UNIVERSIDADE ESTADUAL DE CAMPINAS - UNICAMP

FACULDADE DE ODONTOLOGIA DE PIRACICABA - FOP

Regiane Yatsuda

Cirurgiã-dentista

"Avaliação da atividade anti-cárie dos compostos bioativos isolados das plantas *Mikania laevigata* Schultz Bip. ex Baker e *Mikania glomerata* Sprengel".

Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas, no Programa de Pós-Graduação em Odontologia, para obtenção do título de Doutor em Odontologia, Área de Farmacologia, Anestesiologia e Terapêutica.

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Orientador: 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 21 de Julho de 2006, considerou a candidata REGIANE YATSUDA aprovada.

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PROF. DR. MASAHARU IKEGAKI

PROF. DR. SEVERINO MATIAS DE ALENCAR

Dedico este trabalho

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SUMÁRIO

RESUMO

O uso de plantas medicinais vem crescendo nos últimos anos e isto é devido principalmente as descobertas de suas propriedades biológicas, e entre estas plantas destacam-se as do gênero *Mikania.* Assim, a procura pela descoberta de novos produtos naturais com atividade antibacteriana para a prevenção de doenças bucais e talvez com menores efeitos adversos quando comparados aos produtos industrializados seria muito importante para obtenção de um meio efetivo de controle da formação de um biofilme patogênico. Assim, em estudos anteriores, a fração hexânica das plantas *Mikania laevigata* e *Mikania glomerata* apresentaram atividade antimicrobiana e inibiram a aderência dos estreptococos do grupo mutans. Deste modo, o objetivo geral deste trabalho foi avaliar o efeito antimicrobiano dos compostos isolados das frações hexânicas das plantas *M. laevigata* e *M. glomerata* (guaco) sobre os estreptococos do grupo mutans. Para isso, foram realizados três estudos, sendo o objetivo do estudo 1 analisar a composição química e o efeito antimicrobiano dos compostos isolados e identificados das frações hexânicas das plantas *M. laevigata* e *M. glomerata* sobre o crescimento bacteriano e a aderência celular dos estreptococos do grupo mutans. O objetivo do estudo 2 foi determinar a influência destes compostos isolados na formação e na composição de polissacarídeos de biofilmes de *Streptococcus mutans* UA159 formados em discos de hidroxiapatita, na queda de pH glicolítico e seu efeito na translocação de prótons pela atividade da ATPase. Além disso, foram realizados estudos para analisar seu efeito na atividade de glucosiltransferase (GTF) B em solução (GTF-sol) e em superfície (GTF-sup). O objetivo do estudo 3 foi avaliar as atividades dos compostos isolados da *Mikania* utilizando modelo experimental de cárie em ratos. No estudo 1, os ácidos cupressênico, diterpênico e caurenóico foram os compostos que apresentaram atividade antimicrobiana (CIM entre 2,5-20 µg/mL e CBM entre 2,5-40 µg/mL) e inibição da aderência celular entre 1,25-5 µg/mL, sendo que os compostos espatulenol, caurenol, ácido grandiflórico não apresentaram atividade biológica nas concentrações testadas. No estudo 2, somente os três compostos ativos isolados da *Mikania* foram avaliados na concentração 500 µg/mL. Os resultados demonstraram que os ácidos cupressênico, diterpênico e caurenóico apresentaram efeito na viabilidade bacteriana dos biofilmes, reduziram a produção de ácidos orgânicos (pH final entre 6,4-5,8) e a atividade da ATPase (28-40%). Os compostos também foram potentes inibidores da atividade de GTFs, tanto em solução quanto em superfície de hidroxiapatita, sendo 50-60% de redução para GTF B-sol e 50-

80% de redução para GTF B-surf. Nos biofilmes, o peso seco e a quantidade de polissacarídeos solúveis, insolúveis e intracelulares também foram significativamente reduzidos com o tratamento dos três compostos ($p<0,05$). No estudo 3, a aplicação tópica 2 vezes ao dia dos ácidos cupressênico, diterpênico e caurenóico (500 µg/mL) promoveu a redução na incidência de cárie em superfície lisa e sulcal ($p<0,05$), além da diminuição na porcentagem de infecção por *S. mutans* UA159 pelos ácidos diterpênico e caurenóico, não sendo afetada a microbiota total dessas ratas. Desta forma, concluímos que os ácidos cupressênico, diterpênico e caurenóico isolados da *M. laevigata e M. glomerata* possuem potencial antimicrobiano, inibindo os fatores de virulência dos *Streptococcus mutans* e a cárie em modelo *in vivo*, demonstrando serem promissores agentes anti-cárie e anti-placa.

ABSTRACT

Considering the great use of plants as medicinal substances in the popular medicine, it is critical to investigate their biological and chemical properties in order to not only help to enhance our understanding of the therapeutic potential of these natural products, but also how to make them more effective pharmacological agents. The development of therapeutic agents aimed at disrupting both colonization of the teeth by dental pathogens and the subsequent formation of dental plaque is one of the prime strategies to reduce the incidence of tooth decay. The hexanic fraction of the plants *Mikania laevigata* and *Mikania glomerata* showed antimicrobial activities and inhibit the adherence of mutans streptococci. The overall aim of this study was to evaluate the antimicrobial effect of the isolated compounds of the hexanic fractions of *Mikania laevigata* and *Mikania glomerata* (guaco) on mutans streptococci. Therefore, three studies were carried out. In study 1,the antimicrobial activity of the isolated compounds was assessed by determination of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and inhibition of cell adherence (Adh) to glass surface of mutans streptococci. In study 2, the influence of the isolated compounds were evaluated on viability, development, polysaccharide composition and acidogenicity of *S. mutans* biofilms, on glucans production by purified glucosyltransferases (GTFs) adsorbed to hydroxyapatite beads, and on membrane-associated F-ATPase and glycolytic activities. In study 3, the influence of the isolated compounds on caries development *in vivo* was evaluated. The acids cupressenic, diterpenic and kaurenoic were the compounds most effective in inhibiting the growth of the bacterial strains tested (MIC 2.5-20 μg/ml and MBC 2.5-40 μg/ml) and adherence cells (1.25-5 μg/ml). These three compounds were tested on the concentration of 500 μg/ml on the study 2, and showed antimicrobial activity reducing

the viable cells and the dry-weight of the biofilm treated, and also reduced the soluble, insoluble extracellular and intracellular polysaccharide of this biofilms. The compounds were able to reduce the acid production on the biofilms (final pH between 6.4 and 5.8) and the activity of F-ATPase (28-40%). The activity of the GTF B on solution (50-60%) and on surface (50-80%) of hydroxyapatite was also reduced by the three compounds. In study 3, the topical application twice a day of cupressenic, diterpenic and kaurenoic acids (500 µg/ml) showed cariostatic effect on smooth-surface and sulcal caries, and also showing reduction of the percentage of *Streptococcus mutans* UA159 infection by diterpenic and kaurenoic acids ($p<0.05$), not showing differences on the total microbiology ($p>0.05$). In conclusion, the cupressenic, diterpenic and kaurenoic acids isolated from *M. laevigata* and *M. glomerata* have relevant antimicrobial activity and inhibit the virulence factors of mutans streptococci *in vitro* and *in vivo,* being promising anti-caries and anti-plaque agents.

1. INTRODUÇÃO

A cárie e a doença periodontal são as infecções bacterianas mais comuns nos seres humanos (Loesche, 1986), sendo que a placa dental tem sido extensivamente estudada pela sua relação com estas infecções (Gibbons e van Houte, 1975).

Placa dental é um biofilme bacteriano encontrado naturalmente na superfície dos dentes, apresentando composições bacteriana e bioquímica que podem variar em dependência de fatores intrínsecos e extrínsecos (Marsh, 1992).

O biofilme é benéfico ao hospedeiro na medida em que ajuda a prevenir a colonização de microrganismos exógenos e patógenos. Sua composição é relativamente estável apesar da exposição regular a mudanças no ambiente oral. Entretanto esta estabilidade pode deixar de existir quando ocorrem alterações do meio oral, conduzindo a um desequilíbrio da microbiota bucal residente, favorecendo o estabelecimento de uma população microbiana cariogênica (van Houte, 1994; Loesche, 1986; Marsh, 1992, 1994). Desta forma, bactérias colonizadoras secundárias se aderem aos residentes já aderidos (co-agregação) por interações moleculares específicas. Este processo contribui para a determinação do padrão de sucessão bacteriano, formando um biofilme patogênico, também conhecido como "placa dental patogênica" ou biofilme patogênico (Marcote e Lavoie, 1998).

Um dos fatores de desequilíbrio fundamental para o aparecimento de uma placa dental cariogênica é a dieta rica e freqüente de carboidratos fermentáveis, principalmente a sacarose. Esta dieta promove um aumento da proporção de estreptococos do grupo mutans, uma vez que estes microrganismos apresentam algumas vantagens ecológicas quando da presença deste açúcar no meio bucal, permitindo a sua aderência, colonização e posterior acúmulo na superfície lisa do esmalte dental (Hamada e Slade, 1980; Loesche, 1986). Além disso, a fermentação de carboidratos da dieta pelas bactérias, principalmente sacarose, resulta na produção de ácidos e produtos que inicialmente desmineralizam o esmalte e posteriormente a dentina, sendo que, quando esse processo não é controlado, ocorre à formação das lesões cariosas (Alam *et al.*, 2000; Bowden, 1990). Os estreptococos do grupo mutans possuem a capacidade de metabolizar diversos carboidratos presentes na dieta produzindo ácidos orgânicos, que são liberados na matriz do biofilme. Devido à estrutura do biofilme, o acesso da saliva é limitado, não ocorrendo uma neutralização efetiva do pH ácido, que contribui para desmineralização do esmalte

dental e a seleção de organismos acidúricos (ácidos tolerantes), como o *S. mutans*. Um dos mecanismos desenvolvidos pelos estreptococos do grupo mutans como resposta a acidificação do meio é o aumento da atividade de translocação de prótons pela enzima F-ATPase (H^+-ATPase) (Marquis *et al.*, 2004). Essa enzima presente na membrana desses microrganismos bombeia, em associação com hidrólise do ATP, prótons (H^+) para fora da célula mantendo o meio intracelular mais alcalino que o pH extracelular. A alta tolerância a ácidos por essas bactérias constitui um importante fator de virulência extremamente relacionado à cárie dental. Além disso, as alterações do pH intracelular podem inibir as enzimas glicolíticas sensíveis ao pH mais ácido, reduzindo assim, a capacidade desses microrganismos produzirem ácidos (Marquis *et al.*, 2004).

Os estreptococos do grupo mutans, além de serem acidúricos e acidogênicos, não só fermentam a sacarose como, a partir desta, sintetizam glucanos através das enzimas glucosiltransferases - GTFs (Gibbons e van Houte, 1975; Hamada e Slade, 1980). Atualmente, três GTFs distintas, secretadas pelo *Streptococcus mutans*, estão bem caracterizadas tanto bioquimicamente como ao nível molecular: 1) GTF B - codificada pelo gene *gtfB*, que sintetiza glucanos insolúveis em água tendo ligações glicosídicas principais α (1→3); 2) GTF C - codificada pelo gene *gtfC*, que sintetiza uma mistura de glucanos insolúveis e solúveis, este último apresentando ligações glicosídicas principais α (1→6); e 3) GTF D - codificada pelo gene *gtfD*, que sintetiza basicamente glucanos solúveis (Loesche, 1986; Hanada e Kuramitsu, 1989). A GTF produzida por *S. sanguinis* (GTF Ss) pode também estar envolvida com o desenvolvimento da placa dental. Os glucanos, principalmente os insolúveis em água, têm sido considerados como os principais fatores de aderência e acúmulo de estreptococos cariogênicos sobre a superfície dental (Hamada e Slade, 1980; Rölla *et al.*, 1983; Tanzer *et al.*, 1985; Schilling, 1992). Em acréscimo, tem sido demonstrado que estes glucanos aumentam a porosidade (Dibdin e 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. Assim, estreptococos do grupo mutans e seus glucanos são considerados fatores críticos no desenvolvimento da placa dental cariogênica.

Deste modo, estratégias têm sido estudadas no sentido de prevenir a cárie dental, seja inibindo de forma seletiva o crescimento dos estreptococos do grupo mutans na cavidade bucal ou inibindo seus fatores de virulência. Um agente que eficientemente possuísse propriedades antimicrobianas e inibisse as GTFs e F-ATPase seria extremamente desejável para a prevenção destas doenças bucais (Koo *et al.*, 2002).

Nas últimas décadas têm sido observado um crescente interesse global no aproveitamento da biodiversidade, particularmente no que se refere às plantas medicinais, que têm sido utilizadas em várias áreas da saúde como uma expressiva forma alternativa de tratamento e prevenção (Lewis e Elvin-Lewis, 1977). O comércio de medicamentos fitoterápicos vem crescendo a uma taxa anual média de 15%, sendo que cerca de 30% dos medicamentos comercializados atualmente são originados direta ou indiretamente de produtos naturais, principalmente de plantas (Farnsworth, 1985; Elisabetsky, 1987).

Esse grande consumo atual de medicamentos fitoterápicos pela população decorre basicamente do fato de que representam formas de terapia mais baratas e /ou naturais que aquelas normalmente oferecidas e preconizadas pela indústria farmacêutica e a medicina alopática. Dentro desse contexto, o aumento do uso destas plantas seria de grande utilidade principalmente nos países em desenvolvimento como o Brasil, que ainda possui grande biodiversidade e tem uma posição privilegiada por possuir cerca de 25% da flora mundial (Farnswotrh, 1985).

Dentro dessa perspectiva de aumento do mercado de medicamentos, a participação das plantas medicinais é, sem dúvida, muito importante, particularmente no que tange o desenvolvimento de medicamentos fitoterápicos e a identificação de novas moléculas ou protótipos básicos para geração de novos medicamentos sintéticos. É verdade também, que muitos constituintes de plantas e /ou seus derivados semi-sintéticos constituem uma parcela apreciável dos medicamentos de ponta recém introduzidos no mercado.

Assim, a procura pela descoberta de novos produtos naturais com atividade antibacteriana para a prevenção de doenças bucais e talvez com menores efeitos adversos quando comparados aos produtos industrializados seriam muito importantes para obtenção de um meio efetivo de controle da formação de um biofilme patogênico. Porém, para avaliar a efetividade destes produtos naturais, são necessárias análises progressivas começando com estudos laboratoriais *in vitro*, passando por modelos de estudo *in vivo* e culminando com os estudos clínicos (Ten Cate e Marsh, 1994).

Diante desse contexto, muitos estudos já estão sendo realizados identificando e isolando os princípios ativos presentes nestes extratos vegetais, para que estes novos compostos isolados quimicamente, se efetivos na sua ação antimicrobiana, possam ser sintetizados e utilizados no controle da doença cárie.

