Ramiro Mendonça Murata

Avaliação *in vitro* do efeito do kaempferol e tt-farnesol sobre o biofilme dental – inibição e viabilidade bacteriana.

Dissertação apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção de grau de Mestre em Odontologia, Área de Farmacologia, Anestesiologia e Terapêutica.

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SUMÁRIO

RESUMO

A própolis é um produto resinoso coletado pelas abelhas *Apis mellifera,* e demonstrou inibição de crescimento dos microrganismos orais e atividade nas glucosiltransferases. Muitos compostos vem sendo isolados e identificados na própolis. Entre esses compostos, o kaempferol e *tt*-farnesol demonstraram atividade biológica contra estreptococos do grupo mutans em modelo de células planctônicas. Conseqüentemente este estudo tem como objetivo avaliar o efeito *in vitro* do kaempferol e *tt*-farnesol, isolados ou combinados, sobre a viabilidade (*time-kill)* e inibição da formação do biofilme de patógenos bucais. Os microrganismos utilizados foram os *Streptococcus mutans* UA 159 e *S. sobrinus* 6715. O kaempferol e *tt*-farnesol foram utilizados nas concentrações de 1,33mM isolados e 0,665mM quando associados. O controle positivo foi clorexidina a 1,33mM, equivalente a 0,12%. Os biofilmes foram formados sobre lâminas de vidro durante cinco dias, no teste de viabilidade os biofilmes foram submetidos aos tratamentos com os agentes e suas associações durante 0, 1, 2, 3 e 4 horas (h); sendo os resultados expressos em log N/N_0, sendo N_0 o numero de unidades formadoras de colônias (ufc) formadas pelo controle negativo e N as ufc formadas pelos biofilmes tratados. Para o teste de inibição de formação os biofilmes foram tratados a partir do segundo dia com os agentes e suas associações por 1 minuto 2 vezes/dia por 3 dias; os resultados foram expressos em log ufc/biofilme. Para o teste de inibição de formação foram realizadas as dosagens de polissacarídeos insolúveis (Dubois, 1956) e proteínas totais desse biofilmes (Lowry, 1951). Para todos os teste o n=9, foram realizados os testes estatísticos de ANOVA e Tukey-Kramer HSD. O *tt*-farnesol apresentou ação bactericida em 4 h (log $N/N_0 > 3$) para *S. mutans* e S. *sobrinus* no teste de

viabilidade bacteriana. O kaempferol demonstrou baixa atividade antimicrobiana para o teste de viabilidade bacteriana. Quando associados os compostos apresentaram ação bactericida em 4 h (log $N/N_0 > 3$) para o teste de viabilidade bacteriana. O *tt*-farnesol e o kaempferol, testados isoladamente, no teste de inibição de formação do biofilme não apresentaram efeito bactericida estatisticamente significativo. Mas o *tt*-farnesol e kaempferol+*tt*-farnesol reduziram a quantidade total de polissacarídeos insolúveis e proteínas, sendo esses, estatisticamente diferentes do controle. O kaempferol associado ad *tt*-farnesol, em baixas concentrações apresentaram efeito antimicrobiano, reduzindo significativamente a viabilidade bacteriana e reduzindo os polissacarídeos insolúveis e proteínas totais. Deste modo, a associação kaempferol+*tt*-farnesol demonstrou efeito antimicrobiano sobre biofilmes de estreptococos do grupo mutans e tais componentes podem ser promissores no controle do biofilme dental.

ABSTRACT

Propolis, a resinous hive product collected by *Apis mellifera* bees, has shown *in vitro* inhibitory effects on growth of oral bacteria and activity of glucosyltransferases. Several compounds from propolis have been isolated and identified. Among such compounds, kaempferol and *tt*-farnesol have displayed biological activity against mutans microorganisms and planktonic cells. The purpose of the present study was to evaluate the *in vitro* effects of kaempferol and *tt*-farnesol, alone or in combination on viability (time-kill) and inhibition of biofilm formation. The microorganisms used in this experiment were *S. mutans* UA159 and *S. sobrinus* 6715. The concentrations of kaempferol and *tt*-farnesol were 1.33mM alone and 0.665mM in combination. Chlorhexidine was used as a positive control at concentration of 1.33mM, equivalent to 0,12%. Biofilm formation occurred on glass surface during five days, for the time-kill tests the biofilms were submitted to treatments with the propolis compounds isolated or in combination for 0, 1, 2, 3 and 4 hours (h); the results were expressed in log N/N_0, in that N_0 as ufc formed initially and N the number of recovered biofilms after the treatments. The biofilms were treated from the second day with the compounds and their association for 1 minute twice daily for 3 days for the inhibition of biofilm formation; the results were expressed in log ufc/biofilm. The dosage of insoluble polysaccharides (Dubois, 1956) and total protein of biofilm (Lowry, 1951) was carried out for the inhibion of biofilm formation. ANOVA and Tukey-Kramer HSD were carried out for all tests n=9. *tt*-farnesol showed antibacterial activity after 4 h (log $N/N_0 > 3$) against *S.mutans* and *S.sobrinus* for time-kill test. Kaempferol showed low antimicrobial activity for time-kill test. When in combination, the compounds displayed

antibacterial effect after 4 h (log $N/N_0 > 3$) for time-kill test. Kaempferol and *tt*-farnesol, alone, did not show any statistically significant antibacterial effects for biofilm inhibition test. However, *tt*-farnesol and kaempferol+*tt*-farnesol reduced the total amount of insoluble polysaccharide and protein, being statistically different from the negative control. Kaempferol in combination with *tt*-farnesol, at low concentrations, showed antimicrobial effect, reducing significantly the bacterial viability, amount of insoluble polysaccharide, and amount total protein. In conclusion, the combination of kaempferol+ *tt*-farnesol showed antimicrobial activity against biofilms of mutans microorganisms, being promising compounds against dental biofilm.

