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Atividade antimicrobiana dos extratos da *Rheedia brasiliensis* e potencial anticárie do seu composto bioativo

Dissertação apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção do título de Mestre em Odontologia, área de concentração Farmacologia, Anestesiologia e Terapêutica.

Orientador: Prof. Dr. Pedro Luiz Rosalen

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"O mais importante da vida não é a situação em que estamos, mas a direção para a qual nos movemos."

(Oliver Wendell Holmes)

Resumo

Este estudo avaliou a atividade antimicrobiana de extratos da *Rheedia brasiliensis* contra *Streptococcus mutans* e o potencial anticárie do seu composto ativo isolado. Os extratos hexânicos (EH), acetato de etila e etanólico (concentrações entre 6,25-800 µg/ml) do fruto ("bacupari") foram testados contra *S. mutans* UA159 por meio de determinação das concentrações inibitória mínima (CIM) e bactericida mínima (CBM). Biofilmes de 5 dias de formação foram tratados com os extratos ativos (100 x CIM), por 0, 1, 2, 3 e 4 h, e plaqueados para contagem das UFC/ml (*time kill*). Os extratos ativos foram submetidos à análise química por métodos espectroscópicos, e o composto ativo isolado (0,625-80 µg/ml) foi testado contra células de *S. mutans* por meio de CIM/CBM. A influência do composto isolado (12,5-100 µg/ml) sobre a síntese de glucanos foi avaliada através da atividade da enzima glucosiltranferase (GTF) B em superfície de hidroxiapatita. A ação do composto sobre biofilmes (125 e 250 µg/ml) foi testada com os ensaios de queda de pH e inibição de formação de biofilme. O potencial anticárie do composto (250 µg/ml) foi avaliado em ratas *Wistar* sob alto desafio cariogênico. Os EH (casca e semente) inibiram *S. mutans* em baixas concentrações (CIM:12,5-25 µg/ml) e reduziram células viáveis dos biofilmes após 4 h de exposição. O composto ativo presente foi identificado como a 7-epiclusianona (7-epi), a qual inibiu o crescimento do *S. mutans* (CIM:1,25-2,5 µg/ml), a atividade da GTF B em superfície (48% de inibição) e a produção de ácidos do *S. mutans* em biofilme, porém não reduziu a formação e o acúmulo dos biofilmes. No estudo animal, a 7-epi reduziu a incidência e a severidade de cárie e a microbiota total, mas não diminuiu a porcentagem de *S. mutans.* A 7-epi diminuiu a virulência do *S. mutans* e inibiu o desenvolvimento de cárie *in vivo*, sugerindo ser um agente terapêutico promissor para o controle do biofilme dental cariogênico.

Palavras-chave: *Rheedia brasiliensis*, 7-epiclusianona, *Streptococccus mutans*, fatores de virulência, cárie animal.

Abstract

The present study evaluated the antimicrobial activity of *Rheedia brasiliensis* extracts against *Streptococcus mutans* and the anticaries properties of its isolated bioactive compound. Hexane (HE), ethyl-acetate and ethanolic extracts (concentrations ranging from 6.25 to 800 µg/ml) of *R. brasiliensis* fruits ("bacupari") were tested against *S. mutans* UA159 by determining the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). *S. mutans* 5-days-old biofilms were treated with active extracts (100 x MIC), for 0, 1, 2, 3 e 4 h (time-kill), and plated for colony counting (UFC/ml). Such extracts were submitted to exploratory chemical analyses to isolate and identify the bioactive compound using spectroscopic methods. The compound (0.625-80 µg/ml) was then tested against *S. mutans* cells using MIC/MBC assays. The influence of the bioactive compound (12.5-100 µg/ml) on glucans synthesis was evaluated by testing the activity of glucosyltranferase (GTF) B on hydroxyapatite surface. The effects of the compound (125 e 250 µg/ml) on biofilms were analyzed using glycolytic pH-drop and inhibition of formation assays. The anticaries activity of such compound (250 µg/ml) was evaluated in *Wistar* rats submitted to a high cariogenic challenge. HE (peel and seeds) reduced *S. mutans* cells at low concentrations (MIC:12.5-25 µg/ml) and also biofilm viability after four hours, confirming the presence of the bioactive compound. This compound was identified as 7-epiclusianone (7-epi), which inhibited the *S. mutans* growth (MIC:1.25-2.5 µg/ml), the activity of GTF B (48% of inhibition) and the acid production, not interfering with the formation and accumulation of biofilms. In the animal study, 7-epi additionally reduced the incidence and severity of caries and also the total microbiota, however no reduction on the percentage of *S. mutans* was observed. In conclusion, 7-epi may be considered a promising agent to control cariogenic dental biofilm.

***Keywords*:** *Rheedia brasiliensis*, 7-epiclusianone, *Streptococccus mutans*, virulence factors, animal caries.

Sumário

1 Introdução

Apesar dos avanços na redução de sua prevalência, a cárie dental continua sendo considerada uma das infecções bucais mais comuns, afetando diversas populações no mundo (Bowen, 2002). A cárie ocorre pela ação de bactérias acidogênicas e acidúricas (Hamada & Slade, 1980), especialmente estreptococos do grupo mutans, que interagem numa comunidade mista, formando um biofilme tridimensional na superfície dental (Marsh, 2003). Esses microrganismos organizam-se funcionalmente no interior de uma matriz extracelular e são capazes de modificar o ambiente para favorecer seu crescimento e sua sobrevivência (Davey & O'Toole, 2000).

O *Streptococcus mutan*s, bactéria do grupo mutans associada ao início da doença cárie (Loesche, 1986), possui importantes características fisiológicas reconhecidas como fatores de virulência. Essa bactéria é capaz de metabolizar diversos carboidratos presentes na dieta e de produzir ácidos orgânicos (acidogenicidade) que são liberados na matriz do biofilme. O *S. mutans* possui, também, a capacidade de sobreviver em meio ácido (aciduricidade), através dos mecanismos de translocação de prótons pela enzima F-ATPase (H^+-ATPase), que bombeia prótons para o meio extracelular, e pela provável inibição de enzimas glicolíticas intracelulares, sensíveis ao pH mais ácido, o que pode reduzir a capacidade de produção de ácidos por esses microrganismos (Marquis *et al*., 2004).

Além da acidogenicidade e da aciduricidade, a capacidade de sintetizar glucanos, na presença de sacarose, representa um outro importante fator de virulência associado ao *S. mutans*. Os glucanos são produzidos a partir da ação de três enzimas glucosiltransferases (GTFs): GTF B, responsável pela produção de glucanos insolúveis em água; GTF C, responsável pela produção de glucanos solúveis e insolúveis; e GTF D, responsável, exclusivamente, pela produção de glucanos solúveis (Loesche, 1986). Por estar relacionada à síntese de glucanos insolúveis extracelulares, a GTF B tem sido considerada a enzima de maior importância na patogênese da cárie dental (Yamashita *et al*., 1993). A síntese de glucanos possibilita a aderência do *S. mutans* à superfície dos dentes (Burne *et al.*, 1999; Wunder & Bowen, 1999), permitindo, assim, que a bactéria reduza o pH do biofilme dental a valores inferiores a 5 (Belli & Marquis, 1991), levando à desmineralização dos tecidos dentais e iniciando o processo da cárie (Bowden, 1990).

Além das reconhecidas medidas de prevenção da cárie dental, como os procedimentos mecânicos de remoção do biofilme cariogênico, a adequação da dieta e o uso do flúor (Tinanoff *et al*., 2002), tem-se investigado novas estratégias, buscando-se evitar os prejuízos trazidos pela doença, incluindo dor, comprometimento estético, alto custo do tratamento odontológico e/ou perdas dentais. Dentre as estratégias, tem-se destacado a redução de patógenos específicos relacionados à cárie por meio de agentes antimicrobianos capazes de inibir o crescimento de estreptococos do grupo mutans ou de agir sobre fatores de virulência dessas bactérias (Koo *et al*., 2002; Baehni & Takeuchi, 2003).

A busca por novos agentes com ação sobre patógenos orais tem estimulado a pesquisa com produtos de origem natural, incluindo as plantas medicinais. Uma grande porcentagem das novas moléculas descobertas, com vistas à sua introdução na indústria farmacêutica, provém de constituintes de plantas e/ou derivados semi-sintéticos (Newman & Cragg, 2007). Considerando-se que novos produtos de origem natural com atividade sobre patógenos orais podem ser de grande importância para prevenção e controle de doenças bucais infecciosas, incluindo a cárie dental, estudos recentes apresentaram efeitos de compostos bioativos sobre fatores de virulência de estreptococos do grupo mutans, apresentando potencial antimicrobiano e anticárie (Koo *et al*., 2000; Duarte *et al*., 2003; Duarte *et al*., 2006). Assim, extratos ou substâncias isoladas de plantas medicinais ou de outros produtos encontrados na natureza podem ser pesquisados como alternativas terapêuticas para o controle do biofilme dental cariogênico (Tichy & Novak, 1998).