Como mencionado anteriormente, o uso de plantas medicinais vem crescendo nos últimos anos e isto é devido principalmente as descobertas de suas propriedades

biológicas, e entre estas plantas destacam-se as do gênero *Mikania*. Segundo Cruz e Liberalli (1938), a maioria das espécies pertencentes ao gênero *Mikania* possui emprego na terapêutica popular, merecendo destaque especial àquelas conhecidas pelo nome de "guaco" ou "guaco cheiroso". Oliveira *et al.* (1984) afirmam que o guaco é uma das plantas medicinais mais empregadas no Brasil devido aos seus efeitos farmacológicos: antiespasmódico, analgésico (Ruppelt *et al.*, 1991), antiasmático, antibacteriano (Yatsuda, 2001, 2002, 2003), antifúngico (Davino *et al.*, 1989) antiinflamatório (Ruppelt *et al.*, 1991; Pereira *et al.*, 1994), antiprotozoário, antitussígeno, antialérgico (Fierro *et al.*, 1999), broncodilatador, cicatrizante, expectorante, anti-sifílico, antifebril, antiespasmódico e antiúlcera (Bighetti *et al.*, 2005).

As plantas do gênero *Mikania* foram descritas por Willdenow em 1804, recebendo esta nomenclatura em homenagem ao professor Joseph Gottfried Mikan, de Praga. Para o gênero são citadas cerca de 430 espécies, distribuídas principalmente na América Central e do Sul, sendo 200 espécies no Brasil, distribuídas principalmente nas regiões Sul e Sudeste (King e Robinson, 1987). Apesar de apresentar um grande número de espécies, o gênero *Mikania* foi pouco estudado no Brasil. Diversas espécies do gênero *Mikania* são citadas e empregadas em outros países da América do Sul e Central, todas pertencentes à família Compositae (Ritter *et al.*, 1992).

A espécie *Mikania glomerata* Spreng é a única espécie oficializada na 1ª edição da Farmacopéia Brasileira (da Silva, 1929) e foi identificada por Sprengel em 1826, sendo também conhecida por: *Cacalia trilobata* Vell., *M. amara, M. aspera, M. attenuata, M.scansoria* DC., *M. hederaefolia* DC., *Willoughbya glomerata* (Spreng), *Willoughbya moronoa* (Ktze), sendo popularmente conhecida pelos nomes de guaco, guaco liso, guaco de cheiro, erva de serpente e cipó caatinga (Lucas, 1942; Oliveira *et al.*, 1984). É um subarbusto trepador de ramos lenhosos e de folhas verde-brilhante que, ao serem esfregadas, exalam um forte aroma de baunilha (Vanila) (Oliveira *et al.*, 1984). O seu habitat é nas margens e interiores de matas, adaptando-se muito bem ao cultivo doméstico. Na época da floração torna-se uma planta muito procurada pelas abelhas melíferas. (Ritter *et al.*, 1992; Lucas, 1942; Corrêa, 1952).

A família Asteraceae (antigamente denominada Compositae), à qual pertence esta espécie, é caracterizada quimicamente pela riqueza em produtos do metabolismo secundário (Gibbs, 1974). A *M. glomerata* apresenta vasta variedade de compostos, tais como ácidos orgânicos, açúcares, materiais corantes, resinas, taninos, cumarina, saponinas, o estigmasterol e um éster alifático, cinamoilgrandiflórico, ácido caurenóico

(Oliveira *et al.*, 1984; Santos *et al.*, 1992). No período da floração, agosto a dezembro, ocorre um aumento da concentração dos princípios ativos na planta, decrescendo a produção desses compostos após a floração (Ritter *et al.*, 1992). Dentre os compostos isolados e identificados alguns apresentam atividades farmacológicas destacando os diterpenos da classe dos cauranos e cumarinas (Egan *et al.*, 1990; Rosskopf *et al.*, 1992; Pereira *et al.*, 1994; Ruppelt *et al.*, 1991; Davino *et al.*, 1989).

Apesar das vastas aplicações populares, a literatura traz poucos estudos científicos sobre a ação antimicrobiana do "guaco", principalmente em relação a bactérias orais. Em estudos de Alves *et al.* (1995), o extrato bruto das folhas de guaco (*Mikania cordifolia*) demonstrou atividade antiprotozoária (*Trichomonas vaginalis* e *Trypanosoma cruzi*), *in vitro,* e ação antibacteriana e anticândida. Trabalhos desenvolvidos por Yatsuda *et al.* (2005), demonstraram que os extratos brutos e frações hexânicas da *M. laevigata* e *Mikania glomerata* apresentam atividade antimicrobiana e são capazes de inibir *in vitro* a aderência celular à superfície de vidro.

Deste modo, devido à carência de informações do potencial antimicrobiano das *M. laevigata* e da *M. glomerata* e de seus compostos químicos sobre os patógenos bucais, e os resultados promissores obtidos anteriormente *in vitro* (Yatsuda *et al.*, 2005), os objetivos do presente estudo foram (i) analisar *in vitro* o efeito antimicrobiano dos compostos isolados das frações hexânicas da *Mikania laevigata* e *Mikania glomerata* sobre os estreptococos do grupo mutans em modelo de células planctônicas; (ii) analisar *in vitro* o efeito antimicrobiano dos compostos isolados ativos pré-selecionados da *Mikania* sobre o biofilme de *S. mutans* UA159, além das suas influências em alguns fatores de virulência desse microrganismo; (iii) avaliar os efeitos anticárie desses compostos em modelo *in vivo.*

2. CAPÍTULOS

Esta tese está de acordo com a Informação CCPG 001/98, UNICAMP, que regulamenta o formato alternativo para dissertação e tese, permitindo a inserção de artigos científicos de autoria ou co-autoria do candidato.

Assim sendo, esta tese é composta de 3 estudos que se encontram em fase de submissão em revistas científicas conforme descrito abaixo:

Estudo 1: "*In vitro* antimicrobial activity of purified compounds from *Mikania laevigata* and *Mikania glomerata* on oral pathogens".

Estudo 2: "Influence of Diterpenes from *Mikania* genus plants on virulence and formation of *S. mutans* biofilms".

Estudo 3: 'Effects of isolated compounds from *Mikania* genus plants on caries development in rats".

O estudo 1 foi submetido à revista Journal of Ethnopharmacology, o estudo 2 será submetido a revista FEMS Microbiology Letters, e o estudo 3 será submetido a revista Caries Research.

O objetivo geral deste trabalho foi analisar *in vitro* e *in vivo* a influência dos compostos ativos isolados das plantas *Mikania laevigata* e *Mikania glomerata* sobre os estreptococos do grupo mutans *in vitro* e *in vivo*.

2.1. Estudo 1

In vitro antimicrobial activity of purified compounds from *Mikania laevigata* and *Mikania glomerata* on oral pathogens.

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This material is original and is not previously published, or being considered for publication elsewhere, in either the same or another language. All named authors have seen and agreed to the submitted version of the paper and the person included in the acknowledgements section has agreed to his inclusion. We consider that the present investigation is very important to understand more about the chemical composition and the antimicrobial activity of Mikania genus plant on mutans streptococci, which is primarily involved with dental caries and oral diseases. In the present study we found the active isolated compounds from hexanic fractions of Mikania plants against mutans streptococci and this plant is a promising source for novel antimicrobial agents against oral pathogens. We also declare that this study was performed according to the international, national and institutional rules considering biodiversity rights.

Abstract

The present study evaluated the properties of purified compounds obtained from *Mikania laevigata* and *Mikania glomerata* on bacterial growth and cell adherence. The compounds were chemically isolated from hexanic fraction by flash or silica gel 60/$AgNO_3$ chromatography column using as eluent a mixture hexane - ethyl acetate, in a polarity gradient. The compounds tested were spathulenol, kaurenol, and grandifloric, kaurenoic, diterpenic and cupressenic acids (concentration ranging from 1.25 to 80 μg/mL) and ethanol 10% (v/v) at final concentration as negative control. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and inhibition of adherence on glass surface (Adh) against *Streptococcus mutans* Ingbritt 1600, *S. mutans* UA159, *S. sobrinus* 6715 and 2 recently clinical isolated were determined. The spathulenol, kaurenol and grandifloric acid did not show antimicrobial activity on MIC, MBC and Adh tests; the cupressenic, kaurenoic and diterpenic acids were effective at low values. The cupressenic and kaurenoic acids were the most effectiveness among all tested compounds, with the lowest values of MIC and MBC (2.5 μg/mL and 5 μg/mL, respectivelly). In conclusion, cupressenic, kaurenoic and diterpenic acids showed antimicrobial activity against all microorganisms tested, suggesting that those compounds may have some cariostatic potential.

1. Introduction

Streptococcus mutans and other streptococci are of central importance for the formation of dental plaque and the establishment of conditions that can lead to the development of dental caries (Hamada and Slade, 1980; Tanzer et al., 1985; Loesche, 1986; Schilling and Bowen, 1992; Marsh, 1992; 1994). Several studies confirm a relationship between dental caries and the mutans group streptococci, particularly *S. mutans* and *S. sobrinus* (Quivey et al., 2000). The ability of mutans streptococci to adhere and accumulate on the tooth surface by synthesizing extracellular polysaccharides from sucrose together with their acidogenic and aciduric properties are key virulence factors involved in the development of pathogenic dental plaque related to caries (Hamada and Slade, 1980; Tanzer et al., 1985; Loesche, 1986; Schilling and Bowen, 1992; Marsh, 1992; 1994). Therefore, one of the strategies to prevent the formation and development of dental caries is to control the growth and adherence of mutans streptococci.

Natural products have been used for thousand of years in folk medicine, and are promising sources for prevention of oral diseases, especially dental caries (Wu-Yuan et al., 1988; Cai and Wu, 1996; Park et al., 1998; Koo et al., 1999; Koo et al., 2000; Hwang et al., 2003). Among various medicinal plants used in folk-medicine in Brazil, *Mikania* genus plant, (Asteraceae family) a sub-scrub creeper of woody branches, known popularly as "guaco" (Celeghini et al., 2001), stands out because of its multiple pharmacological properties, especially anti-inflammatory, anti-allergic, analgesic and antimicrobial activities (Corrêa, 1942; Davino, 1989; Ruppelt et al., 1991; Fierro et al., 1999; Paul et al., 2000; Yatsuda et al, 2005; de Moura, 2002). The most studied species are *Mikania laevigata* and *Mikania glomerata.* However, only 10% of *Mikania* species have been chemically studied (Fabbri et al., 1997). Fifteen compounds have been identified and isolated, and the major

compounds are coumarin (Vilegas et al., 1997), coumaric acid, sesquiterpenes and diterpenes (Limberger et al., 2001). Some of these compounds, such as kaurenoic acid derivatives substituted on carbon -15, showed antimicrobial and antifungal activities against some pathogens as described by Davino et al. (1989). Our preliminary studies have shown that hexanic fractions of *M. laevigata* and *M. glomerata* display remarkable antimicrobial activity against mutans streptococci, showing inhibitory concentrations as low as 25 μg/ml (Yatsuda et al., 2005).

Considering the great use of plants as medicinal substances in the Brazilian popular medicine, it is critical to investigate their biological and chemical properties in order to not only help to enhance our understanding of the therapeutic potential of these natural products, but also how to make them more effective pharmacological agents. Thus, in view of the potential of *M. laevigata* and *M. glomerata* against oral pathogens (Yatsuda et al., 2005), the aim of this study was to evaluate the chemical composition and the antimicrobial activity of compounds isolated and identified from hexanic fractions of *M. laevigata* and *M. glomerata* on growth and cell adherence of mutans streptococci.

2. Materials and methods

2.1. Plant material

The aerial parts of *Mikania laevigata* Sch.Bip.ex Baker and *Mikania glomerata* Sprengel were collected from experimental field at the Multidisciplinary Center for Chemical, Biological and Agricultural Researches - CPQBA/UNICAMP, Campinas, SP, Brazil and authenticated by Prof. Dr. Marta Dias Moraes (University of Campinas, SP, Brazil). Voucher specimen of *M. laevigata* and *M. glomerata* are deposited at Herbarium of Biology Institute of UNICAMP at Campinas under number UEC - 102.046 and UEC

102047, respectively. The leaves were allowed to dry under air circulation (40 °C) for three days and ground for uses (Yatsuda et al., 2005 and Bighetti et al., 2005).

2.2. Extraction and isolation of active compounds

Dried and pulverized leaves from *M. laevigata* and *M. glomerata* (400 g) were extracted two times with 2 L of ethanol:water (70:30, v/v) at room temperature. The ethanol solution was concentrated by means of a rotary evaporator yielding 79.1 g and 58.2 g of crude ethanolic extracts of *M. laevigata* (EE-*Ml*) and *M. glomerata* (EE-*Mg*). Forty grams each of the extracts were fractionated with hexane three times in dispersor Ultra-Turrax obtaining the hexane fractions of *M. laevigata* H-*Ml* (5.9g) and *M. glomerata* H-*Mg* (5.6 g). The fractions H-*Ml* and H-*Mg* were further fractionated by dried column chromatography (cellulose 2 X 30 cm) using Si gel 60 (Merck) and chloroform:ethyl acetate (70:30, v/v). The columns were cut in four parts and extracted using chloroform yielding the following fractions: H1-*Ml* (0.45 g), H2-*Ml* (1.80 g), H3-*Ml* (1.86 g), H4-*Ml* (1.50 g); and H1-*Mg* (0.70 g), H2-*Mg* (1.65 g), H3-*Mg* (1.78 g), and H4-*Mg* (1.35 g). The fractions were analyzed by thin layer chromatography and GC/MS; H2-*Ml* and H3-*Mg* displayed the highest content of diterpenic acids derivatives and antimicrobial activity (Rehder, unpublished work). Therefore, the fractions H2-*Ml* and H3-*Mg* were selected for further analysis.

The fraction H2-*Ml* (1.3g) was purified using a silica gel 60 column (0.063-0200 mm) impregnated with 10% $AgNO_3$ and eluted with hexane:ethyl acetate in polarity gradient (90:10, 85:15, 80:20 and 50:50; v/v). Seventy-two fractions were collected and monitored by TLC impregnated with 5% $AgNO_3$ (w/v, in methanol), and eluted with hexane:ethyl acetate (80:20, v/v). The fractions with similar chromatographic pattern

(based on R_f) were combined yielding 4 distinct sub-fractions: F1H2-*Ml* (350mg), F2H2-*Ml* (430mg), F3H2-*Ml* (250 mg) and F4H2-*Ml* (195 mg).

The F2H2-*Ml* was identified as ent-kaur-16-en-19-oic acid (kaurenoic acid) and F4H2-*Ml* as ent-beyer-15-en-19-oic acid (cupressenic acid). The H3-*Ml* (1.6g) was purified by consecutive classic and flash column (silica gel) to give spathulenol (102 mg), kaurenol (54 mg) and grandifloric acid (86 mg).

The fraction H3-*Mg* (1.50g) was purified in flash column using Si gel 60 (230-400 mesh - Merck) and hexane:ethyl acetate (93:7, v/v) as eluent. Sixty fractions were collected and monitored by TLC. Again, the fractions with similar chromatographic pattern (based on R_f) were combined yielding six distinct sub-fractions: F1H3-*Mg* (230mg), F2H3-*Mg* (110mg), F3H3-*Mg* (350 mg), F4H3-*Mg* (250 mg), F5H3-*Mg* (64 mg) and F6H3-*Mg* (295 mg).