1. INTRODUÇÃO

Sabe-se que o desenvolvimento da cárie é intimamente associado a microrganismos de origem bacteriana, que se aderem à superfície dos dentes, formando o biofilme dental (FITZGERALD & KEYES, 1960; HAMADA & SLADE, 1980; GIBBONS, 1984). A constituição do biofilme inicia-se com a formação da película adquirida, através da interação entre proteínas salivares e glicoproteínas sobre a superfície do dente (HAY & MORENO, 1993). Posteriormente, ocorre a colonização de bactérias sob a película adquirida, proporcionando a formação e o crescimento do biofilme dental (GIBBONS & VAN HOUTE, 1975).

Alguns microrganismos, como os estreptococos do grupo mutans possuem características acidúrica e acidogênica. O acúmulo de estreptococos na superfície dental é considerado um dos fatores críticos no desenvolvimento do biofilme cariogênico, devido à produção de ácidos que proporcionam a queda do pH do biofilme dental, aumentando a possibilidade de desmineralização dos tecidos dentais (LOESCHE, 1986).

Além disso, os *Streptococcus mutans* e os *Streptococcus sobrinus* têm a capacidade de se aderirem à película adquirida presente sobre os dentes, através da síntese de polissacarídeos extracelulares produzidos a partir da sacarose (DE STOPELAAR et al., 1971; GIBBONS & VAN ROUTE, 1975; HAMADA & SLATE, 1980). A aderência é facilitada através de glucanos, principalmente os insolúveis em água, que são sintetizados por enzimas chamadas glucosiltransferases, (HAMADA & SLADE, 1980; RÖLLA et al., 1983; TANZER et al., 1985; SCHILLING & BOWEN, 1992), produzidas principalmente pelas bactérias do grupo mutans no biofilme dental (LOESCHE, 1986; HANADA &

KURAMITSU, 1989). Atualmente, a tentativa de inibir as atividades das glucosiltransferases é uma das estratégias visando o controle da formação de um biofilme dental cariogênico (IKENO et al., 1991; PARK et al., 1998; KOO et al., 1999). Tem sido também demonstrado que estes glucanos aumentam a porosidade e mudanças na composição inorgânica da matriz do biofilme tornando-o ainda mais cariogênico (VAN HOUTE, 1994).

Em acréscimo, como relatado até então, a formação do biofilme dental bacteriano é bastante complexa, sendo que vários modelos de estudo para reproduzir este biofilme têm sido propostos. Pesquisas têm demonstrado que esses modelos estão mais próximos de reproduzir as condições reais da cavidade oral, pois em comparação com o modelo de células planctônicas, vastamente utilizado (MA et. al., 1999; DUARTE et. al., 2003), as células do biofilme possuem crescimento diferenciado e metabolismo alterado devido à alta densidade populacional. A maior resistência encontrada nos biofilmes aos agentes antimicrobianos é relacionado às barreiras de difusão, além disso, as células do biofilme possuem mais tempo para se adaptarem ao estresse aplicados por esses agentes (BURNE et al., 1999; SVENSATER,G., 2001).

Deste modo, a inibição do biofilme dental seria importante na prevenção de cárie, seja inibindo o crescimento dos estreptococos do grupo mutans na cavidade bucal ou inibindo a aderência destas bactérias às superfícies dos dentes através da inibição da atividade das glucosiltransferases.

Muitos agentes de origem natural vêm sendo explorados nas últimas décadas (IKENO et al., 1991; OTAKE et al., 1991; OOSHIMA et al., 1993; KOO, H. et al., 2002), devido as suas possíveis ações farmacológicas. Deste modo, tem sido descobertos novos

compostos naturais com atividade anti-glucosiltransferase e/ou antibacteriana. Um dos produtos naturais que vêm se destacando é a própolis, composto resinoso produzido pelas abelhas *Apis mellifera.* Foi demonstrado que o extrato etanólico bruto da própolis (EEP) apresenta atividade antimicrobiana contra estreptococos do grupo mutans e inibe a atividade das glucosiltranferases (IKENO et al., 1991; KOO et al., 2000; DUARTE et al., 2003).

Sabe-se que a composição química da própolis é complexa (TOMÁS-BARBERAN et al., 1993; PARK et al., 1997; BANKOVA et al., 1999; KUJUMGIEV et al., 1999; KOO et al., 1999; MURATA et al., 2001; DUARTE et al., 2003) e formado pôr diterpenos e compostos fenólicos, principalmente os flavonóides, que têm sido considerados como substâncias biologicamente ativas da própolis (GHISALBERTI, 1979; BONHEVI et al., 1994; KOO et al., 1999). Vários componentes da própolis foram identificados, e dentre estes, o kampferol e o *tt*-farnesol (sesquiterpeno) têm demonstrado atividade anti-glucosiltransferase e antimicrobiana em células planctônicas (KOO et al.; 2000; 2002).

