precipitation and/or enzyme inhibition (Elliot et al., 1992; Bravo, 1998). Low molecular weight phenols (e.g. flavonoids), differently from highly polymerized tannins, are unable to precipitate proteins. The enzymatic inhibition is more related to direct enzyme-flavonoid interaction rather than protein precipitation, a view supported by Iio et al. (1984) who examined the specificity of quercetin to α-glucosidase by adding bovine serum albumin ranging from 1 fold to 100 fold the concentration of total protein originally present in the enzyme preparation. The presence of albumin did not affect the inhibitory action of quercetin, suggesting specificity of the flavonoid compound to this enzyme. Additionally, flavones and flavonols (e.g. quercetin), which possess the C2-C3 double bond has been considered the main flavonoids related to inhibition of enzymes (Wheeler and Berry, 1986; Ferriola et al, 1989; Merlos et al., 1991; Eaton et al, 1996).

In regard to Gtf, little is known about the possible mechanism of inactivation of these enzymes by flavonoids and clearly we are pursuing this answer. The results of our study demonstrated that the effectiveness of quercetin varied with the particular Gtf tested and, also, whether the enzyme was free in solution or bound to an experimental pellicle. The reason for these differences of enzymatic inhibition profile needs to be explored in further research.

Recently, Pace et al. (1995) described a method of glycosyltransferase assay using flavonoids, among them quercetin, as substrate. These authors demonstrated that o-glycosyltransferase, isolated and purified from pea seedlings, catalyses the glycosylation of

flavonoids to their glycosides form. These findings may suggest that quercetin is acting competitively with Gtf enzymes, however studies need to be performed using the Gtfs tested in this work before we can conclude that a similar mechanism operates in our system.

In summary, quercetin, a common food ingredient, proved that may have some important implications to reduce dental plaque formation and could be considered one of the active compounds in propolis. It is evident that is highly desirable to search for this class of naturally occurring compounds as inhibitor agents of Gtfs. Our next step is to test other flavonoids identified in propolis and also determine the possible mechanisms of inhibition.

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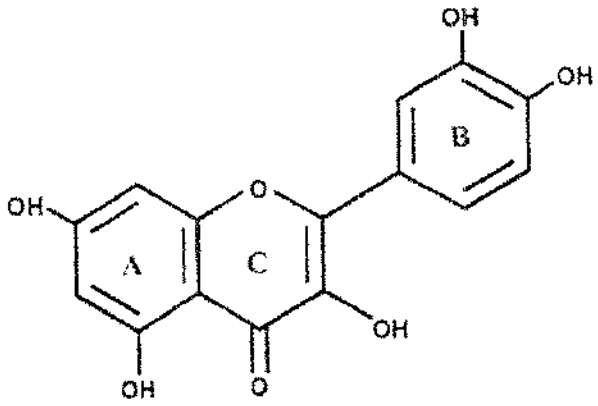

Figure 1. Chemical Structure of Quercetin (3, 3', 4', 5, 7 – pentahydroxyflavone).

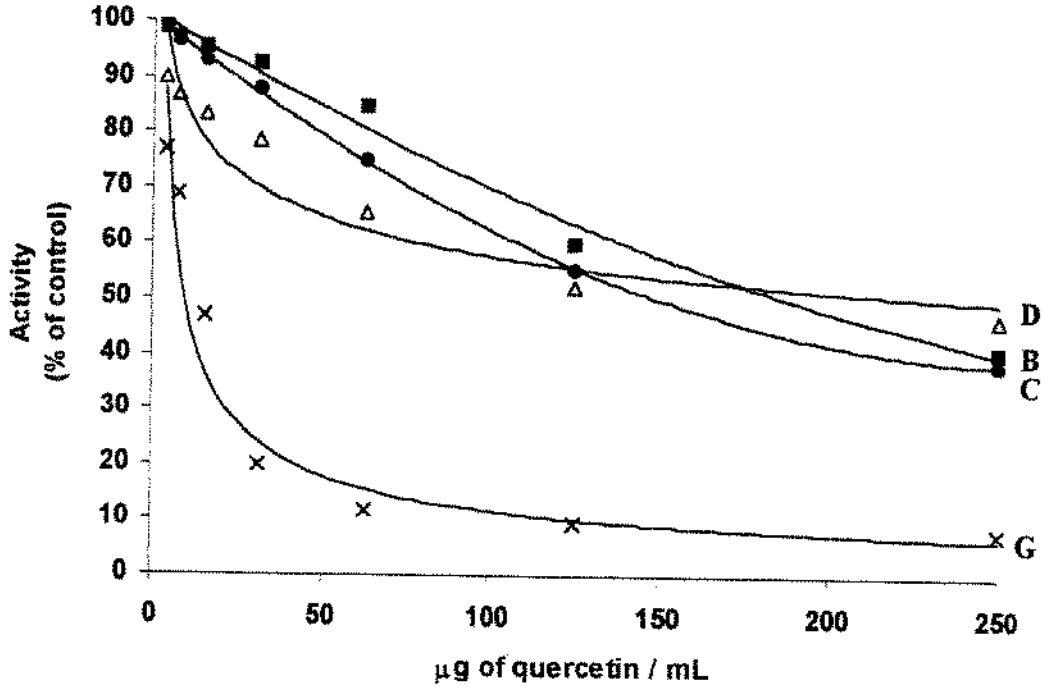

Figure 2. Effect of Quercetin on the Activities of Glucosyltransferases (GTF) in Solution.

[1] The activity of Gtf in solution was determined by incubation in a reaction mixture containing 100 mmol/L ^{14}C-(glucosyl)-sucrose substrate (details in Material and Methods) and different concentrations of quercetin. For the control, ethanol (9.95%, final concentration) was added instead of quercetin.

