RAMIRO MENDONÇA MURATA

Avaliação das atividades anticárie e antiproliferativa da 7-epiclusianona isolada da planta do gênero *Rheedia*

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

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

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RESUMO

As plantas medicinais têm sido utilizadas em várias áreas da saúde como forma alternativa de tratamento e prevenção de doenças. Conseqüentemente, muitos agentes de origem natural vêm sendo explorados, nas últimas décadas, devido as suas possíveis ações farmacológicas. Dentre os produtos naturais que se destacam estão os compostos da planta do gênero *Rheedia*, da família Guttiferae, que têm ações comprovadas contra várias doenças. Assim, o objetivo deste trabalho foi avaliar o potencial antimicrobiano, anticárie e antiproliferativo da 7-epiclusianona (7-epi), benzofenona isolada das plantas do gênero Rheedia. Para isso, foram realizados quatro estudos, cujos objetivos foram: (1) Analisar a influência da 7-epi na formação de biofilmes de *Streptococcus mutans* UA159 em discos de hidroxiapatita, na queda de pH glicolítico e seu efeito na translocação de prótons pela atividade da F-ATPase. Além disso, foram realizados estudos para analisar seu efeito na atividade de glucosiltransferase (GTF) B e C; (2) Determinar a influência da 7-epi, associada ao flúor, na composição de biofilmes de *S. mutans* e a atividades anticárie da 7-epi utilizando modelo experimental de cárie dental em ratos; (3) O efeito antimicrobiano da 7-epi sobre o crescimento dos estreptococos do grupo mutans e (4) Avaliar o potencial antiproliferativo da 7-epi contra células derivadas de tumores malignos humanos. No estudo 1, a 7-epi reduziu a produção de ácidos orgânicos e a atividade da F-ATPase (61,1%±3,0). O composto também foi um potente inibidor da atividade de GTFs (85% de inibição). Nos biofilmes, o peso seco e a quantidade de polissacarídeos insolúveis também foram significativamente reduzidos. No estudo 2, a quantidade de polissacarídeos solúveis, insolúveis e intracelulares em biofilmes, bem como a incidência e a severidade de cárie em superfície lisa e sulco foram significativamente reduzidos pela 7-epi associada ao flúor

(p<0,05). No estudo 3, 7-epi apresentou atividade antimicrobiana (CIM entre 2,5-5 μg/mL e CBM entre 10-20 μg/mL) em baixas concentrações. No estudo 4, a 7-epi apresentou atividade antiproliferativa. Para as células cancerígenas de melanoma (UACC-62), mama resistente (NCI-ADR), ovário (OVCAR) e pulmão (NCI-460) a 7-epi demonstrou-se mais potente que o controle positivo (doxorubicina). Desta forma, concluímos que a 7-epi possui atividade antimicrobiana, anticárie e antiproliferativa, sendo um promissor agente para a prevenção da cárie dental e com potencial atividade anticâncer.

Palavras-Chave: Produtos Biologicos, Produtos com Ação Antimicrobiana, Ensaios de Seleção de Medicamentos Antitumoral, Cárie Dentária.

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 enormous biodiversitiy of Brazil flora appears as an important source of new pharmacological agents. In this way, *Rheedia* genus showed the presence of flavonoids, xanthones and polyprenylated benzophenones with proved actions against important pathogens Overall, aim of this study was to evaluate the antimicrobial and atitumoral effect of the 7-epiclusianone, isolated compound of the *Rheedia* genus plant. Therefore, four studies were carried out. In study 1, the influence of 7-epiclusianone was evaluated on glucans production by purified glucosyltransferases (GTFs), on membrane-associated F-ATPase, on glycolytic activities and insoluble glucans composition/acidogenicity of *S. mutans* biofilmes. In study 2, the influence of 7-epiclusianone, alone or in combination with fluoride, on *S. mutans* biofilms and caries development *in vivo* was evaluated. In study 3, the antimicrobial activity of the 7-epiclusianone was assessed by determination of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) against *S. mutans*. In study 4, the influence of 7-epiclusianone in a differential antiproliferative activity against a human cancer cell lines. In study 1, the synthesis of glucans by glucosyltransferases B and C was remarkably reduced by 7-epiclusianone showing more than 80% inhibition of enzymatic activity at a concentration of 100 μg/ml. The glycolytic pH-drop by *S. mutans* cells was also disrupted by 7-epiclusianone; an effect that can be attributed, in part, to inhibition of

F-ATPase activity (61.1±3.0 % inhibition at 100μg/mL). The extracellular insoluble polysaccharide concentration and acidogenicity of the biofilms were significantly reduced by the test agent (P<0.05). In study 2, biofilms treated with 7-epiclusianone, alone or in combination with F, displayed less biomass and insoluble glucans than the positive control (fluoride) (p<0.05). The biological effect of 7-epiclusianone was greatly enhanced when used in combination with F. Furthermore, the combination of 7-epiclusianone with F was highly effective in preventing caries development in rats. In study 3, 7-epiclusianone was effective in inhibiting the growth of *S. mutans* (MIC 2.5-5 μg/ml and MBC10-20 μg/ml). In study 4, 7-epiclusianone displayed significant cytotoxic activity for all cell lines. The IC_{50}, half maximal inhibitory concentration, of 7-epiclusianone was significantly more potent positive control (doxorubicin) against melanoma (UACC-62), breast-resitant (NCI-ADR), ovarian (OVCAR), and lung (NCI-460) cells. In conclusion, 7-epiclusianone have relevant antimicrobial and atiproliferative activity and are promising anti-caries and antitumoral agents.

Keywords: Biological Products, 7-epiclusianona, Products with Antimicrobial Action, Antitumor Drug Screening Assays, Dental Caries.

SUMÁRIO

1. INTRODUÇÃO

A utilização de plantas medicinais é uma prática generalizada na medicina popular, resultado do acúmulo secular de conhecimentos empíricos sobre o efeito dos vegetais por diversos grupos étnicos. No Brasil, além da assimilação dos conhecimentos indígenas, as contribuições trazidas pelos escravos e imigrantes representam papel importante para o surgimento de uma medicina popular rica e original, na qual a utilização de plantas medicinais ocupa lugar de destaque (CELEGHINI, 1997).

Algumas estimativas revelam a existência de aproximadamente 250.000 espécies de plantas superiores em todo o mundo. Entretanto, dados disponíveis revelam que apenas 17% das plantas foram estudadas quanto ao seu potencial farmacológico (LEWIS & HANSON, 1991; CRAGG & NEWMAN, 1999). As plantas medicinais têm sido utilizadas em várias áreas da saúde como forma alternativa de tratamento e prevenção de doenças (LEWIS & ELVIN-LEWIS, 1994). Existe uma tendência para o aumento do uso destas plantas e isto seria de grande utilidade, principalmente nos países em desenvolvimento, como no Brasil, que apresenta uma grande biodiversidade. Muitos estudos também estão sendo realizados no intuito de identificar e isolar os princípios ativos presentes nestes extratos naturais, para que estes novos compostos isolados quimicamente, se efetivos na sua ação, possam ser sintetizados e utilizados para o tratamento de doenças (ISRAELSON, 1991).

Deste modo, muitos agentes de origem natural vêm sendo explorados nas últimas décadas (VUORELAA *et al*., 2004), devido às suas possíveis ações farmacológicas. Entre os produtos naturais têm-se destacado os extratos e o composto (benzofenona poliprenilada) da planta do gênero *Rheedia,* que fazem parte da família *Guttiferae* e têm demonstrado, pelos estudos químicos, ser possuidor de uma grande diversidade de classes estruturais de compostos químicos. Destacam-se os flavonóides, proantocianinas, xantonas e as benzofenonas polipreniladas, que têm ações comprovadas com princípios ativos contra várias doenças de origem infecciosas, entre outras (DELLE MONACHE *et al*., 1983; 1988). A espécie *Rheedia brasiliensis* ocorre na região Amazônica e nordeste do país. São

cultivadas em todo o território brasileiro sendo conhecida popularmente como bacupari e bacoparé (CORREA, 1978).

Estudos químicos prévios utilizando frutos de plantas do gênero *Rheedia* levaram Santos (1999) a isolar alguns constituintes químicos destas plantas. Do extrato hexânico da polpa foi isolada uma mistura de sitosterol, estigmasterol e sesquiterpenos. O extrato hexânico da casca forneceu a benzofenona tetraprenilada, a 7-epiclusianona [3-benzoil-4-hidroxi-6,6-dimetil-1,5,7-tris(3-metil-2-butenil) biciclo [3.3.1]non-3-ene-2,9-diona] (FIGURA 1), formula $C_{33}H_{42}O_4$ (PM 502), que é uma forma isômera da clusianona, com temperatura fusão de 92-93° C, pureza de 99,8% e rendimento de 0,9% tendo sido elucidada quimicamente por técnicas de Ressonância Magnética Nuclear e difração de raio X (SANTOS, 2001). Posteriormente, a 7-epiclusianona também foi indentificada na espécie *Rheedia brasiliensis* (NEVES *et al*., 2007; ALMEIDA *et al.,* 2008)

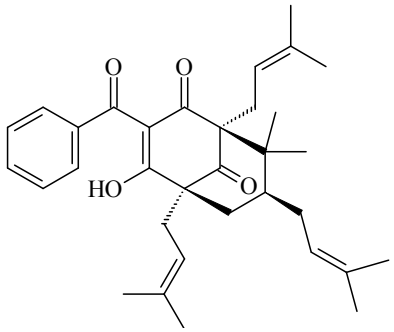

Figura 1. Estrutura química da 7-epiclusianona, uma nova benzofenona tetraprenilada. Adaptado de Santos *et al.,* 1999.

Foram também realizados testes visando avaliar a ação da 7-epiclusianona sobre formas tripomastigotas de *Trypanosoma cruzi*, *in vitro* (ALVES *et al.,* 1999). Os resultados obtidos indicaram redução em 92% de *T. cruzi* nas amostras de sangue contendo baixas concentrações de 7-epiclusianona, entretanto, foi inativo quando no teste *in vivo* (camundongos infectados). Nesse mesmo estudo foi realizado o teste para *Artemia salina*, que é usada para correlacionar a citotoxicidade de uma substância e a 7-epiclusianona

mostrou-se ativa, com promissora ação citotóxica e, portanto, a sua investigação deve ser incrementada com relação à células tumorais.

Nas últimas décadas, a importância do estudo da atividade Antitumoral das plantas medicinais está relacionada com o fato do câncer ser um dos maiores problemas de saúde pública no Brasil e no mundo. Nos Estados Unidos da América, surgem aproximadamente um milhão de novos casos por ano (SICHIERI, 1996). Os Carcinomas da boca correspondem à cerca de 5% das neoplasias malignas do ser humano, afetando lábios, língua, assoalho da boca, palato, gengiva, bochechas e orofaringe. A maioria das neoplasias da boca são carcinomas espinocelulares sendo os principais fatores etiológicos o álcool e o fumo (SCULLY *et al*, 2003). Portanto, a descoberta de novas drogas com efeito antitumoral é uma prioridade, pois numerosas neoplasias malignas não podem ser alcançadas pela cirurgia ou estão além dos limites de segurança da radioterapia, sendo essas drogas úteis como auxiliares da cirurgia/irradiação ou na prevenção de metástase dos tumores primários tratados localmente (WEISS, 1992). Neste sentido a 7-epiclusianona pode ser uma alternativa de droga antiproliferativa a ser investigada.

Em estudos realizados com a planta da família Guttiferae também foram isolados compostos contendo benzofenonas polipreniladas com atividade antimicrobiana *in vitro*, inclusive contra o vírus HIV (DELLE MONACHE *et al.,* 1991; HENRY *et al.,* 1995; OLIVEIRA *et al.,* 1996; ALMEIDA *et al.,* 2008). Estudo prévios (MURATA *et al.,* 2006), avaliou o potencial antimicrobiano do extrato bruto da *Rheedia brasiliensis*, mostrando resultados favoráveis à ação antimicrobiana contra *Sreptococcus mutans*.

Com base nas informações da literatura sobre o potencial farmacológico das plantas da família Guttiferae, o objetivo deste trabalho foi avaliar o potencial Antimicrobiano e Antiproliferativo da 7-epiclusianona, isolados das plantas do gênero *Rheedia*.

2. CAPÍTULOS

Esta tese está de acordo com a Informação CCPG 002/06, UNICAMP, que regulamenta o formato alternativo para dissertação e tese. Assim sendo, esta tese é composta de 4 estudos conforme descrito abaixo:

Estudo 1. Inhibitory effects of 7-epiclusianone on glucan synthesis, acidogenicity and biofilm formation by *Streptococcus mutans*.

Estudo 2. Inhibitory effects of 7-epiclusianone with fluoride on *S. mutans* biofilm and dental caries

Estudo 3. Uso de composto isolado da *Rheedia brasiliensis* na prevenção e/ou tratamento de doenças.

Estudo 4. Antiproliferative effect of poliprenylated benzophenones

O estudo 1 foi submetido para publicação na revista *FEMS Microbiology Letters*, o estudo 2 será submetido à revista *Archives of Oral Biology*, o estudo 3 foi submetido ao Intituto Nacional de Propriedade Industrial (INPI) como pré-requisito para o depósito de patente e o estudo 4 será submetido como *short communication* na revista *Natural Products Research*.

2.1 Estudo 1

INHIBITORY EFFECTS OF 7-EPICLUSIANONE ON GLUCAN SYNTHESIS, ACIDOGENICITY AND BIOFILM FORMATION BY STREPTOCOCCUS MUTANS

Running Title: Influence of 7-epiclusianone on *Streptococcus mutans*.

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ABSTRACT

The aim of this study was to examine the effects of 7-epiclusianone, a new prenylated benzophenone isolated from the plant *Rheedia gardneriana*, on some of the virulence properties of *Streptococcus mutans* associated with biofilm development and acidogenicity. The synthesis of glucans by glucosyltransferases B (GTF B) and C (GTF C) was markedly reduced by 7-epiclusianone showing more than 80% inhibition of enzymatic activity at a concentration of 100 μg/ml. Double-reciprocal analysis (Lineweaver-Burk plots) revealed that the inhibition of GTF B activity was non-competitive (mixed) whereas GTF C was inhibited uncompetitively. The glycolytic pH-drop by *S. mutans* cells was also disrupted by 7-epiclusianone without affecting the bacterial viability; an effect that can be attributed, in part, to inhibition of F-ATPase activity (61.1 ± 3.0 % inhibition at 100μg/mL). Furthermore, topical applications (one-minute exposure, twice daily) of 7-epiclusianone (at 250 μg/mL) disrupted biofilm formation and physiology. The biomass (dry-weight), extracellular insoluble polysaccharide concentration and acidogenicity of the biofilms were significantly reduced by the test agent ($P<0.05$). The data show that 7-epiclusianone disrupts the extracellular and intracellular sugar metabolism of *S. mutans,* and holds promise as a novel naturally occurring compound to prevent biofilm-related oral diseases.

Keywords: 7-epiclusianone, glucosyltransferases, glycolysis, *Streptococcus mutans*, biofilm.