Assim, o objetivo principal do presente projeto é avaliar *in vitro* o efeito isolado e sinérgico do kaempferol e *tt*-farnesol sobre a viabilidade e inibição da formação do biofilme de estreptococos do grupo mutans.

2. ARTIGO

Tendo em vista a possibilidade, segundo deliberação CCPG -001/98 UNICAMP, das teses de Mestrado e Doutorado, a presente tese de mestrado será defendida na forma alternativa sendo que o artigo foi submetido á revista Biological & Pharmaceutical Bulletin (anexo 1).

INFLUENCE OF ISOLATED COMPOUNDS OF PROPOLIS ON MUTANS STREPTOCOCCI BIOFILM

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Running title: Isolated compounds of propolis against mutans streptococci biofilm.

Key words: Propolis, antimicrobial, biofilms, kaempferol, *tt*-farnesol.

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ABSTRACT

Propolis, a resinous hive product collected by *Apis mellifera* bees, has shown *in vitro* inhibitory effects on growth of oral bacteria and activity of glucosyltransferases. Several compounds from propolis have been isolated and identified. Among such compounds, kaempferol and *tt*-farnesol have displayed biological activity against mutans microorganisms and planktonic cells. The purpose of the present study was to evaluate the *in vitro* effects of kaempferol and *tt*-farnesol, alone or in combination on viability (time-kill) and inhibition of biofilm formation. The microorganisms used in this experiment were *S. mutans* UA159 and *S. sobrinus* 6715. The concentrations of kaempferol and *tt*-farnesol were 1.33mM alone and 0.665mM in combination. Chlorhexidine was used as a positive control at concentration of 1.33mM, equivalent to 0,12%. Biofilm formation occurred on glass surface during five days, for the time-kill tests the biofilms were submitted to treatments with the propolis compounds isolated or in combination for 0, 1, 2, 3 and 4 hours (h); the results were expressed in log N/N_0, in that N_0 as ufc formed initially and N the number of recovered biofilms after the treatments. The biofilms were treated from the second day with the compounds and their association for 1 minute twice daily for 3 days for the inhibition of biofilm formation; the results were expressed in log ufc/biofilm. The dosage of insoluble polysaccharides (Dubois, 1956) and total protein of biofilm (Lowry, 1951) was carried out for the inhibion of biofilm formation. ANOVA and Tukey-Kramer HSD were carried out for all tests n=9. *tt*-farnesol showed antibacterial activity after 4 h (log $N/N_0 > 3$) against *S.mutans* and *S.sobrinus* for time-kill test. Kaempferol showed low antimicrobial activity for time-kill test. When in combination, the compounds displayed antibacterial effect after 4 h (log $N/N_0 > 3$) for time-kill test. Kaempferol and *tt*-farnesol, alone, did not show any statistically significant antibacterial effects for biofilm inhibition test. However, *tt*-farnesol and kaempferol+*tt*-farnesol reduced the total amount of insoluble polysaccharide and protein, being statistically different from the negative control. Kaempferol in combination with *tt*-farnesol, at low concentrations, showed antimicrobial effect, reducing significantly the bacterial viability, amount of insoluble polysaccharide, and amount total protein. In conclusion, the combination of kaempferol+ *tt*-farnesol showed antimicrobial activity against biofilms of mutans microorganisms, being promising compounds against dental biofilm.

INTRODUCTION

Mutans streptococci have been implicated as a primary etiological agent of dental caries in animals and humans (Loesche, 1986). The organisms are also involved in the formation and accumulation of dental biofilm (Loesche, 1986). The acidogenic and aciduric properties of *Streptococcus mutans* together with its ability to synthesize extracellular glucans are the major factors for the development and establishment of cariogenic biofilms (Loesche, 1986; Marsh *et al*., 1995).

Chemical analyses of dental biofilm revealed that polysaccharides are one of the major components in the biofilm matrix, especially with the persistent consumption of carbohydrates (Hotz *et al*., 1972). These polysaccharides are synthesized from sucrose chiefly by glucosyltransferases (GTFs) (Rölla *et al*., 1983). GTFs produced by *S. mutans* catalyze the formation of soluble and insoluble glucans, which enhance the pathogenic potential of dental biofilm by promoting adherence and accumulation of cariogenic streptococci on the tooth surface (Schilling and Bowen, 1992). Glucan-rich plaque matrix not only contributes to the bulk of dental plaque but also changes its quality and porosity (Dibdin and Shellis, 1998). Therefore, antimicrobial agents against these oral pathogens or agents that can affect plaque matrix formation and accumulation of cariogenic biofilm communities are some of the strategies for prevention of dental caries and other plaque-related diseases (Koo *et al*., 1999).