[2] The percentage of Gtf activity was calculated considering the control as maximum enzymatic activity.

[3] The data shown are mean values for six replicates.

[4] Gtf B: $y = 0.0009x^2 - 0.4671x + 100.61$ / $r^2 = 0.9993$, Gtf C: $y = 0.0005x^2 - 0.3576x + 101.32$ / $r^2 = 0.9879$, Gtf D: $y = 123.23x^{-0.1643}$ / $r^2 = 0.9108$, Gtf G: $y = 205.84x^{-0.6253}$ / $r^2 = 0.9533$

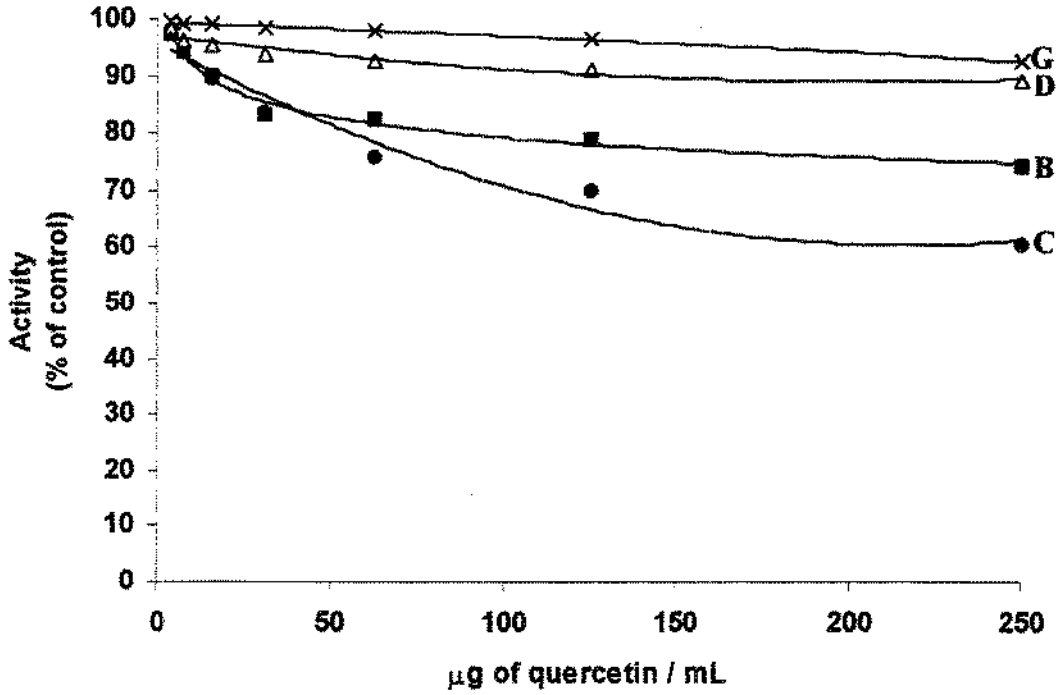

Figure 3. Effect of Quercetin on the Activities of Glucosyltransferases (GTF) Adsorbed onto sHA Surface.

1 The activity of Gtf adsorbed onto sHA surface was determined by incubation in a reaction mixture containing 100 mmol/L ^{14}C-(glucosyl)-sucrose substrate (details in Material and Methods). The sHA beads were exposed to different concentrations of quercetin after Gtf saturation on the sHA surfaces. For the control, ethanol (9.95%, final concentration) was added instead of quercetin.

[2] The percentage of Gtf activity was calculated considering the control as maximum enzymatic activity.

[3] The data shown are mean values for six replicates ± SD.

[4] Gtf B: $y = 106.48x^{-0.0642}$ / $r^2 = 0.9831$, Gtf C: $y = 119x^{-0.113}$ / $r^2 = 0.9606$, Gtf D: $y = 0.0002x^2 - 0.0802x + 97.154$ / $r^2 = 0.909$, Gtf G: $y = -7E\text{-}05x^2 - 0.0166x + 99.368$ / $r^2 = 0.9963$

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Effect of *Apis mellifera* Propolis from Two Brazilian Regions on Caries Development in Desalivated Rats

H. Koo[a] P.L. Rosalen[a] J.A. Cury[a] Y.K. Park[b] M. Ikegaki[b] A. Sattler[c]

[a]Department of Physiological Sciences, Faculty of Dentistry of Piracicaba, State University of Campinas, Piracicaba, [b]Department of Food Science, College of Food Engineering, State University of Campinas, [c]Department of Entomology, Faculty of Agricultural Science, Federal University of Rio Grande do Sul – FEPAGRO, Porto Alegre, Brazil