O Brasil apresenta uma enorme diversidade de plantas medicinais, cujos extratos vegetais têm sido empiricamente utilizados na medicina popular para o tratamento de diversas doenças (Braz-Filho, 1999). Tais aplicações são possíveis graças à ação de substâncias ativas presentes nos extratos utilizados (Newman & Cragg, 2007). Dentro da diversidade química de produtos naturais brasileiros, a família Guttiferae destaca-se pela enorme variedade de gêneros (cerca de 47), tais como *Rheedia*, *Vismia*, *Clusia*, *Cratoxylum*, *Harungana*, *Mesua*, *Hypericum* e *Kielmeyera*, responsáveis pela existência de mais de 1.000 espécies na família (Piccinelli *et al*., 2005).

O gênero *Rheedia* (sinônimo: *Garcinia*) é considerado o de maior número de

espécies da família Guttiferae (Morton, 1987). Análises químicas de partes de plantas desse gênero apresentaram uma composição química variada, com predomínio de derivados fenólicos prenilados e oxigenados (Piccinelli *et al*., 2005), incluindo benzofenonas polipreniladas (Dos Santos *et al*., 1999; Rubio *et al*., 2001). Benzofenonas polipreniladas isoladas da família Guttiferae têm demonstrado potencial antimicrobiano sobre cocos gram-positivos, gram-negativos e fungos (Bakana *et al*., 1987; Rubio *et al*., 1999), *Actinomyces* sp. (Rubio *et al*., 1999), *Staphylococcus aureus* (Hussain *et al*., 1982; Iinuma *et al*., 1996; Santos *et al*., 1996), *Trypanossoma cruzi* (Alves *et al*., 1999), e *Helicobacter pylori* (Chatterjee *et al*., 2003), além de outras propriedades farmacológicas, tais como ações anti-câncer (Ito *et al*., 2003), anti-HIV (Gustafson *et al*., 1992), anti-ulcerativa (Yamaguchi *et al*., 2000) e vasculares (Cruz *et al*., 2007). Entretanto, ainda não há estudos na literatura avaliando a ação antimicrobiana de benzofenonas polipreniladas sobre bactérias causadoras de infecções bucais, incluindo a cárie dental.

Algumas pesquisas envolvendo o gênero *Rheedia* investigaram propriedades biológicas de suas espécies (Weng *et al*., 2004; Dharmaratne *et al*., 2005; Cruz *et al*., 2007); no entanto, existem poucos estudos relacionados à atividade antimicrobiana de extratos ou compostos extraídos desse grupo de plantas. Dos Santos (1996), baseando-se nos estudos prévios de Delle Monache *et al*. (1983), analisou quimicamente o extrato hexânico de uma planta do gênero *Rheedia* e isolou alguns constituintes químicos de partes do seu fruto: o extrato hexânico do pericarpo (casca) apresentou um novo composto poliprenilado, denominado 7-epiclusianona, além de uma fração contendo os sesquiterpenos α-copaeno, α-muuroleno, γ-cadineno e cadineno (Dos Santos *et al*., 1999). A 7-epiclusianona teve seus efeitos biológicos investigados em estudos *in vitro*, demonstrando potencial contra agentes patogênicos, como fitobactérias, *S. aureus* e *T. cruzi* (Santos *et al*., 1996; Alves *et al*., 1999). Estudos *in vivo* (modelo animal) investigaram outras ações da 7-epiclusianona, incluindo potencial antitumoral (Alves *et al*., 1999) e ações vasculares (Cruz *et al*., 2007).

Estudos preliminares recentes realizados pelo nosso grupo de pesquisa avaliaram a atividade antimicrobiana dos extratos da *Rheedia brasiliensis* (sinônimo: *Garcinia brasiliensis*) (Almeida *et al*., 2005; Murata *et al*., 2006), planta nativa da região

amazônica brasileira. Considerada a espécie mais conhecida do gênero *Rheedia* (Morton, 1987) e também chamada de "bacupari", essa planta tem sido utilizada na medicina popular como agente cicatrizante e no tratamento de úlceras pépticas e tumores (Corrêa, 1978).

Extratos hexânicos provenientes do fruto da *R. brasiliensis* demonstraram atividade antimicrobiana contra *S. mutans*, tendo sido capazes de atuar em baixas concentrações (CIM[1]: 12,5-25 µg/ml) (Almeida *et al*., 2005; Murata *et al*., 2006). Os resultados indicaram uma característica apolar para o composto ativo, sugerindo a presença de compostos prenilados, como os encontrados em outra espécie semelhante já analisada quimicamente (Dos Santos *et al*., 1999), incluindo a benzofenona poliprenilada 7-epiclusianona. No entanto, apesar da ação antimicrobiana promissora, os extratos ativos da *R. brasiliensis* ainda não haviam sido quimicamente analisados para identificação de seu composto ativo contra *S. mutans* até a realização do presente estudo

Sendo assim, os objetivos desse estudo foram: 1) analisar a atividade dos extratos do fruto da *Rheedia brasiliensis* contra *Streptococcus mutans*; 2) isolar e identificar o composto bioativo contra *S. mutans*; 3) avaliar a influência do composto bioativo sobre fatores de virulência do *S. mutans*; 4) determinar a capacidade do composto bioativo em prevenir ou reduzir a incidência de cárie dental *in vivo* (modelo animal).

[1] CIM – Concentração Inibitória Mínima.

2 Capítulos

Esta dissertação está baseada na Informação CCPG/002/06/UNICAMP, que regulamenta o formato alternativo para a dissertação de Mestrado e permite a inserção de artigos científicos de autoria ou co-autoria do candidato (Anexo 1).

Desta forma, esta tese é composta de 2 estudos em formato de artigos, sendo 1 já aceito para publicação e outro ainda em fase de submissão, cujos títulos estão descritos a seguir:

2.1 Capítulo 1: "Antimicrobial activity of *Rheedia brasiliensis* and 7-epiclusianone against *Streptococcus mutans*", o qual foi aceito para publicação na revista *Phytomedicine* (Anexo 4).

2.2 Capítulo 2: "Effects of 7-epiclusianone on *Streptococcus mutans* and its anticaries activity in rats", o qual se apresenta em fase de submissão.

2.1. Capítulo 1:

Antimicrobial activity of *Rheedia brasiliensis* and 7-epiclusianone against *Streptococcus mutans*

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Abstract

This *in vitro* study evaluated the antimicrobial activity of extracts obtained from *Rheedia brasiliensis* fruit ("bacupari") and its bioactive compound against *Streptococcus mutans.* Hexane, ethyl-acetate and ethanolic extracts obtained (concentrations ranging from 6.25 to 800 µg/ml) were tested against *S. mutans* UA159 using MIC/MBC assays. *S. mutans* 5-days-old biofilms were treated with the active extracts (100 x MIC) for 0, 1, 2, 3 and 4 hours (time-kill) and plated for colony counting (CFU/ml). Active extracts were submitted to exploratory chemical analyses so as to isolate and identify the bioactive compound using spectroscopic methods. The bioactive compound (concentrations ranging from 0.625 to 80 µg/ml) was then tested using MIC/MBC assays. Peel and seed hexane extracts showed antimicrobial activity against planktonic cells at low concentrations and were thus selected for the time kill test. These hexane extracts reduced *S. mutans* biofilm viability after four hours, certifying of the bioactive compound presence. The bioactive compound identified was the polyprenylated benzophenone 7-epiclusianone, which showed a good antimicrobial activity at low concentrations (MIC: 1.25-2.5 µg/ml; MBC: 10-20 µg/ml). The results indicated that 7-epiclusianone may be used as a new agent to control *S. mutans* biofilms; however, more studies are needed to further investigate the mechanisms of action and the anticariogenic potential of such compound found in *R. brasiliensis*.

Keywords**:** *Rheedia brasiliensis*; Antimicrobial activity; 7-epiclusianone; *Streptococcus mutans*.

Introduction

Despite the advances concerning its prevention and control, dental caries is still considered a public health problem that affects many countries in the world (Bowen, 2002). Dental caries occurs by the action of acidogenic and aciduric bacteria (Hamada and Slade, 1980), which interact with other microorganisms in biofilms on dental surfaces (Marsh, 2003). Because of their role in the process of dental caries disease, some mutans streptococci have been extensively studied regarding their actions in dental biofilm and their virulence factors, including their acid tolerance and synthesis of insoluble glucans (Duarte et al., 2003; Lemos et al., 2005). The *Streptococcus mutans* is an important pathogen related to the initiation of dental caries in animals and humans (Loesche, 1986). It occurs specially because of its capability of sucrose-dependent adhesion and acid production, which leads to the consequent enamel demineralization (Belli and Marquis, 1991; Paes Leme et al., 2006).