The F3H2-*Ml* was identified as ent-kaur-16-en-19-oic acid (kaurenoic acid) and F5H3-*Mg* as ent-kaur-16-en-18-oic acid (diterpenic acid). These compounds were crystallized in hexane or methanol and subjected to physical and spectroscopic analyses. The structure of these compounds was confirmed by comparing their physical and spectral data with those reported in the literature, i.e. for cupressenic acid (Bohlmann et al., 1976; Takahashi et al., 2001), diterpenic acid (Castro e Jakupovic, 1985) and kaurenoic acid (Ohno et al., 1979; Cruz et al., 1992). The optical rotation was measured with a Perkin-Elmer polarimeter and melting points were determined on a Koffer boot place (Eletrothermal 9100 apparatus). The ^{1}H-, ^{13}C-NMR and MS spectra were obtained by INOVA 500 Varian and Hewlett Packard 5890/5970 series II system.

2.3. Test agents

The compounds tested were spathulenol, kaurenol, and grandifloric, kaurenoic, diterpenic and cupressenic acids (concentration ranging from 1.25 to 80 μg/ml). The vehicle (100% ethanol, v/v) was used as negative control. The final concentration of ethanol was 10% (v/v).

2.4. Microorganisms

The microorganisms used in this study were *Streptococcus mutans* Ingbritt 1600, *S. mutans* UA159, *S. sobrinus* 6715 and 4 recently clinical isolated donated by Microbiology Area of the Faculty of Dentistry of Piracicaba and with the approval of the Institutional Ethical Committee of Faculty of Dentistry of Piracicaba, University of Campinas (Napimoga et al., 2004).

2.5. Antimicrobial activity assay

The antimicrobial activity was determined by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in accordance with Koo et al. (2000) and Yatsuda et al. (2005). For MIC determination, the starting inoculum was 5×10^5 cfu/ml and MIC was defined as the lowest concentration of compound that inhibited bacterial growth (no visible growth). For the determination of MBC, an aliquot (50 μl) of all incubated test tubes with concentrations higher than the MIC was subcultured on BHI agar supplemented with 5% of defibrinated sheep blood. The MBC was defined as the lowest concentration that enables no growth on the agar (99.9% killed). Six replicates were made for each concentration of the tested agents for all assays.

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

To assess the bacterial adherence of growing cells of mutans streptococci to a glass surface, microorganisms were grown in BHI broth plus 1% sucrose (w/v) containing the

MIC levels (concentration ranged from 1.25 - 20 μg/ml.) of compounds from *Mikania* (or vehicle control) according to Koo et al. (2000) and Yatsuda et al. (2005). After incubation, the adhered cells were washed resuspended and the adhered cells were spectrophotometrically measured at 550 nm using the procedures outlined by Koo et al. (2000). The inhibition of adherence was defined as the lowest concentration that allowed no visible cell adherence on the glass surface. Six replicates were made for each concentration of the tested agents for all assays.

3. Results

In the present study, the compounds isolated and identified tested were spathulenol, kaurenol, and grandifloric, kaurenoic, diterpenic and cupressenic acids; but in general, at the highest concentration tested of 80 μg/ml, only these three diterpene acids: cupressenic, diterpenic and kaurenoic displayed significant antimicrobial activity. These six compounds were isolated and identified from hexane fractions of *Mikania laevigata* and *M. glomerata*, and their chemical structures determined by spectroscopic methods (Fig. 1).

The MIC and MBC values of cupressenic, diterpenic and kaurenoic acids are shown in Table 1. The cupressenic, diterpenic and kaurenoic acids inhibited the growth of all microorganisms tested, at low concentrations (2.5-20 μg/ml) (Table 1). The cupressenic, diterpenic and kaurenoic acids also showed bactericidal activity on all microorganisms tested at concentrations ranging between 2.5 and 40 μg/ml. The cupressenic acid showed remarkable antibacterial activities displaying the lowest MIC (2.5 μg/ml to 5 μg/ml) and MBC (2.5 μg/ml to 20 μg/ml) values. The cupressenic and kaurenoic acids showed great bactericidal activity (MBC 2.5 μg/ml to 5 μg/ml) nearly to chlorhexidine (gold standard)

that demonstrated MICs of 1-2 μg/ml and an MBC of 8 μg/ml as described by Koo et al., (2002) for mutans streptococci.

The cupressenic, diterpenic and kaurenoic acids were able to inhibit the adherence of mutans streptococci cells to a glass surface at, in general, sub-MIC levels as shown in Table 2; the concentrations of the test agents were ranging between 1.25 and 5μg/ml. The cupressenic acid was the most effective agents, showing inhibitory effects at concentrations as low as 1.25 μg/ml.

4. Discussion and conclusions

The development of therapeutic agents aimed at disrupting both colonization of the teeth by dental pathogens and the subsequent formation of dental plaque is one of the prime strategies to reduce the incidence of tooth decay (Bowen, 1999). In previous studies, the hexane fractions of *M. laevigata* and *M. glomerata* were the most effective in inhibiting the growth of the mutans streptococci strains, showing MIC values between 12.5 – 100 μg/ml and also showed inhibition of cell adherence of mutans streptococci, at sub-MIC concentrations (Yatsuda et al., 2005).

In this investigation, the cupressenic, diterpenic and kaurenoic acids were isolated and identified from the hexane fractions of *M. laevigata* and *M. glomerata* and displayed inhibition of growth and adherence of all the microorganisms tested at low concentration. These compounds may be responsible for the greatest antimicrobial activity of this fraction against mutans streptococci.

Overall, MIC and MBC values for the clinical isolates were almost the same for culture collection strains. This fact was not observed on the previous study where the laboratory strains were more susceptible to antimicrobials than strains recently isolated

(Duarte et al., 2003 and Yatsuda et al., 2005). It is noteworthy that the isolated compounds were still effective against the recently isolated bacteria.

Structurally, the presence of the carboxylic acid in position 19 seemed to be important in the antibacterial activity, because natural and synthetic derivatives with this group blocked or interacting with hydrogen bonds have less or no activity. Ent-kaur-16-en-19-oic acid isolated from the stem bark of *Mitrephora celebica* was identified as the compound responsible for the antimicrobial activity of the plant against methicillin-resistant *Staphylococcus aureus* and *Mycobacterium smegmatis* (Zgoda-Pols *et al.*, 2002). Two spasmolytic diterpene acids, *ent*-beyer-15-en-19-oic acid and *ent*-kaur-16-en-19-oic acid, isolated from the roots of *Viguiera hypargyrea* were active against *Staphylococcus aureus*, *Enterococcus feacalis* and *Candida albicans* (Zamilpa *et al.*,2002).

Furthermore, the cupressenic, diterpenic and kaurenoic acids showed *in vitro* inhibition of sucrose-dependent adherence of mutans streptococci cells to a glass surface at sub-MIC levels. The inhibitory effect of these compounds on bacterial adherence can be related to their action on glucosyltransferases (GTFs), especially on those responsible for insoluble glucans synthesis. The glucans produced by the GTFs are critical for the pathogenic potential of dental plaque by promoting the adherence and accumulation of mutans streptococci on the tooth surface (Yatsuda et al., 2005). However, further studies need to be conducted to confirm their effect on the activity of individual and purified GTF enzymes.

In conclusion, the cupressenic, diterpenic and kaurenoic acids isolated from *Mikania* showed remarkable inhibitory activities against mutans streptococci growth and cell adherence at low concentrations, especially the cupressenic acid. This finding implies that these compounds might be the pharmacologically active compounds from the non-polar fraction, being promising antibacterial agents against oral pathogens. Further studies should

be conducted to examine whether these compounds alone or in combination have any influence on the viability, development and virulence of mutans streptococci biofilms.

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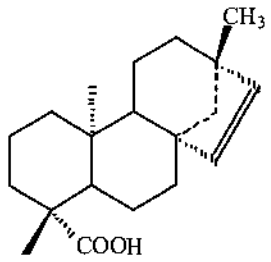
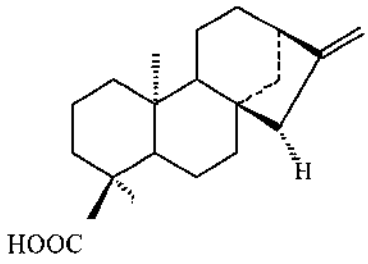
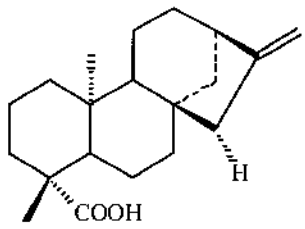
Figure 1. Structures of compounds isolated from *M. laevigata* and *M. glomerata.*



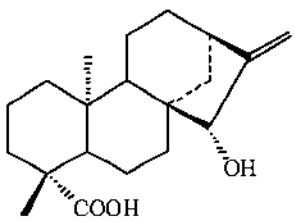
Ent-beyer-15-en-19-oic acid
(Cupressenic acid)



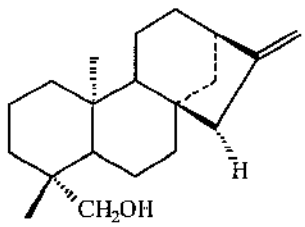
Ent-kaur-16-en-18-oic acid
(Diterpenic acid)



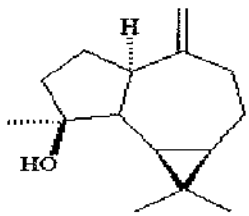
Ent-kaur-16-en-19-oic acid
(Kaurenoic acid)



Grandifloric acid



Kaurenol



Spathulenol

Table 1

Values of Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of cupressenic, diterpenic and kaurenoic acids of *M. laevigata* (*Ml*) and *M. glomerata* (*Mg*) for the mutans streptococci strains.

Microorganisms	Cupressenic acid		Diterpenic acid		Kaurenoic acid	
	MIC[a]	MBC[a]	MIC[a]	MBC[a]	MIC[a]	MBC[a]
S. mutans Ingbritt 1600	2.5	5	10	20	2.5	5
S. mutans UA159	5	20	5	40	5	20
S. mutans D1[b]	5	10	5	10	2.5	5
S. mutans P20[b]	2.5	5	10	20	2.5	5
S. sobrinus 6175	2.5	2.5	5	10	2.5	5
S. sobrinus P7[b]	2.5	5	10	20	5	10
S. sobrinus S2[b]	2.5	5	10	20	5	10

[a] MIC and MBC values are expressed in μg/ml. The concentrations of compounds ranged from 1.25- 80 μg/ml. (n=12)

[b] Recent clinical isolate microorganism and donated by Microbiology Area of the Faculty of Dentistry of Piracicaba.

Table 2

Effect of cupressenic, diterpenic and kaurenoic acids of *M. laevigata* (*Ml*) and *M. glomerata* (*Mg*) on the sucrose-dependent adherence of the mutans streptococci strains.

Microorganisms	*Inhibition of cell adherence*		
	Cupressenic acid	Diterpenic acid	Kaurenoic acid
S. mutans Ingbritt 1600	2.5	2.5	2.5
S. mutans UA159	1.25	5	2.5
S. mutans D1[b]	5	2.5	2.5
S. mutans P20[b]	2.5	5	2.5
S. sobrinus 6175	1.25	5	2.5
S. sobrinus P7[b]	2.5	5	5
S. sobrinus S2[b]	2.5	5	5

[a] Values of the inhibition of cell adherence are expressed in μg/ml. The concentrations of compounds ranged from 1.25 - 20 μg/ml. (n=12)

[b] Recent clinical isolate microorganism.

2.2. Estudo 2

Influence of Diterpenes from *Mikania* genus plants on virulence and formation of *Streptococcus mutans* biofilms

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KEYWORDS: *Mikania* spp., *Streptococcus mutans*, biofilms, glucans, glycolysis.

Abstract

The compounds isolated from hexanic fractions of *Mikania laevigata* and *Mikania glomerata* showed antimicrobial activity and inhibit the adherence of mutans streptococci at low concentration. In the present study, we examined the influence of cupressenic, diterpenic and kaurenoic acids on glucans production by purified glucosyltransferases (GTFs) adsorbed to hydroxyapatite beads; on membrane-associated F-ATPase and glycolytic activities, and on viability, development, polysaccharide composition and acidogenicity of *S. mutans* UA159 biofilms. The test compounds reduced the activity of GTFs B in solution (50 to 60% inhibition) and on a surface (50 to 80% inhibition) ($p<0.05$). The F-ATPase activity of permeabilized cells of *S. mutans* was inhibited by the compounds tested in 28-40% ($p<0.05$). Thus, the compounds reduced the acid production, the dry-weight and polysaccharide by *S. mutans* biofilms. Furthermore, biofilm development and acidogenicity were significantly affected by topical applications of all compounds. Our data show that cupressenic, diterpenic and kaurenoic acids are the active constituents of *Mikania* genus plant against *S. mutans*, and are promising novel natural agents for controlling the development of dental caries.

Introduction

The ability of mutans streptococci to produce extracellular polysaccharides, mainly glucans, has been recognized as a critical virulence factor in the pathogenesis of dental caries; polysaccharides synthesis by oral microorganisms is also associated with plaque (or dental biofilm) formation and accumulation (Hamada & Slade, 1980; Loesche, 1986; Yamashita *et al.*, 1993). Glucans, synthesized from dietary sucrose by glucosyltransferase enzymes, play an essential role in the adherence and accumulation of *Streptococcus mutans* and other oral microorganisms to the tooth surface, leading to formation of cariogenic biofilm communities (Schilling & Bowen, 1992; Marsh & Bradshaw, 1995). Furthermore, *S. mutans* catabolize multiple fermentable dietary carbohydrates producing organic acids efficiently. However, within biofilms effective neutralization cannot occur because of limited access by saliva; and the low pH values in the biofilms matrix contribute to demineralization of tooth enamel and selection of aciduric (acid-tolerant) organisms, such as mutans streptococci. One of the mechanisms by which mutans streptococci have developed to alleviate the influences of acidification is an increased proton-translocating F-ATPase activity in response to low pH (Sturr & Marquis, 1992; Quivey *et al.*, 2000). The high acid tolerance, exhibited by mutans streptococci plays a critical role in their expression of virulence and in the pathogenesis of dental caries (Yamashita *et al.*, 1993). Thus, the development of new mechanisms to eliminate mutans streptococci selectively should be prime targets for any therapeutic agent, which could include inhibition of virulence factors, as glucan production by GTFs and F-ATPase activity.

Among various plant extracts used in Brazilian folk-medicine, those from *Mikania* genus plant (a sub-scrub creeper of woody branches known popularly as "guaco") are of particular interest because of reported multiple pharmacological properties, such as anti-inflammatory and antimicrobial activities (Ruppelt et al., 1991; Corrêa, 1942; Pereira et al., 1994; Celeghini et al., 2001; Yatsuda et al., 2005). Furthermore, results from our previous studies have shown that hexane fractions of *M. laevigata* and *M. glomerata*, exhibit antimicrobial activity against

planktonic cells of mutans streptococci (Yatsuda et al., 2005). The major components in the hexane fractions were coumarin, ent-beyer-15-en-19-oic acid, diterpenic acid, ent-kaur-16-en-19-oic acid and decanoic acid. Recently, we have shown that the ent-beyer-15-en-19-oic acid (cupressenic acid), ent-kaur-16-en-18-oic acid (diterpenic acid) and ent-kaur-16-en-19-oic acid (kaurenoic acid) (Yatsuda, unpublished work) were the most effective compounds, inhibiting the growth of the planktonic cells of mutans streptococci at low concentrations (MIC values ranging from 2.5 to 10 μg/mL). However, their influence on the bacteria growing in biofilms mode, which is likely to occur in the oral cavity, remains to be elucidated.