1. INTRODUCTION

Streptococcus mutans has been regarded as an important microbial agent in the pathogenesis of dental caries although additional acidogenic microorganisms may be involved (Fitzgerald, 1960; Hamada *et al*., 1984; Loesche, 1986; Beighton, 2005). The ability of this bacterium to synthesize extracellular polysaccharides (mainly glucans) from sucrose using glucosyltransferases (GTFs) is a critical virulence factor involved in the formation of a pathogenic biofilm (De Stoppelaar *et al*., 1971; Gibbons & Van Houte, 1975; Hamada & Slade, 1980; Schilling & Bowen, 1992; Yamashita *et al*., 1993). Glucans promote bacterial accumulation on the tooth surface and contribute to the formation of the extracellular polysaccharide matrix, which provides bulk and structural integrity to biofilms (Bowen, 2002; Paes Leme *et al*., 2006). Furthermore, *S. mutans* survives and carries out glycolysis at low pH values attained within the matrix of the biofilms, which results in demineralization of the adjacent dental enamel (Belli & Marquis, 1991; Bowen, 2002). *Streptococcus mutans* has developed mechanisms to alleviate the influences of acidification by increasing proton-translocating F-ATPase activity in response to low pH (Sturr & Marquis, 1992; Quivey *et al*., 2000). F-ATPase transports protons out of cells in association with ATP hydrolysis to maintain intracellular pH more alkaline than the extracellular environment pH (Sturr & Marquis, 1992). Therefore, disruption of the ability of *S. mutans* to utilize sucrose to form acids and glucans could be a precise and selective therapeutic approach to reducing the cariogenicity of this ubiquitous oral pathogen.

Natural products are still major sources of innovative therapeutic agents for infections diseases (Cragg *et al*., 1997; Harvey, 2000; Newman *et al*., 2003). Exploration of biodiversity from rich environments such as in Brazil has led to discovery of many pharmacologically active chemicals (Basso *et al*., 2005). For example, phytochemical investigations of the fruits of *Rheedia gardneriana*, a native plant from Amazon region in Brazil (Corrêa, 1978), resulted in the isolation and identification of several potentially active compounds, including sesquiterpenes, methyl esters of fatty acids (palmitate, estearate, oleate, linoleate), triterpene (oleanolic acid), stigmasterol, sisterol and benzophenones (Santos *et al.,* 1998). Among them, a tetraprenylated benzophenone (7-epiclusianone) has shown several pharmacological activities, including antioxidant and

anti-*Trypanossoma cruzi* (Alves *et al.,* 1999; Cruz *et al.,* 2006) activities. Recently, a bioassay-guided fractionation of *Rheedia gardneriana* has identified 7-epiclusianone as a putative active compound against *S. mutans*, including anti-adherence and antibacterial effects (Murata *et al.,* 2006). Thus, the aim of this study was to investigate further the influence of 7-epiclusianone on *S. mutans* virulence, including glucan synthesis and acid production, and on its ability to form biofilms using our saliva-coated hydroxyapatite disc biofilm model (Koo *et al.,* 2003).

2. MATERIAL AND METHODS

2.1. Plant material

The fruits of *R. gardneriana* Pl. Triana were collected at the Campus of Universidade Federal de Vicosa (UFV), Vicosa, Minas Gerais State, Brazil and identified by a botanist at UFV. A voucher specimen is deposited in the Horto Botanico of UFV (26240). 7-epiclusianone was extracted, isolated and purified from the fruit pericarp as detailed elsewhere (Santos *et al.,* 1998). The substance was identified from the infrared, ultraviolet, mass spectrum and NMR spectral data as confirmed by Santos et al, 1998; the purity level was >98% as determined by HPLC (Santos *et al,* 1998).

2.2. Microorganisms

The bacterial strains used for the production of GTFs were: *Streptococcus anginosus* KSB8, which harbors the *gtfB* gene (for GTF B production) and *S. mutans* WHB 410, in which the *gtfB, gtfD* and *ftf* genes were deleted (for GTF C production). The cloning procedures and construction of *S. anginosus* KSB8 (previously known as *S. milleri* KSB8) and *S. mutans* WHB 410 are described in Fukushima et al. (1992) and Wunder and Bowen (1999), respectively. The *S. mutans* UA159, a proven virulent cariogenic pathogen and the strain selected for genomic sequencing (Ajdic *et al.,* 2002), was used for F-ATPase, glycolytic pH drop and biofilm studies. The cultures were stored at –80^{o} C in tryptic soy broth containing 20% glycerol.

2.3. GTF B and C assays

The GTF B and C enzymes (EC 2.4.1.5) were prepared from culture supernatants and purified to near homogeneity by hydroxyapatite column chromatography as described by Venkitaraman *et al.* (1995) and Wunder & Bowen (1999). GTF activity was measured by the incorporation of [^{14}C]glucose from labeled sucrose (NEN Research Products, Boston, MA, USA) into glucans (Venkitaraman *et al.,* 1995).

Purified GTF B and C (1.0-1.5 units) were mixed with a two-fold dilution series of the test agent (concentrations ranging from 12.5 to 100 μg/ml) or vehicle control (15% ethanol, v/v) and incubated with a [^{14}C]glucose-sucrose substrate (0.2 mCi/ml; 200 mM sucrose, 40 mM dextran 9000, and 0.02% sodium azide in a buffer consisting of 50 mM KCl, 1 mM KPO_4, 1 mM $CaCl_2$, and 0.1 mM $MgCl_2$ at pH 6.5) at 37^{o}C with rocking for 4h as described elsewhere (Koo *et al.,* 2003). GTF activity was measured by the incorporation of [^{14}C]glucose from labeled sucrose (NEN Research Products, Boston, MA, USA) into glucans (Venkitaraman *et al.,* 1995). The radiolabeled glucan was determined by scintillation counting (Venkitaraman *et al.,* 1995).

The mode of inhibition of GTF activity by 7-epiclusianone was examined using Lineweaver-Burk plot (expressed in 1/v *versus* 1/[S]) (Lineweaver & Burk 1934) and the nonlinear regression (Cleland, 1963; Engel, 1981) using the Enzpack 1.4 kinetics software (Biosoft, Ferguson, MO, USA).

2.4. Glycolytic pH-drop and F-ATPase assays

The effects of 7-epiclusianone on glycolysis were measured by standard pH drop with dense cell suspensions (2 mg cell dry-weight/ml) 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 MgCl2), and resuspended in salt solution containing the test agent (12.5 to 100 μg/ml) or vehicle control (15% ethanol, v/v). 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, USA) (Belli *et al.,* 1995). Biocidal activity was

determined by plating aliquots of cell suspension at each time point, and counting the colony forming units/mL; the cells suspension was sonicate twice before plating, each consisting of three 10-s pulses at 5-s intervals, at 50W (Branson Ultrasomics Co., Danbury, CT, USA) (Koo *et al* 2003).

F-ATPase assay was performed using permeabilized cells of *S. mutans* UA159 by subjecting the cells to 10% toluene (v/v) followed by two cycles of freezing and thawing as described by Belli *et al.* (1995). F-ATPase activity was measured in terms of the release of phosphate in the following reaction mixture: 75 mmol of Tris-maleate buffer (pH 7.0) containing 5 mM ATP, 10 mmol MgCl2, permeabilized cells, and the test agent (12.5 to 100 μg/ml) or vehicle control (15% ethanol, v/v). The released phosphate (over a 10-min reaction time) was determined by the method of Bencini *et al.* (1983).

2.5. Biofilm assays

Biofilms of *S. mutans* UA159 were formed on saliva-coated hydroxyapatite discs placed in a vertical position (HAP ceramic – calcium hydroxyapatite, 0.5" diameter – Clarkson Calcium Phosphates, Williamsport, PA) in batch cultures at 37 ^{o}C and 5% CO_2 (Koo *et al.,* 2003). Biofilms of *S. mutans* were formed in ultrafiltered (Amicon 10 kDa molecular weight cut-off membrane; Millipore Co., MA, USA) tryptone-yeast extract broth with addition of 30 mM sucrose (Koo *et al.,* 2003). The biofilms were grown undisturbed for 24 h to allow initial biofilms formation. At this point (24 h-old), the biofilms were treated twice daily (10 a.m. and 4 p.m.) until the fifth day of the experimental period (120 h-old biofilm) with 7-epiclusianone (250μg/mL) or vehicle control (15% ETOH). In our model, biofilms continuously form and accumulate on the hydroxyapatite surface until 120-h of incubation. The biofilms were exposed to the treatments for 1 min, double-dip rinsed in sterile saline solution and transferred to fresh culture medium as detailed elsewhere (Koo *et al.,* 2003). The culture medium was replaced daily. Each biofilm was exposed to the respective treatment a total of eight times. The treated biofilms were analyzed for biomass (dry weight) and bacterial viability (colony forming units – cfu/mg of biofilm dry-weight). The biofilms were subjected to sonication using three 30-s pulses at an output of 7W (Branson Sonifier 150; Branson Ultrasomics Co., Danbury, CT, USA); the homogenized

suspension was plated on blood agar by means of a spiral plater (Eddy Jet; IUL Instruments S.A., Barcelona, Spain). This sonication procedure provided the maximum recoverable counts as determined experimentally (Koo *et al* 2003). The extracellular insoluble polysaccharide was extracted using 1 M NaOH (1 mg biofilm dry weight/0.3 ml of 1 M NaOH) and quantified by colorimetric assays as detailed in Koo *et al.* (2003).

Furthermore, aliquots (0.2 mL) of the culture medium were taken daily at specific time points (4, 8, 12 and 24 hours after medium replacement), and pH values measured by a glass electrode (Futura Micro Combination pH electrode, 5mm diameter, Beckman Coulter, Inc, CA, USA).

2.6. Statistical analyses

Triplicates from at least three separate experiments were conducted in each of the assays. An exploratory data analysis was performed to determine the most appropriate statistical test; the assumptions of equality of variances and normal distribution of errors were also checked. The data were then analyzed using ANOVA, and the F-test was used to determine any difference among the groups. When significant differences were detected, pairwise comparisons were made between all the groups using Tukey's method to adjust for multiple comparisons. Statistical software JMP version 3.1 (SAS Institute, Cary, NC, USA) was used to perform the analyses. The level of significance was set at 5%.

3. RESULTS

The isolation and purification methods used in this study yielded highly purified 7-epiclusianone (≥98% purity) from fruits of *R. gardneriana.*

The effects of 7-epiclusianone on the activity of GTF B and C are shown in Table 1. The test agent effectively reduced the glucan synthesis by GTF B (91.7 ± 4.7 %) and GTF C (84.1 ± 2.8 %) at a concentration of 100 μg/ml. However, 7-epiclusianone displayed distinct inhibitory effects on GTF activity; GTF C was inhibited more lower concentrations of 7-epiclusianone (e.g. 12.5 and 25 μg/ml) than GTF B.

Furthermore, the mechanisms of inhibition of GTF B and C by 7-epiclusianone were investigated by kinetic studies. Figure 1 shows the double-reciprocal plots obtained in

the presence of various concentrations of substrate (sucrose) with or without inhibitor (7-epiclusianone at two different concentrations). The mode of inhibition by 7-epiclusianone was non-competitive (mixed inhibition) for GTF B as indicated by plots with different 1/V axis-intercepts and the lines intersecting below the abscissa (K_m decrease) whereas GTF C activity was inhibited in an uncompetitive manner (parallel lines with different 1/V and 1/[S] axis-intercepts with same slope) (Cleland, 1963; Engel, 1981).

The influence of 7-epiclusianone on glycolytic pH-drop by *S. mutans* UA159 cells in the presence of excess glucose is shown in Figure 2. The acid production by *S. mutans* cells was significantly disrupted by 7-epiclusianone at 50 and 100 μg/ml (P<0.05) without displaying any biocidal activity. In addition, the enzymatic activity of the proton-translocating F-ATPase was partially inhibited by 7-epiclusianone at 50 and 100 μg/ml (Table 2); these same concentrations of the agent also showed significantly higher final pH values in the glycolytic pH drop experiments (Fig. 2).

Lastly, we assessed the effects of 7-epiclusianone on biofilm formation by *S. mutans* on saliva-coated hydroxyapatite surface. Topical applications of 7- epiclusianone (at 250 μg/ml; one-minute exposure, twice daily) significantly reduced the formation and accumulation of *S. mutans* biofilms compared with those treated with the vehicle control (P < 0.05) (Table 3). Treatments with 7-epiclusianone resulted in more than 50% less biomass (dry-weight) than did the vehicle control treatment. The total amount of extracellular insoluble polysaccharides in the biofilms treated with 7-epiclusianone was significantly less that in those treated with vehicle control (P<0.05). Furthermore, 7-epiclusianone also reduced the acidogenic properties of the biofilms as indicated by higher pH-values of the surrounding medium at various time points compared to those from vehicle-treated biofilms (Fig. 3).

4. DISCUSSION

The access to biodiversity is fundamental to expanding the range of natural products to be used in the search for new pharmaceutical drugs or leads (Harvey, 2000; Newman *et al.,* 2003). The results presented in this study revealed a novel naturally occurring

molecule, 7- epiclusianone, that effectively disrupt specific virulence traits of *S. mutans* involved in biofilm formation and acidogenicity.

Initially, we examined the effects of 7- epiclusianone on the activity of glucosyltransferases (GTFs). *S. mutans* produces three GTFs: GTF B, which synthesizes mostly insoluble glucan (α1,3-linked); GTF C, which synthesizes a mixture of insoluble and soluble glucan (α1,6-linked); and GTF D, which synthesizes soluble glucan (Loesche, 1986). GTF enzymes have been shown to be essential virulence factors of *S. mutans* associated with the pathogenesis of dental caries. Mutant strains of this organism defective in *gtf* genes are far less cariogenic than parent strains *in vivo*, especially those defective in *gtfB* and/or *gtfC* (Yamashita *et al.,* 1993). The insoluble glucans synthesized by these enzymes provide the structural integrity and bulk to biofilms (Bowen, 2002). Therefore, the effective inhibition of the activity of GTF B and GTF C by 7-epiclusianione may disrupt the development of virulent biofilms related to dental caries. In order to further understand the inhibitory effects of 7-epiclusianone on GTF, we determined the mode of action of this compound on the enzyme activity. The 7-epiclusianone inhibited GTF B activity in a non competitive manner (mixed inhibition), indicating that the test agent interacted with enzyme and enzyme-substrate complex. On the other hand, the mode of inhibition of GTF C by 7-epiclusianone was uncompetitive in nature suggesting that the inhibitor combines with enzyme-substrate complex only. Nevertheless, the net result of either type of inhibition is the decrease of the V_{max} and K_m of both enzymes by the test agent based on Lineweaver-Burk plots (Engel, 1981). The GTFs catalyze two reactions: the cleavage of sucrose into fructose and an enzyme-bound glucosyl moiety (sucrase activity), and the subsequent transfer of the latter to the C-3/C-6 position of the glucose residue of glucan (transferase activity) or to water (Russell, 1990); thus, GTFs have a catalytic domain and a glucan-binding domain (Mooser & Wong, 1988). It is apparent that the GTF inhibition by 7-epiclusianone does not involve the catalytic domain since it was non- or uncompetitive with the substrate sucrose (Engel, 1981). The effective inhibition of GTF C may involve a tight and irreversible binding to the enzymes once complexed. However, further kinetic and binding assays shall be conducted to elucidate the mechanistic details of the inhibitory effects of 7-epiclusianone on GTF enzymes, including GTF D; analysis of the inhibition

profile of GTF D, which catalyzes a similar reaction but involving distinct glycosidic linkages, would contribute to better understanding of the mechanisms of action of 7-epiclusianone.