Key Words

Caries · Cariostatic effect · Flavonoid compounds · Propolis

Abstract

The purpose of the present study was to evaluate the effect of *Apis mellifera* propolis collected from two regions of Brazil on caries development in desalivated rats. Ethanolic extracts of propolis (EEP) were prepared from crude propolis samples collected in Minas Gerais state (MG), southeastern Brazil, and Rio Grande do Sul state (RS), southern Brazil. The flavonoid composition of EEP was analyzed by high-performance thin-layer chromatography (HPTLC) and reversed-phase high-performance liquid chromatography (HPLC). For the animal study, 30 specific pathogen-free Wistar rats were infected with *Streptococcus sobrinus* 6715 and surgically desalivated. The rats were randomly divided into three groups which were treated with 80% ethanol (control), EEP from MG and EEP from RS. The animals were placed in a König-Höfer programmed feeder and received 17 meals of diet 2000 daily at hourly intervals. The solutions were applied on the rat molars (25 μl on molars of each quadrant) twice a day, by using graduate syringes. After 3 weeks, the animals were killed by CO_2 asphyxiation. For microbial assessment, the left jaw was removed and sonicated in 154 m*M* NaCl solution. Dental caries was evaluated according to Larson's modification of Keyes' system. The HPTLC patterns and HPLC profiles demonstrated that both quality and quantity of flavonoid aglycones of EEP from MG were different compared to EEP from RS. In general, it is apparent that EEP from RS contained the highest concentrations of pinocembrin, chrysin, acacetin and galangin. The group of animals treated with EEP from RS showed the lowest smooth-surface and sulcal caries scores as well as less caries severity in smooth-surface and sulcal lesions, and these data were statistically different when compared with the control group. The group treated with EEP from MG only demonstrated a significant difference in the severity of sulcal lesions when compared to the control group. The percentage of *S. sobrinus* was lower in the groups treated with EEP, but did not differ statistically from the control group. The results showed that the cariostatic effect of propolis depends on its composition, and consequently the region of collection of propolis samples.

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Jaime A. Cury
FOP/UNICAMP
Av. Limeira, 901, Caixa Postal 52
15414-900 Piracicaba, SP (Brazil)
Tel. +55 19 430 5302, Fax +55 19 430 5218, E-Mail jcury@fop.unicamp.br

Mutans streptococci have been implicated as an essential agent in the pathogenesis of dental caries in animal and human experiments [Fitzgerald and Keyes, 1960; Hamada et al., 1984; Loesche, 1986]. The ability of these microorganisms to synthesize extracellular polysaccharides mainly water-insoluble glucan, by action of glucosyltransferase and their acidogenic and aciduric characteristics are the major factors involved in the initiation and progression of dental caries [De Stoppelaar et al., 1971; Gibbons and Van Houte, 1975; Hamada and Slade, 1980; Tanzer et al., 1985; Madison et al., 1991]. Consequently, mutans streptococci have been the prime target for the prevention of dental caries.

Propolis is a resinous hive product collected by *Apis mellifera* bees from tree buds and mixed with secreted beeswax. The propolis is used by bees as glue to seal the opening of the hives. It is known that the ethanolic extract of propolis (EEP) exhibits some pharmacological activities, such as antibacterial antiviral, antifungal, anti-inflammatory, anesthetic and cytostatic properties [Ghisalberti, 1979; Bankova et al., 1989; Marcucci, 1995]. A number of investigations have shown antimicrobial activity of propolis against various pathogenic microorganisms [Lindenfelser, 1967; Methzer et al., 1979; Grange and Davey, 1990; Bonhevi et al., 1994]. Furthermore, earlier reports demonstrated that the EEP inhibited in vitro the growth and the activity of glucosyltransferase of both *Streptococcus mutans* and *Streptococcus sobrinus* [Ikeno et al., 1991; Park et al., 1998], besides reducing dental caries in rats [Ikeno et al., 1991]. Although Ikeno et al. [1991] have shown that propolis reduced caries in animals, the experiment did not consider the control of cariogenic diet and the high cariogenic challenge which could be achieved with programmed feed and desalivated rats [Bowen et al., 1986; Rosalen et al., 1996].

Therefore, pharmacological applications of propolis preparations have led to an increased interest in its chemical composition. So far, polyphenolic compounds, e.g. flavonoid aglycones, phenolic acids and their esters, phenolic aldehydes, have been mainly identified in propolis collected by *A. mellifera* in different regions [Bankova et al., 1992a]. According to Ghisalberti [1979], flavonoid aglycones have been considered the main biological active substance in propolis. However, the composition of propolis is quantitatively and qualitatively variable, depending on the regional plant ecology [Konig, 1985; Greenaway et al., 1990; Bankova et al., 1992b]. Considering that the composition of propolis can affect their biological activity, the aim of the present study was to analyze the flavonoid aglycones of propolis from two Brazilian regions and evaluate the effect of these samples of propolis on dental caries in desalivated rats.

Materials and Methods

Propolis Samples

Crude propolis samples collected by *A. mellifera* were obtained from the states of Minas Gerais (MG; city of Santa Luzia in southeastern Brazil, located between 20° and 25° south latitude) and Rio Grande do Sul (RS; city of Porto Alegre in southern Brazil, located between 30° and 35° south latitude). The samples of propolis were further dehydrated with a low-vacuum pump, and the extracts of the dried propolis were prepared as described by Koo and Park [1997]. The dried propolis samples were ground into fine powder, and 2.0 g of propolis was mixed with 25 ml of 80% aqueous ethanol in a test tube and shaken at 70°C for 30 min. After extraction, the mixture was centrifuged at 8,000 *g* to obtain the supernatants, which were named as EEP.

Reversed-Phase High-Performance Thin-Layer Chromatography of EEP

Precoated plates of silica gel RP-18 $F_{254}S$ (Merck Co., Darmstadt, Germany) were used. Reversed-phase high-performance thin-layer chromatography (HPTLC) of EEP was performed according to the modified method of Vanhalen and Vanhalen-Fastré [1980] and Sabatier et al. [1992]. EEP (10 µl) were applied 12 mm from the lower edge of the plate, and ascending chromatography run with a mobile phase of ethanol:water (55:45 vol/vol) was performed. The detection of flavonoids was carried out by UV radiation at 366 nm.