The reduction in bacterial biofilm or specific pathogens associated with caries lesion using therapeutic agents with action against *S. mutans* and its virulence factors is a very common, effective approach for the prevention and control of caries (Baehni and Takeuchi, 2003).

Many studies on caries-related microorganisms have shown that some natural products can interfere with survival and virulence factors of *S. mutans*. A remarkable anticariogenic potency was also observed for natural bioactive compounds tested *in vitro* and *in vivo* (Koo et al., 2002a, 2003; Yatsuda et al., 2005; Duarte et al., 2006). Thus, medicinal plants are a potential source of biomolecules that must be investigated as an adjunctive therapy to control cariogenic dental biofilms.

The great biodiversitiy of plants found in Brazil might serve as an important source of new pharmacological agents (Basso et al., 2005). The Guttiferae represents a large family of medicinal plants with approximately 1,350 species, many of which are known for their fine-flavored fruits (Campbell, 1996). In addition, some genus, such as *Rheedia*, have many types of substances with various pharmacological properties (Bakana et al., 1987; Delle Monache et al., 1991; Gustafson et al., 1992; Dos Santos, 1996; Yamaguchi et al., 2000; Ito et al., 2003).

Previous studies have reported the presence of flavonoids, xanthones and polyprenylated benzophenones in some species of *Rheedia*, by analyzing their chemical constituents (Delle Monache et al., 1983; Dos Santos et al., 1999). One class of these compounds, the polyprenylated benzophenones, showed good antibacterial activity against important pathogens (Dos Santos, 1996; Alves et al., 1999), suggesting that *Rheedia* is a promising chemical medicinal genus.

Rheedia brasiliensis Planch. & Triana (Syn. *Garcinia brasiliensis* Mart.), considered a very known species of genus *Rheedia*, is a plant native to the Amazon region. Its fruit is popularly known as "bacupari" or "bacoparé" and is widely used in folk medicine and also by natives of that region to prepare candies and medicines (Corrêa, 1978; Morton, 1987).

Since no studies on *R. brasiliensis* activity against oral pathogens were found in the literature, the present study analyzed the activity of *R. brasiliensis* fruit extracts against *Streptococcus mutans* and also the isolation and identification of the bioactive compound obtained from the active fraction.

Materials and methods

Plant material

Extracts and 7-epiclusianone

R. brasiliensis fruits were collected from trees grown under controlled conditions at the herbarium of the University of Viçosa (latitude 20º45'14" south and longitude 42º52'55" west), Minas Gerais, Brazil, where its voucher specimen is deposited (number VIC2604). To obtain the extracts, the peel and the seeds were dried and powdered, and then treated with *n*-hexane at room temperature to allow chemical fractioning based on the polarity gradient of such solvents as hexane, ethyl-acetate and ethanol (peel) and hexane (seed), using the Soxhlet equipment for 24 hours (Dos Santos et al., 1999). Each extract concentration was obtained under reduced pressure to allow peel extracts from hexane, ethyl-acetate and ethanol and seed extract from hexane, which were finally chromatographed as described by Dos Santos et al. (1999). To obtain the bioactive compound, peel hexane extract (PHE) and seed hexane extract (SHE) were

chromatographed on a silica gel column and eluted with crescent polarity mixtures of *n*-hexane/ethyl-acetate and ethyl-acetate/ethanol. Its structure was identified as the polyprenylated benzophenone 7-epiclusianone [m.p. 92-93 ^{o}C (MeOH); $[\alpha]^{25}_{D}$ +77 (*c* 0.1, $CHCl_3$)] (Fig. 1) using several spectroscopic techniques (IR, UV, MS and NMR). The data were compared with those verified in a previous study investigating the chemical structure of this compound (Dos Santos et al., 1998, 1999). The purity (99.85%) of the bioactive compound and the retention time (17.961 minutes in peel hexane extract and 17.959 minutes in seed hexane extract) were determined by HPLC (Shimadzu LC-10A), using a C18 column (150mm-4.6mm) with a 5-µm particle size (Fig. 2). The suitable gradient was achieved using MeOH:acetic acid 5%, pH 3.84, (40:60 v/v) to MeOH 100% for 10 min, with a solvent flow rate of 1.2 mL/min, at 30 °C, λ=254 nm. ClassVP-LC10 software was used for data collection.

For the antimicrobial assays, the extracts and 7-epiclusianone were dissolved in 80% ethanol just prior to the microbiological tests, which were also used to monitor the biological activity of the extracts during the extraction process.

Bacterial strain

The bacterial strain used in this experiment was *Streptococcus mutans* UA159, a proven cariogenic dental pathogen (Ajdic et al., 2002). The cultures were stored at –80 ºC in Brain Heart Infusion (BHI) containing 20% glycerol (v/v).

Antimicrobial activity assays

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The antimicrobial activity of *R. brasiliensis* extracts was first evaluated by determining MIC and MBC, according to the methodology described by Piddock (1990) and Phillips (1991). Concentrations of the extracts tested ranged from 6.25 to 800 µg/ml. 7-epiclusianone was tested in concentrations ranging from 0.625 to 80 µg/ml. MIC was

defined as the lowest concentration of the agents tested showing restricted growth at a lower level than 0.05 at 660 nm (no visible growth).

To determine MBC, an aliquot (50 µl) of each incubated tube containing suspension with extracts and compound at concentrations higher than the MIC was subcultured on BHI agar supplemented with 5% defibrinated sheep blood using a Spiral Plater (Whitley Automatic Spiral Plater). MBC was defined as the lowest concentration that allowed no visible growth on the agar (99.9% killed) (Koo et al., 2000). Three replicates were made for each agent tested and for all assays. Chlorhexidine digluconate 0.12% (v/v) (Sigma®) was used as positive control for determining MIC and MBC.

Bacterial viability in biofilm

Bacterial viability test was carried out before the isolation and identification of the bioactive compound. The peel hexane and seed hexane extracts showed antimicrobial activity against planktonic cells. Thus, the bacterial viability analysis of *S. mutans* biofilms, considering time-kill assay, yielded complementary data for MIC and MBC.

Biofilms were formed on standard glass microscope slides in batch cultures for 5 days (Koo et al., 2002a). The 5-days-old biofilms were exposed to the extracts tested (final concentrations of 100 x MIC) for 0, 1, 2, 3 and 4 hours. Every one hour starting at baseline, the biofilms were removed, suspended in salt solution (50 mM KCl and 1 mM $MgCl_2$, pH 7.0) and subjected to sonication twice, each consisting of three 10-s pulses, at 5-s intervals, at 50 W. Then, the homogenized suspension was serially diluted (10^1, 10^2, 10^3 and 10^4) and plated on Tryptic Soy Agar (TSA) using a Spiral Plater (Whitley Automatic Spiral Plater). The plates were incubated in 5% CO_2, at 37 ºC, for 48 h, and then colony forming units per ml (CFU/ml) were quantified.

Killing curves were constructed by plotting values in the ordinate label: N_0 stands for the original number of CFU/ml; N stands for the number after the times of exposure. The assays were made in duplicate on at least three different occasions. A bactericidal effect was defined as a decrease in the CFU/ml (log $N/N_0 > 3$) from initial viable counts at baseline (Koo et al., 2002b). Chlorhexidine digluconate 0.12% (v/v) (Sigma®) was used as positive control for the time-kill assay.

Results

Effect of agents on planktonic cells

The effect of the *R. brasiliensis* fruit extracts on *S. mutans* planktonic cells is shown in Table 1. Among the extracts tested, the peel hexane and seed hexane extracts presented a potential antibacterial activity against *S. mutans* at low concentrations (seed hexane extract – MIC: 12.5-25 µg/ml/ MBC: 50-100 µg/ml; peel hexane extract – MIC: 12.5-25 µg/ml/ MBC: 25-50 µg/ml). The ethanol and ethyl-acetate peel extracts displayed no inhibitory activity against the microorganism tested.

The isolated bioactive compound 7-epiclusianone presented lower MIC/MBC values (1.25-2.5 µg/ml and 10-20 µg/ml, respectively) than those found for the active extracts (Table 1). Chlorhexidine showed MIC/MBC values of 1-2 µg/ml and 8 µg/ml, respectively.

