UNIVERSIDADE ESTADUAL DE CAMPINAS - UNICAMP
FACULDADE DE ODONTOLOGIA DE PIRACICABA - FOP

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Cirurgião Dentista

Avaliação das atividades antibacteriana, anticárie e antiinflamatória dos compostos isolados da própolis vermelha

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

Orientador: Prof. Dr. Pedro Luiz Rosalen
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Este exemplar corresponde à versão final da Tese defendida pelo aluno, e orientada pelo Prof. Dr. Pedro Luiz Rosalen

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Dedico este trabalho..

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RESUMO

A própolis brasileira destaca-se entre os produtos naturais por ser considerada uma rica fonte para a descoberta de novos compostos bioativos. Entre as própolis brasileiras, destaca-se a vermelha (tipo 13), a qual apresentou atividade antimicrobiana, antioxidante e antitumoral, sendo que seu principal constituinte químico são as isoflavonas, diferentemente de qualquer outro tipo de própolis brasileira. Portanto, o objetivo deste trabalho foi avaliar as propriedades antibacteriana, anticárie e antiinflamatória da própolis vermelha, identificando os possíveis compostos ativos responsávelis por estas atividades biológicas. Este objetivo foi atingido por meio das seguintes metodologias: 1- fracionamento bioguiado do extrato etanólico da própolis vermelha (EEP); 2- identificação e isolamento dos compostos ativos, 3- avaliação do potencial anticárie dos compostos ativos utilizando modelos: a) *in vitro* de inibição de biofilme oral monoespécie nos níveis molecular, estrutural e bioquímico; b) de cárie animal submetido a um severo desafio cariogênico; e 4- avaliação das propriedades anti-inflamatórias do EEP e de seus compostos isolados. Como resultado do fracionamento bioguiado, foram isolados e identificados 2 compostos: neovestitol e vestitol. Para os testes antimicrobianos e anti-cárie foi utilizada a fração 4.2 que continha os dois compostos isolados enquanto que para os testes anti-inflamatórios os compostos foram testados separadamente. A fração inibiu o desenvolvimento de biofilme em discos de hidroxiapatita na concentração de 800 µg/mL, através da inibição da formação dos polissacarídeos e do teor protéico. Essa inibição foi observada através de: a) análise dos dados bioquímicos (redução de 40% da quantidade de políssacarídeos e 20% da quantidade protéica); b) redução da atividade das enzimas produtoras de políssacarídeos (glucanos), as glucosiltransferases B, C e D em aproximadamente 60%; c) redução da biomassa do biofilme e espessura da matrix de políssacarídeos extracelular sem afetar a viabilidade bacteriana, observada através da análise computadorizada das imagens obtidas com o microscópio confocal laser; d) alteração da expressão de 59 genes (52 *dowregulated* e 6 *upregulated*) de *S. mutans* observadas por microarray, sendo que os resultados dos genes: *copY*, *copA*, *copZ*, *sloA*, *gtfD* e *amyA* foram validados por qRT-PCR. A fração com os dois compostos também foi efetiva em reduzir a incidência e severidade das lesões de cárie em estudo animal sem afetar a viabilidade bacteriana. Em relação à atividade anti-inflamatória, tanto EEP quanto neovestitol e vestitol foram capazes de reduzir a migração leucocitária no processo inflamatório assim como inibiram a produção de citocinas pró-inflamatórias. Portanto, o fracionamento bioguiado revelou-se um processo eficaz para obtenção de novos compostos bioativos e tanto o EEP quanto seus compostos isolados (neovestitol e vestitol) apresentaram atividade anti-cárie e anti-inflamatória, revelando-se compostos promissores para o desenvolvimento de novos medicamentos.