Reversed-Phase High-Performance Liquid Chromatography of EEP

A quantitative analysis of the flavonoids was performed by reversed-phase high-performance liquid chromatography (HPLC) with a chromatograph equipped with YMC PACK ODS-A column. The mobile phase was acetic acid:methanol:water (5:75:60 by vol) [Bankova et al., 1982], the flow rate was 1 ml/min, and detection was done with a diode array detector. The flavonoids were identified by time retention and use of internal standards. The quantities of flavonoids in the EEP were calculated by using standards of flavonoids (Extrasynthese A.A. Co., Genay Cedex, France). The authentic standards of flavonoids used in this experiment were: quercetin, kaempferol, apigenin, isorhamnetin, rhamnetin, pinocembrin, sakuranetin, isosakuranetin, chrysin, acacetin, galangin, kaempferide and tectochrysin.

Microorganism

S. sobrinus 6715 was used in this research. The microorganism was kindly donated by Dr. William H. Bowen from the Center for Oral Biology, University of Rochester, N.Y., USA.

Caries in Rats

Thirty specific pathogen-free 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 (Difco Laboratories, Detroit, Mich., USA) and mitis salivarius agar plus bacitracin (Sigma, St. Louis, Mo., USA) using a described technique [Bowen et al., 1988a]. The rats were infected with *S. sobrinus* 6715 when aged 21, 22 and 23 days. They were fed pellet chow, diet 2000 [Keyes, 1958] and 5% sucrose in drinking water ad libitum until 25 days of age to establish infection by *S. sobrinus*. Oral swabs from the rats were streaked on mitis salivarius agar plus streptomycin (Sigma) to confirm the infection.

When aged 25 days, the rats were desalivated by ligation of the parotid ducts, and the sublingual and submandibular glands were removed [Bowen et al., 1988a, b]. On the following day, the rats were randomly divided into three groups of 10 animals each and placed in a König-Höfer programmed feeder [König et al., 1968]. At hourly intervals beginning at 4 p.m., 17 meals consisting of diet 2000 of approximately 400 mg were offered to each animal. Sterile distilled water was provided ad libitum. From day 26 until the end of the experiment on day 48, 100 µl of EEP or ethanol 80% were applied to the molars of the rats (25 µl on molars of each quadrant) twice daily using graduate syringes at 9 a.m. and at 2 p.m. The groups were divided as follows: group1 received 80% aqueous ethanol (control group), group 2 received EEP from RS state (southern Brazil) and group 3 received EEP from MG state (southeastern Brazil).

The designed experiments, as described above, continued for 3 weeks. All animals were weighed weekly and their physical appearance noted daily. The number of meals consumed by each animal was also recorded daily. The rats were killed by CO_2 asphyxiation and decapited 60–90 min after the last meal. The left jaw was aseptically dissected and sonicated in 5.0 ml of 154 m*M* sterile NaCl solution, using a low-power ultrasonic bath for 20 min with 10-second pulses (5-second intervals). The suspensions obtained were analyzed for microbial assessment. Aliquots of the suspension were streaked on mitis salivarius agar (Difco) plus streptomycin (Sigma) to estimate the *S. Sobrinus* population and on blood agar (blood agar base plus 5% sheep blood) to determine the total cultivable flora. All of the jaws were defleshed, and the teeth were prepared for caries scoring by means of Larson's modification of Keyes' system [Larson, 1981]. The determination of caries score was blind by codification of the jaws and was done by 1 calibrated examiner.

Statistical Analysis

Data were subjected to ANOVA, the Tukey-Kramer HSD test for all pairs, using software for statistical visualization, JMP version 3.1 [SAS Institute Inc, 1989]. Analysis of certain outcome measures was done with transformed values of the measures in order to stabilize variances; smooth-surface and sulcal caries scores were expressed as proportions of their maximum possible values (124 and 56, respectively), and the arcsine transformation was applied. The significant level was 5%.

Results

The two samples of propolis analyzed in this experiment were selected from our previous survey of southeastern and southern Brazilian propolis [partially published, Park et al., 1997]. One representative sample from southeastern Brazil (MG) and another from southern Brazil (RS), which demonstrated the highest total flavonoid concentration, in vitro antimicrobial action and inhibition of crude glucosyltransferase activity were selected for the present study.

The chromatograms of reversed-phase HPTLC and reversed-phase HPLC of propolis samples from MG (southeastern Brazil) and RS (southern Brazil) are shown in figure 1 and 2, respectively. It was found that the HPTLC pattern

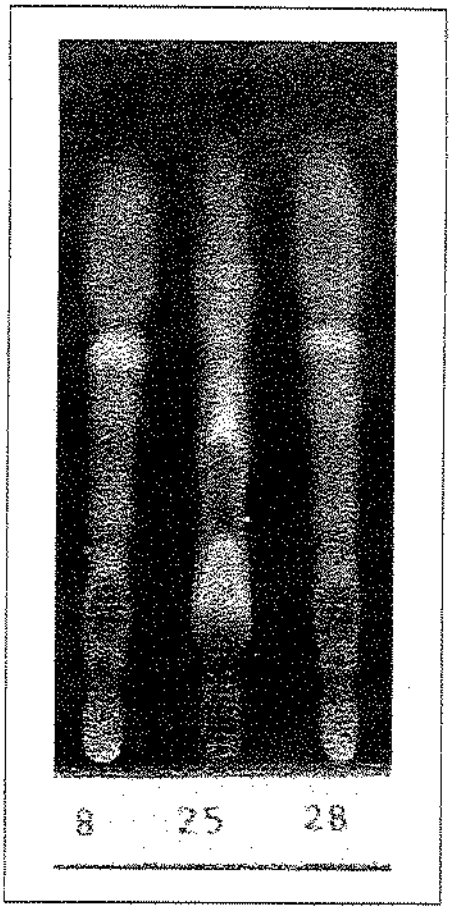

Fig. 1. Reversed-phase HPTLC of propolis from two different regions in Brazil. 8.28 = Propolis from MG (southeastern Brazil); 25 = propolis from RS (southern Brazil).