Effect of extracts on biofilm viability

Among the extracts tested for antimicrobial activity, only the bioactive extracts, such as peel hexane and seed hexane extracts, were tested in a biofilm model using the time-kill analysis (Fig. 3). These extracts, at a concentration of 100 x MIC (2500 µg/ml or 2.5 mg/ml), reduced viable counts of *S. mutans* UA159. However, only the seed hexane extract was able to decrease the log $N/N_0 > 3$ units after 4 h exposure. Chlorhexidine 0.12%, showed total bactericidal effect after 2 h exposure (Fig. 3).

Discussion

It is well known that most of the new drugs discovered in the last few decades have originated from the nature (Newman and Cragg, 2007). Chemical constituents obtained from medicinal plants and other natural products have been increasingly used to treat many infectious diseases. Since dental caries is considered one of the most common infectious disease of mankind (Smith, 2002), many studies have been aimed at identifying compounds from natural products against mutans streptococci, specially *Streptococcus*

mutans (Tichy and Novak, 1998; Badria and Zidan, 2004; Yatsuda et al., 2005; Duarte et al., 2006).

The present study showed that hexane extracts obtained from peel and seeds of *Rheedia brasiliensis* fruit, a member from Guttiferae family, showed a potential activity against *S. mutans* at low concentrations, showing the same MIC values. According to Rios et al. (1988), natural extracts that exhibit activity at concentrations lower than 100 µg/ml could have great antimicrobial potential, since the active compounds can be isolated and used at lower concentrations.

Of all the species identified in Brazil, the Guttiferae family is outstanding for its great number of pharmacological properties and bioactive compounds from its species. Some polyprenylated benzophenone derivatives obtained from Guttiferae have been reported as having potential antimicrobial activity against gram-positive and gram-negative cocci, mycobacteria and fungi (Bakana et al., 1987), *Staphylococcus aureus* (Iinuma et al., 1996; Dos Santos et al., 1996), *Trypanossoma cruzi* (Alves et al., 1999) and *Helicobacter pylori* (Chatterjee et al., 2003). However, Guttiferae antimicrobial activity against oral microorganisms had never been investigated.

Our study showed that, in addition to their ability to inhibit planktonic cells, the hexane extracts tested reduced the colony forming units of *S. mutans* biofilms, with the seed hexane showing bactericidal activity. Microorganisms in biofilms are known to be more resistant to antimicrobial agents than are cells in suspension (planktonic state), and are more complex and similar to the biofilms in the conditions of oral cavity (Lewis, 2001; Bowen, 2002; Marsh, 2003), showing that these results might be of great importance for anticaries studies. However, none of the extracts was as effective in reducing colony forming units as chlorhexidine 0.12%, a clinically proven antimicrobial agent (Marsh, 1992).

Since the other extracts tested displayed no antibacterial activity and the hexane extracts could reduce the viability of *S. mutans* biofilms in time-kill assay, the bioactive compound present in both peel and seed hexane extracts was isolated and identified in our study. Delle Monache et al. (1983) were the first to investigate the genus *Rheedia* (Guttiferae) and its chemical composition. Dos Santos et al. (1999) have isolated the new

polyprenylated benzophenone, denominated 7-epiclusianone. This compound has been reported as having good antimicrobial activity against *Staphylococcus aureus* and phytobacteria (Dos Santos, 1996).

The present study identified 7-epiclusianone as the bioactive compound, with MIC values approximately 10 times lower than those observed for the active hexane extracts, having more complexes in their composition (7-epiclusianone present in a diluted form) and similar to those observed for chlorhexidine. 7-epiclusianone is not a synthetic substance, with its origin exclusively from the nature. This new biological property of 7-epiclusianone is of great relevance in dentistry due to its antibacterial activity against *S. mutans*.

In conclusion, our results showed that the polyprenylated benzophenone 7-epiclusianone, obtained from the *R. brasiliensis* fruit, could be used as an agent to control cariogenic microorganisms and prevent dental caries. However, further studies are needed to investigate the underlying mechanisms of action and anticariogenic potential of 7-epiclusianone.

Acknowledgements

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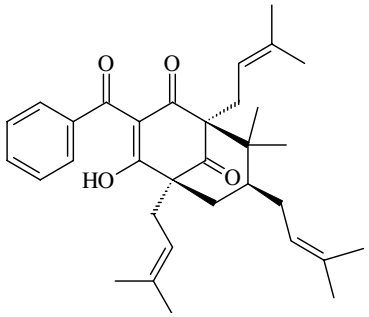

Fig. 1. Chemical structure of 7-epiclusianone, a polyprenylated benzophenone.

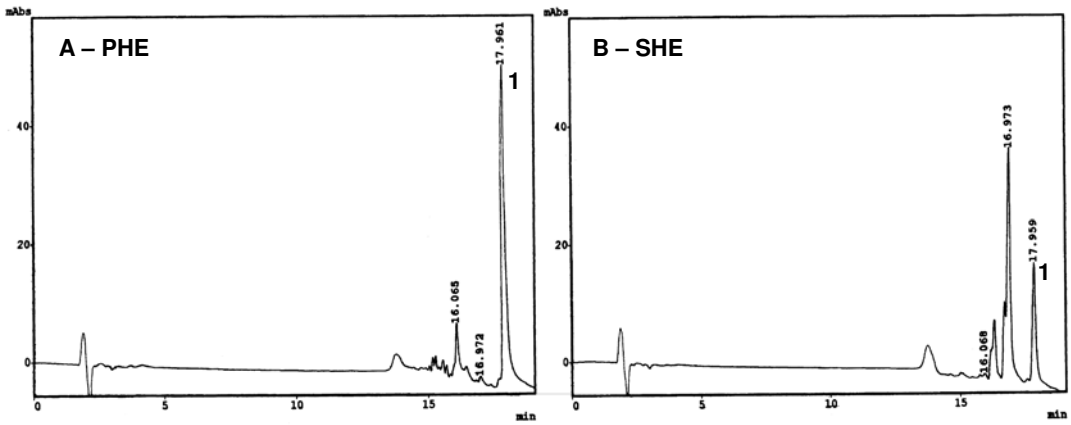

Fig. 2. HPLC chromatograms of hexane extracts from *Rheedia brasiliensis* fruit. A – PHE: peel hexane extract; B – SHE: seed hexane extract; 1 – 7-epiclusianone (retention time: 17.961 min in PHE and 17.959 min in SHE).

Table 1. MIC and MBC results from *Rheedia brasiliensis* fruit extracts and 7-epiclusianone.

Microorganism	EXTRACTS (µg/ml)								COMPOUND (µg/ml)	
	Seed hexane		Peel hexane		Peel ethanol		Peel ethyl-acetate		7-epiclusianone	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
S. mutans UA159	12.5-25	50-100	12.5-25	25-50	>800	>800	>800	>800	1.25-2.5	10-20

MIC and MBC values for chlorhexidine (positive control) were 1-2 µg/ml and 8 µg/ml, respectively.

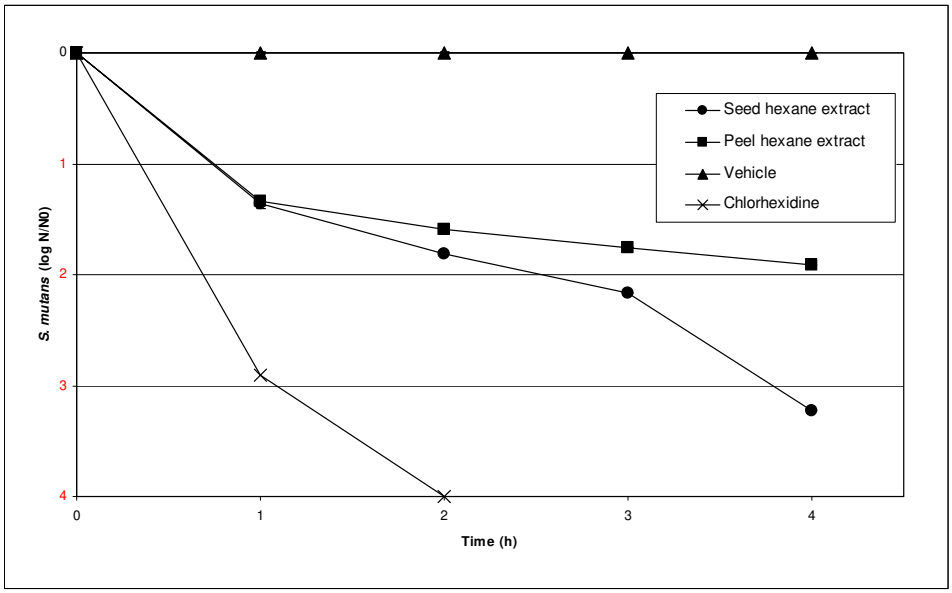

Fig. 3. Results from time-kill study with the hexane extracts and negative (vehicle) and positive (chlorhexidine) controls.