ABSTRACT

The Brazilian propolis stands out among the natural products because it is considered a rich source for discovering new bioactive compounds. Among Brazilian propolis, the red one (type 13) exhibited antimicrobial, antitumor and antioxidant activities. Moreover, the main chemical constituents are the isoflavones, which are distinctive from other types of Brazilian propolis. Therefore, this study aimed to evaluate the antibacterial, anticaries and anti-inflammatory properties of red propolis, identifying the putative active(s) compound(s) that could be responsible for the biological activities. This objective was reached through the following methods: 1 – bioassay-guided fractionation of the ethanolic extract of propolis (EEP), 2 - identification and isolation of active compounds, 3 - evaluation of the anticaries potential of active compounds using the following models: a) *in vitro* monospecies biofilm and analyzed in the molecular, structural and biochemical levels b) *in vivo* rodent model of dental caries using a severe cariogenic challenge, and 4 - evaluation of anti-inflammatory properties of EEP and isolated compounds. As a result of bioassay-guided fractionation two compounds were isolated and identified: neovestitol and vestitol. A fraction 4.2 contains mostly these two compounds, was used for antimicrobial and anti-caries evaluations. For anti-inflammatory evaluation, the compounds were tested separately. The bioactive fraction at 800 µg/mL decreased the development of biofilm on hydroxyapatite discs, by reducing the formation of extracellular polysaccharides (EPS) and the protein content. This inhibition was observed through four different methodologies: a) analysis of biochemical data (reduction of 40% of EPS and 20% of protein content), b) reduction of approximately 60% of activity of enzymes glucosiltransferases B, C and D that produces polysaccharides (ie. Glucans), c) reduction of biofilm biomass and thickness of EPS-matrix without affecting bacterial cell viability, as determined by confocal laser microscopy and computational analysis; d) disruption of expression of 59 genes (52 upregulated and 7 dowregulated) as determined by microarray, *copY*, *copZ*, *sloA*, *gtfD* and *amyA* were validated by qRT-PCR. Also, fraction containing vestitol and neovestitol was effective in reducing the incidence and severity of caries *in vivo* without affect viability of bacterial cells. In relation to anti-inflammatory activity, EEP, neovestitol and vestitol were able to reduce leukocyte migration during inflammation process and also, inhibited pro-inflammatory cytokines release. Therefore, the bioassay-guided fractionation identified bioactive compounds, which along with EEP exhibited anti-caries and anti-inflammatory activities. The agents may be promising compounds for the development of new drugs against oral diseases.

SUMÁRIO

1. INTRODUÇÃO

A cárie dental é um dos principais indicativos que determinam o estado fisiopatológico da cavidade bucal de um ser humano, sendo uma doença multifatorial, envolvendo aspectos microbianos (HOROWITZ, 2004).

Um dos fatores etiológicos mais importantes da cárie é a formação de biofilme patogênico (por meio de uma matrix extracelular), o qual produz ácidos e produtos citotóxicos que levam, respectivamente, a desmineralização do esmalte dental e/ou inflamação gengival (MARSH, 2003).

A cárie dental ocorre pela ação de bactérias produtoras de polissacarídeos extracelulares, acidogênicas e acidúricas (HAMADA & SLADE, 1980), que interagem com saliva, carboidratos da dieta e outros microrganismos orais, permitindo a formação de um biofilme na superfície dos dentes (MARSH, 2003). O *Streptococcus mutans*, associado ao início da doença cárie (LOESCHE, 1986), possui uma importante capacidade produção de glucanos na presença de sacarose, contribuindo com a formação de um biofilme rico em polissacarídeos e altamente acidogênico, causando a desmineralização do esmalte dental (BOWEN & KOO, 2011).

O *S. mutans* tem sido intensamente estudado quanto aos seus fatores de virulência, entre eles, a tolerância ao meio ácido favorecida pela atividade de deslocamento de prótons pela ATPase (BURNE *et al.,* 1999; QUIVEY *et al.*, 2000) e a síntese de glucanos através das enzimas glicosiltransferases - GTFs (GIBBONS & van HOUTE, 1975; HAMADA e SLADE, 1980; MARSH 2004; BOWEN & KOO, 2011). Atualmente, três GTFs distintas, secretadas pelo *S. mutans*, estão bem caracterizadas molecular e bioquimicamente: 1) Gtf B - codificada pelo gene *gtfB*, que sintetiza glucanos insolúveis em água tendo ligações

glicosídicas principais α (1→3); 2) Gtf C - codificada pelo gene *gtfC*, que sintetiza uma mistura de glucanos insolúveis e solúveis, este último apresentando ligações glicosídicas principais α (1→6); e 3) Gtf D - codificada pelo gene *gtfD*, que sintetiza basicamente glucanos solúveis (LOESCHE, 1986; HANADA e KURAMITSU, 1989; VACCA-SMITH e BOWEN, 1998). Os glucanos, principalmente os insolúveis em água, têm sido considerados como os principais fatores de aderência e acúmulo de estreptococos cariogênicos sobre a superfície dental (HAMADA e SLADE, 1980; ROLLA *et al.*, 1983; TANZER *et al.*, 1985; SCHILLING e BOWEN, 1992). Uma vez acumulado, o consumo frequente de sacarose (e outros carboidratos fermentáveis) levam a produção de uma matriz extracelular rica em polissacarídeos, auxiliando na formação de biofilme altamente coeso e adesivo. Paralelamente, o metabolismo dos açúcares pelos microrganismos acidogênicos levam a acidificação do microambiente do biofilme, contribuindo para o predomínio de bactérias acidúricas. Assim, estreptococos do grupo mutans e glucanos são considerados fatores críticos no desenvolvimento do biofilme dental cariogênico.