and the HPLC profile of the sample of EEP from MG were clearly different from the one from RS. In the EEP from MG, 7 flavonoid aglycones were identified: quercetin, kaempferol, isorhamnetin, pinocembrin, sakuranetin, acacetin and kaempferide, while the EEP from RS presented 11 flavonoids: quercetin, kaempferol, apigenin, rhamnetin, pinocembrin, sakuranetin, chrysin, acacetin, galangin, kaempferide and tectochrysin. These findings demonstrated that the qualities of flavonoid aglycones were different between the EEP from southeastern Brazil and southern Brazil. A quantitative analysis of the flavonoid aglycones is shown in table 1. In general, propolis from RS contains higher concentrations of pinocembrin, chrysin, acacetin and galangin than propolis from MG.

In the animal experiment, the rats remained in apparent good health during the 3-week experiment. The animals gained weight and the number of meals consumed daily during the dental caries experiment is demonstrated in table 2.

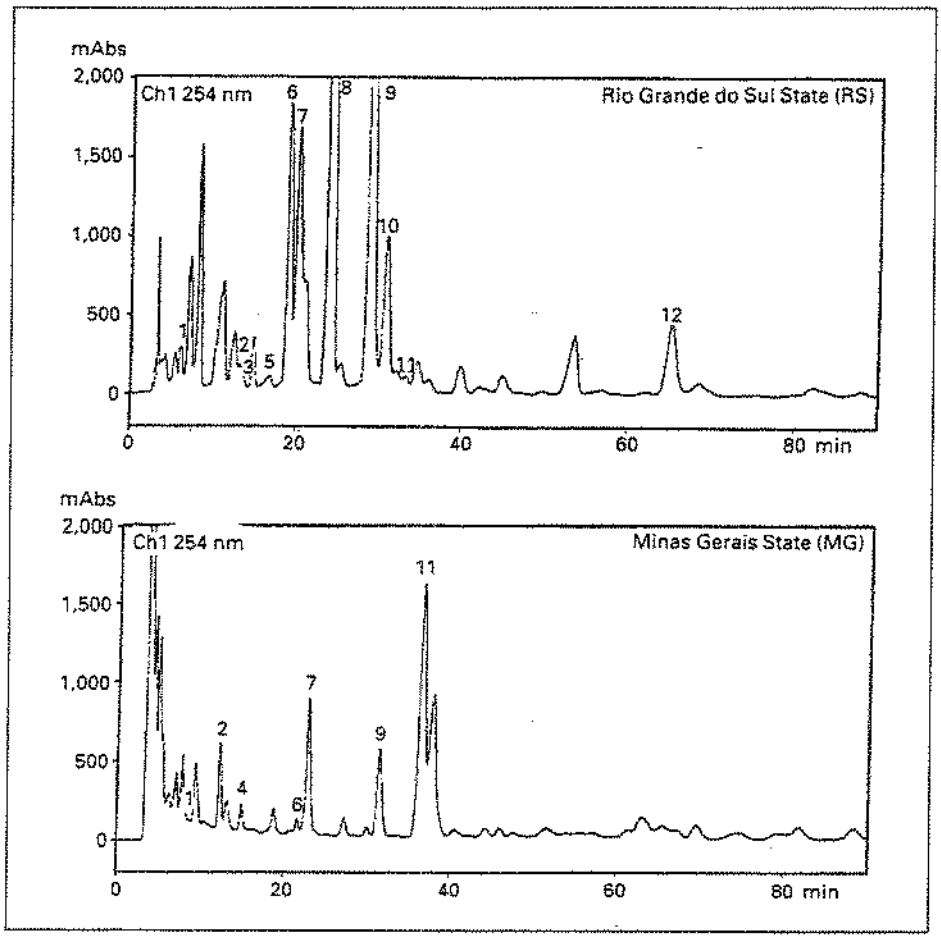

Fig. 2. Reversed-phase HPLC of propolis from RS and MG. 1 = Quercetin; 2 = kaempferol; 3 = apigenin; 4 = isorhamnetin; 5 = rhamnetin; 6 = pinocembrin; 7 = sakuranetin; 8 = chrysin; 9 = acacetin; 10 = galangin; 11 = kaempferide; 12 = tectochrysin.

Table 1. Quantitative analysis of propolis from two different regions of Brazil

Flavonoids	Propolis[a]	
	RS southern Brazil	MG southeastern Brazil
Quercetin	2.0	0.9
Kaempferol	0.6	2.4
Apigenin	0.5	–
Isorhamnetin	–	1.1
Rhamnetin	0.8	–
Pinocembrin	16.8	1.8
Sakuranetin	25.0	19.6
Isosakuranetin	–	–
Chrysin	21.9	–
Acacetin	28.8	4.3
Galangin	9.4	–
Kaempferide	1.1	22.2
Tectochrysin	3.3	–

[a] The values are the media of duplicate analysis. All results represent mg/g of propolis.

Table 2. Weight gain and number of meals consumed daily by desalivated rats: means (SD)

Treatments	Weight gain[a,b], g	Meals[a], n
Ethanol 80% (control)	13.96 (5.84)	14.82 (0.64)
EEP from RS (southern Brazil)	15.15 (7.94)	15.10 (0.72)
EEP from MG (southeastern Brazil)	18.07 (6.98)	14.89 (0.88)

[a] There was no significant difference between any of the groups for weight gain ($p = 0.452$) and number of meals consumed daily ($p = 0.677$). ANOVA, comparison for all pairs using Tukey-Kramer HSD.