In this context, propolis, a resinous substance collected by the *Apis mellifera* bee (Ghisalberti., 1979), has shown anti-caries and anti-plaque properties *in vitro* and *in vivo* (Koo *et al*., 1999; 2002a; 2002b; Duarte *et al*., 2003). Several compounds have been

identified in propolis, and three distinct chemical groups have been reported to be present: (1) flavonoid aglycones, (2) cinnamic acid derivatives, and (3) terpenoids (Bankova *et al*., 1995; Banskota *et al*., 1998; Burdock *et al*., 1998; Park *et al*., 1998; Tazawa *et al*., 1998). Recently, Koo *et al*. (2002c) reported that some of the compounds from propolis displayed biological activities on GTFs and mutans streptococci growth. It has been found that kaempferol (3,4,5,7-tetrahydroxyflavone) is an effective inhibitor of GTFs. The *tt*-farnesol, a natural sesquiterpene alcohol (3,7,11-trimethyl-2, 6,10-dodecatrien-1-ol), showed inhibition of growth and metabolism of mutans streptococci (Koo *et al*., 2002c). In addition, *tt*-farnesol showed cariostatic properties in rats without significant effects on the microbial viability in the animals' mouth (Koo *et al*., 2002b).

Considering the effects of kaempferol and *tt*-farnesol on GTFs and bacterial membrane, the purpose of this study was to evaluate the influence of these agents, alone or in combination, on viability, accumulation and composition of mutans streptococci biofilms.

MATERIALS AND METHODS

Test compounds. Kaempferol and *tt*-farnesol were obtained from Extrasynthese Co. (Genay-Sedex, France); the compounds were verified by means of HPLC and GC/MS as standard procedures performed by the company for purity (99%) and authenticity. Chlorhexidine (CHX), used as a positive control, was purchased from Sigma-Aldrich Co. (St. Louis, MO). Kaempferol and *tt*-farnesol were dissolved in ethanol:dimethyl sulfoxide (DMSO) (1:4, v/v) just prior to carrying out the assays. The biofilms were treated with *tt*-

farnesol, Kaempferol, either alone or in combination, and Chlorhexidine to determine whether these agents could adversely affect further biofilm development and accumulation. The concentrations (see Table 1) were chosen based on our previous concentration to response data (Koo *et al*., 2002c), and also to compare with our positive control 0.12% CHX (equivalent to 1.33 mM), which is a clinically proven anti-plaque agent. Appropriate solvent controls were always included.

Biofilm growth. The microorganisms used were *S. mutans* UA159 and *S. sobrinus* 6715. The bacterial inoculum was prepared as detailed elsewhere (Koo *et al*., 2002). Biofilms were formed on standard glass microscope slides (Micro slides, Glass Técnica Com., São Paulo, SP, Brazil) in batch cultures for maximum of 5 days (Ma *et al*., 1999). Mutans Streptococci were grown in tryptone-yeast extract broth with the addition of 1% (w/v) sucrose and incubated at 37 ^{o}C and 10% CO_2. Typically, 5-day-old biofilms yield approximately 10^8 colony forming units (CFU) per mm^2 of slide (Ma *et al*., 1999).

Time-kill assays. The killing assays were performed according to Ma *et al*., 1999 and Koo *et al*., 2002. The 5 day-old biofilms were treated with one of the test agents described above. After 1, 2, 3 and 4 hours of treatment, the biofilms were removed from test solutions, washed and suspended in salt solution (Ma *et al.,*1999), and subjected to sonication by a Vibra Cell (Sonics & Material Inc) (6 pulses of 10 seconds with 5-second intervals, at 40 watts). This sonication procedure provided the maximum recoverable counts as determined experimentally (Koo *et al*., 2002a). The homogenized suspension was serially diluted (10^{-1} to 10^{-5}) and plated on tryptic soy agar (Oxoid LTD, Basingstoke, UK)

by means of a spiral plater (Whitley Automatic Spiral Plater, DW Scientific). The plates were incubated at 10% CO_2 at 37 ^{o}C for 48 h, to determine viable-cell counts. Killing curves were constructed by plotting values in the ordinate label, N_0 stands for the original number of colony – forming units per mL and N stands for the number after the indicated times of exposure. All of the assays were carried out in quadruplicate on at least three different times. A bactericidal effect was defined as a > 3 log N/N_0 decreased in the CFU/mL from initial viable counts, at time zero (Koo *et al*., 2002). The potential for drug carryover to produce false low viability counts was minimized by washing biofilm slides in salt solution (6 times).

Inhibition of biofilm formation. The biofilms were grown for 48 h to allow initial bacterial deposition incubated in 10% CO_2 at 37 ^{o}C for 48 h. At this point (48 h old), the biofilms were treated twice daily until the 5th day of the experimental period (120 h-old biofilms). The biofilms were exposed to the treatments with the test agents or vehicle control for one minute, followed by double-dipped rinse in sterile salt solution, and transferred to fresh culture media. The treatments were repeated 6 h later, except the culture medium replacement. Following the second one minute exposure, the biofilms were incubated undisturbed for 18 h. The experimental procedure was performed in the third (72 h old) and fourth (96 h) day. The final exposure to the test agents occurred when the biofilms reached 108 h; they were incubated an additional 18 h and harvested at the age of 120 h (5th day of the experimental period). Each biofilm was exposed to the respective treatment six times. Our preliminary data showed that the vehicle control (one-minute exposure, twice-daily) allowed the continued formation of biofilm. Biofilm assays were