2.2 Capítulo 2:

Effects of 7-epiclusianone on *Streptococcus mutans* and its anticaries activity in rats

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Abstract

The aim of this study was to evaluate the effects of 7-epiclusianone on virulence factors of *Streptococcus mutans* and its anticaries activity. 7-epiclusianone (7-epi) was obtained from peel hexane extract (PHE) of *Rheedia brasiliensis* fruits and dissolved in a phosphate buffer containing 15% ethanol (vehicle). The influence of 7-epi (at 12.5, 25, 50 and 100 µg/ml) on glucosyltranferase (GTF) B activity was evaluated using the surface-adsorbed enzyme. The effects of 7-epi (at 125 and 250 µg/ml) on *S. mutans* UA159 biofilms were analyzed using glycolytic pH-drop and inhibition of formation assays. PHE (at 500 µg/ml) and 15% ethanol were used as active and vehicle controls, respectively. The animal study used *Wistar* rats, under cariogenic challenge, treated twice daily with 7-epi (250 µg/ml) as well as the positive (250 ppm F) and vehicle controls. 7-epi (100 µg/ml) reduced the activity of GTF B (48% of inhibition). The acids produced by *S. mutans* biofilms were reduced by 7-epi, and the difference was statistically different from vehicle control considering all times evaluated at the higher concentration tested ($p<0.05$). The compound showed no significant effect on formation and accumulation of biofilms when compared to vehicle control ($p>0.05$). In the animal study, 7-epi reduced the incidence and severity of smooth-surface and sulcal caries and the total microbiota ($p<0.05$), but not the *S. mutans*. The caries reduction probably occurred because of the action of 7-epi against virulence factors of *S. mutans*. In conclusion, 7-epi may be used as a new phamarcological agent to prevent and control dental caries; however; further studies are needed to invetigate its toxicological effects.

Keywords: 7-epiclusianone, *Streptococcus mutans*, glucosyltransferase B, biofilm, dental caries, animal study.

Introduction

Dental caries is a common infectious disease associated with microorganisms, such as mutans streptococci, capable of forming a complex biofilm on tooth surfaces. This biofilm formation occurs as a three-dimensional consortium of microorganisms functionally organized within an extracellular matrix (Marsh, 2003). Inside the biofilm, mutans streptococci can modify their environment to optimize their growth and metabolism (Davey & O'Toole, 2000).

Streptococcus mutans is considered the bacterium having the most important role in dental caries initiation and development (Loesche, 1986; Van Houte, 1994). In the presence of sucrose, this oral pathogen is capable of producing acids and synthesizing glucans. The acid production can reduce the pH of dental biofilm to low levels, leading to tooth demineralization; the synthesis of glucans occurs by the action of extracellular B, C and D glucosyltransferases (GTFs). GTF B has been considered one of the most important, since it is responsible for the synthesis of insoluble glucans (Yamashita *et al*., 1993). Synthesis of glucans allows bacteria to attach to dental surfaces and provides the dental biofilm with a stable matrix (Schilling & Bowen, 1992).

These physiological characteristics of *S. mutans*, as well as its ability to survive in acid environments (Quivey *et al.*, 2000) and to respond to acid stress by changing its protein expression (Welin *et al*., 2004; Svensäter *et al*., 2000), have been reported in previous studies investigating the virulence factors of *S. mutans*.

Besides the mechanical removal of biofilm and the control of sugar intake, some natural products have been used as antimicrobial agents against virulence factors of *S. mutans* in an attempt to find new pharmacological agents to prevent and control dental caries (Baehni & Takeuchi, 2003). Studies investigating natural compounds as antimicrobial agents against *S. mutans* and other mutans streptococci have shown an interference with virulence factors of such microorganisms and a reduction on caries development *in vivo* (Koo *et al*., 2002, 2003; Duarte *et al.*, 2006).

In a recent study, the polyprenylated bezophenone 7-epiclusianone was isolated from the fruits of *Rheedia brasiliensis* Planch. & Triana (Syn. *Garcinia brasiliensis* Mart.) (Almeida *et al*., 2008, *in press*), a plant from Guttiferae family native to the Amazon region

(Corrêa, 1978; Morton, 1987). Its activity against planktonic cells of *S. mutans* was investigated, showing antimicrobial properties (minimum inhibitory concentration of 1.25-2.5 µg/ml) (Almeida *et al.*, 2008, *in press*). However, it is important to evaluate the mechanisms of action of 7-epiclusianone against *S. mutans*, such as its effects on virulence factors, and to verify the anticariogenic potential of such compound. The aim of this study was to investigate the influence of 7-epiclusianone on surface-adsorbed GTF B and on the acidogenicity and formation of *S. mutans* biofilms. We also analyzed the influence of this compound on caries development in rats subjected to a high cariogenic challenge.

Materials and Methods

Plant material

R. brasiliensis is cultivated at the herbarium of the University of Viçosa (latitude 20º45'14" south and longitude 42º52'55" west), Minas Gerais, Brazil (voucher specimen number: VIC2604). 7-epiclusianone [m.p. 92-93 ^{o}C (MeOH); $[\alpha]^{25}_{D}$ +77 (*c* 0.1, $CHCl_3$)] (Fig. 1) was obtained from the peel (pericarp) hexane extract (PHE) as previously described (Dos Santos *et al.*, 1999; Almeida *et al.*, 2008, *in press*). 7-epiclusianone (purity 99.85%) and PHE were dissolved in a phosphate buffer containing 15% ethanol (v/v – vehicle) just prior to the tests. Different concentrations of 7-epiclusianone (7-epi) were used to test its effect on GTF B activity (12.5 to 100 µg/ml) and for biofilm assays (125 and 250 µg/ml). Biofilm assays also tested PHE at 500 µg/ml and 15% ethanol (v/v) as active and vehicle control, respectively. For the animal study, the highest concentration of 7-epi was used (250 µg/ml) and 250 ppm F and 15% ethanol were used as positive and vehicle control, respectively.

Bacteria

The bacterium strain used for the production of glucosyltranferase B was the *Streptococcus anginosus* KSB8, which harbors the *gtfB* gene. *Streptococcus mutans* UA159 was the oral pathogen used for the biofilm assays and animal study. The cultures were stored at –80 ºC in Brain Heart Infusion (BHI) containing 20% glycerol (v/v).

Saliva

In the present study, the hydroxyapatite beads (Macro-Prep Ceramic Hydroxyapatite Type I, 80 µm, Bio-Rad®, Hercules, CA) and discs (HAP ceramic – calcium hydroxyapatite, 0.5" diameter ceramic – Clarkson Calcium Phosphates, Williamsport, PA) were coated in saliva, which was stimulated (paraffin film) and collected from one donor (Ethics Comittee in Research of the School of Dentistry of Piracicaba – State University of Campinas – Protocol No 012/2007). The saliva was clarified by centrifugation (9.500 *g*, 4 ºC, 10 min) and diluted (1:1) in adsorption buffer (50 mM KCl, 1 mM KPO_4, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$, pH 6.5), supplemented with the protease inhibitor phenylmethylsulfonyl-fluoride (PMSF) at a final concentration of 1 mmol/l. For GTF assays, the saliva was also supplemented with sodium azide (0.02%, final concentration) (Koo *et al*., 2000).

Glucosyltranferase (GTF) B activity on surface assay

The GTF B enzymes were purified to near homogeneity by hydroxyapatite column chromatography as described by Venkitaraman *et al*. (1995). GTF activity was measured by the incorporation of [^{14}C] glucose from labeled sucrose (NEN Research Products, Boston, Mass., USA) into glucans. The GTF B enzyme added to each sample was equivalent to the amount required to incorporate 1-1.5 µmol of glucose over the 4-h reaction. To evaluate the activity of GTF B on surface, we used hydroxyapatite beads coated with clarified whole saliva free of GTF activity in the presence or absence (control) of 7-epi (12.5, 25, 50 and 100 µg/ml), as described by Schilling & Bowen (1988). This evaluation was carried out in triplicate in at least three different experiments.

Biofilm assays

For biofilm assays, the saliva-coated hydroxyapatite discs were placed in a vertical position in batch cultures of *S. mutans* UA159, containing an ultrafiltered (Amicon 10 kDa molecular weight cut-off membrane, Millipore Co., MA, USA) tryptone-yeast extract with sucrose (1%, final concentration), at 37 ºC, 5% CO_2 (Koo *et al*., 2003). Biofilms were first grown for 24 h to allow initial formation. At this point and each day,

they were transferred to fresh culture medium (glycolytic pH-drop assay) and also treatments (inhibition of biofilm formation assay) until the fifth day, according to the experiments described below. All assays were done in duplicate on at least three different experiments.