Weight gains were not significantly different among the control and propolis groups ($p = 0.452$). There was also no significant difference among the groups in the number of meals consumed daily ($p = 0.677$).

Table 3 shows the percentage of *S. sobrinus* 6715 recovered from the jaws of the rats, which was calculated from

Table 3. Effects of EEP on percentage of *S. sobrinus* 6715: means (SD)

Treatments	Infection by *S. sobrinus*[a], %
Ethanol 80% (control)	17.57 (12.68)
EEP from RS (southern Brazil)	10.49 (8.21)
EEP from MG (southeastern Brazil)	11.30 (7.48)

[a] There was no significant difference among any of the treatments ($p = 0.232$). ANOVA, comparison for all pairs using Tukey-Kramer HSD.

Table 4. Effects of EEP on caries development (smooth surface and sulcal) in desalivated rats: means SD, Keyes' scores

Treatments	Smooth surface	Sulcal
Ethanol 80% (control)	88.10(12.07)[a]	53.10 (2.96)[a]
EEP from RS (southern Brazil)	65.60 (13.81)[b,c]	47.10 (2.85)[b,c]
EEP from MG (southeastern Brazil)	75.22 (10.84)[a,c]	49.56 (3.91)[a,c]

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.

Table 5. Effects of EEP on caries severity (smooth surface and sulcal) in desalivated rats: means (SD), Keyes' scores

Treatments	Smooth-surface severity		Sulcal severity	
	Ds	Dm	Ds	Dm
Ethanol 80% (control)	84.20 (12.50)[a]	11.00 (5.10)[a]	43.40 (2.95)[a]	14.90 (4.58)[a]
EEP from RS (southern Brazil)	61.60 (13.00)[b,c]	3.20 (1.62)[b]	38.60 (2.22)[b]	4.80 (1.81)[b]
EEP from MG (southeastern Brazil)	73.44 (10.03)[a,c]	8.22 (4.71)[a]	39.78 (4.06)[b]	8.44 (3.78)[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.

the total cultivable microflora and *S. sobrinus* population counted in blood agar and mitis salivarius agar plus streptomycin, respectively. Although the propolis-treated groups presented lower infection by *S. sobrinus* when compared with the control group, the difference was not statistically significant ($p = 0.232$).

Smooth-surface and sulcal caries scores are shown in table 4. The animals treated with EEP from RS presented the lowest scores for both smooth-surface and sulcal caries and significant difference was observed when compared with the control group, but no statistical difference was noted when compared with the animals treated with EEP from MG. Nevertheless, the animals treated with EEP from MG demonstrated no statistical difference in either smooth-surface or sulcal caries scores when compared with the control group ($p < 0.05$). Thus, only propolis from RS (southern Brazil) was able to reduce caries on both smooth and sulcal surfaces when compared to the control group ($p < 0.05$).

The severity of smooth-surface lesions (table 5) followed a similar pattern to that of smooth-surface caries scores. The animals treated with EEP from RS demonstrated less caries severity (Ds and Dm) than other treatments, and a significant difference was observed when compared with the control group. However, the Ds scores of the animals treated with EEP from RS did not differ significantly from those of the animals treated with EEP from MG. A significant difference was observed in Dm scores between the animals treated with EEP from RS and MG. Again, the animals treated with EEP from MG showed no statistical difference in severity of smooth-surface caries scores when compared with the control group. The severity of sulcal lesions (table 5) was significantly lower in both EEP-treated animals than in the control group. Although the animals treated with EEP from RS showed less caries severity (Ds and Dm) in sulcal lesion, no statistical difference was observed when compared with those treated with EEP from MG. Therefore, only the animals treated with EEP from RS (southern Brazil) demonstrated less severity on both smooth-surface and sulcal caries when compared with the control group, although the animals treated with EEP from MG (southeastern Brazil) also presented significantly lower severity scores for the sulcal lesions.

Discussion

Propolis has been widely used in folk medicine and has recently found application in clinics. It is a very complex mixture, containing mainly phenolics [Bankova et al., 1992a]. The flavonoid aglycones have been considered the major phenolic compounds of EEP and are associated with the beneficial effects of propolis [Ghisalberti, 1979; Grange and Davey, 1990; Bonhevi et al., 1994]. However, the composition of these biological active compounds in propolis is variable depending on the origin of the sample [Bankova et al., 1992b].

The findings of HPTLC and HPLC analysis demonstrated that patterns of chromatograms of EEP from MG (southeastern Brazil) were different when compared with propolis from RS (southern Brazil). These different patterns are due to different flavonoids present in propolis, as previously reported [Park et al., 1997]. Apigenin, rhamnetin, chrysin, galangin and tectochrysin were identified in EEP from RS and were not detected in EEP from MG. Nevertheless, EEP from MG contains isorhamnetin, which was not found in EEP from RS. The results of quantitative analysis of flavonoids in propolis demonstrated that propolis from RS contained high concentrations of pinocembrin, chrysin, acacetin and galangin. However, propolis from MG presented high concentrations of kaempferide. Therefore, both quality and quantity of flavonoid aglycones of propolis were different between samples from southeastern Brazil and southern Brazil, probably due to different plant ecology.