Considering the promising antibacterial effects of the compounds from *M. laevigata* and *M. glomerata*, the purpose of this study was to evaluate the actions of these agents on (i) glucans production by purified glucosyltransferases (GTFs) adsorbed to hydroxyapatite beads (ii) membrane-associated F-ATPase and glycolytic activities, and (iii) viability, development, polysaccharide composition and acidogenicity of *S. mutans* biofilmes

Materials and Methods

Plant material. In this study, the compounds from hexane fraction of *M. laevigata (Ml)* and *M. glomerata* (*Mg*) were isolated and identified. The aerial parts of *Mikania laevigata* Schultz Bip. and *Mikania glomerata* Sprengel were collected from experimental field of the Center for Chemical, Biological and Agricultural Research - CPQBA/UNICAMP, Brazil. Voucher specimen of *M. laevigata* (*Ml*) and *M. glomerata* (*Mg*) is deposited at Herbarium of Biology Institute of UNICAMP under number UEC - 102.046 and UEC - 102047.

Extraction and isolation of active compounds. Dried and pulverized leaves from *M. laevigata* and *M. glomerata (*400 g) were extracted two times with 2 L of ethanol:water (70:30, v/v) at room temperature. The ethanol solution was concentrated by means of a rotary evaporator yielding 79.1 g and 58.2 g of crude ethanolic extracts of *M. laevigata* (EE-*Ml*) and *M. glomerata* (EE-*Mg*). Forty grams of the extracts were fractionated with hexane three times in dispersor

Ultra-Turrax obtaining the hexane fractions of *M. laevigata* H-*Ml* (5.9g) and *M. glomerata* H-*Mg* (5.6 g). The fractions H-*Ml* and H-*Mg* were further fractionated by dried column chromatography (cellulose 2 X 30 cm) using Si gel 60 (Merck) and chloroform:ethyl acetate (70:30, v/v). The columns were cut in four parts and extracted using chloroform yielding the following fractions: H1-*Ml* (0.45 g), H2-*Ml* (1.80 g), H3-*Ml* (1.86 g), H4-*Ml* (1.50 g); and H1-*Mg* (0.70 g), H2-*Mg* (1.65 g), H3-*Mg* (1.78 g), and H4-*Mg* (1.35 g). The fractions were analyzed by thin layer chromatography and GC/MS; H2-*Ml* and H3-*Mg* displayed the highest content of diterpenic acids derivatives and antimicrobial activity (Rehder, unpublished work). Therefore, the fractions H2-*Ml* and H3-*Mg* were selected for further analysis.

The fraction H2-*Ml* (1.3g) was purified using a silica gel 60 column (0.063-0200 mm) impregnated with 10% $AgNO_3$ and eluted with hexane:ethyl acetate in polarity gradient (90:10, 85:15, 80:20 and 50:50; v/v). The fractions with similar chromatographic pattern (based on R_f) were combined yielding 4 distinct sub-fractions: F1H2-*Ml* (350mg), F2H2-*Ml* (430mg), F3H2-*Ml* (250 mg) and F4H2-*Ml* (195 mg). The fraction H3-*Mg* (1.50g) was purified in flash column using Si gel 60 (230-400 mesh - Merck) and hexane:ethyl acetate (93:7, v/v) as eluent. Again, the fractions with similar chromatographic pattern (based on R_f) were combined yielding six distinct sub-fractions: F1H3-*Mg* (230mg), F2H3-*Mg* (110mg), F3H3-*Mg* (350 mg), F4H3-*Mg* (250 mg), F5H3-*Mg* (64 mg) and F6H3-*Mg* (295 mg). The F2H2-*Ml* (R_f = 0.43), F4H2-*Ml* (R_f = 0.21), F3H3-*Mg* (R_f = 0.42), and F5H3-*Mg* (R_f = 0.50) were the most active sub-fractions (Rehder, unpublished work). These compounds were crystallized in hexane and subjected to physical and spectroscopic analyses. The optical rotation was measured with a Perkin-Elmer polarimeter and melting points were determined on a Koffer boot place (Eletrothermal 9100 apparatus). The ^{1}H-, ^{13}C-NMR and MS spectra were obtained by INOVA 500 Varian and Hewlett Packard 5890/5970 series II system. The F2H2-*Ml* was identified as ent-kaur-16-en-19-oic acid (kaurenoic acid) and F4H2-*Ml* as ent-beyer-15-en-19-oic acid (cupressenic acid) whereas F3H3-*Mg* was identified as ent-kaur-16-en-19-oic acid (kaurenoic acid) and F5H3-*Mg* as ent-kaur-16-en-18-oic acid

(diterpenic acid); the structure of the compounds were confirmed by comparing their physical and spectral data with those reported in the literature, i.e. for cupressenic acid (Takahashi *et al.*, 2001; Bohlmann *et al.*, 1976), diterpenic acid (Castro, 1985) and kaurenoic acid. (Cruz *et al.*, 1992; Ohno *et al.*, 1979).

Our previous study determined the minimum inhibitory concentration (MIC) of compounds ent-beyer-15-en-19-oic acid (cupressenic acid) (5-10 μg/mL), ent-kaur-16-en-18-oic acid (diterpenic acid) (2.5-5 μg/mL) and ent-kaur-16-en-19-oic acid (kaurenoic acid) (5-10 μg/mL) against mutans streptococci. The concentrations of the isolated compounds used in this study were 100 times of their MIC values, as the inhibitory concentration for biofilms as showed in previously studies (Yatsuda *et al.*, 2002), being determinate the concentration of 500 μg/mL or 1.65 mM for all compounds. Vehicle control (20% ethanol, v/v) was used as a negative control.

Bacterial strains. *Streptococcus mutans* UA159, a virulent cariogenic pathogen and the strain selected for genomic sequencing (Ajdic *et al.*, 2002), was used for F-ATPase, glycolytic pH drop, proton permeability and biofilms studies. For the production of GTF B, *Streptococcus milleri* KSB8 was used, which harbors the *gtfB* gene from *S. mutans* GS-5 (for GTF B production) (Wunder & Bowen, 1999).

Glucosyltransferases (GTFs) assays. The GTF B enzyme (EC 2.4.1.5) was obtained from culture supernatants of *S. milleri* KSB8, and purified to near homogeneity by hydroxyapatite column chromatography as described by Venkitaraman *et al.* (1995) and Koo *et al.* (2000). GTF activity was measured by the incorporation of [^{14}C] glucose from labeled sucrose (NEN Research Products, Boston, Mass., USA) into glucans. The GTF enzyme added to each sample for all assays was equivalent to the amount required to incorporate 1 to 1.5 μmol of glucose over the 4-h reaction. The activity of GTF was determined either in solution or adsorbed to hydroxyapatite beads (Macro-Prep Ceramic Hydroxyapatite Type I, 80 μm, Bio-Rad®) coated with clarified whole saliva (sHA) (free of GTF activity) in the presence or absence (control) of the test

compounds as described elsewhere. The concentrations of the test compounds were 100 times of the MIC values (500 μg/ml).

Biofilms assays. Biofilms of *Streptococcus mutans* UA159 were formed on saliva-coated hydroxyapatite discs placed in a vertical position (HAP- ceramic – calcium hydroxyapatite, 0.5" diameter ceramic - Clarkson Calcium Phosphates, Williamsport, PA) in batch cultures at 5% CO_2 and 37 °C (Koo *et al.*, 2003; Chatfield *et al.*, 2005; Duarte *et al.*, 2006). The biofilms were grown on tryptone-yeast extract broth with addition of sucrose undisturbed for 24 h to allow initial biofilm formation. At this point (24 h old), the biofilms were treated twice daily (one-minute exposure) until the 5th day of the experimental period (126 h-old biofilms) with each of the test compounds (at 500 μg/mL) or vehicle control. Each biofilm was exposed to each compound a total of eight times. The treated biofilms were analyzed for biomass (dry-weight) and bacterial viability (colony forming units – CFU mg^{-1} of biofilm dry-weight). The polysaccharide composition (extracellular water-soluble and insoluble glucans, and intracellular iodophilic polysaccharide) was determined by colorimetric assays and scintillation counting as detailed by Koo *et al.*, (2003). The acid production by the biofilms that were treated with the test agents (or, control) was determined by glycolytic pH drop after addition of glucose solution (final concentration of 1%, w/v) by means of a glass electrode (Futura Micro Combination pH electrode, 5mm diameter, Beckman Coulter, Inc, CA) (Belli, *et al.*, 1995).

Glycolytic pH-drop assay. The effects of the compounds on glycolysis were measured by standard pH drop with dense cell suspensions as previously described by Belli et al. (1995). Cells of S. mutans UA159 from suspension cultures were harvested, washed once with salt solution (50mM KCl plus 1mM $MgCl_2$), and resuspended in salt solution containing the test agents. The pH was adjusted to 7.2 with 0.1M KOH solution, sufficient glucose was added to give a concentration of 1% [weight in volume (w/v)], and the decrease in pH was assessed by means of a glass electrode over a period of 2 h (Futura Micro Combination pH electrode, 5mm diameter, Beckman Coulter, Inc., CA) (Belli et al., 1995).

Proton Permeability. Intact biofilms of *S. mutans* UA159 grow for 5 days being treated with the test compounds or ethanol 20% (v/v), as described before. On the 5 day the biofilms were transferred to a solution with the pH adjusted to 4.4 with HCl w/ 50 mM KCl. The pH was read at following intervals of 10 min. The increase in pH was assessed with glass electrode overtime (up to 2 h) (Marquis et al., 2004).

F-ATPase assay. ATPase (Adenosine 5'-triphosphate disodium salt, grade I, n° A2383, Sigma) assay was performed using permeabilized cells of *S. mutans* UA159 prepared as described by Belli *et al.* (1995). F-ATPase activity was assayed in terms of the release of inorganic phosphate in 100 mMol of Tris-maleate buffer (pH 7.0) containing 10 mMol $MgCl_2$, permeabilized cells and the test agents cupressenic, diterpenic and kaurenoic acids or vehicle control. The released phosphate was then determined by the method of Bencini *et al.*, (1983).

Statistical analyses. All of the assays were done in triplicate on at least three different experiments. The data were analyzed using one-way analysis of variance (ANOVA), pairwise comparison was made between all the groups using Tukey-Kramer HSD method to adjust for multiple comparisons, using statistical software JMP version 3.1 (SAS Institute Inc., 1989). When no parametric data was found, Kruskal-Wallis test was used to compare all pairs in the statistical software BioEstat 3.0 (2003). The level of significance was at 5%.

Results and discussion

Cupressenic, diterpenic and kaurenoic acid were isolated and identified from hexane extracts of *Mikania laevigata* and *M. glomerata*, and their chemical structures determined by spectroscopic methods (Fig. 1).

The compounds cupressenic, diterpenic and kaurenoic acids at 1.65 mM showed significant inhibitory effects on formation and further accumulation of the biofilms. The influence of the test compounds on the mutans streptococci biofilms are shown in Table 1. The biofilms treated with the compounds did not reduce the viability of the biofilms as assessed by the

determination of CFU mg^{-1} of biofilm dry-weight compared to the vehicle control. Furthermore, the biofilms treated with the three compounds exhibited almost 50% less biomass (dry-weight) than those treated with vehicle control alone (Table 1, $p<0.05$). Table 1 also reveals that the content of polysaccharide, not only extracellular glucans (water-soluble and insoluble), but also intracellular iodophilic polysaccharide in the biofilms was significantly reduced at 50% by cupressenic, diterpenic and kaurenoic acids (1.65 mM) compared to those treated with the vehicle control. Although, our mono-species biofilm model does not mimic the complex microbial community found in dental plaque, it is more advantageous when examining specific actions of test agents on *S. mutans* physiology, especially on the glucan-mediated processes involved in the biofilm development (Duarte *et al.*, 2006). Based on these results, it appears that the major biological activity of the compounds in reducing the accumulation of the biofilms is related to their effects on the synthesis of insoluble polysaccharides and on the IPS accumulation.

The effects of the test compounds on synthesis of glucans are shown by the activity of GTF B in Figures 2 and 3. The test compounds reduced the activity of GTFs B in solution (50 to 60% inhibition) and on a surface (50 to 80% inhibition). The kaurenoic acid displayed the most potent inhibition of GTF B activity in surface (80% inhibition) ($p<0.05$) and on solution (60% inhibition). GTFs display distinct physical and kinetic properties whether they are in solution or adsorbed to a surface (Venkitaraman *et al.*, 1995); surface-adsorbed GTFs display an increased resistance to the most common inhibiting agents (Koo *et al.*, 2002; Wunder & Bowen, 1999). Nevertheless, all the compounds showed more inhibition of GTF B in surface than in solution. The activities of GTFs in solution could be diminished in this study because their enzymatic activities are affected by the acidic pH of the test agents (near pH 6.5-5.5), which is below the optimum pH for the enzymes in solution (?pH 6.5). In contrast, surface-adsorbed GTFs are active over a much broader pH range (pH 4.7–7.5) (Schilling & Bowen, 1988). The inhibition of GTF B has many implications for bacterial adherence and biofilms development because large proportion of the insoluble glucans (α1,3-linked glucans) synthesized by these surface-adsorbed enzymes is

retained on the pellicle promoting accumulation of *S. mutans* and other cariogenic bacteria on the tooth surface, and contributing to the formation of the matrix of the biofilms (Schilling & Bowen, 1992). By diminishing the synthesis of glucans, the compounds tested have had significant impact on the further development and accumulation of the biofilms.

Other mechanism of action may be related to the ability of diterpenoids to inflict damage to the cell membrane, which can indirectly affect the glucan synthesis by *S. mutans* (Marquis *et al.*, 2003). Enzyme secretion by bacterial cells is generally coupled to Δp, the proton-motive force, across the cell membrane; by increasing the proton permeability of the membrane, the Δp would be affected, and thereby the secretion of GTFs and the glucans synthesis may be diminished (Marquis *et al.*, 2003). Citoplasmatic acidification caused by these agents could also disrupt the glycolytic acid production and induce the formation of intracellular iodophilic polysaccharides (IPS), a glycogen-like storage polymer (Hamilton, 1990). The IPS provide *S. mutans* with an endogenous source of carbohydrate which can be metabolized when exogenous fermentable substrate has been depleted in the oral cavity (Hamilton, 1977); as a result, IPS can promote the formation of dental caries by prolonging the exposure of tooth surfaces to organic acids, with a concomitant lower fasting pH in the matrix of the plaque (Tanzer *et al.*, 1976). It is showed in this study that, by disrupting the accumulation of IPS, the agents are also reducing the acidogenicity of the biofilms.