performed in quadruplicate in at least 3 different experiments. At the end of the experimental period, the biofilms were dip-washed twice, then gently swirled in salt solution to remove loosely adherent material. The biofilms were placed in 30 mL of sterile salt solution, and the glass surfaces were gently scraped by means of sterile spatula to harvest adherent cells. The removed biofilms were subjected to sonication by a Vibra Cell (Sonics & Material Inc) (6 pulses of 10 seconds with 5-second intervals, at 40 watts). An aliquot (0.1 mL) of the homogenized suspension was serially diluted (10^{-1} to 10^{-5}) and plated on tryptic soy agar by means of a spiral platter (Autoplate model 3000, Spiral Biotech, Inc., Bethesda, MD). The plates were incubated at 10% CO_2 at 37 ^{o}C for 48 h, and then the number of CFU was determined. Curves of inhibition of formation of biofilm that will be described at log N/N_0. A bactericidal effect was defined as a > 1 log N/N_0 decreased from initial viable counts; at time zero (Koo *et al*., 2003). The potential for drug carryover to produce false low viability counts was minimized by washing biofilm slides in salt solution (6 times). All of the assays were carried out in quadruplicate on at least three different times. The biofilms obtained of these test was dried in P_2O_2 for 48 h and used to determine the dry-weight, total protein and insoluble polysaccharide.

Protein assays. The kit Bio-Rad DC Protein Assay® (Bio-Rad Laboratories, USA) (BIO-RAD Laboratories publication No. 28EG, 1978) was used to determine the total protein concentration in each sample testes based on Lowry method. To determine total protein in biofilms, first the proteins were extracted of 10 mg of dried biofilm from the inhibition of biofilm formation, using 1 mL of reagent A (Bio-Rad DC Protein Assay®) and being incubated to 60°C for 15 min. An aliquot of 100 µL was retired of this tube and

replaced at another with 500 µL of reagent A and 4 mL of reagent B (Bio-Rad DC Protein Assay®). After 15 min, the absorbance of the samples was measured at 750 nm. For the standard curve was used bovine serum albumin (Bio-Rad DC Protein Assay ®) (Lowry *et al*., 1951). The results are expressed in µg of protein in 1 mg of dried-weight biofilm.

Polysaccharide analyses. The dried biofilm was used for polysaccharide analysis with a modification of the proportion of solutions in the use of dry-weight biofilm. Thus, 0.5 M HCl was added to each tube at 0.1 mL/mg proportion of biofilm dried-weight. After extraction for 3 h at room temperature under constant agitation, the same volume of TISAB II, pH 5.0 (containing 20 g of NaOH/L), was added to the tube as buffer (Cury *et al*., 2000). The samples were centrifuged for 1 minute and the supernatant discarded. To the precipitate 1.0 N NaOH (0.2 mL/mg biofilm dry-weight) was added. The samples were homogenized (11,000 g) for 1 min and maintained under agitation for 3 h at room temperature and the concentration of insoluble polysaccharide was determined in the supernatant. Insoluble polysaccharide was determined colorimetrically (Dubois *et al.*, 1956). The results are expressed in µg of protein in 1 mg of dried-weight biofilm.

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

RESULTS

Effects of test agents on Time-kill. The results of the time-kill kinetic studies are summarized in Figure 1 and 2. The kaempferol at 1.33 mM showed slight antibacterial activity. In contrast, *tt*-farnesol was bactericidal against *S. mutans* UA 159 and *S. sobrinus* 6715 after 4 hour exposure. The kaempferol combined with *tt*-farnesol was also effective in killing biofilms of *S. mutans* UA 159 and *S. sobrinus* 6715.

Effects of test agents on biofilm accumulation. The population of viable cells recovered from the biofilms (*S. mutans* UA 159 and *S. sobrinus* 6715) after treatment is shown in Table 2 and 3. The biofilms treated with *tt*-farnesol (1.33mM) and combination of kaempferol + *tt*-farnesol (0,665 mM each) showed significantly lower number of recoverable viable cells compared to vehicle control (1.0 to 2.5 $\log_{10}$ decrease in cfu/biofilm). The positive control CHX 1.33mM dramatically affected the viability of *S. mutans* showing more than 8-$\log_{10}$ decrease in cfu/biofilm.

Effects of test agents on dry-weigth, polysaccharide and protein composition of the biofilms. The total amount of dry-weigth, polysaccharides and protein in the biofilm after treatment (120 h-old biofilms) is found in Tables 2 and 3. The biofilms treated with *tt*-farnesol and kaempferol + *tt*-farnesol, resulted in a significantly lower concentration of polysaccharides in the biofilms (Table 2 and 3, $P<0.05$). The CHX (1.33 mM) treatment stalled further accumulation of the biofilms. Furthermore, the concentration of protein in the biofilms treated with the *tt*-farnesol and kaempferol + *tt*-farnesol was significantly lower than that in the biofilms treated with vehicle control (Table 2 and 3, $P<0.05$).

DISCUSSION

Biofilm is an organized microbial community which displays enhanced resistance to biocides and antimicrobial agents, and also has the ability to adapt to various stress conditions (Burne *et al.*, 1996; Svensäter *et al.*, 2001; Lewis, 2001).