Several studies have reported that some flavonoids present biological activity against oral microorganisms. Iio et al. [1984] reported that quercetin and chrysin inhibited in vitro activity of glucosyltransferase and glucan formation, respectively. Isoflavanones, e.g. dihydrogenistein, dihydrobiochanin A and ferreirin, isolated from heartwood of *Swartzia polyphylla*, demonstrated antibacterial activity against *Streptococcus mutans* and *S. sobrinus* [Osawa et al., 1992]. Recently, Cai and Wu [1996] showed that kaempferol and myricetin, isolated from *Syzygium aromaticum*, have antibacterial activity against *S. mutans* in vitro. In addition, pinocembrin, galangin, sakuranetin and pinobanksin have been recognized as antimicrobial flavonoids in propolis [Villanueva et al., 1964; Villanueva et al., 1970; Metzner et al., 1979; Ghisalberti, 1979; Dimov et al., 1992]. The flavonoids are ubiquitous in the plant kingdom, including most edible vegetables and fruits, and it is now generally accepted that the vast majority of flavonoids have low toxicity and are a common component in the human diet [Hertog et al., 1992; Tsuchiya et al., 1996].

The chemical analysis performed on the EEP from RS (fig. 2, table 1) confirmed the presence of these bioactive flavonoids, such as quercetin, kaempferol, pinocembrin, sakuranetin, chrysin and galangin, whereas in the EEP from MG, quercetin, kaempferol, pinocembrin and sakuranetin were detected. However, while galangin and chrysin were found only in the propolis from RS, the concentration of pinocembrin was 9 times higher in this propolis than in that from MG. Our previous study [Park et al., 1998] demonstrated that propolis from RS presented the highest antimicrobial activity (against *S. mutans*) and in vitro inhibition of glucosyltransferase.

The model of experimental dental caries used in this study provided the severest cariogenic challenge available by desalivation of the rats and cariogenic diet with 56% sucrose (diet 2000). Furthermore, the feeding of the cariogenic diet for the rats was controlled because there is overwhelming evidence, from animal and human experiments, that the level of caries is directly related to the frequency of sucrose ingestion [Guggenheim and Regolati, 1972; Newbrun and Frostell, 1978; Bowen et al., 1980]. No statistical difference was detected in the number of meals consumed daily per animal among control and propolis-treated groups. Thus, all of the animals received the same cariogenic challenge caused by the diet. The animals treated with both propolis showed a reduction of infection by *S. sobrinus*, but the difference was not statistically significant when compared with the control group. Nevertheless, dental caries decreased in rats which were given propolis, mainly those treated with the EEP from RS (southern Brazil). It is interesting to note that only the EEP from RS reduced smooth-surface and the severity of smooth-surface caries, mainly Dm, which was reduced about 74.5% when compared with the control group. Although the EEP from MG reduced smooth-surface dental caries in relation to the control group, it was not statistically significant (table 4). These data suggest that the reduction in number of smooth-surface caries lesions by propolis from RS (southern Brazil) was related, mainly, to inhibition of glucosyltransferase activity rather than antimicrobial action against *S. sobrinus*. The glycosyltransferase-catalyzed synthesis of water-insoluble glucans from dietary sucrose is known to contribute to the pathogenic potential of *S. mutans* and *S. sobrinus* [Hamada and Slade, 1980; Tanzer et al., 1985]. Although glucan production is not required for initial attachment of *S. mutans* to surfaces, evidences from several studies suggest that sucrose-dependent adherence and accumulation of cariogenic streptococci are critical processes in the development of pathogenic dental plaque, especially on smooth surface [Gibbons, 1984; Hamada and Slade, 1980]. The propolis

from RS inhibited glucosyltransferase to a higher extent than propolis from MG in vitro [Park et al., 1998], thus the best result in reducing smooth-surface caries in the group treated with EEP from RS can be explained.

The EEP from MG also did not reduce sulcal caries as much as the EEP from RS. Nevertheless, both propolis reduced the severity of sulcal caries (Ds and Dm severity), even when the animals were under high cariogenic challenge. The retention capacity of the sulcal region of the rat molars probably permitted accumulation of propolis extracts. Therefore, more concentration of flavonoid compounds could be acting in this region, and consequently, reducing the progression of dental caries.

It was found that the effect of propolis samples from two Brazil regions on reduction of dental caries in rats was not the same. Only the EEP from RS (southern Brazil) reduced both smooth-surface and sulcal caries when compared to the control group, while the EEP from MG (southeastern Brazil) reduced only the severity of sulcal caries. This can be attributed to the qualitative and quantitative differences of bioactive flavonoids, e.g. quercetin, kaempferol, pinocembrin, sakuranetin, chrysin and galangin between the propolis samples. It is evident (fig. 2, table 1) that the EEP from RS contains more bioactive flavonoid compounds than the EEP from MG; however, further investigations on the chemical characterization should be carried out before any final conclusion, due to other flavonoid aglycones and related compounds in propolis which could be involved in antimicrobial action and inhibition of glucosyltransferase activity. As shown in the reversed-phase HPLC chromatograms (fig. 2), four large peaks of unknown compounds were detected in the RS propolis profile, as well as another large peak in the MG propolis profile. For this reason, the next step of our research is to isolate each fraction and attempt to examine their antibacterial action and inhibition of glucosyltransferase activity, as well as to identify these unknown compounds. Ikeno et al. [1991] suggested that the active agent in the EEP from China and Japan was cinnamic acid because of its antimicrobial action (against *S. mutans*) and inhibition of glucosyltransferase activity, although they did not quantify this compound in the propolis. Nevertheless, according to Amoros et al. [1992] and Bonhevi et al. [1994], the activity of propolis against microorganisms is more related to the synergistic effect of flavonoids (and other phenolic compounds) than the individual compounds. Thus, the cariostatic effect of EEP in rats was probably due to a synergistic effect of bioactive flavonoids, which were more exalted in the propolis from RS.