Glycolytic pH-drop

The acid production by *S. mutans* biofilms exposed to the test agents was evaluated by glycolytic pH-drop assay (Belli *et al.*, 1995). 5-days-old biofilms (without daily treatments) were washed in salt solution (50 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, pH 7.0) and exposed to 7-epi (125 and 250 µg/ml) and active (PHE – 500 µg/ml) and vehicle (15% ethanol) controls for 0, 1, 2 and 3 h. The initial pH (0 h) of the solutions containg biofilms and the test agents was titrated with 0.25 M KOH to a pH just above 7.2. The effects of 7-epi on glycolytic pH-drop were measured in presence of excess glucose, which was added to a final concentration of 1% (v/v) to allow bacteria to produce acids. The pH values were monitored with an Orion pH electrode attached to Orion $290A^+$ pHmeter. The pH values were measured every 1 h and until the third hour.

Inhibition of biofilm formation

To evaluate the effect of the test agents on biofilm formation, 24h-old biofilms (initial formation period) were treated with 7-epi (125 and 250 µg/ml), PHE (500 µg/ml) or vehicle control (15% ethanol) twice a day (10 a.m. and 4 p.m.) until the fifth day of experiment. The biofilms were exposed to the treatments for 1 min, double-dip rinsed in sterile 0.9% NaCl and transferred to fresh culture medium (Koo *et al.*, 2003). At the fifth day, the treated biofilms were removed from discs (ultrasonic bath), serially diluted and plated in Tryptic Soy Agar (TSA). The plates were incubated in 5% CO_2, at 37 ºC, for 48 h, and then colony forming units per ml (cfu/ml) were quantified. In addition, an aliquot (1 ml) of the diluted suspension was used for determining the biomass (dry weight).

Animal study

This study was approved by the Ethical Committee on Animal Research at the State University of Campinas – UNICAMP (Protocol No 963-1) and was performed as described elsewhere (Bowen *et al*., 1988; Koo *et al*., 2003).

To conduct the experiment, 36 specific pathogen-free female *Wistar* rats, aged 19 days, were purchased from CEMIB/UNICAMP (Campinas, Brazil). Rats were first screened for indigenous mutans streptococci as previously described (Bowen *et al.*, 1988). When aged 20, 21, 22 and 23 days, they were infected with bacterial suspensions obtained from 3-days-old *S. mutans* biofilms formed on glass microscope slides (Koo *et al*., 2003).

At age 25 days, the animals were randomly divided into three groups, according to the treatment (12 animals each group): 1) 7-epi 250 μg/ml; 2) 250 ppm F (positive control) - concentration chosen based on data from previous studies (Koo *et al*., 2003; Koo *et al*., 2005); 3) 15% ethanol (v/v) (vehicle control for all groups). Their molars were treated using a camel hair brush twice daily (9 a.m. and 2 p.m.) until the last day of experiment (5 weeks). The rats, which were placed in individual cages, were provided with cariogenic diet 2000 (Keyes, 1959) and 5% sucrose water *ad libitum*. The animals were weighed weekly, also observing their physical appearance daily.

At the end of the 5-week experimental period, the rats were killed by CO_2 asphyxiation. The lower left jaw was aseptically dissected, suspended in 5.0 ml of a sterile 0.9% NaCl solution and sonicated (six 10-s pulses at 5-s intervals, at 40 watts, Vibra Cell, Sonics & Material Inc.). Aliquots (50 μl) of the suspensions were plated on blood agar and on Mitis Salivarius Agar plus bacitracin (Sigma®), using a Spiral Plater (Whitley Automatic Spiral Plater), to determine, respectively, the number of total microorganisms and *S. mutans* populations. The smooth-surface and sulcal caries and their severities were evaluated according to Larson's modification of Keyes' system (Larson, 1981). The determination of caries score was blinded and performed by one calibrated examiner.

Statistical analyses

The data were subjected to ANOVA and Tukey-Kramer test to adjust for multiple comparisons, using JMP version 3.1 software for statistical visualization. The level of significance was set at 5%.

Results

GTF B activity on surface

The effects of 7-epi on glucan synthesis are shown in Table 1. In the highest concentration tested, the test compound reduced the activity of GTF B on surface with approximately 50% of inhibition of activity. The activity of the enzyme was diminished when the doses were increased (Table 1).

Biofilm assays

Glycolytic pH drop

The acid production by *S. mutans* biofilms was affected by 7-epi, as weel as by its origin fraction (PHE 500 µg/ml), in both concentrations tested (125 and 250 µg/ml). After 3 h exposure to test agents, biofilms had pH values higher than 5.5. In absence of 7-epi, the final pH values were lower than 5.0 after 3 h (4.7 for vehicle control). 7-epi at 250 µg/ml reduced the acid production by *S. mutans* biofilms and was statistically different from vehicle control in all hours tested ($p<0.05$) (Fig. 2). 7-epi at 125 µg/ml and PHE at 500 µg/ml inhibited the acid production after the second hour when compared to the vehicle ($p<0.05$) and they were not statistically different from 7-epi at 250 µg/ml ($p>0.05$).

Inhibition of biofilm formation

The biofilms treated with 7-epi at 250 µg/ml and PHE at 500 µg/ml showed slightly lower numbers of recoverable viable cells when compared to the vehicle control, but the difference was not statistically significant (Table 2, $p>0.05$). All agents could not reduce statistically the accumulation of biomass (dry weight) of *S. mutans* biofilms at the concentrations tested (Table 2, $p>0.05$), although we observed a reduction with 7-epi at 250 µg/ml.

Animal study

In the present study, the animals maintained a good health during all the experiment. The weight gain of animals was not statistically significant among 7-epi,

positive (250 ppm F) and vehicle control (15% ethanol) groups ($p>0.05$) (data not shown). The effects of the treatments on the incidence and severity of smooth-surface caries and sulcal caries are shown in Table 4. 7-epi reduced the total caries of smooth-surface and total sulcal caries and the severity of caries in dentin when compared to the vehicle control ($p<0.05$), but the positive control (250 ppm F) showed better results for all parameters related to the incidence and severity of caries than 7-epi. The total microbiota was reduced by treatment with 7-epi and 250 ppm F when compared to the vehicle ($p<0.05$) (Table 3). However, there was no difference regarding the number and percentage of *S. mutans* among the groups tested ($p>0.05$) (Table 3).

Discussion

The high incidence of dental caries in some populations of the world and all damages caused by this infectious disease have required new strategies to its prevention and control (Antunes *et al*., 2004). Studies on compounds isolated from natural products have reported antibacterial activity against mutans streptococci, also reducing caries in animals (Koo *et al*., 2003a; Duarte *et al*., 2006).

The present study was aimed at investigating the polyprenylated benzophenone 7-epiclusianone (7-epi), focusing on its antimicrobial activity and anticaries effect. This compound, obtained from *Rheedia brasiliensis* fruits (Guttiferae family), showed actions against important virulence traits of *Streptococcus mutans*, by reducing its ability to sinthesize insoluble glucans and produce acids, and also reduced de incidence of caries in rats.

The synthesis of insoluble glucans (α1,3-linked glucans) by GTF B, specially surface-adsorbed enzyme, facilitates the adherence of *S. mutans* and contributes to the formation of biofilm matrix (Schilling & Bowen, 1992). This matrix makes it difficult for antimicrobial agents to act against biofilms (Koo *et al*., 2002; Wunder & Bowen, 1999). Even at low concentrations (12.5-100 µg/ml), 7-epi could reduce the activity of GTF B, probably affecting the *S. mutans* adherence as a consequence. Previous studies focusing on effects of other clinical agents, including chlorhexidine (0.12%) and fluoride (250 ppm), on

GTF B showed negligible or moderate inhibitory results (Wunder & Bowen, 1999; Koo *et al*., 2003a).

The concentrations used for 7-epi (125 and 250 µg/ml) to evaluate its activity against *S. mutans* biofilms were approximately 100 times higher than the MIC values obtained for the compound tested, as previously described by Marsh (1992) concerning other antimicrobial agents. When compared to those reported in previous studies on natural products (Koo *et al*., 2003a, 2003b; Duarte *et al*., 2006), the 7-epi concentrations tested were considered low and were choosen just to avoid problems with solubility (7-epi is a weak acid with non-polar characteristics) and toxicological effects.