O acúmulo de biofilme iniciará um processo inflamatório nos tecidos moles adjacentes à estrutura dental, com conseqüente produção de citocinas, as quais exercerão efeito quimiotáxico sobre os leucócitos. O processo de migração leucocitária é mediado por inúmeras citocinas, entre as quais podemos citar TNF-α e IL-1β (citocinas inflamatórias) e IL-10 (citocina antiinflamatória) (JONES *et al.,* 1991). Os neutrófilos são os principais tipos de leucócitos envolvidos na defesa do organismo e no processo inflamatório e sua migração dos vasos sanguíneos para os tecidos lesados é crucial na defesa do organismo contra a inflamação (MALECH & GALLIN, 1987).

Embora os neutrófilos apresentem um papel protetor na inflamação, a destruição tecidual é conseqüência de sua migração excessiva, o que gera a dor de origem

inflamatória. Esse sintoma é o principal motivo que leva as pessoas a procurarem tratamento especializado e seu controle e prevenção tem sido alvo de intensas pesquisas (NAPIMOGA *et al.,* 2008).

Portanto, a busca de novas drogas que bloqueiem a infiltração dos neutrófilos em diferentes modelos de inflamação é intensa na literatura (HÉBERT, 2000; ALENCAR *et al*., 1999; ASSREUY *et al.,* 1999, NAPIMOGA *et al.,* 2007). Os produtos naturais têm sido pesquisados como fonte alternativa de descoberta de novas drogas que possam controlar as doenças inflamatórias e o biofilme cariogênico (NEWMAN 2003; TEIXEIRA *et al*., 2006; Jeon *et al.,* 2011).

Entre os produtos naturais, a própolis, um produto resinoso, coletado de diversas partes das plantas como brotos, botões florais e exudatos resinosos por abelhas africanizadas *Apis Mellifera* (SILVA *et al.,* 2008), tem sido empregada popularmente como agente terapêutico na medicina alternativa como agente anticárie e antiinflamatório, dentre outros.

A própolis brasileira tem atraído interesse científico devido à descoberta de novas drogas e obtenção de patentes como o CAPE (éter fenílico do ácido cafeico) (SU *et al.,* 1994), Apigenina, *tt*-Farnesol (KOO *et al.,* 2003, Artepelim C (3,5-diprenil-4-ácido hidroxicinâmico), assim como várias patentes de aplicação e uso terapêutico (KIMOTO *et al.,* 1998).

Considerando a própolis brasileira como uma rica fonte de compostos químicos bioativos, devido à biodiversidade da vegetação do nosso país, já foi possível classificá-la em 12 tipos distintos, sendo que as própolis dos tipos 3 (estado do Rio Grande do Sul), 6 (estado da Bahia) e 12 (estado de Minas Gerais) têm sido bastante estudadas e apresentam atividade antimicrobiana e anticárie (PARK *et al*., 2000). A própolis do tipo 12 apresentou

atividade antinflamatória (PAULINO *et al.,* 2003) e a própolis originária do sul do Brasil apresentou propriedades antiinflamatórias em estudos *in vitro* através de vários mecanismos de ação, entre eles o do óxido nítrico (PAULINO *et al.,* 2002).

A própolis brasileira chamada de vermelha e classificada como tipo 13, oriunda da região de Alagoas (SILVA *et al.,* 2008), apresentou forte atividade antimicrobiana contra *S. mutans, S. aureus, S. sobrinus* e *A. naeslundii.* Além disso, foram isolados e identificados diversos compostos químicos desse tipo de própolis (RIGHI *et al.,* 2011; PICCINILLE *et al.,* 2011). Entre esses compostos, foram identificadas, pela primeira vez em própolis brasileiras, 4 isoflavonas, compostos com reconhecidas propriedades antimicrobianas, antiinflamatória, antifúngicas e anticâncer (SOBY *et al.,* 1997; WANG *et al.,* 2000; MILITAO *et al.,* 2005, 2006; RUFER and KULLING, 2006; ALENCAR *et al.,* 2007).

Recentemente, o extrato bruto da própolis vermelha inibiu a proliferação de linhagens cancerígenas de células leucêmicas em testes *in vitro* (FRANCHI *et al.,* 2011) demosntrando assim o potencial dessa própolis para a descoberta de novos agentes com diversas atividades biológicas.

Assim, o objetivo principal deste trabalho foi avaliar as propriedades antibacteriana, anticárie e antiinflamatória da própolis vermelha, identificando os compostos ativos responsáveis por estas atividades biológicas.

2. PROPOSIÇÃO

Os objetivos do presente trabalho foram:

1) Identificar as frações bioativas da própolis vermelha, que apresentam propriedades antibacteriana, anticárie e antiinflamatória;

2) Isolar e identificar os compostos existentes na própolis vermelha e relacioná-los com as atividades antibacteriana, anticárie e antiinflamatória;

3) Avaliar os efeitos dos compostos bioativos isolados da própolis vermelha nos fatores de virulência dos microrganismos envolvidos no processo de cárie dental nos níveis molecular, estrutural e bioquímico e;

4) Avaliar os efeitos dos compostos bioativos isolados da própolis vermelha sobre a migração de leucócitos e inibição de citocinas.