In conclusion, the results of the present study show that EEP exert cariostatic effects on dental caries in rats, mainly the EEP from RS, even in conditions of high cariogenic challenge provided by desalivated rats and cariogenic diet. Probably, the effects of propolis would have been more if the cariogenic challenge had been less severe. Therefore, the use of propolis to prevent dental caries is promising. Nevertheless, the flavonoid composition of propolis was qualitatively and quantitatively different depending on the region of collection of propolis, and consequently, the effects of propolis on dental caries were also different using animal model. These data are important considering that Brazil and many other countries do not have any kind of guidelines or standardization for quality control of crude propolis used to manufacture oral hygiene products.

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Anexo 2 - Documento Informativo e Termo de Consentimento

INFORMAÇÕES E CONSENTIMENTO PÓS-INFORMAÇÃO PARA PESQUISA CLÍNICA.

Nome do Voluntário:.....

Endereço: .....

Fone para contato:

Cidade: CEP:

As informações contidas neste prontuário foram fornecidas Hyun Koo (Doutorando em Biologia e Patologia Buco-Dental - FOP/UNICAMP) e Prof. Dr. Jaime A. Cury (Orientador), objetivando firmar acordo escrito mediante o qual, o voluntário da pesquisa autoriza sua participação, com pleno conhecimento da natureza dos procedimentos e riscos a que se submeterá, com a capacidade de livre arbítrio e sem qualquer coação.

1. Título do trabalho experimental:

Avaliação do efeito de um enxaguatório bucal com própolis na formação da placa dental

2. Objetivo: Avaliar um enxaguatório bucal contendo própolis, verificando o efeito na formação da placa dental (estimulada com uma solução de sacarose) durante 3 dias.

3. Justificativa: Os resultados preliminares *in vitro* e em animais sugerem que a própolis apresenta um grande potencial como agente anti-placa e anti-cárie. Para a confirmação destes resultados é necessário a realização de um estudo em humanos, uma vez que os parâmetros analisados anteriormente não representam com exatidão a complexidade da cavidade bucal.

4. Procedimentos da fase experimental: O estudo será do tipo cruzado em 2 etapas de 3 dias, nas quais 6 voluntários saudáveis que não estejam utilizando medicamentos (ex: antibióticos) utilizarão enxaguatórios bucais com própolis. Como controle os voluntários utilizarão um enxaguatório bucal com os mesmos ingredientes, porém sem própolis. Os enxaguatórios serão colocados em frasco âmbar e codificados.

Após cada etapa os voluntários serão avaliados com relação ao índice de placa de acordo com o método de Silness e Löe. Antecedendo os experimentos e entre as fases os voluntários utilizarão

dentifrício placebo de agentes anti-placa e antimicrobianos por um período de pelo menos sete dias, para eliminar o possível efeito residual dos tratamentos.

5. Desconforto ou Riscos esperados: Os voluntários podem sentir desconforto ao bochechar devido a presença de etanol (20%) nos enxaguatórios bucais.

6. Informações: O voluntário tem garantia que receberá respostas a qualquer pergunta ou esclarecimento de qualquer dúvida quanto aos procedimentos, riscos, benefícios e outros assuntos relacionados com a pesquisa. Também os pesquisadores supracitados assumem o compromisso de proporcionar informação atualizada obtida durante o estudo, ainda que esta possa afetar a vontade do indivíduo em continuar participando.

7. Retirada do consentimento: O voluntário tem a liberdade de retirar seu consentimento a qualquer momento e deixar de participar do estudo.

8. Aspecto Legal: Elaborados de acordo com as diretrizes e normas que regulamentam pesquisas envolvendo seres humanos, atendendo à Resolução n. 196, de 10 de outubro de 1996, do Conselho Nacional de Saúde / Ministério da Saúde - Brasília - DF.

9. Garantia do Sigilo: Os pesquisadores asseguraram a privacidade dos sujeitos quanto aos dados confidenciais envolvidos na pesquisa.

10. Local da Pesquisa: A pesquisa será desenvolvida na Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas - UNICAMP, localizada à Avenida Limeira, 901- Caixa Postal 52; CEP 13.414.900 - Piracicaba -SP

12. Telefones dos pesquisadores para contato:
Michel H. Koo - 430 5303
Prof. Jaime - 430 5302 / Res. 433 4736/433 9685

13. Consentimento Pós-informação:

Eu....., certifico que tendo lido as informações acima e suficientemente esclarecido(a) de todos os ítens pelo cirurgião dentista Hyun Koo (Doutorando em Biologia e Patologia Buco-Dental - FOP/UNICAMP) e Prof. Dr. Jaime A. Cury (Orientador), estou plenamente de acordo com a realização do experimento. Assim, eu autorizo a execução do trabalho de pesquisa, exposto acima, em mim.

Piracicaba, de 1997.

Nome (por extenso).....

Assinatura:.....

1 via : instituição

2 via : voluntário

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FACULDADE DE ODONTOLOGIA DE PIRACICABA

D E C L A R A Ç Ã O

Declaro para os devidos fins que o Projeto "Avaliação do potencial anti-cárie e anti-placa da própolis de *Apis mellifera provenientes de duas regiões do Brasil*", do CD **Hyun Koo**, tendo como orientador o Prof. Jaime Aparecido Cury, teve seu início em 03/03/1997 e término em 10/12/1998, antes da instalação do Comitê de Ética em Pesquisa, 19/09/96, Portaria do Diretor 09/97, razão pela qual não foi submetido ao referido Comitê.

Piracicaba, 17 de setembro de 1999

Prof. Dr. Antonio Wilson Sallum
DIRETOR