The effects of 7-epiclusianone on acidogenic and aciduric properties of *S. mutans* were examined by glycolytic pH-drop assays and F-ATPase activity. The *S. mutans* can survive and carry out glycolysis at low pH which can lead to the demineralization of the adjacent dental enamel leading to formation of carious lesions (Loesche, 1986). *S. mutans* cells rapidly degrade glucose and lower the pH value of the suspension until they can no longer maintain a cytoplasmatic pH compatible with activity of glycolytic enzymes. Acid sensitization can be rapidly seen in glycolytic pH-drop experiment in which cells are given excess glucose. Thus, the rate of pH drop reflects acidogenic capacities of the cells, while final pH values of the suspensions reflect acid tolerance. The presence of 7-epiclusianone (at 50 and 100 μg/ml) affected both the acid production and acid tolerance of *S. mutans* cells as indicated by higher final pH values in the pH-drop experiments. The test agent sensitized the cells to acidification to the point that the final pH values were significantly higher (0.7-0.9 units) than those in the presence of vehicle control ($P<0.05$). The effects may be related to disturbances of the net membrane permeability to protons based on the pH-drop curves (Fig. 2) and lack of any biocidal activity (or effects on growth rate within 2 hours of incubation) by 7-epiclusianone at the concentrations tested. Protons from the environment diffuse inward across the cell membrane after acidification of the suspension but can be extruded by the F-ATPase of the cell membrane. Thus, the F-ATPase protects *S. mutans* against environmental acid stress by regulating pH homeostasis, which is critical for the optimum function of glycolysis in *S. mutans* (Sturr & Marquis, 1992). Enolase and other enzymes of the glycolytic pathway and the sugar transport system are sensitive to cytoplasmic acidification (Belli *et al.,* 1995). Our data suggest that one of the mechanisms by which 7-epiclusianone modulates the acidogenicity of *S. mutans* involve partial inhibition of proton-translocating F-ATPase activity. Overall, the F-ATPase sensitivities to 7-epiclusianone agree well with the pH drop data. Furthermore, weak acids, such as 7-epiclusianone (Santos *et al.,* 1999), are known to cause acidification of the cytoplasm of cells in acid environment by acting as a transmembrane proton transporter (Marquis *et al.,*

2003). The combination of weak-acid effect and inhibition of F-ATPase may have affected the ΔpH across the membrane, which could disrupt the glycolyis by *S. mutans* cells. Whether, 7-epiclusianone can inhibit the glycolytic enzymes directly awaits further evaluation.

Streptococcus mutans in the mouth are primarily in plaque biofilms, so we assessed the effects of short-term topical application (one-minute exposure, twice daily) on biomass and extracellular insoluble polysaccharides content of *S. mutans* biofilms formed on apatic surface covered by salivary pellicle. The regimen of one-minute exposure and daily treatments was selected for this experiment to simulate the likely exposure of test agents at clinical level. Higher level of the agent was used for biofilms because of higher biomass densities of biofilms and previous findings that biofilms are less sensitive to 7-epiclusianone than cells in suspension, depending on biomass concentration; the test concentration of 250 μg/ml was selected based on our preliminary dose-response studies and lack of solubility of higher concentrations in the vehicle solution (Murata *et al.,* 2006).

Viability of the biofilms (as assessed by determination of colony forming units/mg of biofilm dry-weight) was not impacted by topical applications of 7-epiclusianone (data not shown) likely due to the brief exposure to the agent and higher bacterial densities in biofilms. Nevertheless, 7-epiclusianone significantly disrupted the accumulation and polysaccharide composition of *S. mutans* biofilms compared with the control, reducing both the biomass and total amount of insoluble polysaccharides. The insoluble polysaccharides, which are comprised of mostly 1→3 and 1→6 linkages, and branch points of 3,4-, 3,6- and 3,4,6-linked glucose (synthesized mostly by GTF B and C), are the major components of the extracellular polysaccharide matrix, which are associated with the development, bulk and cariogenicity of dental biofilms (Bowen, 2002; Paes Leme *et al*., 2006). The reduction of the biomass of biofilms treated with 7-epiclusianone is proportional to that of extracellular insoluble polysaccharides in the biofilm matrix. This observation is consistent with the effective inhibition of GTF B and C observed in this study suggesting that disruption of insoluble glucan synthesis is one of the mechanisms by which the test agent reduced biofilm formation and accumulation. Although *S. mutans* cells with biofilms were

less sensitive to the test agent than those in suspensions, the rate of biofilm acid production was still inhibited even with brief exposures to 7-epiclusianone, which agrees at some extent with the pH-drop studies using cells of *S. mutans* in suspension.

Overall, the data show that the 7-epiclusianone is a promising naturally occurring agent displaying multiple inhibitory effects which may be working in concert to inhibit the development and acidogenicity of *S. mutans* biofilms *in vitro*. The putative pathways by which 7-epiclusianone affect *S. mutans* virulence may involve at least three routes: (1) inhibition of glucan synthesis, particularly those synthesized by GTF C, and (2) disruption of acid production and (3) acid tolerance. We are currently pursuing the potential anti-biofilm and cariostatic properties of 7-epiclusianone *in vivo*.

5. ACKNOWLEDGEMENT

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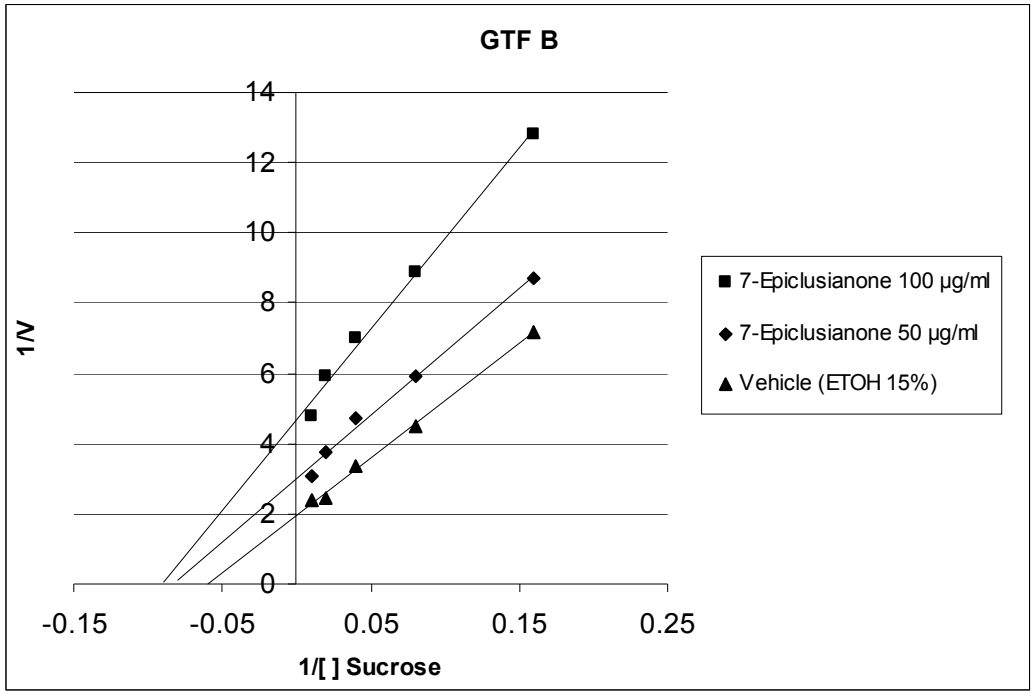
Table 1. Effects of 7-epiclusianone on the activities of glucosyltransferases B (GTF B) and C (GTFC).

7-epiclusianone (μg/mL)	GTF B (% of inhibition)	GTF C (% of inhibition)
100.0	91.7 ± 4.7	84.1 ± 2.8
50.0	74.1 ± 5.3	73.2 ± 5.0
25.0	11.5 ± 2.1	68.6 ± 5.2
12.5	n.d.	62.3 ± 6.9

The percentage of inhibition was calculated considering the vehicle control as 100% GTF activity. Values (s.d., $n = 9$) from each of the concentrations tested are significantly different from vehicle control ($P<0.05$, ANOVA, comparison for all pairs using Tukey test), except at 12.5 μg/ml for GTF B ($P>0.05$).

n.d., not detected.



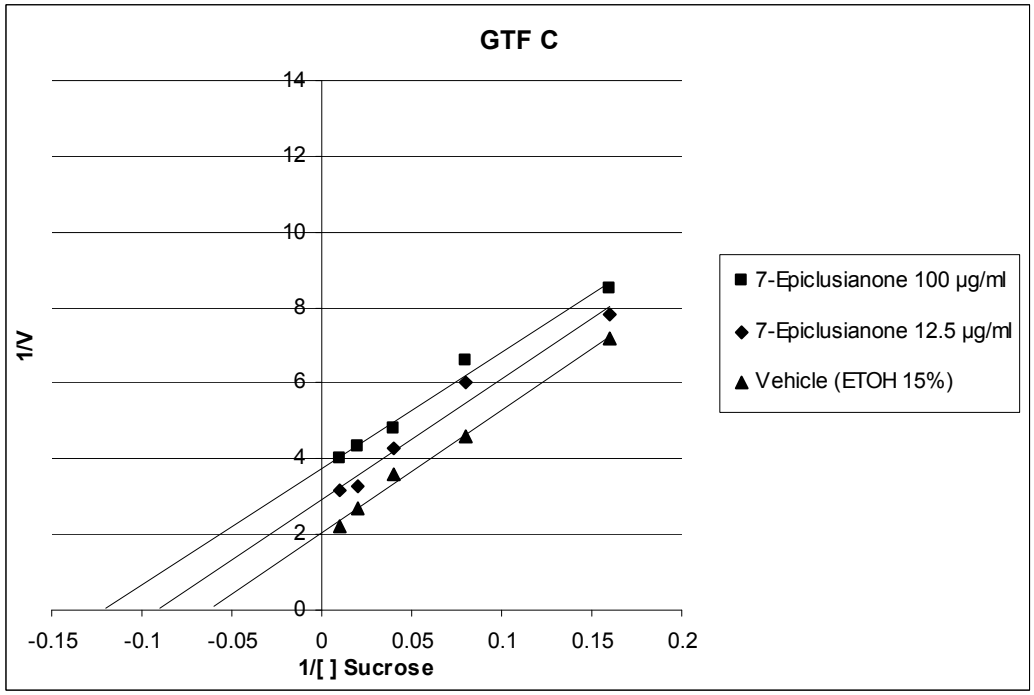


Figure 1. Double-reciprocal (Lineweaver-Burk) plots of GTF activity versus the amount of sucrose in the presence of 7-epiclusianone or vehicle control.

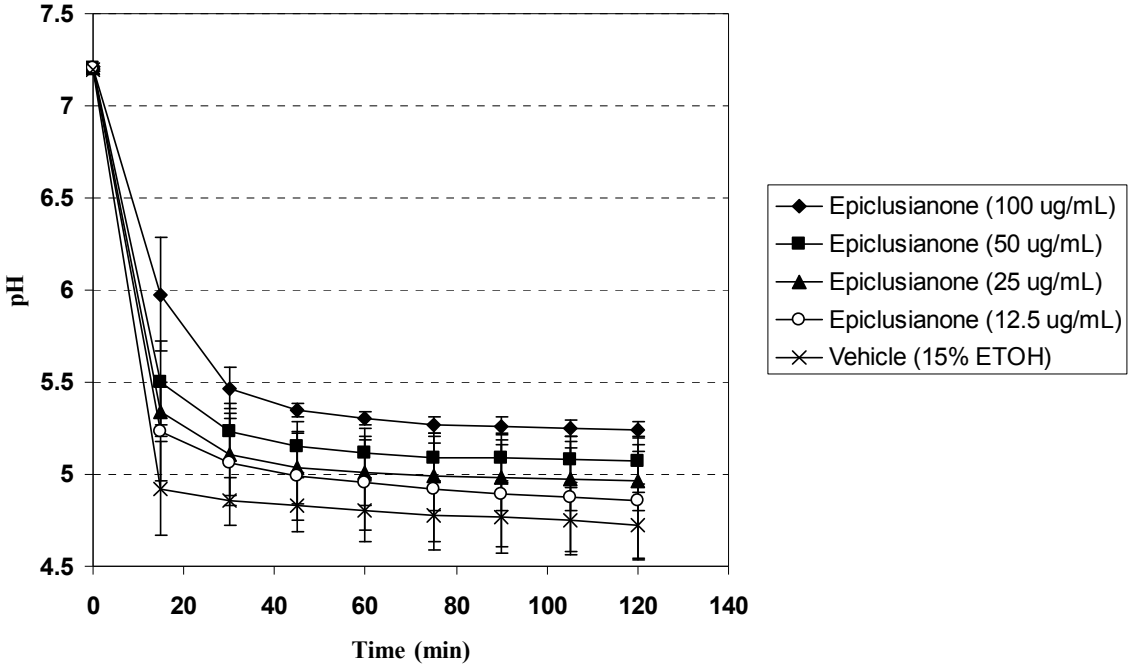


Figure 2. Influence of 7-epiclusianone on glycolytic pH-drop of *S. mutans* UA159 in suspensions. Values (s.d., n=9) from vehicle control, 7-epiclusianone at 50 and 100 µg/ml are significantly different from each other at all time points (P<0.05, ANOVA, comparison for all pairs using Tukey test).

Table 2. Influence of the 7-epiclusianone on F-ATPase of permeabilized cells of *S. mutans* UA159.

7-epiclusianone (μg/mL)	F-ATPase (% of inhibition)
100.0	61.09 ± 3.0
50.0	15.40 ± 6.7
25.0	n.d.
12.5	n.d.

The percentage of inhibition was calculated considering the vehicle control as 100% F-ATPase activity. Values (s.d., n = 9) from 7-epiclusianone at 50 and 100 μg/ml are significantly different from vehicle control (P<0.05, ANOVA, comparison for all pairs using Tukey test).

n.d., not detected.

Table 3. Biomass (dry-weight) and total amount of extracellular insoluble polysaccharides in the biofilms after treatments.