3. PREÂMBULO DOS CAPÍTULOS

Esta Tese está de acordo com a Informação CCPG/002/06, UNICAMP, de 13/09/2006 (anexo I), que regulamenta o formato alternativo para dissertação e tese, permitindo a inserção de artigos científicos de autoria ou co-autoria do candidato.

Assim sendo, cada capítulo corresponderá a um artigo científico distinto totalizando três capítulos, sendo que os 3 artigos encontram-se em fase de submissão em revistas científicas. A seguir a sequência dos capítulos:

Capítulo 1: "Isolation and identification of bioactive isoflavonoid with antimicrobial and anti-inflammatory properties from Brazilian Red Propolis." Autores: Bruno B. Silva; Severino M. Alencar; Masaharu Ikegaki; Gil Valdo J. Silva; Marcelo H. Napimoga; Hyun Koo, Pedro L. Rosalen. Revista a ser submetido: *Separation and Purification Technology* (Fator de Impacto = 2,775), em fase de submissão.

Capítulo 2: "**Anti-biofilm and anti-caries properties of purified fraction of the Brazilian red propolis**". Autores: Bueno-Silva, B; Koo, H; Wood, M.; Alencar, SM, Ikegaki, M; Rosalen, PL. Revista: Plos One (Fator de Impacto = 4,411), em fase de submissão.

Capítulo 3: "**Vestitol and neovestitol isolated from Brazilian red propolis decreased neutrophils migration on inflammatory process**" Autores: Bueno-Silva, B; Alves, CF; Franchin, M.; Koo, H Alencar, SM, Ikegaki, M; Napimoga, M.H., Rosalen, PL. Revista: Inflammation Research (Fator de Impacto = 2, 004), submetido (Anexo V).

4. CAPÍTULO 1

Isolation and identification of bioactive isoflavonoids with antimicrobial and anti-inflammatory properties from Brazilian Red Propolis.

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Abstract

Brazilian propolis is noteworthy because it was possible to isolate many compounds with different biological activities. Among them, red propolis is notable due to its antioxidant and antimicrobial properties. Thus, the objective of this study was to isolate and identify the major bioactive compound with antimicrobial and anti-inflammatory activities from Brazilian red propolis. This objective was achieved using the following methodologies: 1- A bioassay-guided fractionation, focusing in isolation and identification of new chemical molecules with biological activities was carried out; 2- Analysis by high resolution mass spectrometry (Q-TOF-MS/MS) and Nuclear Magnetic Resonance (NMR) were conducted in order to identified the purified compound obtained through bioassay-guided fractionation; 3-Minimal Inhibitory and Bactericidal Concentrations (MIC and

MBC, respectively) against *S. mutans*, *S. sobrinus*, *S. aureus*, and *A. naeslundii* were performed to evaluate antimicrobial properties of isolated compounds; and 4- Experimental procedure to evaluate neutrophil migration was executed to verify anti-inflammatory properties of compounds. As a result, two compounds were isolated and identified as Neovestitol (2',4'-dihydroxy-7-methoxyisoflavan) and Vestitol (2',7-diidroxi-4'metoxiisoflavan). Neovestitol showed MIC ranging from <6.25 µg/mL to 25-50 µg/mL and MBC ranging from 25-50 µg/mL to 50-100 µg/mL, while Vestitol showed MIC ranging from 25-50 µg/mL to 50-100 µg/mL and MBC ranging from 25-50 µg/mL to 50-100 µg/mL. Regarding anti-inflammatory properties, Neovestitol was able to inhibit neutrophil migration (vs. vehicle control) at dose of 10 mg/Kg, while vestitol inhibited at dose of 1, 3, and 10 mg/kg without statistical difference between these three doses. Concluding, both compounds showed biological properties, such as antimicrobial and anti-inflammatory, and seem to be promising targets of Brazilian red propolis to further investigations.

Keywords: isolation, red propolis, vestitol, neovestitol

1. Introduction

Brazilian propolis has attracted scientific interest due to the discovery of new drugs, such as CAPE (caffeic acid phenyl ester) (Su *et al.,* 1994), apigenin, *tt*-farnesol (Koo *et al.,* 2003), artepillin C (3,5-diprenyl-4-hydroxycinnamic acid), as well as more than 250 patents related to its applications and therapeutic uses (Kimoto *et al*., 1998).

Among Brazilian propolis, red propolis (13^{th} type) showed remarkable antioxidant and antimicrobial activities (Alencar *et al.,* 2007; Silva *et al.,* 2008). Also, *Dalbergia ecatosphyllum*, from leguminosae family, rich in isoflavonoids, was determined as its botanical origin (Silva *et al.,* 2008).