The permeability of cells membrane to protons was increased by the cupressenic, kaurenoic and diterpenic acids when in comparison to the control as shown on the data of Fig. 4B. This effect is confirmed when pH rises after butanol addition, that disrupted the cell membrane, showing a rapid rise in pH, indicative of loss of ΔpH between cytoplasm and the environment. Thus, the acidification of the cytoplasm might sensitize the glycolysis to acidification, inhibiting the growth and inducing the death of the bacteria. The cupressenic, diterpenic and kaurenoic acids were able to reduce the acid tolerance of the *S. mutans* on biofilms as indicated by the final pH values between 6.4 and 5.8 in pH-drop experiments (Fig. 4A), while the final pH of biofilms

treated by the vehicle control values are near 4.5 ($p<0.05$), slightly below the critical pH for enamel dissolution (pH 5.0-5.5). The compounds not only slowed or almost stopped the glycolysis but also reduced the production of acids, affecting the final pH value, and affecting both acidogenesis and acid tolerance of these bacteria on the biofilm. Whether, this compounds can actually prevent enamel demineralization awaits further evaluation since the final pH (5.8-6.4) values are still higher than the critical pH for enamel dissolution (pH 5.0-5.5). And also, kaurenoic acid induced a more potent inhibition than the other compounds tested.

The aciduricity of these microorganisms is attributed in large part of the possession of a proton-extruding F-ATPase that is expressed at higher levels than in many oral bacteria and that functions well at pH values of 5.0 and below, allowing these organisms to maintain adequate ΔpH when the external pH falls to 4.0 and lower. In addition, *S. mutans* is capable to of mounting an adaptative acid tolerance response that renders to the organisms to be less susceptible to lethal acidification and is associated with enhanced glycolytic capacities and increased activity of the proton-translocating F-ATPase. The Figure 5 shows that the F-ATPase activity of permeabilized cells of *S. mutans* was inhibited by the compounds tested in 28-40% ($p<0.05$). The tests compound appears to slow glycolysis as well as inhibiting the FATPase and sensitizing the glycolysis to acidification.

The compounds can be acting reducing the acid tolerance by two actions: inhibiting the synthesis of ATP or/and inhibition of F-ATPase. Probably these diterpenes acids can be acting as an inhibitor of other types of ATPase in *S. mutans* that eliminate intracellular H^+ produced by these bacteria. Wilkens *et al.*, (2002) showed that kaurenoic acid has bactericidal activity against gram–positive bacteria, and his activity can be affected by the composition and pH of the culture medium. Since a weak acid could transport protons across the membrane, one possible mechanism of action of kaurenoic acid is the alteration of the electron transport and subsequently the oxidative phosphorylation is the disruption of the cytoplasmatic membrane by physical interaction (Wilkens *et al.*, 2002).

Although the toxicology of these compounds was not studied here, it is known that extracts of *M. glomerata* and *M. laevigata* are devoid of mutagenic and toxic effects *in vitro* and *in vivo* (Fernandes & Vargas, 2003; da Silveira *et al.*, 2003). Most of the currently available anti-plaque agents are broad-spectrum antimicrobials with negligible effects on glucans synthesis or permeability of the membrane. In contrast, kaurenoic, cupressenic and diterpenic acids offer promising avenues to explore development of therapeutic agents for plaque-related diseases based on inhibition of the synthesis of glucans, and changes in permeability of cell membrane (Wunder & Bowen, 1999). Additional studies will be conducted to evaluate their potential as anti-caries and anti-plaque agents using *in vitro* multispecies biofilm and *in vivo* models. The putative mechanisms of action of these natural compounds are being investigated.

Acknowledgements

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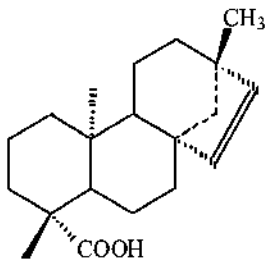
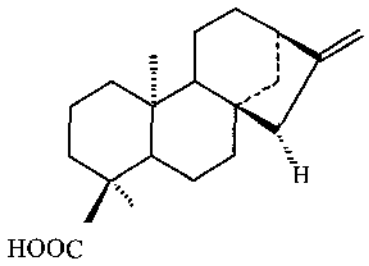
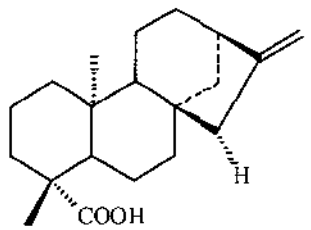
Figure 1. Structures of compounds isolated from *M. laevigata* and *M. glomerata.*



Ent-beyer-15-en-19-oic acid
(Cupressenic acid)



Ent-kaur-16-en-18-oic acid
(Diterpenic acid)



Ent-kaur-16-en-19-oic acid
(Kaurenoic acid)

Table 1. *Streptococcus mutans* UA159 biofilm composition (dry-weight, viability, and total amount of polysaccharides) after treatments with the compounds at 1.65 mM or vehicle control (ethanol 20%).

Treatments	Dry-weight mg	c.f.u. ($\times 10^{10}$)	Alkali-soluble glucan mg	Soluble Glucan mg	Iodophilic Polysaccharide mg
Cupressenic Acid	4.4 ± 1.1^{b}	0.68 ± 0.63^{a}	1.14 ± 0.38^{b}	0.22 ± 0.05^{b}	0.24 ± 0.07^{b}
Diterpenic Acid	5.6 ± 1.0^{b}	1.15 ± 1.52^{a}	1.55 ± 0.40^{b}	0.21 ± 0.04^{b}	0.29 ± 0.09^{b}
Kaurenoic Acid	4.8 ± 1.3^{b}	0.76 ± 1.23^{a}	1.36 ± 0.47^{b}	0.21 ± 0.02^{b}	0.31 ± 0.10^{b}
Vehicle Control	8.0 ± 1.5^{a}	1.59 ± 0.84^{a}	2.67 ± 0.53^{a}	0.49 ± 0.14^{a}	0.79 ± 0.20^{a}

Values in the same column followed by different superscripts are significantly different from each other ($p < 0.05$, ANOVA, comparison for all pairs using Tukey's test).

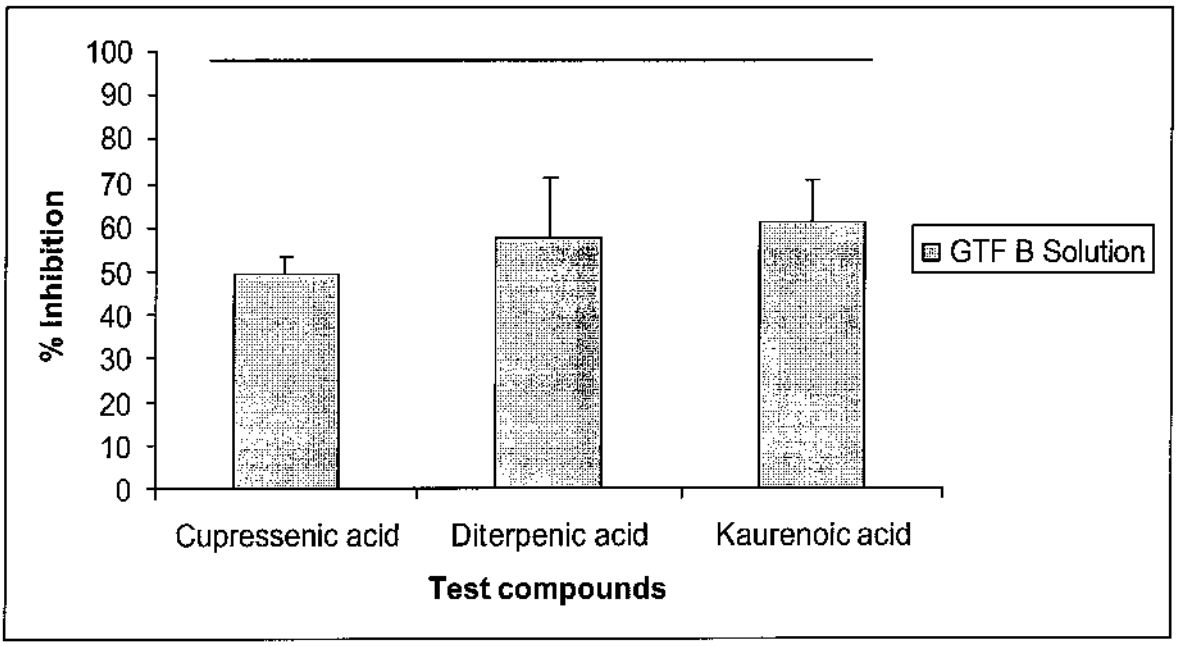


Fig. 2. Influence of *Mikania* diterpenes on the activities of Glucosyltransferase B in Solution. The percentage of inhibition was calculated considering the negative control (vehicle control, 20% ethanol) as 100% GTF activity. Values (SD, N = 9) connected by lines are not significantly different from each other ($p>0.05$, ANOVA, comparison for all pairs using Tukey test).

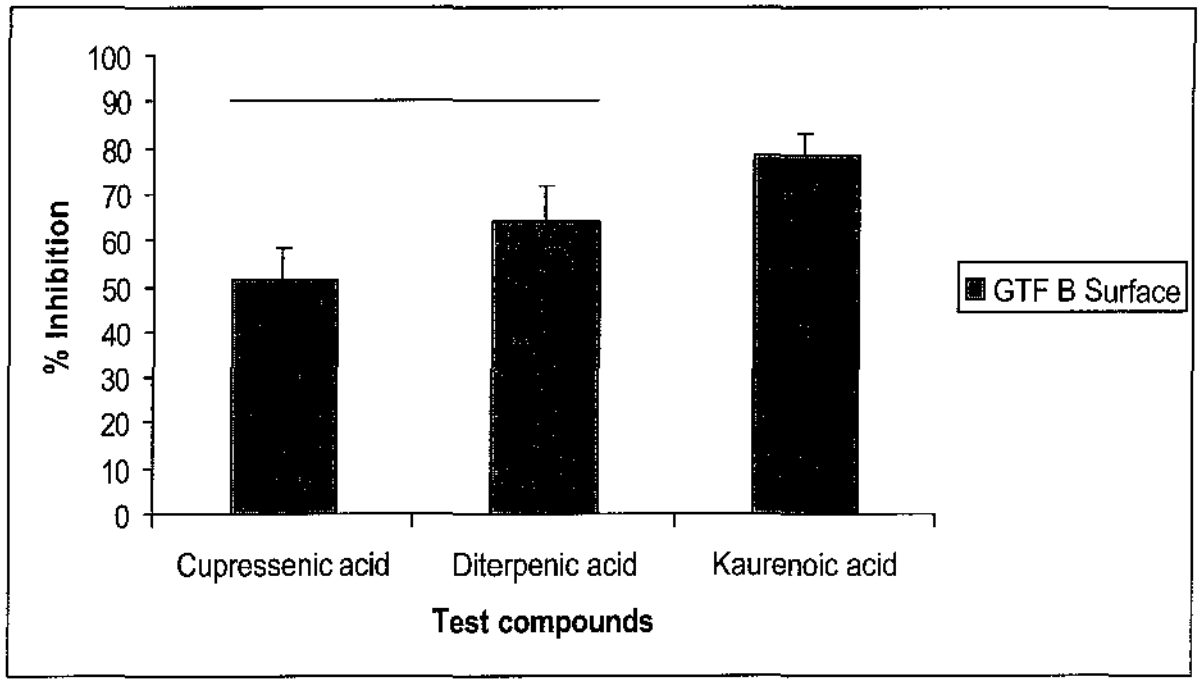


Fig. 3. Influence of *Mikania* diterpenes on the activities of Glucosyltransferase B adsorbed onto a saliva-coated hydroxyapatite surface. The percentage of inhibition was calculated considering the negative control (vehicle control, 20% ethanol) as 100% GTF activity. Values (SD, N = 9) connected by lines are not significantly different from each other ($p>0.05$, ANOVA, comparison for all pairs using Tukey test).

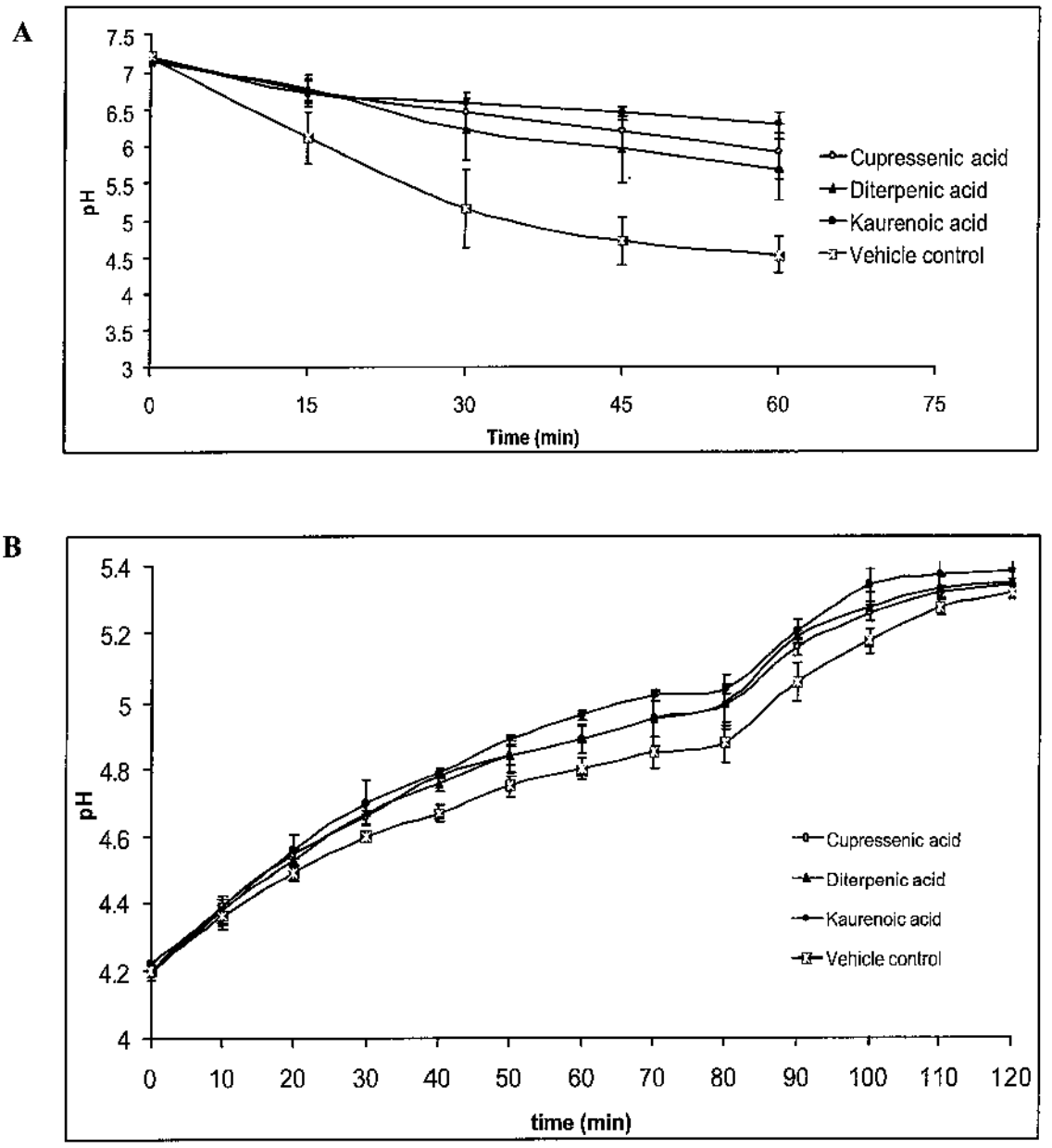


Fig. 4. Influence of *Mikania* diterpenes on Glycolytic pH drop (A) and on Proton permeabilities (B) of *S. mutans* UA159 in Biofilms. Values (SD, N = 9).