Treatments	Dry- weight (mg)	Extracellular insoluble polysaccharides (mg)
7-Epiclusianone (250 μg/ml)	2.2 ± 0.2	0.86 ± 0.16
Vehicle control (15% ethanol, v/v)	5.0 ± 0.1	1.67 ± 0.24

Values (s.d., $n = 9$) from vehicle control and 7-epiclusianone treatments are significantly different from each other ($P<0.05$, ANOVA, comparison for all pairs using Tukey test)

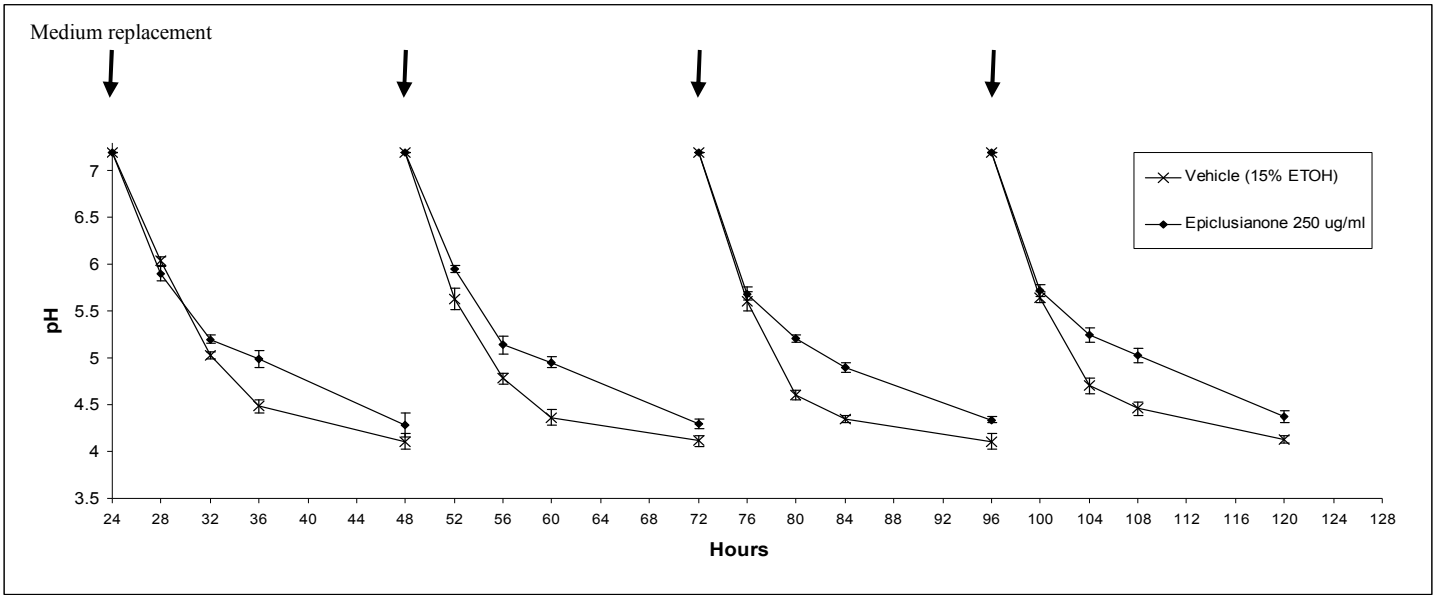


Figure 3. Influence of 7-epiclusianone on the pH values in the culture medium during *S. mutans* biofilm formation. The medium was replaced daily with fresh medium. The pH values (n = 9) were determined after 4, 8, 12 and 24 h of incubation.

2.2 Estudo 2

Inhibitory effects of 7-epiclusianone associated with fluoride on *S. mutans* biofilm and dental caries

Running Title: Influence of 7-epiclusianone on caries.

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ABSTRACT

7-epiclusianone is a naturally occurring agent that affects the development of biofilm-related oral diseases. Fluoride (F) interferes physicochemically with caries development and also exhibits antibacterial activity. We examined whether the association of 7-epiclusianone (7-epi) enhance the anti-caries properties of F by acting cooperatively on the expression of virulence of *Streptococcus mutans* biofilm and development of caries in rats. Biofilms of *S. mutans* UA159 were treated topically twice daily as follows: (1) 7-epi 250 μg/mL; (2) 7-epi 250 μg/mL + 125 ppm F; (3) Ethanol 15% v/v (vehicle control) and (4) 250 ppm F (positive control). *In vivo* study, 48 Wistar SPF rats were infected with *Streptococcus mutans* UA159 and treated twice daily with 7-epi, alone or in combination with F. Furthermore, from each rat 14 organs/tissue were removed for toxicological investigation (hematoxylin and eosin stain). Biofilms treated with 7-epi, alone or in combination with F, displayed less biomass and insoluble glucans than the positive control ($p<0.05$). The combination of 7-epi with F was highly effective in preventing caries development in rats and results were comparable with those observed with in positive control ($p>0.05$). Histologically, no abnormality was observed. In conclusion, the combination of 7-epi with fluoride may represent a potentially useful alternative approach to prevent caries disease, by reducing concentration of F without any toxicological effect.

1. INTRODUCTION

Colonization of tooth surfaces by mutans streptococci is associated with the etiology and pathogenesis of dental caries in humans (Loesche, 1986; Beighton, 2005). Glucans, synthesized from dietary sucrose by glucosyltransferases (GTFs), enhance the pathogenic potential of dental plaque by promoting the adherence and accumulation of cariogenic streptococci on the tooth surface, and by contributing to the bulk and structural integrity of plaque (Tanzer *et al.,* 1985; Yamashita *et al*., 1993). Furthermore, *S. mutans* survives and carries out glycolysis at low pH values attained within the matrix of the biofilms, which results in demineralization of the adjacent dental enamel (Belli and Marquis, 1991; Bowen, 2002).

Fluoride, in various vehicles, is the most effective anti-caries agent known (Clarkson, 2000; NIH, 2001). Nevertheless, dental caries remains a significant problem in many countries, including the United States, and continues at a high level in susceptible subpopulations, especially among economically underprivileged children (NIH, 2001). Fluoride exerts its major effect by reducing demineralization and enhancing remineralization of early caries lesions (Dawes and Ten Cate, 1990). Furthermore, there is a plethora of evidence which shows that fluoride can affect the biological activities of cariogenic streptococci (Hamilton, 1990; Marquis *et al.,* 2003).

Enhancement of the protective effects of fluoride by the inclusion of substances which affect the virulence of cariogenic bacteria and/or enhance the antibacterial effects of fluoride offers an attractive route to reducing the prevalence of dental caries. It is generally accepted that the effectiveness of fluoride can be enhanced when it is combined with additional cariostatic agents (NIH, 2001). Recently, we have shown that extracts of *Rheedia brasiliensis* exhibited antibacterial effects against *S. mutans*, and 7-epiclusianone was identified as the putative bioactive compound and displaed multiple inhibitory effects which may be working in concert to inhibit the development and acidogenicity of *S. mutans in vitro* (Murata *et al.,* 2006; Almeida *et al*., 2008). In this study, we followed an alternative approach, using 7-epiclusianone to enhance the biological effects of fluoride against *S. mutans* biofilm (*in vitro*) and development of caries in rats.

2. MATERIAL AND METHODS

2.1. Test agents

The fruits of *Rheedia brasiliensis* 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 was deposited (#VIC2604). 7-epiclusianone (7-epi) was extracted, isolated and purified from the fruit pericarp as detailed elsewhere (Almeida *et al*., 2008; Santos *et al.,* 1999). The substance was identified from the infrared, ultraviolet, mass spectra data and the NMR spectra data and confirmed by comparison with data from literature (Almeida *et al*., 2008; Santos *et al.,* 1999); the purity level was >98% as determined by HPLC (Almeida *et al*., 2008; Santos *et al.,* 1999). Sodium fluoride (NaF) was purchased from sigma Sigma-Aldrich Co. (St. Louis, MO, USA). For this study, we tested 7-epi (250 μg/ml), alone or in combination with NaF (125 ppm F), vehicle control was 15% ethanol (v/v) and the NaF at 250 ppm has known clinical effectiveness and served as positive control (Koo *et al.,* 2005).

2.2 Biofilm assays

Biofilms of *S. mutans* UA159 were formed on saliva-coated hydroxyapatite discs placed in a vertical position (HAP ceramic – calcium hydroxyapatite, 0.5" diameter – Clarkson Calcium Phosphates, Williamsport, PA) in batch cultures at 37^{o} C and 10% CO_2 (Koo *et al.,* 2003). Biofilms of *S. mutans* were formed in ultrafiltered (Amicon 10 kDa molecular weight cut-off membrane; Millipore Co., MA, USA) tryptone-yeast extract broth with addition of 30 mM sucrose (Koo *et al.,* 2003). The biofilms were grown undisturbed for 24 h to allow initial biofilms formation. At this point (24 h-old), the biofilms were treated twice daily (10 a.m. and 4 p.m.) until the fifth day of the experimental period (120 h-old biofilm). The biofilms were exposed to the treatments for 1 min, double-dip rinsed in sterile saline solution and transferred to fresh culture medium as detailed elsewhere (Koo *et al.,* 2003). The culture medium was replaced daily. Each biofilm was exposed to the respective treatment a total of eight times. The treated biofilms were analyzed for biomass (dry weight), polysaccharide composition and protein content by colorimetric assays as detailed in Koo *et al.* (2003).

2.3 Animal study

The animal experiment was reviewed and approved by the Ethical Committee on Animal Research at the State University of Campinas – UNICAMP (Protocol # 963-1) and was performed according to methods described previously (Koo *et al.*, 2002). At weaning, pups aged 21 days were infected by *S. mutans* UA159, and randomly placed into 4 groups of 12 animals, and their teeth were treated topically by means of a camel's hair brush twice daily, as follows: (1) 7-epi 250µg/ml, (2) 7-epi + 125 ppm F, (3) vehicle control (15% ethanol, as negative control), or (4) 250 ppm F (as positive control). Each group of 12 animals was provided with diet 2000 (which contains 56% sucrose) and 5% sucrose water *ad libitum*. The animals were weighed weekly, and their physical appearance was noted daily. The experiment proceeded for 5 weeks, at the end of which the animals were sacrificed by CO_2 asphyxiation and microbiological assessment and the scored caries were assayed by using the modified Keyes method caries evaluation (Larson 1981). Samples of heart, lung, pancreas, spleen, liver, stomach, intestine, kidney, ovary, brain, eyes, tong, palatal tissue and submandibular glands were taken and fixed with formalin 12% for 24–48 h. Thereafter, the tissue samples were dehydrated in a graded alcohol series and embedded in paraffin. Five µm sections were cut in a microtome and stained with hematoxylin and eosin to be examined under a lighted microscope.

2.4 Statistical Analyses

For the *in vitro* studies, the data were analyzed by ANOVA, and the F-test was used to detect difference between and among the groups. When significant differences were detected, pair wise comparisons were made among all the groups by Tukey's method to adjust for multiple comparisons. The data from animal study were subjected to ANOVA and Tukey-Kramer Honest Standard Deviation (HSD) test for all pairs. Statistical software JMP version 3.1 (SAS Institute, Cary, NC, USA) was used to perform the analyses. The level of significance was set at 5% for both studies.

3. RESULTS

The biomass, polysaccharides and protein compositions of the biofilms treated with test agents are shown in Table 1. Short-term daily topical application of 7-epi and 7-epi + 125 ppm F diminished significantly the formation and accumulation of *S. mutans* biofilms *in vitro*, compared with the control ($p < 0.05$). The 7-epi and 7-epi + 125 ppm F treatments resulted in a approximately 50% reduction in biomass and the amount of insoluble and soluble extracellular polysaccharides in the biofilms were significantly less than in those treated with control solutions ($p < 0.05$), protein was affected similarly. None of the test agents displayed bactericidal activity. 7-epi did not display significant effect on the amount of intracellular iodophilic polysaccharides. In contrast, the amount of iodophilic polysaccharide was drastically reduced by F, alone or in combination (7-epi + 125 ppm F).

In the animal study, the rats remained in apparent good health during the 5 weeks of the experiment and the weight gains [80.8 g (±15.1) to 87.3 g (±16.3)] were not significantly different among the treatment groups ($p > 0.05$). The percentage of *S. mutans* UA 159 recovered from the jaws (Table 2) of the animals treated with 7-epi, alone or in combination with F, did not differ statistically from that of the control group ($p > 0.05$). However, the group treated with 7-epi displayed significantly lower counts of total populations compared with the vehicle control ($p < 0.05$). The effects of the treatments on the incidence and severity of smooth-surface caries are shown in Table 3. Both, 7-epi and 7-epi + 125 ppm F, treatments significantly reduced the incidence of total smooth-surface and sulcal caries compared with the vehicle control group ($p < 0.05$) and 7-epi +125 ppm F did no differ from 250 ppm F group (positive control). In addition, the severity of the smooth-surface and sulcal caries were reduced by 7-epi and 7-epi + 125 ppm F ($p < 0.05$). The macroscopic and histological analysis did not show any alteration on the organ or tissues of the groups treated with the compounds when it was compared with the control groups (data not shown).

4. DISCUSSION

The mechanisms that F promotes the anti-caries effects are known and involve at least two routes: (1) acidifies the cytoplasm of *S. mutans* and inhibits intracellular enzymes

(Marquis *et al.*, 2003) and (2) promotes the remineralization of teeth and enhancing the tooth resistence by fluorapatite creation (Ten Cate, 1999). However, the fluoride therapy carry a risk of dental fluorosis (Mascarenhas 2000). In order to reduce the side effects of F, are accepted that the effectiveness of fluoride can be enhanced when it is combined with cariostatic agents (NIH, 2001).

The findings in our study suggest that 7-epi acts in conjuction with low concentrations of F (125 ppm) on the virulence of *S. mutans*. The combination with 7-epi +125 ppm F caused, *in vitro*, significant changes in the soluble, insoluble and intracellular polysaccharide content of the biofilms. The insoluble polysaccharides are extracellular glucans which are essential for the adherence, accumulation and structural integrity of the biofilms (Bowen 2002). The reduction of the amount of biomass and insoluble polysaccharides content in the biofilms is consistent with the effective inhibition of GTFs by 7-epi, observed previously (Murata *et al*., 2006). The GTFs enzymes are responsible for the synthesis of insoluble glucans, which are critical in the expression of virulence in the pathogenesis of dental caries (Yamashita *et al.*, 1993). Furthermore, the association of 7-epi +125 ppm F and the positive control caused significant changes in the intracellular polysaccharide (IPS) content of the *in vitro* biofilms. Koo *et al*., 2005 suggest that the *S. mutans* biofilms with the least amount of IPS had low acidogenicity, because the IPS provides an endogenous source of carbohydrate which can be metabolized when the exogenous fermentable substrate has been depleted in the oral cavity (Hamilton, 1976).

Analysis of the *in vitro* experiment showed that topical application twice a day of 7-epi and 7-epi + 125 ppm F significantly reduced incidence and severity of caries, and the association resulted in enhanced cariostatic properties which did no differ from the positive control. The putative pathway by which 7-epi + 125 ppm F affect the cariogenicity of *S. mutans* may be related with the biological effects of 7-epi. According to previously studies (Murata *et al.*, unpublished data) 7-epi inhibited the acid production, acid tolerance and affected F-ATPase activity of *S. mutans* cells. The F-ATPase, membrane associated enzyme, pumps protons (H+) out of the cell in association with ATP hydrolysis to maintain intracellular pH more neutral than extracellular pH. Therefore, mutans streptococci survive and carry out glycolysis at low extracellular pH values commonly found in the plaque's

matrix; this ability to tolerate an acidic environment is critical for the expression of virulence by these cariogenic organisms. By inhibiting the activity of F-ATPase, 7-epi could disrupt intracellular pH which would also affect the pH-sensitive glycolitic enzymes, thereby reducing the ability of the microorganisms to produce acids. Thus, 7-epi acting cooperatively with fluoride could reduce the acid production in the biofilm without affecting the percentage of total microorganisms. In addition, details of the toxicology of 7-epi, alone or associated with F, were investigated and no alterations or adverse reactions in our animal study were observed. Also, there is no evidence in the literature showing that 7-epi has potential toxicity.

In conclusion, the combination of these novel agents with fluoride may represent a potentially useful alternative approach to the current chemotherapeutic strategies to prevent caries disease. It has been demonstraded that it is possible reduce the concentration of F without necessarily reducing the therapeutic effect or increasing the toxicological effects.

5. ACKNOWLEDGEMENT

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Table 1. Effects of 7-epiclusianone (7-Epi), fluoride (F) and associations on the composition of *Streptococcus mutans* UA159 biofilm.