Corroborating the findings above, several flavonoids and isoflavonoids, such as vestitol, neovestitol, biochanin A and others, were identified and isolated from Brazilian red propolis (Oldoni *et al.,* 2011; Piccinelli *et al.,* 2011). In addition, it is well-known that

isoflavonoids have many pharmacological effects (e.g. antiviral, antimicrobial, anti-inflammatory, etc.) (Cornwell *et al.,* 2004). Lately, prevention of coronary heart disease, osteoporosis, and different cancer forms, such as breast, prostate, and colon cancer, has been connected to the intake of foods and food additives containing isoflavones, and, this way, isoflavonoids have been considered more helpful for human health than flavonoids (Koblovská *et al.,* 2008).

Altogether, these findings suggest that isoflavonoid has recently increased its acceptance as a health food supplement and is used extensively in diets around the world, where it is applied to improve health and prevent diseases such as inflammation, diabetes, and cancer (Koblovská *et al.,* 2008). Therefore, isoflavonoids have become an important class of flavonoids, being the theme of scientific researches of high quality.

Thus, this study aimed to isolate and identify the major bioactive compound with antimicrobial and anti-inflammatory activities from Brazilian red propolis.

2. Material and Methods

In order to reach a bioactive compound in Brazilian propolis, an experimental procedure based on bioassay-guided fractionation was designed, focusing on the isolation and identification of a new chemical molecule with antimicrobial and anti-inflammatory activities (Jeon *et al.,* 2011). Each step of the study is showed in Figure 1.

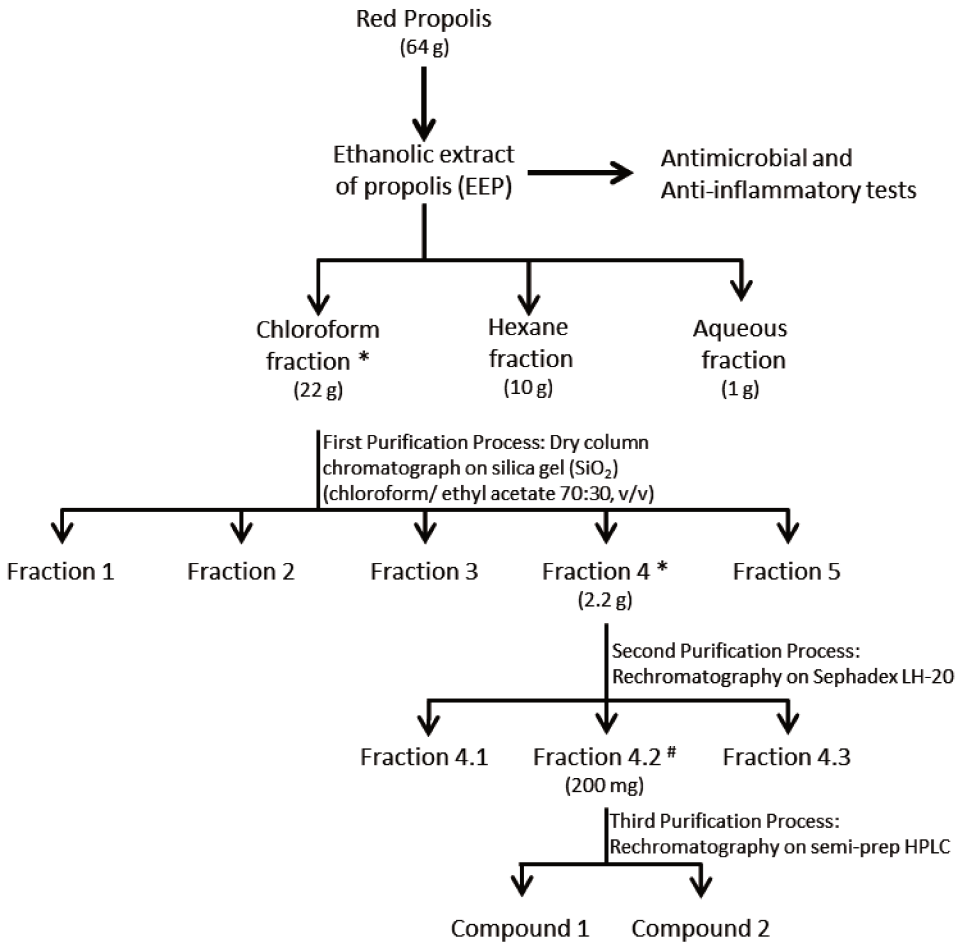

Figure 1: Experiment design of bioassay-guided fractionation in order to reach bioactive compounds. Antimicrobial tests: Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) against *S. mutans*, *S. sobrinus*, *S. aureus*, and *A. naeslundii.* Anti-inflammatory test: evaluation of neutrophil migration.
* Best antimicrobial and anti-inflammatory activities when compared with others from same line; # Easier to dissolve when compared with others from the same line.