Our results demonstrated that *tt*-farnesol, a novel promising control plaque agent (Koo *et al.,* 2003) had a bactericidal effect on mutans streptococci biofilms. This finding is in agreement with previous reports suggesting that the chemical structure and the lipophilic properties of the *tt*-farnesol may cause changes in the permeability and fluidity of the membrane, thereby affecting the bacterial viability (Ramage *et al*., 2002; Koo *et al*., 2003). Also, these effects were observed on other microorganisms, such as *Streptomyces tendae* and *Saccharomyces cerevisiae*, but not on *Escherichia coli* (Machida *et al.,* 1999). In the present study, *tt*-farnesol was able to inhibit the biofilm accumulation when applied topically twice daily (one-minute exposure, twice daily). Furthermore, it also affected the organic composition of biofilms by reducing their biomass, total protein and alkali soluble polysaccharides ($p<0.05$).

Moreover, kaempferol alone did not demonstrate significant antimicrobial effect in time-kill and inhibition of biofilm formation tests. This finding is in agreement with a recent study where kaempferol demonstrated to have slight antimicrobial effect (MIC > 800 μg/mL), however showed inhibitory effect on GTFs C and D (Koo *et al*., 2002).

The combination of kaempferol and *tt*-farnesol was significantly more effective in reducing the biofilm accumulation than *tt*-farnesol alone ($p<0.05$), even if in low

concetration (0.665 mM each compound). Furthermore, the combination of agents was the only treatment in low concentration (0.665 mM) that significantly decreased the concentration of insoluble polysaccharides and total protein.

When the compounds were tested in association an improved antimicrobial effect was observed. This improved effect might be related with the combination of action mechanisms with which each compound can affect mutans streptococci biofilm. It is known that *tt*-farnesol has disrupted bacterial membrane function that decreases the population of microorganisms in biofilm (Ramage *et al*., 2002; Koo *et al*., 2003) and kaempferol acts on GTFs C and D, inhibiting the adherence of mutans (Koo *et al*., 2002), consequently reducing the biomass of biofilms.

Clearly, the influence of kaempferol + *tt*-farnesol on this monospecies biofilm model has many implications on their therapeutic potential in the context of the complex biochemical composition of human dental plaque. First and foremost, these agents remarkably affected the synthesis of polysaccharide in the plaque matrix, further bacterial accumulation, and disrupted oral microorganisms that do not form polysaccharides. Furthermore, it has been shown that a glucan matrix is the main factor for a high cariogenicity of dental plaque (as a microbiologically and biochemically heterogeneous biofilm) formed in the presence of sucrose *in vivo* (Cury *et al*., 2000).

In this study, the data obtained showed that a brief exposure (one-minute, twice daily) of kaempferol and *tt*-farnesol caused significant inhibition on bacterial accumulation, reducing biomass and polysaccharide and protein contents of mutans streptococci biofilms. The topical application of the test agents in combination may have been sufficient to affect

the rate of the glucan synthesis, and consequently, reduced the accumulation and the biomass of the biofilms (Koo *et al*., 2003).

In conclusion, the association of kaempferol + *tt*-farnesol, at low concentration, significantly influenced mutans biofilm formation showing an improved bactericidal effect, accomplished biofilm matrix change, probably due to the combination of different mechanism interactions. These two natural compounds combined might be a promising source of new agents that may prevent dental caries and other oral diseases, however studies involving animal models are worth performing to evaluate their effects *in vivo*.

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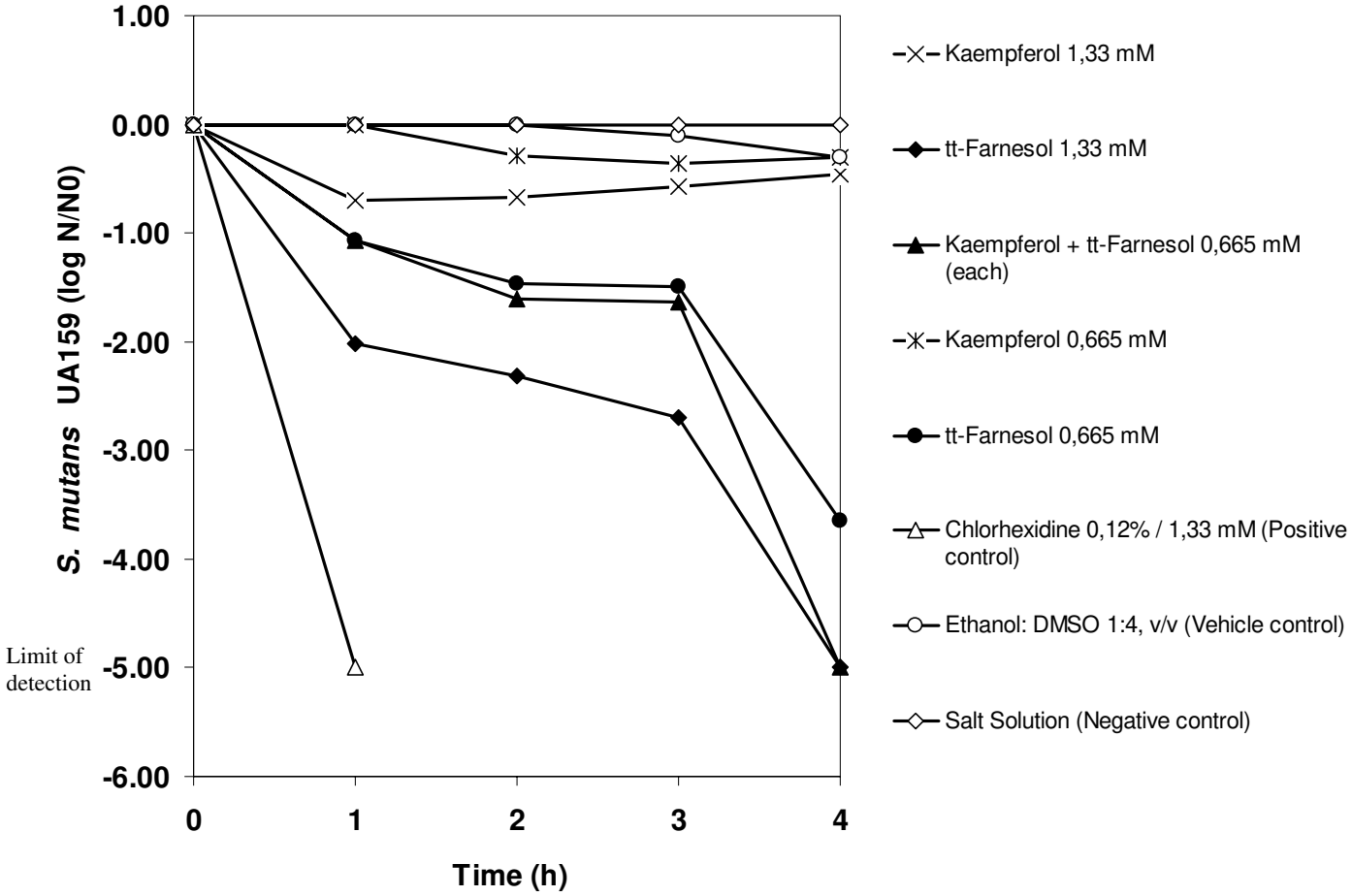
Table 1. Tested compounds used in biofilm treatments.