Treatments*	Dry-weight (mg)	Total Protein (mg)	Insoluble Polysaccharide (mg)	Soluble Polysaccharide (mg)	Iodophilic Polysaccharide (mg)
Vehicle control	5.0 (0.1)[a]	1.9 (0.4)[a]	1.6(0.2)[a]	1.8 (0.2)[a]	0.65(0.01)[a]
7-Epi 250 μg/mL	2.2 (0.2)[b]	0.7 (0.2)[b]	0.8(0.1)[b]	0.9 (0.1)[b]	0.70(0.02)[a]
7-Epi 250 μg/mL+125 ppm F	2.2 (0.2)[b]	0.6 (0.1)[b]	0.9(0.1)[b]	0.9 (0.2)[b]	0.43(0.01)[b]
Positive control (250 ppm F)	4.6 (0.2)[a]	2.0 (0.5)[a]	1.7(0.2)[a]	1.9 (0.1)[a]	0.42(0.01)[b]

Values (SD, N = 12) with same letter are not significantly different from each other (P > 0.05, ANOVA, comparison for all pairs using Tukey test).
*Twice daily with one-minute exposure for each treatment

Table 2. Effects of 7-epiclusianone (7-Epi), fluoride and associations after five-week experiment on oral microbiota in rats: means (SD).

Treatment	Total microorganisms ($x10^5$ CFU/mL)	*Streptococcus mutans* UA159 ($x10^5$ CFU/mL)	*Streptococcus mutans* UA159 (%)
Vehicle control	4.0 (3.0)[a]	2.9 (2.5)[a]	61.5 (22.1)[a]
7-Epi 250 μg/mL	1.6 (0.7)[b]	1.1 (0.4)[a]	76.9 (32.4)[a]
7-Epi 250 μg/mL+125 ppm F	3.0 (1.1)[a]	1.7 (0.8)[a]	56.1 (17.2)[a]
Positive control (250 ppm F)	2.0 (0.6)[a,b]	1.2 (0.3)[a]	61.9 (13.6)[a]

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

Table 3. Effects of 7-epiclusianone (7-Epi), fluoride and associations on caries development (smooth-surface, sulcal and dentin severity) in rats, after five-week experiment: means (SD) of Keyes' scores modify by Larson.

Treatment	Smooth-Surface				Sulcal			
	Total	Severity			Total	Severity		
		Ds	Dm	Dx		Ds	Dm	Dx
Vehicle control	**76.2(10.1)[a]**	**42.5(11.8)[a]**	**18.3(7.4)[a]**	**6.4(7.2)[a]**	**46.5(4.3)[a]**	**38.2(2.8)[a]**	**30.8(5.1)[a]**	**21.0(7.7)[a]**
7-Epi 250 μg/mL	**62.9(7.4)[b]**	**25.5(7.8)[b]**	**3.3(4.8)[b]**	**1.9(3.5)[b]**	**39.7(5.6)[b]**	**24.8(5.3)[b]**	**14.7(5.2)[b]**	**5.3(4.5)[b]**
7-Epi 250 μg/mL+ 125 ppm F	**33.2(2.6)[c]**	**21.0(7.7)[b]**	**0.3(0.5)[b]**	**0.0(0.0)[b]**	**25.7(3.0)[c]**	**13.4(4.7)[c]**	**2.2(1.9)[c]**	**0.6(1.0)[c]**
Positive control (250 ppm F)	**34.2(4.5)[c]**	**22.5(6.5)[b]**	**0.5(0.8)[b]**	**0.0(0.0)[b]**	**28.5(3.1)[c]**	**15.7(3.6)[c]**	**2.7(1.5)[c]**	**0.3(0.8)[c]**

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

2.3 Estudo 3

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Uso de composto isolado da *Rheedia brasiliensis* na prevenção e/ou tratamento de doenças

Relatório Técnico submetido a INPI como requisito para solicitação de registro de patente.

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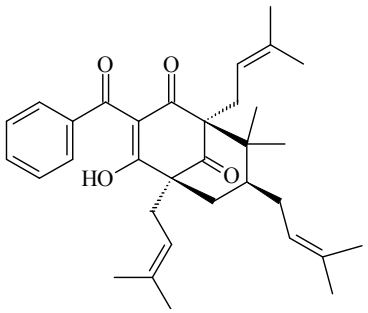
Piracicaba
2006

Uso de composto isolado da *Rheedia brasiliensis* na prevenção e/ou tratamento de doenças.

A presente invenção se refere ao uso de um composto isolado da *Rheedia brasiliensis,* planta conhecida com o nome de Bacupari liso, Bacuri ou Bacuripari, como agente antimicrobiano no tratamento e prevenção de doenças. Mais especificamente, a presente invenção se refere ao potencial antimicrobiano da 7-epiclusianona e a sua utilização na formulação de produto(s) para a prevenção e/ou tratamento de cáries dentais, inibição da formação da placa dental (biofilme), gengivites, estomatites e ulcerações.

A 7-epiclusianona [3-benzoil-4-hidroxi-6,6-dimetil-1,5,7-tris(3-metil-2-butenil)biciclo[3.3.1]non-3-ene-2,9-diona], isolada da *Rheedia brasiliensis*, é uma nova forma isômera da $C_{33}H_{42}O_4$, combinação identificada por análise de difração de Raios X, conforme fórmula estrutural abaixo:

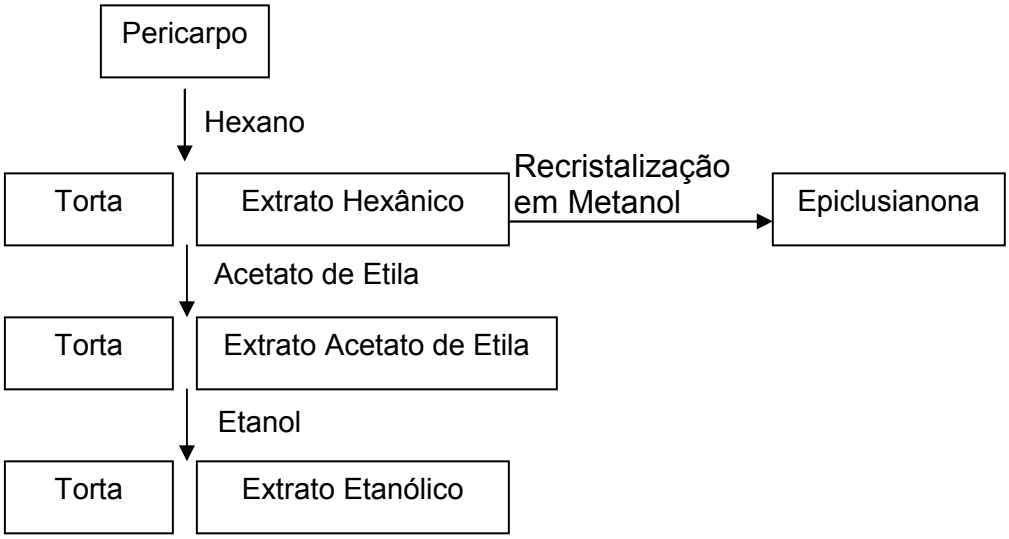


Os frutos maduros de Rheedia brasiliensis são coletados e o pericarpo é submetido à extração sucessiva com hexano, etanol e acetato de etila em aparelho de Soxhlet por 24 horas (SANTOS et al., Estudo químico dos frutos de Rheedia gardneriana Pl. e Tr. e aplicações biológicas dos seus constituintes. Viçosa: UFV, 1996; Epi-clusianone: a new natural product derivative of bicyclo[3.3.1]nonane-2,4,9-trione. Acta Cryst. C54, Pag. 1990-1992, 1998; 7-Epiclusianona, a nova benzofenona tetraprenilada e outros constituintes químicos dos frutos de Rheedia gardneriana. Quím. Nova, vol.22, no.5, p.654-660, 1999).

Os componentes extraídos são concentrados sob pressão reduzida em evaporador-rotatório, obtendo-se assim, extrato hexânico (REH), extrato de acetato de etila (REA) e o

extrato etanólico (REE) e o do epicarpo. Do extrato hexânico do epicarpo de *R. brasiliensis* é isolado o composto 7–epiclusianona (componente principal) (SANTOS *et al.,* Estudo químico dos frutos de *Rheedia gardneriana* Pl. e Tr. e aplicações biológicas dos seus constituintes. Viçosa: UFV, 1996; Epi-clusianone: a new natural product derivative of bicyclo[3.3.1]nonane-2,4,9-trione. Acta Cryst. C54, Pag. 1990-1992, 1998; 7-Epiclusianona, a nova benzofenona tetraprenilada e outros constituintes químicos dos frutos de *Rheedia gardneriana*. Quím. Nova, vol.22, no.5, p.654-660, 1999).

O fluxograma de obtenção dos extratos e do composto 7-epiclusianona é apresentado na Figura 1.



Cada extrato obtido é submetido à cromatografia de camada delgada e posteriormente purificado por cromatografia em coluna utilizando como eluente misturas de hexano, acetato de etila e etanol em ordem crescente de polaridade, sendo as frações reagrupadas e segundo análise por cromatografia em camada delgada e comparação com os respectivos padrões (SANTOS *et al.,* Estudo químico dos frutos de *Rheedia gardneriana* Pl. e Tr. e aplicações biológicas dos seus constituintes. Viçosa: UFV, 1996; 7-Epiclusianona, a nova benzofenona tetraprenilada e outros constituintes químicos dos frutos de *Rheedia gardneriana*. Quím. Nova, vol.22, no.5, p.654-660, 1999).. Do extrato hexânico é obtido a 7-epiclusianona (componente principal) através do processo de recristalização em metanol (ou etanol). Sua pureza é checada pelo ponto de fusão e análise cromatográfica

analítica (SANTOS *et al.,* Estudo químico dos frutos de *Rheedia gardneriana* Pl. e Tr. e aplicações biológicas dos seus constituintes. Viçosa: UFV, 1996; Epi-clusianone: a new natural product derivative of bicyclo[3.3.1]nonane-2,4,9-trione. Acta Cryst. C54, Pag. 1990-1992, 1998; 7-Epiclusianona, a nova benzofenona tetraprenilada e outros constituintes químicos dos frutos de *Rheedia gardneriana*. Quím. Nova, vol.22, no.5, p.654-660, 1999).

A cavidade bucal, de modo similar a outros sítios do corpo humano apresenta uma microbiota natural com composição característica que, na maioria das vezes, coexiste de modo harmônico com o hospedeiro. Entretanto, a maioria dos indivíduos sofrem, em algum período de sua vida, episódios localizados de doenças bucais causados por um desequilíbrio na composição da microbiota bucal residente. As manifestações clínicas deste desequilíbrio incluem a cárie dental e a doença periodontal. Um dos fatores etiológicos mais importantes das doenças cárie e doença periodontal são os microrganismos de origem bacteriana. Estes formam um biofilme patogênico que se adere sobre a superfície dental, de modo a produzir ácidos e produtos citotóxicos que levam, respectivamente, à desmineralização do esmalte e/ou inflamação gengival (MARSH, Microbial ecology of dental plaque and its significance in health and disease. Adv Dent Res, Washington, 8: 263-71, 1994).

Um dos fatores de desequilíbrio fundamental para o aparecimento de um biofilme cariogênico é a dieta rica e freqüente de carboidratos fermentáveis, principalmente a sacarose. Esta dieta promove um aumento da proporção de estreptococos do grupo mutans, como *Streptococcus mutans* e *Streptococcus sobrinus*, uma vez que estes microrganismos apresentam vantagens ecológicas quando na presença de sacarose no meio bucal. Pois os estreptococos do grupo mutans, além de serem acidogênicos e acidúricos, não só fermentam a sacarose, como a partir dessa, sintetizam polissacarídeos extracelulares (HAMADA, S.; SLADE, H.D. Biology, immunology and cariogenicity of *Streptococcus mutans* and dental caries prevention. J Dent Res, Washington, v.63, p.407-11, 1980; GIBBONS, R. J. Adherence interactions that may affect microbial ecology in the mouth. J Dent Res, Washington, v. 63, p. 378-85, 1984).

Deste modo, muitos agentes de origem natural vêm sendo explorados nas últimas décadas (KOO *et al*. Effect of new variety of *Apis mellifera* propolis on mutans

streptococci. Current Microbiology. Estados Unidos da América, v 41, p.192-198, 2000), devido as suas possíveis ações farmacológicas. Entre os produtos naturais têm-se destacado os extratos e o composto (7-epiclusianona) da planta do gênero *Rheedia,* que fazem parte da família *Guttiferae* e têm demonstrado, pelos estudos químicos, ser possuidor de uma grande diversidade de classes estruturais de compostos químicos. Destacam-se os flavonóides, proantocianinas, xantonas e as benzofenonas polipreniladas, que têm ações comprovadas com princípios ativos contra várias doenças de origem infecciosas, entre outras (BRAZ-FILHO *et al*., Xantones of *Rheedia gardneriana*. Phytochemistry 9, 673, 1970; DELLE MONACHE *et al*., Chemical investigation of the genus *Rheedia*. II Prenylated xanthone from *Rheedia gardneriana*. J. Nat. Prod. 46, 655, 1983; Nemoronosol, a derivative of tricycle-[4.3.1.0]-decane-7-hodroxy-2,9-dione from *Vismia decipiens*. Phytochemistry 27, 2305, 1988). Neste contexto, a 7-epiclusianona, um produto natural inexplorado, é um valioso composto bioativo.

A determinação da atividade antimicrobiana da 7-epiclusianona compreende as seguintes etapas:

a) Preparo da Suspensão Bacteriana

O Estreptococos do Grupo Mutans (*Streptococcus mutans* UA 159) é inicialmente reativado a partir da sua cultura original (mantida em meio líquido BHI contendo 20% de glicerol, a -80^0C ou liofilizadas) e posteriormente cultivado em placas de BHI ágar contendo 5% de sangue de carneiro desfibrinado estéril, e incubados nas seguintes condições: temperatura de 37^0C, em atmosfera de 10% CO_2, por 18-24 horas. Colônias isoladas foram suspensas em 5mL de solução de NaCl 0,89% esterilizadas até atingir uma turbidez equivalente a 0,5 da escala de McFarland. A suspensão assim obtida é utilizada para as determinações descritas a seguir.

b) Concentração Mínima Inibitória (CIM)

A determinação do CIM foi realizada em meio líquido, pela técnica de macro-diluição. Para determinação do CIM em meio líquido, a suspensão bacteriana, como descrita no item anterior, é homogeneizada com meio líquido BHI na proporção 1:1000 (v/v) obtendo-se uma concentração bacteriana em torno de 1-2 x 10^5 ufc/ml (PINDOK, Techniques used for the determination of antimicrobial resistance and sensitivity in

bacteria. J Appl Bacteriol, Oxford, 68: 307-18, 1990; PHILLIPS, A guide to sensitivity testing. J Antimicrob Chemother, London, 27: 1-50, 1991). Após homogeneizada, 4,96 ml são transferidos em tubos de vidro esterilizados, sendo posteriormente pipetados 40µl dos extratos da *R. brasiliensis* e o composto isolado 7–epiclusianona (com concentração final variando de 12,5 a 800µg/ml) ou controle (80% etanol, v/v). Os tubos são, em seguida homogeneizados com auxílio de um vortex em baixa velocidade e incubados nas mesmas condições descritas no item anterior.