2.1. Extraction and isolation of bioactive compounds

Brazilian red propolis were collected from the mangrove region in Marechal Deodoro, a city in the vicinity of Maceio, Alagoas, in northeastern Brazil; this region has a wet tropical climate (SL 09.40 and WL 35.41). A red propolis sample (64 g) was extracted with 80% ethanol (300 mL) in a water bath at 70ºC for 30 min and, after filtration, yielded the ethanolic extract of propolis (EEP). The EEP was further fractionated by liquid-liquid extraction with hexane and chloroform yielding 10 g of hexane fraction (Hex-fr), 22 g of

chloroform fraction (Chlo-fr), and 1 g of aqueous fraction (Aqu-fr). The active Chlo-fr (22 g) was subjected to open dry column chromatography on normal phase silica gel (particle size: 0.0063 - 0.2mm; pore size: 60 Å; pore volume: ~0.8cm^3/g; and specific surface area: 500m^2/g) and eluted with a solvent mixture of chloroform/ethyl acetate (70:30, v/v) to afford five major fractions. The fractions obtained were monitored by thin layer chromatography (TLC) using the anisaldehyde reagent (4-methoxybenzaldehyde, acetic acid, sulphuric acid: 1.0:48.5:0.5), followed by heating at 100ºC for 5 min. Fluorescent substances were visualized under ultraviolet (UV) light at the wavelengths of 254 and 366 nm. Sub fractions 1 and 5 showed no activity and negligible activities were found in fractions 2 and 3. The most bioactive fraction (4) was chromatographed over a Sephadex LH-20 column (5X30cm) using methanol to yield three bioactive fractions. Fractions 4.2 and 4.3 were active; however, fraction 4.3 was rejected because it was impossible to be dissolved in buffer solution routinely used to perform antimicrobial and anti-inflammatory tests, such as AB buffer, phosphate buffer, DMSO, sodium chloride. Thus, fraction 4.2 was purified by semi-preparative reverse-phase HPLC [Shimadzu PREP-ODS (H) 250X20mm column eluted with a gradient starting with CH_3OH:H2O (65:35) to CH_3OH:H_2O(95:5) in 35 min, flow rate 3 mL/min] and yielded two active compounds.

2.2. Identification of chemical structure of the isolated bioactive compound

2.2.1. Analysis by high resolution mass spectrometry (Q-TOF-MS/MS)

The system of mass spectrometry (MS) used was a quadrupole-time of flight (Q-TOF) (UltrOTOF-Q, Bruker Daltonics, Billerica, MA, USA) with electrospray ionization (ESI) in full scan positive and negative modes. The analysis parameters were: capillary voltage: 3.9 KV; gas temperature: 150ºC, nebulizer gas flow: 4 L/h. Introduction of sample into the spectrometry system was made in split mode at a ratio of 1:3.

2.2.2. Nuclear Magnetic Resonance (NMR)

For NMR analysis, bioactive compounds were isolated under low pressure for complete removal of solvent and, then, dissolved in CD_3OD. The ^{1}H and ^{13}C NMR spectra were obtained at 500 and 100 MHz, respectively (Bruker DRX500). As internal standard, tetramethylsilane (TMS) was used.

2.3. Antimicrobial tests

Antimicrobial tests consisted in the determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the EEP, its fractions, and isolated compounds. The tested microorganisms were *Streptococcus mutans* UA159*, Streptococcus sobrinus* 6715*, Staphylococcus aureus* ATCC25923, and *Actinomyces naeslundii* ATCC 12104. The methodology described by Koo *et al*. (2000) was modified into a micro technique, in which 190 µL of BHI broth with inoculum (1-2 x 10^5 UFC/mL) and 10 µL of samples of EEP, fractions, isolated compounds, and control solution were dispensed onto a microplate. Concentrations of the tested extracts for MIC ranged from 12.5 to 800 µg/mL, and bacterial growth was assessed by adding 0.01% resazurin stain (Aldrich). Ethanol (4%) was used as the control vehicle. MIC values were defined as the lowest concentration of a given extract that could inhibit bacterial growth. An aliquot (30 µL) of concentrations higher than MIC was cultured on BHI agar supplemented with 5% defibrinated sheep blood for 18-24 hours, at 37ºC, with 10% CO_2 to determine MBC. MBC was the lowest concentration that allowed no visible bacterial growth on agar (Koo *et al.,* 2000).

2.4. Anti-inflammatory test

Anti-inflammatory test consisted of evaluating neutrophil migration to peritoneal cavity after treatment with EEP and isolated compounds.