Test Compounds	**Concentration / (mM)**
Kaempferol	1.33
tt-Farnesol	1.33
Kaempferol +*tt*-farnesol	0.665*
Kaempferol	0.665
tt-farnesol	0.665
Chlorhexidine (Positive control)	1.33 (0.12%)
ethanol:dimethyl sulfoxide (DMSO) (Vehicle control)	(1:4, v/v)
Salt solution - (Negative control)	(50mM KCl and 1mM $MgCl_2$, pH7.0)

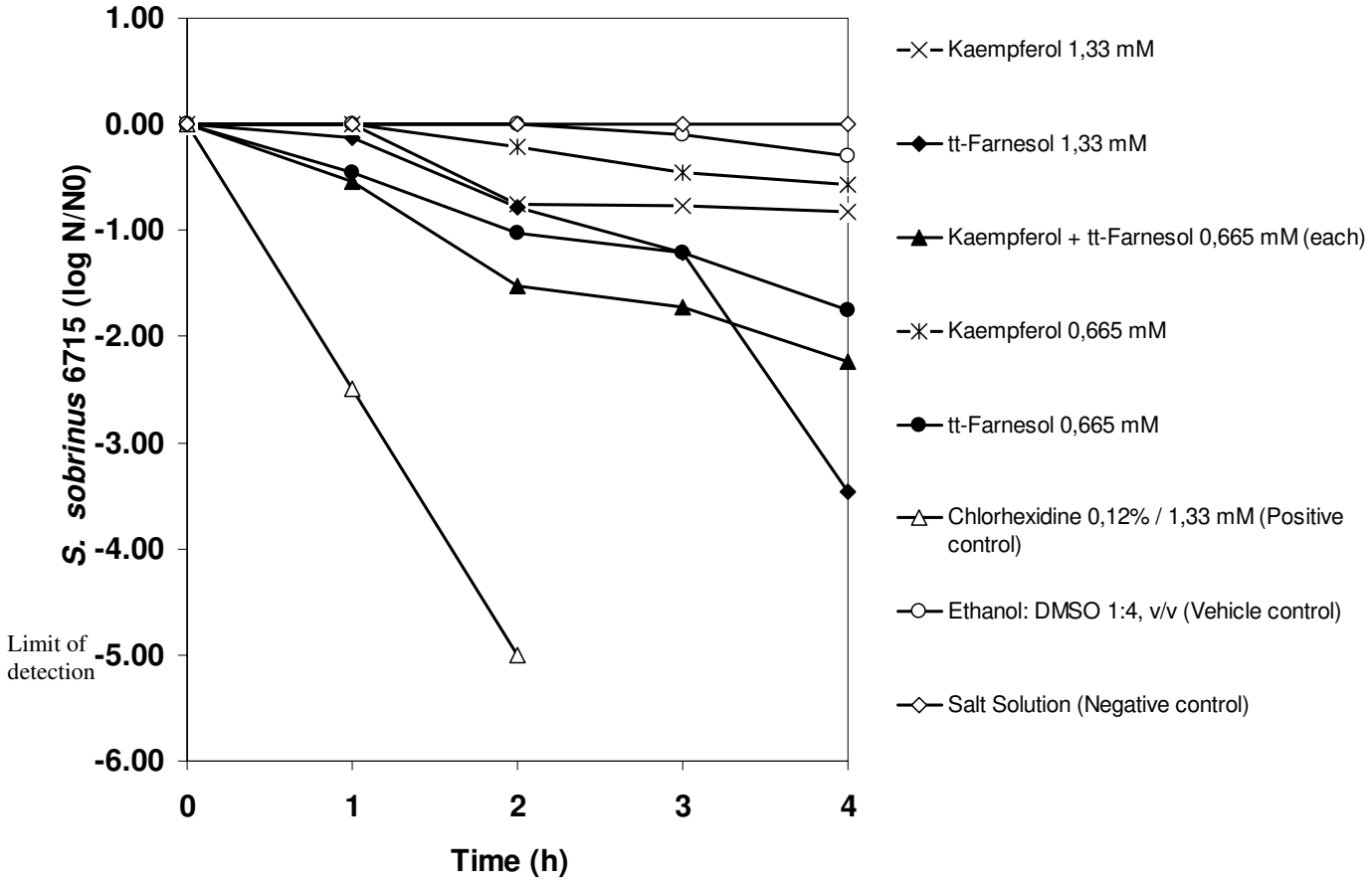
* Concentration for each compound

Figure 1. Time-kill curves for *S. mutans* UA 159 by isolated compounds of propolis and controls.



In ordinate label, N_0 stands for the original number of colony-forming units per ml and N stands for the number after the indicated times of exposure (n=9).

Figure 2. Time-kill curves for *S. sobrinus* 6715 by isolated compounds of propolis and controls.



In ordinate label, N_0 stands for the original number of colony-forming units per ml and N stands for the number after the indicated times of exposure (n=9).

Table 2. Dry-weight, and total amount of polysaccharides, and protein in *S. mutans* UA 159 biofilms after treatments.

Test Compounds	Dry-weight (mg)	Polysaccharide (µg/mg of biofilm)	Protein (µg/mg of biofilm)	Average cfu recovered after treatments (log_{10} cfu/Biofilm)
Kaempferol (1.33mM)	26.9±3.4 ab	490±34 a	211±12 a	10.6±0.5 a
tt-Farnesol (1.33mM)	23.8±2.8 b	430±28 b	180±17 b	9.6±0.3 b
Kaempferol + *tt*-farnesol (0.665 mM, each)	24.6±3.1 b	422±41 b	186±13 b	9.4±0.7 b
Kaempferol (0.665 mM)	31.7±1.4 a	488±31 a	274±22 a	10.8±0.5 a
tt-Farnesol (0.665 mM)	29.8±1.2 a	490±39 a	278±19 a	9.9±0.4 ab
Chlorhexidine (Positive control) (1.33mM)	0.0±0.0 c	0.0±0 c	0.0±0 c	0.0±0.0 c
ethanol:dimethyl sulfoxide 1:4, v/v (DMSO) (Vehicle control)	30.8±1.8 a	490.7±37 a	275±27 a	11.0±0.7 a
Salt solution (Negative control) 50mM KCl and 1mM $MgCl_2$	33.9±2.6 a	533.9±43 a	274±26 a	11.3±0.5 a