In the present study, 7-epi was observed to interfere with acids produced by *S. mutans* biofilms as well as to maintain pH at levels higher than those (5.0-5.5) known to cause enamel demineralization. This compound at concentrations of 125 µg/ml and 250 µg/ml reduced acid production starting at the 2-hour period when compared to the vehicle ($p<0.05$), using the glycolytic pH-drop test. This reduction in acid production might be related to the ability of 7-epi to inhibit the F-ATPase enzyme, which allows bacteria to survive in acid enviroments, or even to the inhibition of glycolytic enzymes by 7-epi, suggesting that further studies are needed to investigate such hypothesis. Since biofilm acts as a barrier to antibiotic diffusion (Stewart, 1996), 7-epi was observed to have good activity against acid production by *S. mutans*.

However, this compound could not reduce the accumulation and formation of *S. mutans* biofilms treated twice daily, suggesting that the action of 7-epi, at the concentrations tested, might be related to virulence factors of *S. mutans* (activity of GTF B and acid production) and not to its bactericidal effect. Similar results were found in the literature concerning the action of natural products against the virulence of mutans streptococci as test agents showed no effect on the viability of bacterial biofilms (Duarte *et al*., 2006).

7-epi was able to reduce the incidence and severity of caries in rats submitted to cariogenic challenges, without a reduction in *S. mutans* when compared to the vehicle control ($p>0.05$), confirming the results obtained with the *in vitro* tests. The group treated with 250 ppm F presented lower incidence and severity of caries than 7-epi and showed the

same pattern for total microbiota and *S. mutans* as 7-epi. The best results for fluoride, the most effective anticaries agent (Clarkson, 2000), might be attributed to its additional physicochemical actions (Hamilton, 1990). Similar data were observed in a previous study evaluating the effects of fluoride and natural products on dental caries in rats (Koo *et al*., 2003a).

Some anticaries agents, when compared to some bioactive compounds, were observed to be more efficient in preventing dental caries in rats (Koo *et al*., 2003); however, it is well known that there are differences among drugs concerning their mechanism of action as well as their substantivity (Dawes & Ten Cate, 1990; Jones, 1997).

Based on the *in vitro* and *in vivo* results obtained in the present study, it could be suggested that the anticaries activity of 7-epi might be related to a reduction on *S. mutans* virulence, and not to its bactericidal activity. Substances that act on *S. mutans* virulence factors could be used to control dental caries or even to enhance the anticariogenic effect of other recognized agents, such as fluoride (Koo *et al*., 2003a; Koo *et al*., 2005).

In conclusion, 7-epi could be considered a promising agent to prevent and control dental caries. Further studies are needed to investigate its activity against multispecies biofilms as well as its toxicological effects.

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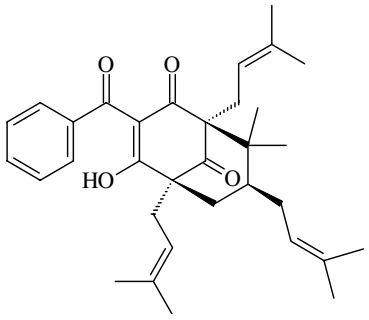

Fig. 1. Chemical structure of 7-epiclusianone, a polyprenylated benzophenone obtained from *Rheedia brasiliensis*.

Table 1. Effects of 7-epiclusianone (7-epi) on the activity of GTF B on surface.

Concentration of 7-epi (µg/ml)	**GTF B (% of inhibition)**
100	48.0 ± 1.8
50	33.4 ± 4.7
25	26.6 ± 4.7
12.5	9.2 ± 6.2

The percentage of inhibition was calculated considering the vehicle control (15% ethanol) as 100% glucosyltransferase activity (n=9).

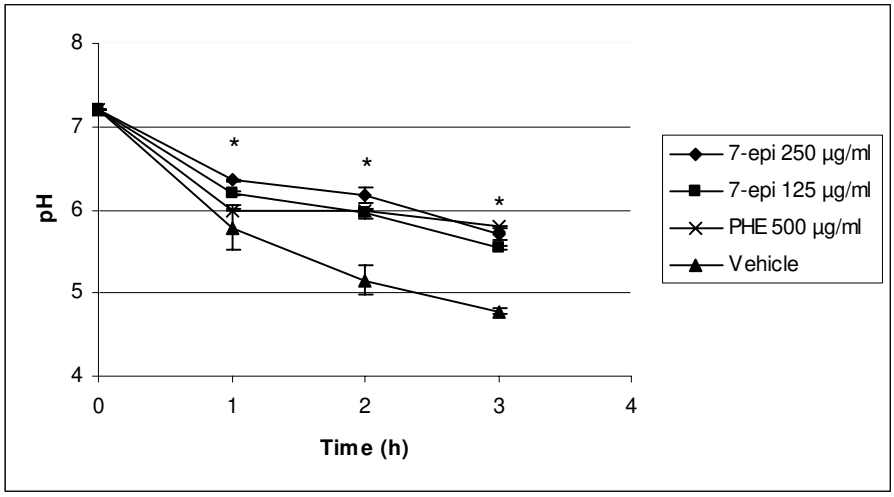

Fig. 2. Influence of 7-epiclusianone (7-epi) and its active (peel hexane extract – PHE) and vehicle (15% ethanol) controls on glycolytic pH-drop in *S. mutans* UA159 biofilms.

* Indicates a statistically significant difference among vehicle control (15% ethanol) and each treatment ($p<0.05$) (n=6).

Table 2. Influence of test agents on *Streptococcus mutans* UA159 biofilms formation (dry-weight and viability): means (±SD).

Treatments	**Dry-weight (mg)**	**cfu/ml ($\times 10^6$)**
Vehicle	6.5 ± 1.9	1.5 ± 0.7
7-epi 250 µg/ml	5.2 ± 1.8	1.1 ± 0.3
7-epi 125 µg/ml	6.2 ± 1.7	1.5 ± 0.4
PHE 500 µg/ml	6.9 ± 2.0	0.9 ± 0.4

No statistical differences were found for dry-weight (biomass) and cfu/ml among the treatments ($p>0.05$, ANOVA, comparison for all pairs using Tukey-Kramer HSD) (n=6).

Table 3. Effects of 7-epiclusianone (7-epi) and positive (250 ppm F) and vehicle (15% ethanol) controls after five-week experiment on oral microbiota in rats: means (±SD).

Treatment	**Total microorganisms (x10^4 cfu/ml)**	***Streptococcus mutans* UA159 (x10^4 cfu/ml)**	***Streptococcus mutans* UA159 (%)**
Vehicle	3.6 (1.7) [a]	2.6 (1.9) [a]	63.8 (21.3) [a]
7-epi 250 µg/ml	1.8 (0.7) [b]	1.2 (0.5) [a]	66.7 (17.2) [a]
250 ppm F	1.9 (0.5) [b]	1.6 (0.3) [a]	62.4 (14.1) [a]

Values followed by the same letters (vertical) are not significantly different from each other ($p>0.05$) (n=12). ANOVA, comparison for all pairs using Tukey-Kramer HSD.

Table 4. Effects of 7-epiclusianone (7-epi) and respective controls on caries development (smooth-surface and sulcal caries) and severity (Ds, dentin exposed; Dm, 3/4 of the dentin affected; Dx, dentin loss) in rats, after five-week experiment: means (±SD) of Keyes' scores modified by Larson.

GROUP	Smooth-Surface				Sulcal			
	TOTAL	Severity			TOTAL	Severity		
		Ds	Dm	Dx		Ds	Dm	Dx
Vehicle	78.6 [a]	44.8 [a]	19.9 [a]	7.0 [a]	47.1 [a]	38.9 [a]	30.5 [a]	22.6 [a]
	(5.8)	(8.1)	(7.0)	(7.2)	(4.0)	(3.0)	(5.3)	(5.4)
7-epi 250 µg/ml	63.9 [b]	25.7 [b]	3.4 [b]	2.1 [b]	40.6 [b]	25.2 [b]	14.9 [b]	5.4 [b]
	(6.7)	(8.1)	(5.0)	(3.6)	(5.0)	(5.4)	(5.4)	(4.7)
250 ppm F	34.8 [c]	22.8 [b]	0.5 [b]	0.0 [b]	28.6 [c]	16.1 [c]	2.6 [c]	0.4 [c]
	(4.1)	(6.7)	(0.8)	(0.0)	(3.3)	(3.4)	(1.6)	(0.7)

Values followed by the same letters (vertical) are not significantly different from each other ($p>0.05$) (n=12). ANOVA, comparison for all pairs using Tukey-Kramer HSD.

3 Conclusões

No presente estudo, concluiu-se que a 7-epiclusianona extraída da *Rheedia brasiliensis* foi capaz de atuar sobre fatores de virulência do *Streptococcus mutans* e de reduzir cárie dental em modelo animal, sugerindo que este composto possa ser um possível adjunto terapêutico no controle da cárie dental. Estudos toxicológicos são necessários para que o uso da 7-epiclusianona continue sendo considerado.