O CIM foi considerado a menor concentração dos compostos onde não houve crescimento bacteriano visível, ou seja, uma leitura da absorbância a 660 nm menor que 0,05.

c) Determinação da Concentração Bactericida Mínima (CBM)

Os tubos incubados para determinação da CIM em meio líquido são utilizados para determinação da CBM (PINDOK, Techniques used for the determination of antimicrobial resistance and sensitivity in bacteria. J Appl Bacteriol, Oxford, 68: 307-18, 1990; PHILLIPS, A guide to sensitivity testing. J Antimicrob Chemother, London, 27: 1-50, 1991). Uma alíquota (50µl) é inoculada, com auxílio da "Spiral-plater" (Whittley Automatic Spiral Plater da DW Scientific), em placas de BHI ágar suplementado com 5% de sangue de carneiro. E posteriormente as placas são incubadas nas mesmas condições descritas no item anterior. A CBM foi considerada a menor concentração dos compostos onde não houve crescimento celular sobre a superfície do ágar inoculado (99,9% de morte microbiana).

d) Determinação da Atividade das GTF B e C

As enzimas GTF B e C são obtidas de sobrenadantes de culturas bacterianas e purificadas utilizando uma coluna de hidroxiapatita, com descrito por VENKITARAMAN *et al.* (1995) (VENKITARAMAN, A.R. et al. Characterization of glucosyltransferase B, GtfC, and GtfD in solution and on the surface of hydroxyapatite. J dent. Res., Washington, v.74, p.1694-1701, 1995). A atividade das GTFs são medidas pela incorporação de [^{14}C] glicose proveniente de sacarose marcada (NEN Research Products, Boston, Mass., USA) nos glucanos (Koo H, *et al*. Effect of new variety of Apis mellifera Propolis on mutans

streptococci. Current Microbiology. Estados Unidos da America: , v.41, p.192 - 198, 2000; VENKITARAMAN, A.R. et al. Characterization of glucosyltransferase B, GtfC, and GtfD in solution and on the surface of hydroxyapatite. J dent. Res., Washington, v.74, p.1694-1701, 1995). As enzimas GTF adicionadas a cada amostra para todas as análises é equivalente à quantidade requerida para incorporação de 1 a 1,5 μmol de glicose após 4h de reação.

Para as análises de GTF em solução, GTF B ou C é adicionada a 7–epiclusianona (concentrações variando de 6,25 a 25 μg/ml) e incubadas com substrato de ^{14}*C-(glucosyl)-sucrose* (0,2 μCi/ml; 200,0 mmol/l de sacarose, 40 μmol/l de dextrano 9000, 0,02% de azida sódica em *adsorption buffer*, pH 6,5) para uma concentração final de 100 mmol/l de sacarose (volume final de 300 μl). Para o controle, a mesma reação é feita com etanol (concentração final de 10% v/v) substituindo os extratos teste. As amostras são incubadas a 37^{o}C sob agitação, por 4 h. Após a incubação, 1 mL de etanol a baixa temperatura (-20^{o}C) é adicionado às amostras e estas são armazenadas por 18h a 4^{o}C para precipitação dos glucanos. A quantidade de glucanos produzidos a partir da sacarose marcada pode então ser determinada por cintilação (Koo H, et al. Effect of new variety of *Apis mellifera* Propolis on mutans streptococci. Current Microbiology. Estados Unidos da America: , v.41, p.192 - 198, 2000; VENKITARAMAN, A.R. et al. Characterization of glucosyltransferase B, GtfC, and GtfD in solution and on the surface of hydroxyapatite. J dent. Res., Washington, v.74, p.1694-1701, 1995).

Os resultados obtidos na presente invenção demonstram o potencial antimicrobiano da epiclusianona, que inibiu significativamente o crescimento microbiano em células planctônicas e em biofilme. Para os resultados de CIM e CBM a epiclusianona apresentou uma excelente atividade antimicrobiana, contra *S. mutans* UA 159, a partir da concentração de 2,5 μg/mL, conforme mostrado na Tabela 1.

Tabela 1. Resultados de CIM e CBM

Microrganismo	7- Epiclusianona		Extrato Hexânico do epicarpo da *R. brasiliensis*		Extrato Hexânico da Semente da *R. brasiliensis*	
	CIM	CBM	CIM	CBM	CIM	CBM
***S. mutans* UA 159**	**2,5-5**	**10-20**	**12,5-25**	**0-100**	**12,5-25**	**25-50**

A epiclusianona apresentou-se promissora na inibição da GTF B e C em solução inibindo a GTF B (92.8 ± 3.3%) em baixas concentrações, conforme Tabela 2.

Tabela 2. Resultados da inibição sobre GTF B e GTF C

7-epiclusianona	GTF B (% de inibição)	GTF C (% de inibição)
62,5 µg /ml	70.4 ± 4.4	71.2 ± 2.4
125,0 µg /ml	85.2 ± 5.1	79.8 ± 4.1
250,0 µg /ml	92.8 ± 3.3	90.8 ± 5.3

Assim, a epiclusianona é promissora no tratamento e prevenção de cáries dentais, inibição da formação da placa dental (biofilme), gengivites, estomatites e ulcerações.

Formulações orais com a epiclusianona devem incluir um veículo adequado que disperse satisfatoriamente o princípio ativo (epiclusianona). A composição oral pode levar a forma de uma pasta, creme ou gel dental, um pó, uma solução (por exemplo, colutório bucal), uma suspensão, uma emulsão, uma pastilha, um veículo excipiente aderente a mucosas (por exemplo, Periochip®), um tablete, um filme absorvível, uma goma (por exemplo, balas e/ou goma de mascar). A composição também pode ser em fio dental embebido com a epiclusianona.

A escolha do veículo dependerá, em parte, da forma de apresentação que a composição oral levará. O veículo deverá solubilizar satisfatoriamente a epiclusianona e ser farmacêuticamente aceitável para administração oral. Normalmente, o veículo incluirá

como componente(s) principal(is) a seguinte formulação: água, glicerina, Álcool como etanol, sorbitol, polipropileno glicol, etc, DMSO, polímeros, derivados de celulose, corantes, etc.

Quando a água é empregada, utilizar preferencialmente água destilada e deionizada. Normalmente a água pode corresponder de 10 a 85 por cento da composição oral, dependendo da formulação.

Veículos, como polímeros, podem incluir copolímeros de polivinilmetiléter com anidrido maléico ou veículo semelhante aos polímeros.

Além da composição oral, a epiclusianona, pode-se incluir vários elementos aditivos, sem limitação, como: agente abrasivo, um agente emulsificante, um umectante, um agente cariostático, agente flavorizante, agente adoçante, agente dessensibilizante, agente de anti-cálculo (anti-tártaro), agente clareador, surfactante, preservativo, agente de opacificante, agente de coloração (corantes), agente de proteção, ou qualquer combinação.

Agentes abrasivos, geralmente, são empregados nas composições de dentifrício. Reativos abrasivos utilizados, sem limitação, são: óxido de alumínio, hidróxido de alumínio, fosfato dibásico de cálcio, desidratado ou hidratado, sílica gel, zirconosilicato, sílica anidra, aluminosilicato, metafosfato de sódio insolúvel, carbonato de magnésio, sulfato de cálcio, resinas sintéticas, ou qualquer combinação. Geralmente podem ser empregados abrasivos em quantia efetiva de aproximadamente 20 a 90 por cento de peso, mais tipicamente aproximadamente 20 para aproximadamente 60 por cento de peso para dentifrícios.

Agentes geleificantes podem ser usados em várias composições para ajudar o processamento. Agentes emulsificantes utilizados, sem limitação, são: carragenana, carragenina, carboximetilcelulose, sais de alginatos alcalinos (ex.como alginato de sódio), gomas, álcool de polivinil, ou material semelhante. Geralmente, o agente geleificante é empregado na proporção de 0,3 a 5 por cento de peso.

Umectantes também podem ser empregados nas composições orais, particularmente dentifrícios, géis e enxaguatórios orais. Umectantes normalmente utilizados, sem limitação, são: sorbitol, glicerina, propileno glicol, 1,3-butileno glicol, polietileno glicol, xilitol, maltitol, lactiol, ou semelhantes. O agente umectante pode estar

presente em uma quantia aproximada de 5 a 90 por cento de peso.

Podem ser adicionados agentes cariostáticos em cada forma da composição oral. Agente cariostáticos normalmente utilizados, sem limitação, são: fluoreto de sódio, fluoreto de estanho, monofluorfosfato de sódio, trimeta-fosfato de sódio, triclosan, caseina, ou qualquer combinação. O agente cariostáticos pode estar presente em uma quantia aproximada de 0,01 a 3 por cento de peso.

Agente de flavorizante é utilizado nas composições orais para melhorar o sabor e aroma da composição oral com a epiclusianona e, assim, aumentar a probabilidade do uso desse composto. Agente flavorizante pode ser essências (por exemplo óleo de menta, menta, wintergreen (*Gaultheria procumbens*), sassafras, cravo-da-índia, salva, eucalipto, canela, limão e laranja, salicilato de metila, etc.) ou adoçante (por exemplo sacarose, sucralose, lactose, maltose, xilitol, ciclamato de sódio, perilartina, aspartame, sacarina, etc.). Os agentes flavorizantes podem estar presentes, individualmente ou coletivamente, em uma quantia de 0,1 a 10 por cento de peso.

Dessensibilizantes podem ser introduzidos em algumas composições orais com a epiclusianona, sendo utilizados no tratamento dentes sensíveis a choque térmico, substâncias químicas, etc. Agentes dessensibilizantes utilizados, sem limitação, são: citrato de potássio, cloreto de potássio, tartarato de potássio, bicarbonato de potássio, oxalato de potássio, nitrato de potássio e sais de estrôncio. Os dessensibilizantes podem estar presentes, individualmente ou coletivamente, em uma quantia de 0,1 a 5 por cento de peso.

Podem ser incorporados agentes anti-cálculo (anti-tártaro) em algumas formulações orais com a epiclusianona. Agente anti-cálculo utilizados, sem limitação, são: sais de pirofosfatos alcalinos, polímeros que contém hipofosfito, fosfato orgânico, citato-fosfato e as possíveis combinações. O agente de anti-cálculo pode estar presente, individualmente ou coletivamente, em uma quantia de 0,1 a 5 por cento de peso.

Os agentes clareadores podem ser empregados em algumas formas de apresentação oral com a epiclusianona. Agente clareadores utilizados são: peróxido de uréia, peróxido de cálcio, perborato de sódio e peróxido de hidrogênio. Os clareadores podem ser empregados em quantias de 0,5 a 5 por cento de peso.

Os surfactantes também pode ser empregado nas várias composições orais com a

epiclusianona. Qualquer variedade de surfactante pode ser utilizado sem limitações, como por exemplo os aniônicos, não aniônicos, catiônicos e zwitterionicos ou anfóteros, ou qualquer combinação. Exemplos de surfactantes aniônicos: sulfato lauril de sódio, sarcosinato de lauroil de sódio, alfa-olefina sulfonato, taurato, monoglicerídeo lauril sulfato, monoglicerídeo lauril sulfonato ou qualquer combinação. Surfactantes não iônicos incluem: Tween, lauroil dietanol amida, estearil monoglicerídeo, ésteres de ácido graxo com sacarose, ésters de ácido graxo com lactose, ésters de ácido graxo com lactitol, ésters de ácido graxo com maltitol, polioxietileno monoestearato de sorbitano, ou qualquer combinação. Surfactantes anfóteros incluem, sem limitação, betaina e surfactantes do tipo aminoácido. Os surfactantes podem está presente entre 0,5 a 15 por cento de peso.

Podem ser utilizados os agentes de ligação para dá forma a apresentações do tipo tabletes ou pastilha. Tais agentes de ligação incluem carboximetil-celulose de sódio, goma xantana, goma arábica, etc. Esses agentes podem está presentes entre 0,5 a 50 por cento de peso que dependem da forma da composição oral.

Podem ser utilizados preservativos para preservar as propriedades da composição oral durante o armazenamento. Um preservativo utilizado é o benzoato (por exemplo, beozoato de sódio, sorbato de sódio etc.), que também possui atividade cariostática.

Podem ser incluídos na composição oral agentes opacificantes. Um agente opacificante utilizado é o dióxido de titânio, um pó branco que aumenta a opacidade das composições. O dióxido de titânio pode estar presente em uma quantia de 0,25 a 5 por cento de peso.

Os corantes também podem ser acrescentados à composição oral da presente invenção. O corante pode estar na forma de solução aquosa, em aproximadamente 1 por cento do corante em solução aquosa. A solução colorante pode estar presente em uma quantia de 0,001 a 5 por cento de peso.

A composição oral também pode incluir tampões e sais para tamponamento do pH da composição oral, promovendo sua estabilidade.

Reivindicações

1. Uso de composto isolado da *Rheedia brasiliensis* **caracterizado** pelo fato do composto isolado ser a epiclusianona, como agente antimicrobiano em formulações orais para a prevenção e/ou tratamento de cáries dentais, inibição da formação da placa dental (biofilme), de glucosiltransferases, gengivites, estomatites e ulcerações.

2. Uso de composto isolado da *Rheedia brasiliensis,* de acordo com a reivindicação 1, **caracterizado** pelo fato do composto isolado ser a 7-epiclusianona com atividade antimicrobiana

3. Uso de composto isolado da *Rheedia brasiliensis*, de acordo com as reivindicações 1 e 2, **caracterizado** por ser em formulações orais antimicrobianas, em forma de pasta, creme ou gel dental, pó, solução por exemplo, colutório bucal, suspensão, emulsão, pastilha, veículo excipiente aderente a mucosas, tablete, filme absorvível, goma ou fio dental embebido com a epiclusianona.

4. Uso de composto isolado da *Rheedia brasiliensis*, de acordo com as reivindicações 1 e 2, **caracterizado** pelo fato das formulações orais incluírem, além da epiclusianona como agente antimicrobiano, abrasivo, emulsificante, umectante, um cariostático, flavorizante, adoçante, dessensibilizante, anti-cálculo (anti-tártaro), clareador, surfactante, preservativo, opacificante, agente de coloração (corantes), agente de proteção, ou qualquer combinação.

5. Formulação oral **caracterizada** por conter a 7- epiclusianona em uma quantidade terapeuticamente efetiva para a prevenção e/ou tratamento de cáries dentais, inibição da formação da placa dental (biofilme), de glucosiltransferases, gengivites, estomatites e ulcerações.

6. Formulação oral, de acordo com a reivindicação 5, **caracterizada** por conter a 7-epiclusianona como agente antimicrobiano.

7. Epiclusianona **caracterizada** por ser isolada da *Rheedia brasiliensis* e conter atividade antimicrobiana.

8. Método de tratamento **caracterizado** por prevenir e/ou tratar cáries dentais, formação da placa dental (biofilme), glucosiltransferases, gengivites, estomatites e ulcerações, a partir da administração via oral de uma quantidade terapeuticamente efetiva de 7-epiclusianona.

2.4 Estudo 4

Antiproliferative effect of poliprenylated benzophenones

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ABSTRACT

The objective of this study was to investigate the antiproliferative activity of garciniaphenone and 7-epiclusianone, prenylated benzophenones isolated from *Rheedia brasiliensis*, against human cancer cell lines. The cell lines from melanoma (UACC-62), breast (MCF-7), breast resistant (NCI-ADR), lung/non-small cells (NCI460), ovarian (OVCAR 03), prostate (PC03), kidney (786-0), lung (NCI-460) and tongue (CRL-1624 and CRL-1623) were grown in RPMI with 5% of fetal bovine serum to be investigated in presence of benzophenones. For the antiproliferative assay the cells were exposed of garciniaphenone and 7-epiclusianone in a range of different concentrations from 0.25 to 250.0 μg/mL, at 37° C, 5% of CO_2, 48 h. The cell proliferation was determined by spectrophotometric quantification (540 nm) of the cellular protein content using sulforhodamine B. Garciniaphenone displayed significant cytostatic activity for all cell lines while, 7-epiclusianone showed dose dependent cytotoxic activity. The IC_{50}, half maximal inhibitory concentration, of 7-epiclusianone was significantly more potent than garciniaphenone in all cases, except for squamous cell carcinoma (CRL-1624 and CRL-1623). The chemical structure of 7-epiclusianone has a C-5 prenyl that is not present in the garciniaphenone structure, which might explain the reduced antiproliferative effect presented by garciniaphenone. In conclusion, garciniaphenone and 7-epiclusianone have shown antiproliferative activity against all tumor cell lines tested and the presence of C-5 prenyl at 7-epiclusianone structure could be important for increasing this antiproliferative activity.