2.4.1. Experimental procedure to evaluate neutrophil migration

Male Balb/c mice (20–25 g) were housed in temperature controlled rooms (22–25°C) with *ad libitum* access to water and food. All experiments were conducted in accordance with National Institutes of Health guidelines for the welfare of experimental animals and with the approval of the institutional Committee for Ethics in Animal Research (protocol number: 1484-1). The animals were used only in a single experimental group. For the determination of neutrophil migration to peritoneal cavity, EEP, fractions, and isolated compounds were administered by subcutaneous injection 15 min before (1, 3, or 10 mg/kg) the administration of inflammatory stimuli by intraperitoneal injection of carrageenan at 500 µg/cavity in naive mice. Mice were killed 4 h after the challenge (carrageenan) administration and the peritoneal cavity cells were harvested by washing the cavity with 3 mL of phosphate buffered saline (PBS) containing 1 mM EDTA. The volumes recovered were similar in all experimental groups and equated to approximately 95% of the injected volume. Total counts were performed in a cell counter (COULTER A CT; Coulter, Miami, FL, USA), and differential cell counts (100 cells total) were carried out on cytocentrifuge (Cytospin 3; Shandon Lipshaw, Pittsburgh, Pennsylvania, USA) slides stained with Rosenfeld. The results are presented as the number of neutrophils per cavity (Napimoga *et al*., 2007).

3. Results and Discussion

3.1 Chemical analysis

Compounds **1** and **2** were obtained from chloroform fraction of Brazilian red propolis (type 13th) by semi-preparative HPLC. Compound **1** showed a parent ion $[MH]^-$, obtained according to data from the mass spectrum in negative mode of 271.0977 m/z and molecular formula as $C_{16}H_{15}O_4$ (Figure 2).

Compound **2** was identified based on findings of Oldoni *et al.*(2011) and was identified as Vestitol (2',7-diidroxi- 4'metoxiisoflavan).

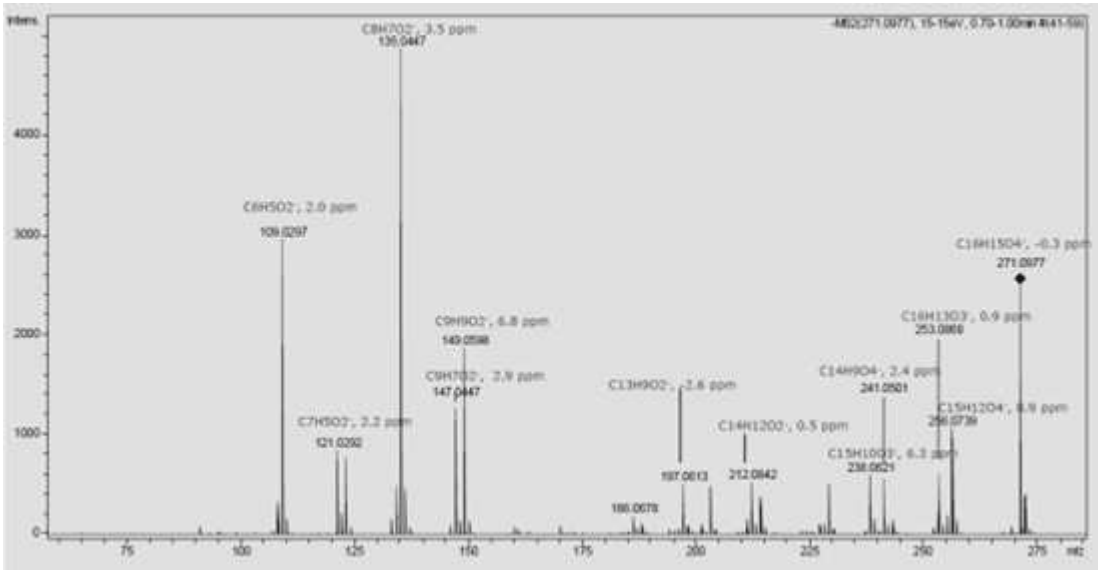

Figure 2: Spectrum obtained by ESI-Q-TOF-MS in negative mode of the bioactive compound isolated from propolis

The ^{13}C NMR spectrum of compound **1** displayed 16 distinct signals, which is in agreement with the molecular formula deduced from the mass spectrum. The DEPT-135 spectrum showed 10 hydrogen-bound carbon atoms, including two methylene groups. ^{1}H NMR and ^{1}H-^{1}H COSY spectra allowed the identification of three distinct spin systems: two aromatic and another one consisting of two methylene coupled to a methine group. Heteronuclear correlations (HMQC and HMBC) were used next for a complete assignment of the structure. Table 1 summarizes the ^{1}H and ^{13}C NMR data of compound **1**.