4 Referências*

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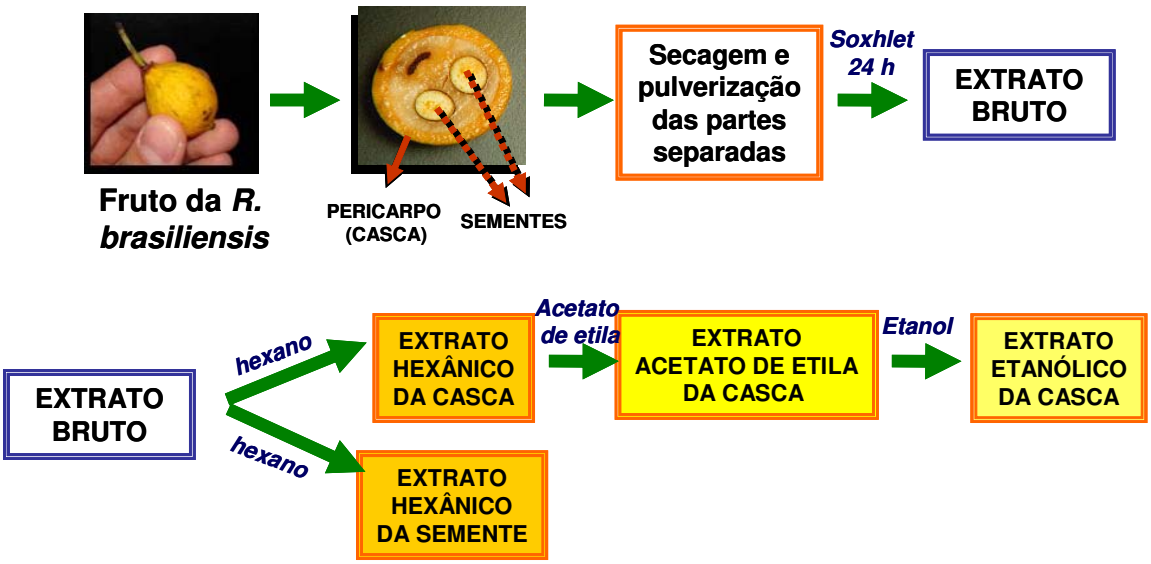
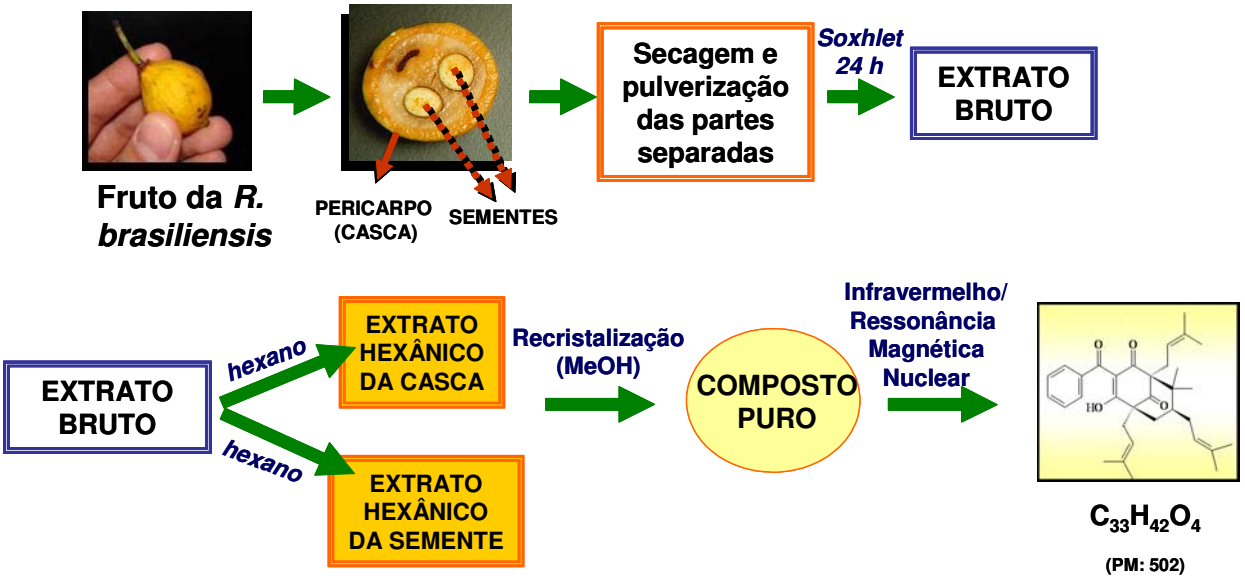
5 Apêndice

Desenhos esquemáticos dos métodos de obtenção dos extratos e da 7-epiclusianona a partir do fruto da *Rheedia brasiliensis* (bacupari).

➢ **Obtenção dos extratos:**



➢ **Obtenção da 7-epiclusianona:**



6 Anexos

6.1 Anexo 1: Resolução do formato alternativo para defesa da dissertação de mestrado.

INFORMAÇÃO CCPG/002/06

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

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

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

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

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

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

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

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

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

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

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

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

§ 3º - A gráfica da Unicamp imprimirá os exemplares solicitados com capa padrão. Os exemplares solicitados serão encaminhados à Unidade em, no máximo, cinco dias úteis.

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

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Campinas, 13 de setembro de 2006

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

6.2 Anexo 2: Certificado do Comitê de Ética em Pesquisa.

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

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "**Análise antimicrobiana e proteômica de biofilmes de Streptococcus mutans tratados com uma benzofenona poliprenilada extraída da Rheedia brasiliensis**", protocolo nº **012/2007**, dos pesquisadores **LUCIANA SALLES BRANCO DE ALMEIDA e PEDRO LUIZ ROSALEN**, satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 11/04/2007.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project "**Antimicrobial and proteomic evaluation of Streptococcus mutans biofilms treated with a polyprenylated benzophenone from Rheedia brasiliensis**", register number **012/2007**, of **LUCIANA SALLES BRANCO DE ALMEIDA and PEDRO LUIZ ROSALEN**, comply with the recommendations of the National Health Council – Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 11/04/2007.

Profa. Cinthia Pereira Machado Tabchoury
Secretária
CEP/FOP/UNICAMP

Prof. Jacks Jorge Júnior
Coordenador
CEP/FOP/UNICAMP

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

6.3 Anexo 3: Certificado do Comitê de Ética na Experimentação Animal.

Universidade Estadual de Campinas
Instituto de Biologia

UNICAMP

CEEA-IB-UNICAMP

Comissão de Ética na Experimentação Animal
CEEA-IB-UNICAMP

CERTIFICADO

Certificamos que o Protocolo nº **963-1**, sobre **"AVALIAÇÃO DAS ATIVIDADES ANTITUMORAL, ANTIMICROBIANA E DO CONTROLE DA DOR, RELACIONADAS ÀS PATOLOGIAS ORAIS DAS PLANTAS DA FAMÍLIA GUTTIFERAE"** sob a responsabilidade de **Prof. Dr. Pedro Luiz Rosalen / Ramiro Mendonça Murata / 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 22 de março de 2006.

CERTIFICATE

We certify that the protocol nº **963-1**, entitled **"EVALUATION OF THE ANTITUMORAL, ANTIMICROBIAL AND OF THE CONTROL OF THE PAIN ACTIVITY, RELATED TO THE ORAL PATHOLOGIES OF FAMILIA GUTTIFERAE'S PLANTS"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on March 22, 2006.

Campinas, 22 de março de 2006.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

Fátima Alonso
Secretária Executiva

CEEA/IB – Unicamp
Caixa Postal 6109
13083-970 Campinas, SP – Brasil

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6.4 Anexo 4: E-mail de confirmação de aceite do artigo referente ao Capítulo 1.

Date: Dec 07, 2007

To: "Pedro Luiz Rosalen" rosalen@fop.unicamp.br

From: "Phytomedicine" h.wagner@cup.uni-muenchen.de

Subject: Your Submission

Ms. Ref. No.: PHYMED-D-07-00281R1
Title: Antimicrobial activity of Rheedia brasiliensis and 7-epiclusianone against Streptococcus mutans
Phytomedicine

Dear Dr. Rosalen,

I am pleased to inform you that your paper "Antimicrobial activity of Rheedia brasiliensis and 7-epiclusianone against Streptococcus mutans" has been accepted for publication in Phytomedicine.

Thank you for submitting your work to Phytomedicine.

Yours sincerely,

Silvia Bächer
Editorial Office
Phytomedicine

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Piracicaba, 29/02/2008

Autor: LUCIANA SALLES BRANCO DE ALMEIDA
RG n.º 35360595-6

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6. 6 Anexo 6: Declaração da revista *Phytomedicine* referente a direitos autorais.

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