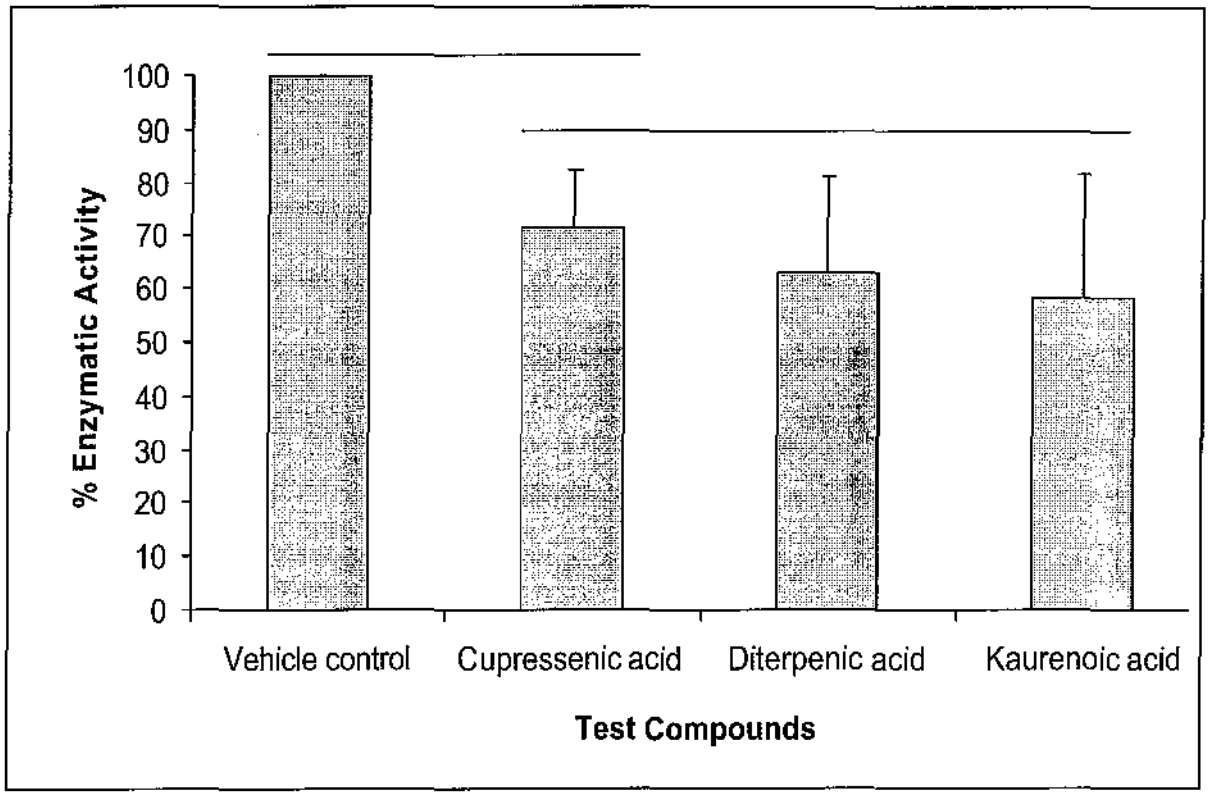


Fig. 5 Influence of *Mikania* diterpenic acids on F-ATPase of permeabilized cells of *S. mutans* UA159. The percentage of inhibition was calculated considering the vehicle control as 100% F-ATPase activity. Values (SD, N = 9) connected by lines are not significantly different from each other ($p>0.05$, ANOVA, comparison for all pairs using Tukey test).

2.3. Estudo 3

Effects of isolated compounds from *Mikania laevigata* and *Mikania glomerata* on caries development in rats

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Abstract

This study aimed to evaluate the influence of cupressenic, diterpenic and kaurenoic acids from hexanic fractions of *Mikania laevigata* and *M. glomerata* on caries development in rats. The rats were infected with *S. mutans* UA159 and fed with cariogenic diet 2000 *ad libitum*. The rats were treated topically twice a day with each of the compounds at 500µg/ml, chlorexidine 0.12% and vehicle control (20% ethanol) for 5 weeks. After the experimental period, the microbial composition of their dental plaque and their caries scores were determined. The animals were weighed weekly, and their physical appearance was noted daily. At the end of the 5-week experimental period, the rats were killed by CO_2 asphyxiation and the tissues were removed to macroscopic and histological assays. The results showed that cupressenic, diterpenic and kaurenoic acids significantly reduced the incidence of smooth-surface and sulcal caries *in vivo,* without displaying a reduction of the percentage of total microorganisms in the animals' plaque ($p < 0.05$). Overall, the animals treated with the compounds and the chlorexidine did not show any statistical difference in either severity of smooth-surface or sulcal caries when compared to the control ($p > 0.05$). The data confirm that the cariostatic properties of the compounds isolated of *Mikania* genus plant can be related to their effects on glucosyltransferases, on acid production and on acid tolerance of cariogenic streptococci.

Keywords: *Plants; Dental caries, Diterpenes, S. mutans*

1. Introduction

Natural products have been one of the most successful sources for the isolation and discovery of novel therapeutic agents (Cragg *et al.*, 1997; Harvey, 2000). Among various plant extracts used in Brazilian folk-medicine, those from *Mikania* genus plant (a sub-scrub creeper of woody branches known popularly as "guaco") are of particular interest because of reported multiple pharmacological properties, such as anti-inflammatory and antimicrobial activities (Ruppelt *et al.*, 1991; Corrêa, 1942; Pereira *et al.*, 1994; Celeghini *et al.*, 2001; Yatsuda *et al.*, 2005). Furthermore, results from our previous studies have shown that hexane fractions of *M. laevigata* and *M. glomerata*, exhibit antimicrobial activity against planktonic cells of mutans streptococci (Yatsuda *et al.*, 2005). The major components in the hexane fractions were coumarin, ent-beyer-15-en-19-oic acid, diterpenic acid, ent-kaur-16-en-19-oic acid and decanoic acid. Our previous study determined the minimum inhibitory concentration (MIC) of compounds ent-beyer-15-en-19-oic acid (cupressenic acid) (5-10 μg/ml), ent-kaur-16-en-18-oic acid (diterpenic acid) (2.5-5 μg/ml) and ent-kaur-16-en-19-oic acid (kaurenoic acid) (5-10 μg/ml) against mutans streptococci. Recently, we have shown that the ent-beyer-15-en-19-oic acid (cupressenic acid), ent-kaur-16-en-18-oic acid (diterpenic acid) and ent-kaur-16-en-19-oic acid (kaurenoic acid) were the most effective compounds, inhibiting the synthesis of glucans and diminishing the acid production and acid tolerance of *Streptococcus mutans* UA159 biofilms (Yatsuda, unpublished work). The cupressenic, diterpenic and kaurenoic acids in this study also affected the glucosyltransferases B and F-ATPase activities, disturbing the biofilm accumulation *in vitro* of mutans streptococci at concentrations of 500 μg/ml or 1.65 mM(Yatsuda, unpublished work). This microorganism metabolizes sucrose by the GTF that catalyze the formation of soluble and insoluble a-linked glucans from sucrose. The insoluble glucan promotes the firm adherence of bacteria to the tooth surface. The presence of these glucans along with the microbial aggregation and accumulation of acids are critical in the development of dental caries and other oral diseases (Hamada *et al.*, 1984).

Considering the promising effects on the virulence of the compounds from *M. laevigata* and *M. glomerata*, the purpose of this study was to evaluate the actions of cupressenic, diterpenic and kaurenoic acid on caries development *in vivo*.

2. Materials and methods

Plant material. In this study, the compounds from hexane fraction of *M. laevigata (Ml)* and *M. glomerata* (*Mg*) were isolated and identified. The aerial parts of *Mikania laevigata* Schultz Bip. and *Mikania glomerata* Sprengel were collected from experimental field of the Center for Chemical, Biological and Agricultural Research - CPQBA/UNICAMP, Brazil. Voucher specimen of *M. laevigata* (*Ml*) and *M. glomerata* (*Mg*) is deposited at Herbarium of Biology Institute of UNICAMP under number UEC - 102.046 and UEC - 102047.

Extraction and isolation of active compounds. Dried and pulverized leaves from *M. laevigata* and *M. glomerata (*400 g) were extracted two times with 2 L of ethanol:water (70:30, v/v) at room temperature. The ethanol solution was concentrated by means of a rotary evaporator yielding 79.1 g and 58.2 g of crude ethanolic extracts of *M. laevigata* (EE-*Ml*) and *M. glomerata* (EE-*Mg*). Forty grams of the extracts were fractionated with hexane three times in dispersor Ultra-Turrax obtaining the hexane fractions of *M. laevigata* H-*Ml* (5.9g) and *M. glomerata* H-*Mg* (5.6 g). The fractions H-*Ml* and H-*Mg* were further fractionated by dried column chromatography (cellulose 2 X 30 cm) using Si gel 60 (Merck) and chloroform:ethyl acetate (70:30, v/v). The columns were cut in four parts and extracted using chloroform yielding the following fractions: H1-*Ml* (0.45 g), H2-*Ml* (1.80 g), H3-*Ml* (1.86 g), H4-*Ml* (1.50 g); and H1-*Mg* (0.70 g), H2-*Mg* (1.65 g), H3-*Mg* (1.78 g), and H4-*Mg* (1.35 g). The fractions were analyzed by thin layer chromatography and GC/MS; H2-*Ml* and H3-*Mg* displayed the highest content of diterpenic acids derivatives and antimicrobial activity (Rehder, unpublished work). Therefore, the fractions H2-*Ml* and H3-*Mg* were selected for further analysis.

The fraction H2-*Ml* (1.3g) was purified using a silica gel 60 column (0.063-0200 mm) impregnated with 10% $AgNO_3$ and eluted with hexane:ethyl acetate in polarity gradient (90:10,

85:15, 80:20 and 50:50; v/v). Seventy-two fractions were collected and monitored by TLC impregnated with 5% $AgNO_3$ (w/v, in methanol), and eluted with hexane:ethyl acetate (80:20, v/v). The fractions with similar chromatographic pattern (based on R_f) were combined yielding 4 distinct sub-fractions: F1H2-*Ml* (350mg), F2H2-*Ml* (430mg), F3H2-*Ml* (250 mg) and F4H2-*Ml* (195 mg).

The fraction H3-*Mg* (1.50g) was purified in flash column using Si gel 60 (230-400 mesh - Merck) and hexane:ethyl acetate (93:7, v/v) as eluent. Sixty fractions were collected and monitored by TLC. Again, the fractions with similar chromatographic pattern (based on R_f) were combined yielding six distinct sub-fractions: F1H3-*Mg* (230mg), F2H3-*Mg* (110mg), F3H3-*Mg* (350 mg), F4H3-*Mg* (250 mg), F5H3-*Mg* (64 mg) and F6H3-*Mg* (295 mg).

The F2H2-*Ml* (R_f = 0.43), F4H2-*Ml* (R_f = 0.21), F3H3-*Mg* (R_f = 0.42), and F5H3-*Mg* (R_f = 0.50) were the most active sub-fractions (Rehder, unpublished work). These compounds were crystallized in hexane and subjected to physical and spectroscopic analyses. The optical rotation was measured with a Perkin-Elmer polarimeter and melting points were determined on a Koffer boot place (Eletrothermal 9100 apparatus). The ^{1}H-, ^{13}C-NMR and MS spectra were obtained by INOVA 500 Varian and Hewlett Packard 5890/5970 series II system. The F2H2-*Ml* was identified as ent-kaur-16-en-19-oic acid (kaurenoic acid) and F4H2-*Ml* as ent-beyer-15-en-19-oic acid (cupressenic acid) whereas F3H3-*Mg* was identified as ent-kaur-16-en-19-oic acid (kaurenoic acid) and F5H3-*Mg* as ent-kaur-16-en-18-oic acid (diterpenic acid); the structure of the compounds were confirmed by comparing their physical and spectral data with those reported in the literature, i.e. for cupressenic acid (Takahashi *et al.*, 2001; Bohlmann *et al.*, 1976), diterpenic acid (Castro & Jakupovic, 1985) and kaurenoic acid. (Cruz & Roque, 1992; Ohno *et al.*, 1979).

Test agents. The concentrations of the isolated compounds used in this study were 100 times of their MIC values, being determinate the concentration of 1.65 mM (500 µg/ml) for all compounds. Vehicle control (20% ethanol, v/v) was used as a negative control. Chlorhexidine at 0.12% (1.33 mM) was used as a positive control (gold standard).

Bacterial strains. The bacterial strains used in this study was *Streptococcus mutans* UA159, a virulent cariogenic pathogen and the strain selected for genomic sequencing (Ajdic *et al.*, 2002).

Animal study. The animal experiment was performed as previously described by Bowen *et al.* (1988) and Koo *et al.* (2005) and was approved by the Ethical Committee on Animal Research at the State University of Campinas (UNICAMP), which is in accordance with the internationally accepted principles for laboratory animal use and care. To conduct the animal study, 50 Wistar female rat, specific pathogen and mutans-free, 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, MI, USA) and mitis salivarius agar plus bacitracin (Sigma, St. Louis, MO, USA), according to Bowen *et al.* (1988) When aged 21, 22 and 23 days, the rats were infected with S. mutans UA 159, which is a proven virulent cariogenic oral pathogen (Adjic *et al.*, (2002). They were fed pellet chow, diet 2000 (Keyes, 1958) and 5% sucrose in drinking water *ad libitum* until 25 days of age to establish the infection by *S. mutans*. Oral swabs from the animals were streaked on mitis salivarius agar plus bacitracin (MSB) (Sigma) to confirm the infection, and re-infected if necessary. At age 25 days, the rats were placed randomly into 5 groups of 10 animals, and their molars were treated topically using a camel hair brush twice a day Koo *et al.* (1999) from the 26^{th} day (first day of the experiment) to the 62^{nd} day (the end of the fifth week of the experiment) as follows: (1) the vehicle control ethanol 20%; or (2) cupressenic acid 1.65 mM; or (3) diterpenic acid 1.65 mM; or (4) kaurenoic acid 1.65 mM; or (5) chlorhexidine 1.33 mM. Diet 2000 and sterilized distilled water were provided *ad libitum*. The animals were weighed weekly, and their physical appearance was noted daily. At the end of the 5-week experimental period, the rats were killed by CO_2 asphyxiation. The left jaw was aseptically dissected and transferred to 5.0 ml of 0.89% sterile NaCl solution, and sonicated using a Vibra Cell (Sonics & Material Inc; six 10 s pulses with 5 s intervals at 40 W) Koo *et al.* (2005). The suspensions obtained were used for microbial assessment. Aliquots (50 μl) of the suspensions were plated on blood agar, to

determine total cultivable flora, and on MSB (Sigma), to estimate *S. mutans* populations, using a spiral plater (Whitley Automatic Spiral). Macroscopic and histological effects of the compounds were evaluated on liver, lung, tongue, oral mucosa, stomach, spleen, kidney, salivary glands, brain, pancreas, eyes. For the histological study, the tissues were fixed in paraffin and colored with hematoxylin and eosin. The jaws were de-fleshed and the teeth prepared for caries scoring. Caries on the smooth and sulcal surfaces and its severity (*D*s, dentin exposed; *D*m, 3/4 of the dentin affected) was evaluated by means of Larson (1981) modification of the Keyes system.