Keywords: Benzophenones, Garciniaphenone, 7-epiclusianone, cancer, antiproliferative.

1. INTRODUCTION

Cancer is a major public health problem in developed and developing countries (Jemal *et al.,* 2005). Therefore, new compounds with antiproliferative activity have an important role in the prevention and treatment of cancers (Cragg & Newman, 1999). Natural products are still major source of innovative therapeutic agents for cancer diseases (Mukherjee *et al*., 2001). Approximately 61% of new antitumor drugs approved by the Food and Drug Administration or comparable entities in other countries were natural products or derived from natural products (Vuorelaa *et al*., 2004).

The large biodiversity within the territory of Brazil is an important source for obtaining pharmacologically active chemicals (Basso *et al*., 2005). In this context, *Rheedia brasiliensis*, commonly known as "bacupari", is used in Brazilian folk medicine as a wound healing agent, for peptic ulcers and urinary and tumor diseases treatments (Correa, 1978). The Guttiferae family presents remarkably diversity of metabolites. For instance, the oxygenated and prenylated phenol derivatives (Delle Monanche *et al*., 1991), including xanthones (Bennett & Lee 1989), flavonoids (Crichton & Waterman 1979), phenolic acids (Gunatilaka *et al*., 1982) and benzophenones (Rubio *et al*., 2001).

Phytochemical investigations of the fruits of *Rheedia brasiliensis* resulted in isolation and identification of new potentially active benzophenones, including garciniaphenone and 7-epiclusianone (Derogis *et al*., 2007). Chemically, garciniaphenone does not contain the C-5 prenyl when compared to 7-epiclusianone (Derogis *et al*., 2007), which may affect the biological activities. Furthermore, a tetraprenylated benzophenone (7-epiclusianone) has shown several pharmacological activities, including antioxidant (Cruz *et al*., 2006) and anti-*Trypanossoma cruzi* (Alves *et al*., 1999).

However, investigation against tumor cells has not been carried out for garciniaphenone and 7-epiclusianone. Therefore, the purpose of the present study was to investigate the differential antiproliferative activity of garciniaphenone and 7-epiclusianone on human cancer cell lines.

2. MATERIAL AND METHODS

2.1 Plant Material and Extract Preparation

The fruits of *Rheedia brasiliensis* 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 state, Brazil, where its voucher specimen is deposited (#VIC2604). Dried and powdered *R. brasiliensis* fruit pericarps (1000 g) were extracted by maceration with 3.0 L of n-hexane at room temperature, filtered and then dried, using a rotary evaporator under reduced pressure at 45°C. 7-epiclusianone was extracted, isolated and purified from the fruit pericarp as detailed elsewhere (Santos *et al.,* 1999). The garciniaphenone was isolated by recrystallization for several times using methanol solutions as detailed elsewhere (Derogis *et al.,* 2007). Both substances were identified from the infrared, ultraviolet, mass spectra data and the NMR spectra data and confirmed by comparison with data from literature (Santos *et al,* 1999, Derogis *et al.,* 2007).

2.2 Antiproliferative assay

Following the National Cancer Institute (NCI – U.S. National Institutes of Health) preclinical antitumor drug discovery, melanoma (UACC-62), breast (MCF-7), breast-resistant (NCI-ADR), lung/non-small cells (NCI460), ovarian (OVCAR 03), prostate (PC03), kidney (786-0) and lung (NCI-460) cell lines were used. Also included in this study was squamous cell carcinoma, isolated from the tongue (CRL-1624 and CRL-1623), obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Since multidrug resistance phenomenon is a relevant therapeutic problem, we have also evaluated the antiproliferative activity of our compounds against NCI-ADR (breast cells) which expressing multiple drugs resistance phenotype. Stock cultures were grown in RPMI 1640 media (Gibco-BRL, Life Technologies) supplemented with 5% fetal bovine serum (Nutricell Nutrientes Celulares, Campinas, SP, Brazil). Gentamicine (Schering-Plough) (50 μg/mL) was added to the assay cultures. The antiproliferative assay was done as described by Skehan *et al* (1990), briefly, cells distributed in 96-well plates (100 μL cells/well) were

exposed to various concentrations of Garciniaphenone and 7-epiclisianone in DMSO (Sigma) (0.25, 2.5, 25.0 and 250.0 μg/mL) at 37 °C, 5% of CO_2 for 48 h. The final concentration of DMSO did not affect the cell viability. A 50% of trichloroacetic acid solution was added and after incubation for 30 min at 4 °C, washing and drying. The cell proliferation was determined by spectrophotometric quantification (540 nm) of the cellular protein content using sulforhodamine B. The relative potency was based on the drug concentration that inhibited cell growth by 50% (IC_{50}) [calculated from $[(T-T_0)/(C-T_0)] \times 100 = 50$, which is the drug concentration resulting in a 50% reduction in the net cell number of drug-treated cultures relative to the increase in control cultures during the drug incubation period]. This was determined from concentration-effect relationships using GraphPad prism 2.01 (GraphPad Software, San Diego, CA).

3. RESULTS

In this study, garciniaphenone and 7-epiclusianone were isolated from dried fruit of *R. brasiliensis*. These substances, as the main constituent of the hexane extract, were yellow crystalline solid and the melting point for garciniaphenone was 105-107 °C and 92-93 °C for 7-epiclusianone. The purity of the compounds garciniaphenone and 7-epiclusianone were 98.6 and 99.85%, respectively. The chemical structure showed that 7-epiclusianone has in C-5 a prenyl group (see Figure 1) (Santos *et al*., 1998; Derogis *et al*., 2007).

The antiproliferative properties of garciniaphenone and 7-epiclusianone against the human cell lines were shown in Figure 2A-B. Garciniaphenone displayed significant cytostatic activity (growth inhibition) for all cell lines at 2.5 μg/mL while 7-epiclusianone, in the same concentration, showed cytotoxic activity for ovarian (OVCAR), kidney (786-0) and breast-resistant (NCI-ADR) cells. This cytotoxic activity was dose dependent and at concentrations ≥ 25.0 μg/mL, death could be observed in all cell lines. The relative potency was based on IC_{50} (see Table 1) and it is noteworthy that 7-epiclusianone and garciniaphenone have shown a potent aniproliferative effect against melanoma (UACC-62), breast-resitant (NCI-ADR), ovarian (OVCAR), and lung (NCI-460) cells when compared

with positive control (doxorubicin). Furthermore, 7-epiclusianone was more potent than garciniaphenone, except for tongue cell lines (CRL1624 and CRL1623).

4. DISCUSSION

Natural products have been one of the most successful sources for the discovery of new therapeutic agents (Harvey, 2000). In an effort to explore the large biodiversity within the territory of Brazil, several projects have aimed at identifying new naturally occurring molecules of therapeutic value (Basso *et al*., 2006). In this context, new benzophenones, garciniaphenone and 7-epiclusianone (Derogis *et al*., 2007), were identified from *R. brasiliensis*, a native fruit from Brazil that is commonly used in traditional medicine for treatments of inflammatory diseases (Corrêa, 1978). Phytochemical investigations of the fruits of *R. brasiliensis*, resulted in the isolation and identification of several potentially active compounds, including sesquiterpenes (Santos *et al.,* 1998) and benzophenones (Santos *et al.,* 1998; Derogis *et al.,* 2007). Among them, a tetraprenylated benzophenone (7-epiclusianone) has shown several pharmacological activities, including antioxidant and anti-*Trypanossoma cruzi* (Alves *et al.,* 1999; Cruz *et al.,* 2006) activities.

In our study, garciniaphenone, a new benzophenone, and 7-epiclusianone have shown a very broad spectrum of antiproliferative effects against various cancer cell lines. A remarkable difference in citotoxic activity was observed for the breast (MCF-7), prostate (PCO3) and kidney (786-0) cancer cell lines, with 7-epiclusianone being approximately more than 10 times potent than garciniaphenone. Furthermore, both compounds have shown effect for breast cancer cells that express multidrug resistance phenotype (NCI-ADR) since that acquired drug resistance is a factor in the failure of chemotherapy in breast cancer (Panasci *et al*., 1996). These results are in accordance with recent studies which demonstrated that benzophenone from the *Garcinia spp.* (Hong *et al*.,2006) and *Garcinia hanburyi* (Yang *et al*., 2007) has been shown to possess anticarcinogenic activity. The putative pathway by which 7-epiclusianone affects the proliferation of cancer cells may suggest that garcinones are involved in the prostaglandins biosynthesis inhibition which interfere in the COX-2 activity (Yamakuni *et al.,* 2006) and this is significant due to the fact that COX-2 inhibitors promote the reduction of: (1) carcinogen agents production

(Plastaras *et al*., 2000); (2) proliferative actions (Evans & Kargman 2004); and (3) pro-apoptopic actions (Zhang *et al*., 2000).

Our initial structure-activity relationship studies were focused on the effects of prenyl group on both chemical structures. Tanaka *et al.*, (2004) showed that garciniaphenone can be prenylated, forming 7-epiclusianone. The garciniaphenone does not contain the C-5 prenyl subsistent when looking at structure 7-epiclusianone, resulting in a decreased lipophilic character (Derogis *et al*., 2007). Thus, the garciniaphenone capacity to bind and penetrate into the cells is lower than 7-epiclusianone, which might explain the reduced antiproliferative effect presented by garciniaphenone when compared with 7-epiclusianone. However, the IC_{50} values from squamous cell carcinoma lines (CRL1624 and CRL1623) have not shown relationship with structure-activity, since garciniaphenone was more potent than 7-epiclusianone.

In conclusion, garciniaphenone and 7-epiclusianone have shown antiproliferative activity against all tumor cell lines tested and the presence of C-5 prenyl at 7-epiclusianone structure could be important for increasing this antiproliferative activity.

5. ACKNOWLEDGEMENT

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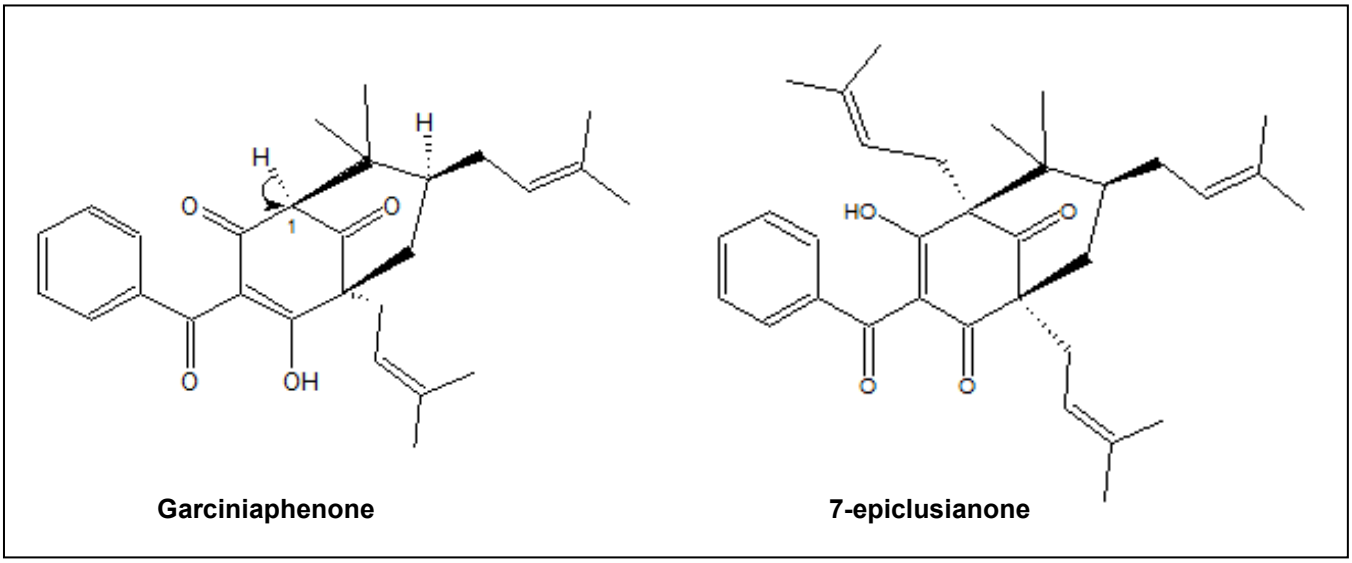
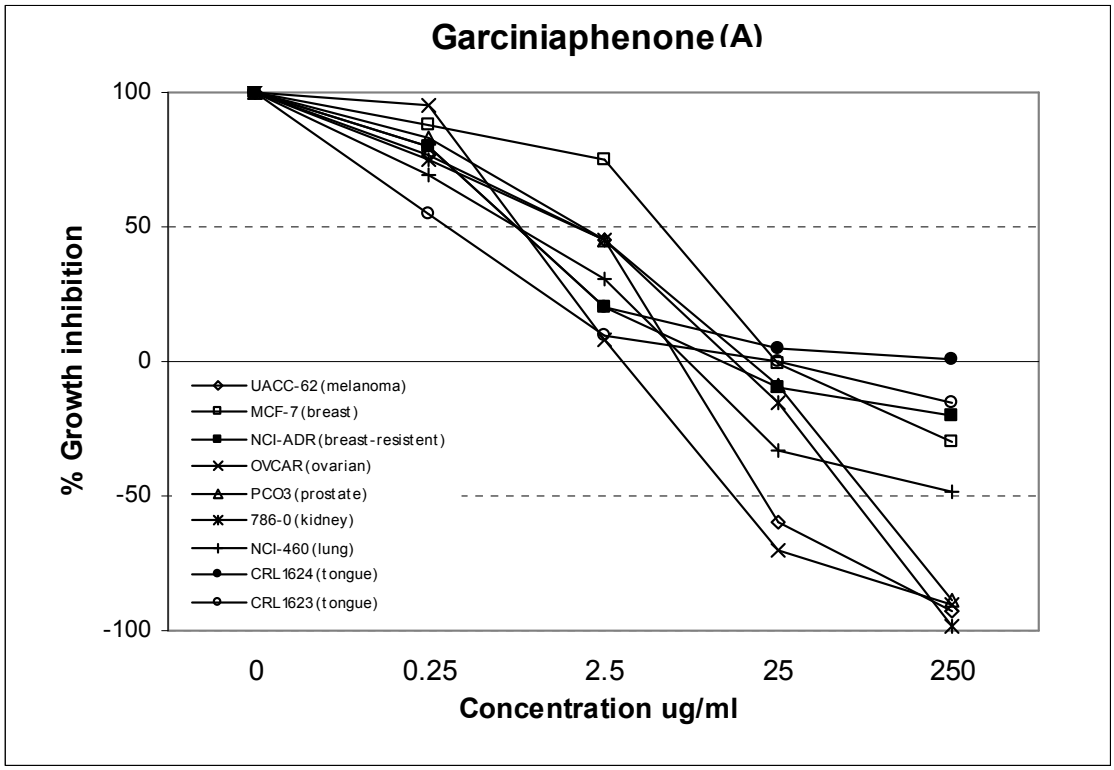


Figure 1. Chemical structure of Garciniaphenone and 7-epiclusianone (Santos *et al*, 1999, Derogis *et al.,* 2007).