***Values in the same column followed by the same letters are not significantly different from each other (*P*< 0.05, Anova, comparison for all pairs using Tukey-Kramer test, n=9).**

Table 3. Dry-weight, and total amount of polysaccharides, and protein in *S. sobrinus* 6715 biofilms after treatments*.

Test Compounds	Dry-weight (mg)	Polysaccharide (µg/mg of biofilm)	Protein (µg/mg of biofilm)	Average cfu recovered after treatments (log_{10} cfu/Biofilm)
Kaempferol (1.33mM)	10.8±1.6 ab	419±37 a	192±23 a	7.6±0.5 a
tt-Farnesol (1.33mM)	7.2±1.3 b	375±41 b	164±21 b	6.9±0.2 b
Kaempferol + *tt*-farnesol (0.665 mM, each)	7.7±1.8 b	363±43 b	151±17 b	6.7±0.4 b
Kaempferol (0.665 mM)	12.7±1.3 a	421±38 a	202±24 a	8.0±0.6 a
tt-Farnesol (0.665 mM)	13.5±1.2 a	432±31 a	196±18 a	7.6±0.3 ab
Chlorhexidine (Positive control) (1.33mM)	0.0±0.0 c	0±0 c	0±0 c	0.0±0.0 c
ethanol:dimethyl sulfoxide 1:4, v/v (DMSO) (Vehicle control)	12.4±1.3 a	435±49 a	259±27 a	7.8±0.9 a
Salt solution (Negative control) 50mM KCl and 1mM $MgCl_2$	12.9±2.1 a	457±42 a	264±32 a	8.1±0.8 a

***Values in the same column followed by the same letters are not significantly different from each other (*P*< 0.05, Anova, comparison for all pairs using Tukey-Kramer test, n=9).**

CONCLUSÕES

Concluímos que o Kaempferol apresenta baixa atividade antimicrobiana e o *tt*-farnesol apresenta significativo potencial antimicrobiano na redução da viabilidade bacteriana e na inibição da formação de biofilme de microrganismos do grupo mutans.

Os compostos Kaempferol e *tt*-farnesol quando associados em baixas concentrações apresentaram potencial antimicrobiano sobre a redução da viabilidade bacteriana e na inibição da formação de biofilme de microrganismos do grupo mutans.

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