Statistical analyses

The animal study data were subjected to ANOVA, Tukey—Kramer HSD test for all pairs using JMP Version 3.1 software for statistical visualisation. Smooth surface and sulcal caries scores were expressed as proportions of their maximum possible values (124 and 56, respectively). The significance level was 5%.

3. Results

The rats remained in apparent good health during the 5-week experiment. Weight gains (Table 1) did not show statistical difference when compared to among the treatment groups and the control ($p>0.05$). The macroscopic and histological did not show any alteration on the tissues of the groups treated with the compounds and the control (data not shown).

The effects of chlorhexidine, cupressenic, kaurenoic and diterpenic acids after five-week experiment on oral microbiota in rats are showed on Table 2. For the total cultivable flora, the groups treated with the acids and chlorhexidine did not show significant difference. The percentage of *Streptococcus mutans* UA159 recovered from the rat oral cavity was calculated from the total cultivable flora and the *Streptococcus mutans* population. The group treated with the cupressenic acid did not show any significant effects on the level of infection by *Streptococcus mutans* compared to that of the control group ($p>0.05$), although lower counts for

Streptococcus mutans population were observed in the groups treated with the chlorhexidine, diterpenic and kaurenoic acids.

The effects of the treatments on the incidence of smooth-surface and sulcal caries score are shown in Table 3. The animals treated with cupressenic, diterpenic and kaurenoic acids showed significant cariostatic properties on smooth-surface caries. The cupressenic, diterpenic and kaurenoic acids were able to affect, under a high cariogenic challenge, the incidence of smooth surface caries, when compared to the control group ($p<0.05$), not differing statistically to the chlorhexidine group ($p>0.05$). For the total sulcal caries, diterpenic and kaurenoic acids showed statistically significant difference when compared to the control group ($p<0.05$), not differing statistically to chlorhexidine group that showed the lowest score ($p>0.05$). The group treated with the cupressenic acid did not show significant difference to the control group and to the others treatments.

In general, the animals treated with the compounds and the chlorexidine did not show any statistical difference in either severity of smooth-surface or sulcal caries when compared to the control ($p>0.05$) (data not shown). The groups treated with the acids cupressenic, diterpenic and kaurenoic did not show statistical difference when compared among them for the incidence of smooth-surface and sulcal caries score ($p>0.05$).

4. Discussion and conclusions

Dental caries development involves a series of events in the biofilm on the tooth surface, where bacterial interactions with diet occur. There is a general consensus that the frequent consumption of carbohydrates, mainly sucrose, can result in the emergence of cariogenic microorganisms, such as mutans streptococci (Fitzgerald and Keyes, 1960; Hamada *et al.*, 1984; Loesche, 1986). Glucans, synthesized from dietary sucrose by glucosyltransferases (GTFs), are essential for *Streptococcus mutans* and other oral microorganisms to adhere and to accumulate on the tooth surface, leading to the formation of cariogenic biofilm communities (Schilling and Bowen, 1992; Marsh and Bradshaw, 1995). Furthermore, the formation of acid

end-products through the metabolism of carbohydrates by acidogenic microorganisms within these biofilms is an important factor in the development of dental caries (Svensater *et* al., 2003). The essential process involves demineralization of the tooth structure by high concentrations of organic acids (van Houte, 1994). *Streptococcus mutans* has been implicated as the primary etiological agent because of its relatively high numbers in plaque prior to the appearance of carious lesions, its ability to degrade carbohydrates rapidly with the formation of abundant acid and its ability to induce a tolerance to low pH environments (Svensater *et* al., 2001). Therefore, mutans streptococci and/or their virulence factors should be prime targets for any therapeutic agent aimed at preventing dental biofilm related diseases, such as dental caries.

The *in vivo* experiment in this study showed that topical application twice a day of cupressenic, diterpenic and kaurenoic acids (500 μg/ml) showed cariostatic effect on smooth-surface and sulcal caries, and have also showed reduction of the percentage of *Streptococcus mutans* UA159 infection by diterpenic and kaurenoic acids.

The findings of this study confirm and extend previous observations on the anti-caries properties of cupressenic, diterpenic and kaurenoic acids isolated from the hexanic fractions of *M. laevigata* and *M. glomerata*. The compounds in previous studies (Yatsuda, unpublished work) showed antimicrobial activity against mutans streptococci either in planktonic cells or on biofilm model. The biofilms treated with these compounds (1.65 mM) showed reduced the dry-weight compared to the vehicle control. In addition, the study reveals that the content of polysaccharide, not only extracellular glucans (water-soluble and insoluble), but also intracellular iodophilic polysaccharide in the biofilms was significantly reduced by a half by cupressenic, diterpenic and kaurenoic acids (Yatsuda *et al.*, unpublished work). Furthermore, the compounds reduced the activity of GTF B in solution and on a surface of hydroxyapatite beads (Yatsuda *et al.*, unpublished work). Thus, by diminishing the synthesis of glucans, the compounds tested have had significant impact on the adherence of bacteria to the tooth surface, consequently on the further development and accumulation of the biofilms, affecting the progress of dental caries.

In our previous study (Yatsuda *et al.*, unpublished work), the compounds also increased the permeability of cells membrane to protons and reduced the F-ATPase activity of permeabilized cells of *S. mutans* UA159, affecting both acidogenesis and acid tolerance of these bacteria on the biofilm (Marquis *et al.*, 2004). Whether, this compounds can actually prevent enamel demineralization awaits further evaluation since the final pH (5.8-6.4) values are still higher than the critical pH for enamel dissolution (pH 5.0-5.5). These effects can occur as a result of the structure of these acids compounds that have the presence of the carboxylic acid in position 19 that seemed to be important in the antibacterial activity, because natural and synthetic derivatives with this group blocked or interacting with hydrogen bonds have less or no activity (Wilkens *et al.*, 2002).

In this study, we also demonstrated that the tissues of the rats treated with the compounds maintained normal, as showed on the macroscopic and histological assays; and also, the weight gains did not differ to the control group, showing that the animals were healthy and eating regular during the 5- week experiment.

Considering the reduction of smooth-surface and sulcal caries, the lack of effect on total microorganisms but reduction on *Streptococcus mutans* levels in the animals' plaque, the data in this study suggest that the cariostatic properties of cupressenic, diterpenic and kaurenoic acids are related to inhibition of virulence factors rather than to antimicrobial action. Consequently, cupressenic, diterpenic, and kaurenoic acids offer promising avenues to explore development of therapeutic agents for plaque-related diseases based on inhibition of some of the critical virulence factors associated with the pathogenesis of dental caries, including acid production, F-ATPase and GTF activities.

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Table 1 – Weight gain by rats treated daily with cupressenic, diterpenic and kaurenoic acids, after 5 week experiment: means (SD).

Treatment	Weight gain (g)
Ethanol 20%	101.6 (20.5)[a]
Chlorhexidine 0,12%	99.4 (10.4)[a]
Cupressenic Acid	106.3 (13.6)[a]
Diterpenic Acid	98.2 (7.9)[a]
Kaurenoic Acid	106.1 (35.3)[a]

There was no significant difference between any of the groups for weight gain (p=0.846) (n=10). ANOVA, comparison for all pairs using Tukey-Kramer HSD

Table 2 – Effects of cupressenic, kaurenoic and diterpenic acids after five-week experiment on oral microbiota in rats: means (SD).

Treatment	Total microorganisms (x10^5 CFU/mL)*	*S. mutans* UA159 (x10^5 CFU/mL)*	*S. mutans* UA159 (%)**
Ethanol 20%	17.1(14.6)[a]	1.6(1.2)[a]	11.1(3.9)[a]
Chlorhexidine 0,12%	8.2(1.0)[a]	0.3(0.1)[b]	3.7(1.5)[b]
Cupressenic Acid	9.8(5.4)[a]	1.1(0.9)[a,b]	11.3(8.6)[a]
Diterpenic Acid	16.1(7.1)[a]	1.0(0.6)[a,b]	8.0(5.7)[b]
Kaurenoic Acid	10.9(2.5)[a]	1.0(1.1)[a,b]	8.5(8.5)[b]

*Values followed by the same superscripts are not significantly different from each other (p>0.05) (n=10). *ANOVA, comparison for all pairs using Tukey-Kramer HSD.

**Nonparametric Wilcoxon/ Kruskal-Wallis tests.

Table 3 – Effects of cupressenic, diterpenic and kaurenoic acids (500 μg/ml) on total caries development (smooth surface and sulcal) in rats, after five-week experiment: means (SD) of Keyes' scores.

Treatment	Total Smooth-surface	Total Sulcal
Ethanol 20%	55.8 (16.3)[a]	46.7(5.7)[a]
Chlorhexidine 0,12%	32.9 (6.7)[b]	30.6(6.4)[b]
Cupressenic Acid	38.0 (5.9)[b]	40.8(9.5)[a,c]
Diterpenic Acid	24.2 (11.4)[b]	34.6(6.0)[b,c]
Kaurenoic Acid	38.0 (12.7)[b]	34.9(9.5)[b,c]

Values followed by the same superscripts are not significantly different from each other (p>0.05) (n=10). ANOVA, comparison for all pairs using Tukey-Kramer HSD.

3. CONCLUSÕES

Este estudo confirma que os ácidos cupressênico, diterpênico e caurenóico, isolados das frações hexânicas obtidas do extrato hidroalcoólico da *Mikania laevigata* e *Mikania glomerata*, possuem atividade antimicrobiana inibindo a formação do biofilme de *Streptococcus mutans* UA 159 e também reduzem a produção de polissacarídeos intracelulares e extracelulares insolúveis nesses biofilmes quando tratados 2 vezes ao dia pelos compostos na concentração de 500 µg/ml. Além disso, os compostos também afetaram os fatores de virulência dos *S. mutans*, como as enzima GTF B e F-ATPase. Desta forma, foram alterados a produção de polissacarídeos, a acidogenicidade e a aciduricidade desses *S. mutans*, reduzindo a sua viabilidade. Essas atividades biológicas dos compostos podem explicar a redução da incidência de cárie dental em superfície e sulcal de ratos demonstrada no experimento *in vivo*. Nesse experimento, houve redução da porcentagem de *S. mutans* sem alteração do microbiota total, demonstrando seletividade desses compostos na atividade antimicrobiana a *S. mutans*. Além disso, os compostos não provocaram alterações macroscópicas ou histológicas nos tecidos dos ratos, nem perda de peso ou atividade dos animais. Deste modo, ácidos cupressênico, diterpênico e caurenóico, isolados da fração hexânica da *Mikania laevigata* e *M. glomerata*, são promissores agentes anti-cárie.

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✓ Resultados dos cromatogramas obtidos por Cromatografia gasosa acoplada a espectro de massa.

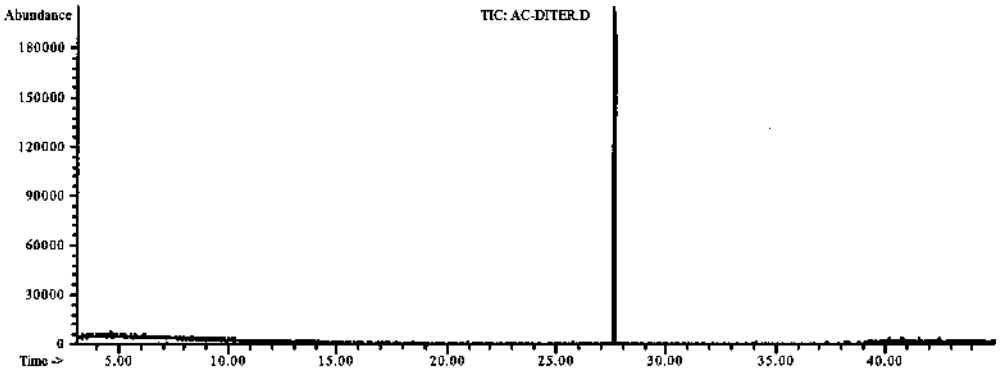


Figura 1: Ácido diterpênico metilado

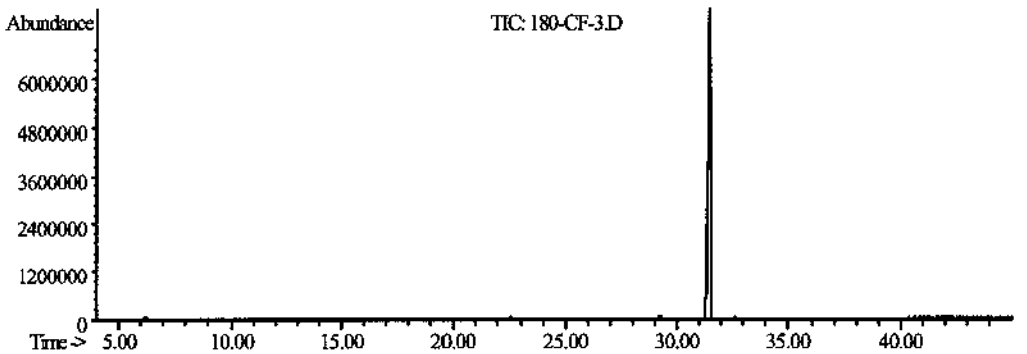


Figura 2: Ácido caurenóico metilado

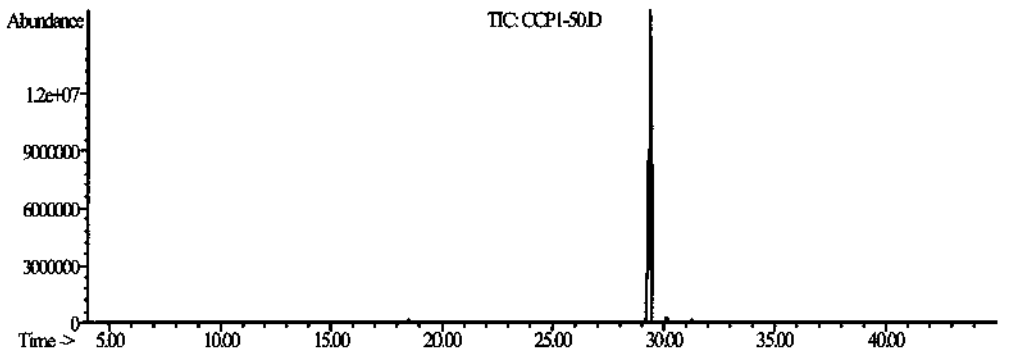
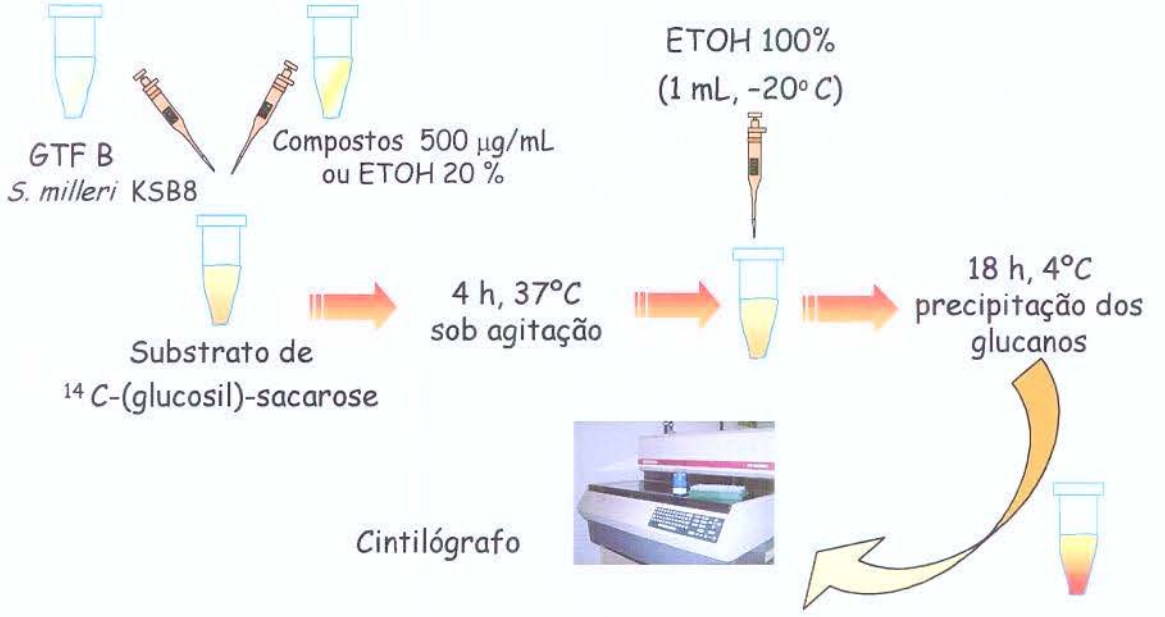