Garciniaphenone (A)
100
50
0
-50
-100
% Growth inhibition
UACC-62 (melanoma)
MCF-7 (breast)
NCI-ADR (breast-resistent)
OVCAR (ovarian)
PCO3 (prostate)
786-0 (kidney)
NCI-460 (lung)
CRL1624 (tongue)
CRL1623 (tongue)
0
0.25
2.5
25
250
Concentration ug/ml

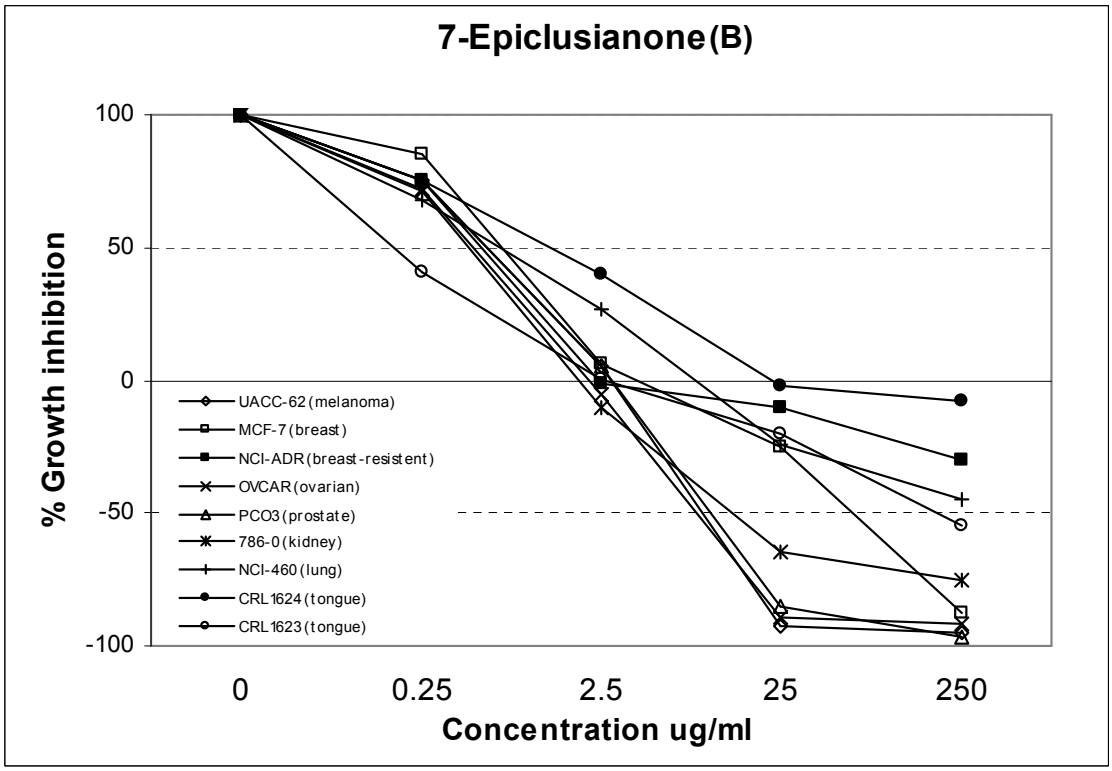

7-Epiclusianone (B)
100
50
0
-50
-100
% Growth inhibition
UACC-62 (melanoma)
MCF-7 (breast)
NCI-ADR (breast-resistent)
OVCAR (ovarian)
PCO3 (prostate)
786-0 (kidney)
NCI-460 (lung)
CRL1624 (tongue)
CRL1623 (tongue)
0
0.25
2.5
25
250
Concentration ug/ml

Figure 2. Effect of garciniaphenone (A) and 7-epiclusianone (B) on the growth of different cancer cell lines. Positive values in relation to y axis correspond to cytostatic activity while negative values refer to cytotoxic activity.

Table 1. Comparison of IC_{50} values of garciniaphenone and 7-epiclusianone against cancer cell lines.

	IC_{50} (μg/ml)[a]		
Cell Line	Garciniaphenone	7-epiclusianone	Doxorubicin[b]
UACC-62	2.9	2.5	5.2
MCF-7	24.5	2.3	2.4
NCI-ADR	2.1	2.0	31.2
OVCAR	2.4	2.4	4.3
PC)3	24.8	2.5	20.2
786-0	24.9	2.2	2.1
NCI-460	2.6	2.4	3.6
CRL1624	1.8	2.3	1.4
CRL1623	1.9	2.2	1.2

[a] IC_{50} values (concentration that elicits 50% inhibition) were determinated from nonlinear regression analysis by GraphPad Prism software ($r^2 > 0.9$). [b] Doxorubicin was employed as positive control.

3. CONCLUSÕES GERAIS

- A 7-epiclusianona apresenta atividade contra os fatores de virulência do *Streptococcus mutans,* reduzindo a atividade das glucosiltransferases, F-ATPase bem como a produção de ácidos desses microrganismos;

- A 7-epiclusianona afeta a produção de polissacarídeos extracelulares insolúveis em biofilmes bem como reduz a incidência de cárie dental em superfície e sulcal de ratos, sendo um promissor agente anticárie e anti-placa;

- A 7-epiclusianona possue atividade antiproliferativa contra células cancerígenas humanas, sendo um promissor agente anticâncer.

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5. ANEXOS

Anexo 1 - Resolucão do formato alternativo para defesa da tese de doutorado.

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 nivel (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 nivel (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 caracteristico 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 "impar" 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 à 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.

§ 5º - As dissertações e teses serão reproduzidas no padrão frente e verso, exceção feita às páginas iniciais e divisões de capítulos; dissertações e teses com até 100 páginas serão reproduzidas no padrão apenas frente, exceção feita à página que contém a ficha catalográfica.

§ 6º - As páginas fornecidas para inserção deverão ser impressas em sua forma definitiva, ou seja, apenas frente ou frente/verso.

§ 7º - O custo, em reais, de cada exemplar produzido pela gráfica será definido pela Administração Superior da Universidade.

Artigo 5º - É obrigatória a entrega de dois exemplares para homologação.

Artigo 6º - Esta Informação entrará em vigor na data de sua publicação, ficando revogadas as disposições em contrário, principalmente as Informações CCPG 001 e 002/98 e CCPG/001/00.

Campinas, 13 de setembro de 2006

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

Anexo 2 – Declaração do autores.

Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas cientificas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada "Avaliação das atividades anticárie e antiproliferativa da 7-epiclusianona isolada da planta do gênero *Rheedia*", não infrigem os dispositivos da Lei n.o. 9.610/98, nem o direito autoral de qualquer editora.

Autor. RG. 29508841-2

Orientador. RG. 11185975

Anexo 3 – Certificado do Comitê de Ética em Pesquisa.

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

Telefone: (19) 3788-6359
Telefax: (19) 3788-6356
E-mail: ceea@cemib.unicamp.br
http://www.ib.unicamp.br/institucional/ceea/index.htm

Anexo 4 – E-mail de submissão do artigo.

Data:	Mon, 10 Dec 2007 15:58:59 -0500 (EST)
De:	femsle@fems-microbiology.org
Para:	ramirofop@yahoo.com
Assunto:	Manuscript submitted - FEMSLE-07-12-1277

10-Dec-2007

Dear Mr. Ramiro Murata,

The manuscript you submitted to our journal, INHIBITORY EFFECTS OF 7-EPICLUSIANONE ON GLUCAN SYNTHESIS, ACIDOGENICITY AND BIOFILM FORMATION BY STREPTOCOCCUS MUTANS, by Murata, Ramiro; Almeida, Luciana; yatsuda, regiane; Santos, Marcelo; Nagem, Tanus; Rosalen, Pedro; Koo, Hyun, has been uploaded to Manuscript Central.

As the submitting author, you will receive future communications via e-mail. Your manuscript number is FEMSLE-07-12-1277.

We thank you for submitting your work for publication in one of the FEMS Microbiology journals.

Please be sure to save the word processing and graphics files from your manuscript. You may need them later again for production purposes if your manuscript is accepted.

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Again, thank you for the submission of your manuscript.

Best wishes,

Dr Andrea O´Brien
FEMS Editorial Administrator

On behalf of Chief Editor,
Dr Jeff Cole
FEMS Microbiology Letters

Please note: It is the responsibility of the corresponding author (or submitting author if different) that all authors of this manuscript are informed about its submission and subsequent progress.

Anexo 5 – Pedido de depósito de patent

Protocolo

Número (21)

(Uso exclusivo do INPI)

DEPÓSITO
Pedido de Patente ou de Certificado de Adição

depósito / /

Espaço reservado para etiqueta (número e data de depósito)

Ao Instituto Nacional da Propriedade Industrial:

O requerente solicita a concessão de uma patente na natureza e nas condições abaixo indicadas:

1. Depositante (71):

1.1 Nome: **UNIVERSIDADE DE SÃO PAULO - USP**

1.2 Qualificação:

1.3 CNPJ/CPF (se houver): **6302553000004**

1.4 Endereço completo: **Av. Prof. Almeida Prado, 1.280 - Cidade Universitária - Butantã - SP - 05508-070**

1.5 Telefone: **(11) 3091.4474**
FAX: **(11) 3091.0922**

☐ Continua em folha anexa

2. Natureza:

☒ 2.1 Invenção ☐ 2.1.1 Certificado de Adição ☐ 2.2 Modelo de Utilidade

Escreva, obrigatoriamente e por extenso, a Natureza desejada: **INVENÇÃO**

3. Título da Invenção, do Modelo de Utilidade ou Certificado de Adição (54):

"USO DE COMPOSTO ISOLADO DA RHEEDIA BRASILIENSIS NA PREVENÇÃO E / OU TRATAMENTO DE DOENÇAS"

☐ Continua em folha anexa

4. Pedido de Divisão do pedido nº. , de

5. Prioridade Interna – O depositante reivindica a seguinte prioridade

Nº de depósito Data de Depósito **(66)**

6. Prioridade - O depositante reivindica a(s) seguinte(s) prioridade(s):

País ou organização de origem	Número do depósito	Data do depósito

☐ Continua em folha anexa

7. Inventor (72):

☐ Assinale aqui se o(s) mesmo(s) requer(em) a não divulgação de seu(s) nome(s)

(art. 6º § 4º da LPI e item 1.1.do Ato Normativo nº 127/97))

7.1 Nome: **SEVERINO MATIAS DE ALENCAR**

7.2 Qualificação: **Prof. Universitário, brasileiro, casado, R.G. 3.682.343, CPF. 550.118.024-34**

7.3 Endereço: **Rua Heitor Vila Lobos, 750, apto 143 – Piracicaba – SP**

7.4 CEP: **03420-130** 7.5 Telefone **(11) 3091.4474**

Formulário nº 1.01 – Depósito de Pedido de Patente ou Certificado de Adição (fl.1/2):

☒ Continua em folha anexa

8. Declaração na forma do item 3.2. do Ato normativo nº 127/97:

☐ em anexo

9. Declaração de divulgação anterior não prejudicial (Período de graça):

☐ em anexo

10. Procurador (74):

10.1 Nome e CPF/CGC: **MARIA APARECIDA DE SOUZA 121.846.178-06**

10.2 Endereço completo: **Av. Prof. Almeida Prado, 1.280 - Cidade Universitária - Butantã - SP - 05508-070**

10.3 CEP: **05508-070** 10.4 Telefone **(11) 3091.4474**

11. Documentos anexados (assinale e indique também o número de folhas):

(Deverá ser indicado o nº total de somente uma das vias de cada documento)

☒	11.1 Guia de Recolhimento	1 fls.	☒	11.5 Relatório Descritivo	9 fls.
☒	11.2 Procuração	1 fls.	☒	11.6 Reivindicações	1 fls.
☐	11.3 Documentos de prioridade	fls.	☒	11.7 Desenhos	1 fls.
☐	11.4 Doc. de contrato de Trabalho	fls.	☒	11.8 Resumo	1 fls.
☒	11.9 Outros (especificar): **Aut. Inventor**				8fls.
	11.10 Total de folhas anexadas:				22fls.

12. Declaro, sob pena da Lei, que todas as informações acima prestadas são completas e verdadeiras

São Paulo, 31 de julho de 2006.
Local e Data

Assinatura e Carimbo
MARIA APARECIDA DE SOUZA
Procuradora

Formulário nº 1.01 – Depósito de Pedido de Patente ou Certificado de Adição (fl.2/2):

7. Inventor:

7.1. Nome: SEVERINO MATIAS DE ALENCAR.

7.2. Qualificação: Brasileiro, casado, professor universitário, portador do R.G. nº. 3.682.343 e do C.P.F. nº. 550.118.024-34, residente e domiciliado a Rua Heitor Vila Lobos, 750, apto 143 – Piracicaba – SP – 3420-130.

7. Inventor:

7.1. Nome: JAIME APARECIDO CURY

7.2. Qualificação: Brasileiro, casado, professor universitário, portador do R.G. nº. 4.329.838 e do C.P.F. nº. 157.357.299-34, residente e domiciliado a Rua Moraes Barros, 1510, apto 21 – Piracicaba – SP – 13419-240.

7. Inventor:

7.1. Nome: PEDRO LUIZ ROSALEN

7.2. Qualificação: Brasileiro, casado, professor universitário, portador do R.G. nº. 11.185.975 e do C.P.F. nº. 030.958.228-80, residente e domiciliado a Rua Octávio Almeida Mello, 215, Terras de Piracicaba I – Piracicaba – SP – 13403-845.

7. Inventor:

7.1. Nome: WILLIAM HENRY BOWEN

7.2. Qualificação: Irlandês, casado, professor universitário, portador do PASSAPORTE nº. 156352209, residente e domiciliado a 315 Victor Egypt Road, Victor, NY 14564.

7. Inventor:

7.1. Nome: HYUN KOO

7.2. Qualificação: Alemão, casado, professor universitário, portador do R.G. nº. 36.144.932-X e do C.P.F. nº. 030.958.228-80, residente e domiciliado a 45 Oak Lane, Rochester, 14610, NY, Estados Unidos da América.

7. Inventor:

7.1. Nome: MARCELO HENRIQUE DOS SANTOS

7.2. Qualificação: Brasileiro, casado, professor universitário, portador do R.G. nº. 4.623.651 e do C.P.F. nº. 644.358.306-04, residente e domiciliado a Rua Gonçalves Dias, 200, Jardim São Carlos – Alfenas – MG – 37130-000.

7. Inventor:

7.1. Nome: TANUS JORGE NAGEM

7.2. Qualificação: Brasileiro, casado, professor universitário, portador do R.G. nº. M-15920. e do C.P.F. nº. 016.093.946-15, residente e domiciliado a Rua Tulipa, 357, Esplanada – Belo Horizonte – MG – 30280-200.

7. Inventor:

7.1. Nome: RAMIRO MENDONÇA MURATA

7.2. Qualificação: Brasileiro, solteiro, cirurgião-dentista, portador do R.G. nº. 29.508.841-2 e do C.P.F. nº. 274.119.818-77, residente e domiciliado a Rua Quinze de Novembro, 216, apto 21, Centro – Piracicaba – SP – 13400-970.