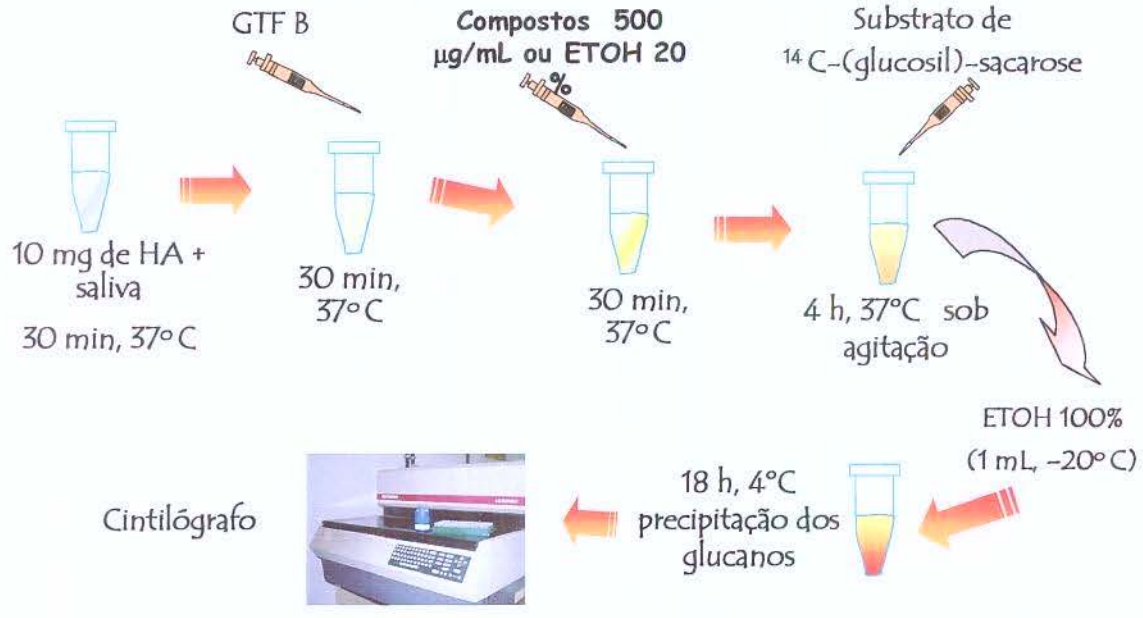
Figura 3: Ácido cupressênico metilado

✓ Atividade de GTF B em solução



(VENKITARAMAN, 1995; KOO *et al.*, 2000)

✓ Atividade de GTF B aderida à superfície de hidroxiapatita



(VENKITARAMAN, 1995; KOO *et al.*, 2000)

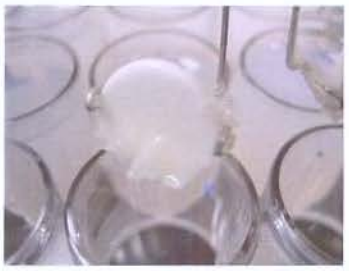
✓ Inibição da formação do biofilme

discos de HA

Saliva, 30 min, 37°C, sob agitação

LMW/ 1% de sacarose + inóculo de *S. mutans* UA159

Tratamentos 2 x dia, 1 min
Compostos 500 µg/mL ou ETOH 20 %



Biofilme de 5 dias

S. mutans UA159

NaCl 0,89%

UFC/mL

Polissacarídeos:

- ✓ Solúvel;
- ✓ Insolúvel;
- ✓ Intracelular.

(KOO et al., 2003; DUARTE *et al.*, 2006)

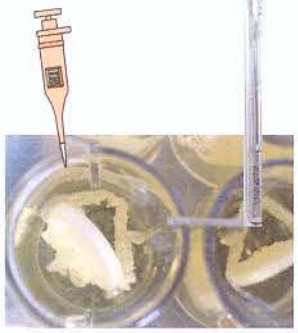
✓ Permeabilidade a prótons

Butanol (80 min)

Biofilme de 5 dias

S. mutans UA159

pH ajustado para 4,2



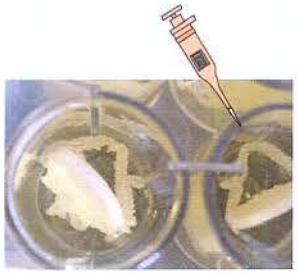
Leitura pH
10/10 min

✓ Queda de pH (via glicolítica)

Glucose 1%

Biofilme de 5 dias

S. mutans UA159



pH ajustado para 7,2

Leitura pH por
2 h

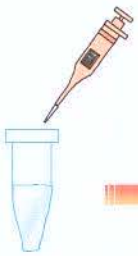
(BELLI *et al.*, 1995)

✓ Inibição da enzima F-ATPase

Compostos 500 µg/mL
ou ETOH 20 %

+

ATPase



S. mutans UA 159
permeabilizados

10 mMol $MgCL_2$

ATP —ATPase→ ADP + Pi

Leitura liberação
fosfato

(BENCINI *et al.*, 1983)

✓ Desenvolvimento de cárie em Modelo experimental com ratas


- Comitê de ética: # 609-1 UNICAMP
- 50 ratas Wistar spf: 19 dias de idade - CEMIB/ UNICAMP

- Verificação presença de estreptococos do grupo mutans (microbiota indígena) - MSA e MSB
- 21°, 22° e 23° dia: ratas infectadas c/ *S. mutans* UA 159
- Alimentação com dieta 2000 c/ 56% sacarose
- Água destilada, deionizada estéril com 5% sacarose
- 23° dia: verificação da infecção *S. mutans*
  - meio mitis salivarius c/ bacitracina - MSB
- Dividido em 5 grupos aleatoriamente:

etanol 20%
digluconato de clorexidina a 0,12%
ácido cupressênico
ácido diterpênico
ácido caurenóico

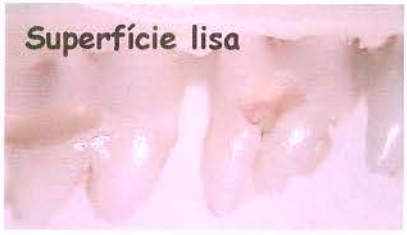
n = 10

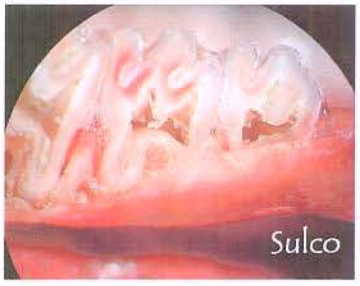
Concentração 500 μg/mL

2 x dia, 5 semanas

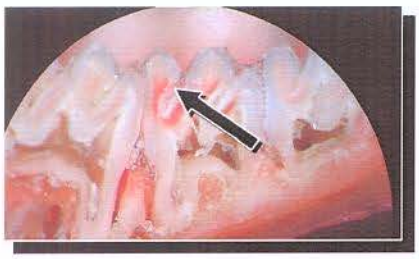
Maxilas e mandíbulas

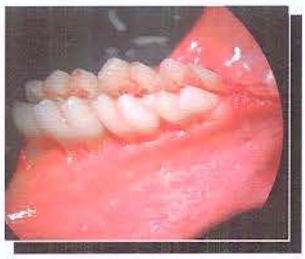
Incidência e severidade

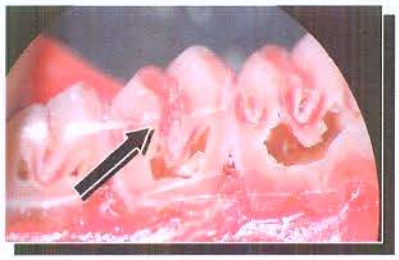




Índice de cárie (Keyes modificado por Larson, 1981)







Asfixia por CO_2

mandíbula dissecada

Decapitação

mandíbula removida

Sonicação em soro fisiológico

Inoculação em MSB e ágar sangue

48-72 h, 37°C, 10% CO_2

Apêndice 1

- Certificado do Comitê de Ética em Experimento Animal, IB-UNICAMP

Universidade Estadual de Campinas
Instituto de Biologia

Comissão de Ética na Experimentação Animal
CEEA-IB-UNICAMP

CERTIFICADO

Certificamos que o Protocolo nº 609-1, sobre "AVALIAÇÃO DAS ATIVIDADES ANTICÁRIE E ANTIPLACA DOS COMPOSTOS ISOLADOS E QUIMICAMENTE IDENTIFICADOS DA PLANTA MIKANIA GLOMERATA" sob a responsabilidade de Prof.Dr. Pedro Luiz Rosalen/Regiane Yatsuda está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em reunião de 03 de Outubro de 2003.

CERTIFICATE

We certify that the protocol nº 609-1, entitled "EVALUATION OF THE ANTI-CARIES AND ANTIPLAQUE ACTIVITIES OF THE ISOLATED AND CHEMICALLY IDENTIFIED COMPOUNDS OF THE PLANT MIKANIA GLOMERATA", is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the Institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on October 3, 2003.

Campinas, 03 de Outubro de 2003.

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

Fátima Alonso
Secretária - CEEA/IB/UNICAMP

UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE BIOLOGIA
CIDADE UNIVERSITÁRIA ZEFERINO VAZ
CEP -13.083-970 - CAMPINAS - SP - BRASIL

TELEFONE 55 19 3788 6309
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Apêndice 2

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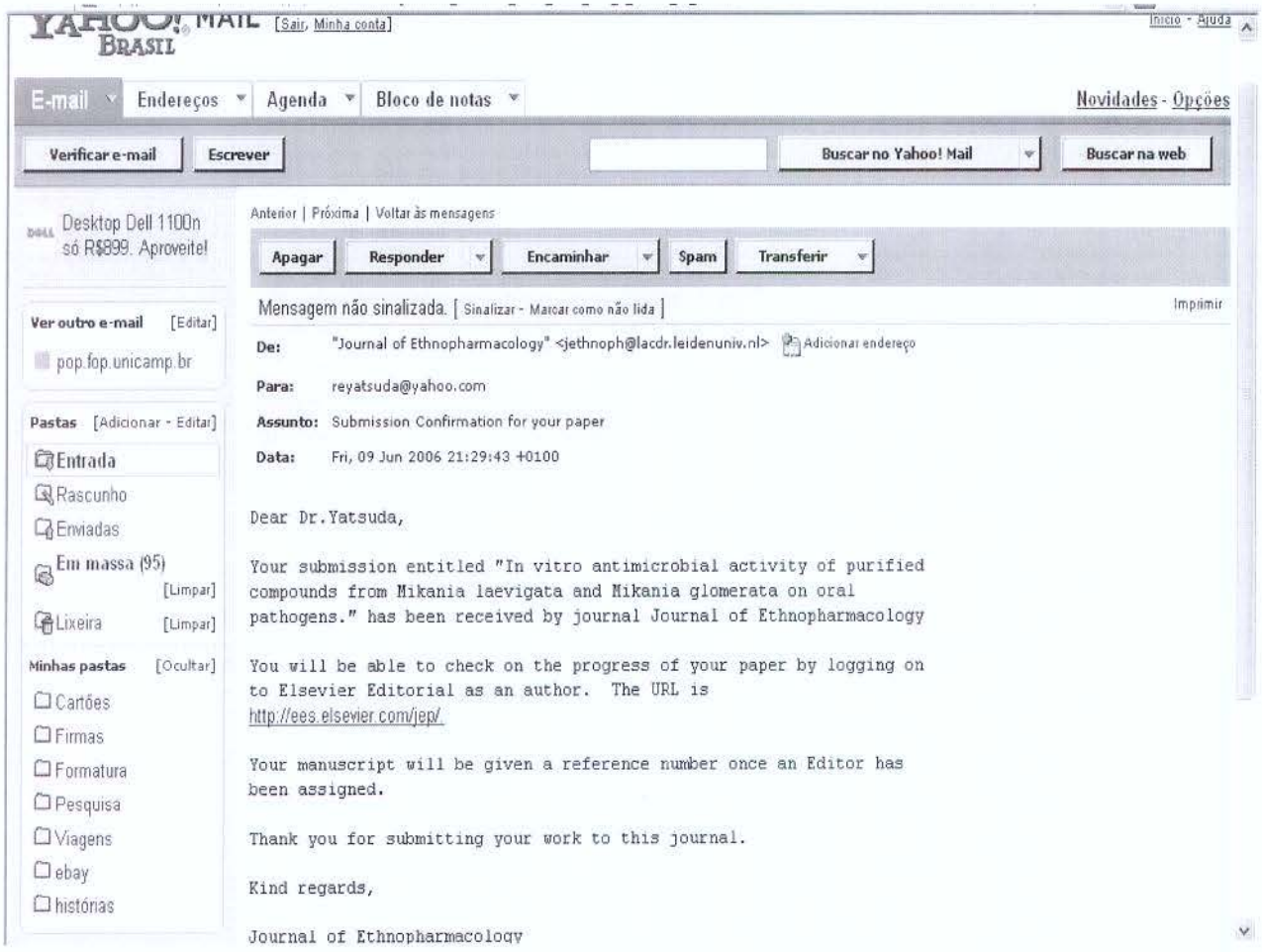

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