Table 1: ^{13}C and ^{1}H NMR data of bioactive compound **1** (125 and 500 MHz, respectively)

Carbon atom	^{13}C NMR ($CDCl_3$, 125 MHz)	^{1}H NMR ($CDCl_3$, 500 MHz)	HMBC correlations
2	69.91	4.04 (dd, $J_{2ax-2eq}$=10,4Hz; J_{2ax-3}=10,0Hz, H-2ax) 4.32 (ddd, $J_{2eq-4eq}$ =2,0; J_{2eq-3}=3,4; $J_{2ax-2eq}$=10,4, H-2eq)	H-3; H-4ax; H-4eq;
3	31.75	3.50 (dddd, J_{2eq-3}=3,4Hz; J_{3-4eq}=5,5Hz; J_{2ax-3}=10,0Hz, J_{3-4ax} =10,4Hz, H-3)	H-2ax; H-2eq; H-4ax; H-4eq; H-3´; H-6´;
4	30.35	2.91 (ddd, $J_{2eq-4eq}$=2,0Hz; J_{3-4eq}=5,5Hz; $J_{4ax-4eq}$=15,7Hz, H-4eq) 2.98 (dd, J_{3-4ax-}=10,4Hz; $J_{4ax-4eq}$ =15,7Hz, H-4ax)	H-2ax; H-2eq; H-3; H-5
4a	114.41		H-3; H-4ax; H-4eq; H-6
5	130.18	6.98 (d, J_{5-6}=8,4Hz, H-5)	H-4ax; H-4eq
6	107.29	6.48 (dd, J_{5-6}=8,4Hz; J_{6-8}=2,5Hz, H-6)	H-4ax; H-5; H-8
7	159.14		H-5; H-6; H-8; OCH_3
8	101.46	6.42 (d, J_{6-6}=2,5Hz, H-8)	H-4aq; H-5; H-6
8a	155.22(*)		
1´	120.08		H-2ax; H-2eq; H-3; H-4ax; H-4eq; H-3´; H-5´
2´	154.36		H-3;
3´	103.13	6.31 (d, $J_{3´-5´}$=2,3Hz, H-3´)	H-5`; H-6´
4´	155.11(*)		
5´	108.02	6.38 (dd, J=8,3 Hz; $J_{3´-5´}$=2,3Hz, H-5´)	H-3´
6´	128.44	6.95 (d, $J_{5´-6´}$=8,3Hz, H-6´)	H-3
OCH3	55.35	3.77	

(*) these values may be swapped)

Compound **1** was identified as 2',4'-dihydroxy-7-methoxyisoflavan (neovestitol) by two techniques: high resolution mass spectrometry (Q-TOF-MS/MS) and NMR. Neovestitol was also identified in previous works but none of them used such accurate techniques as used in the present work (Ingham 1976, Cuesta-Rubio *et al.,* 2007, Campo Fernández *et al.,* 2008, Oldoni *et al.,* 2011). The structure of this compound is shown in Figure 3.

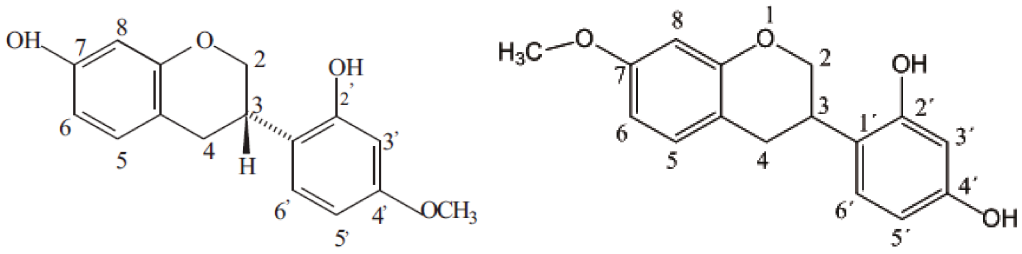

Figure 3: Chemical structure of bioactive compounds isolated from Brazilian red propolis – vestitol and neovestitol

Antimicrobial and anti-inflammatory activities of EEP, chloroform, and hexane fractions, and isolated compounds from Brazilian red propolis were reported here. As mentioned the pharmacological activities were used in this investigation as a bioguide of the chemical studies in order to pinpoint the target compounds responsible for antimicrobial and anti-inflammatory effects.

3.2. Antimicrobial activity

Results of antimicrobial tests of EEP, chloroform, and hexane fractions, and both isolated compounds confirmed the antimicrobial activity of EEP and showed that chloroform faction was more active than hexane fraction (Table 2). EEP showed MIC values ranged from <6.25 µg/mL (*S. sobrinus*) to 100-200 µg/mL (*S. mutans* and *S. aureus*), and MBC ranged from 25-50 µg/mL (*S. mutans* and *A. naeslundii*) to 200-400 µg/mL (*S. aureus*). Chloroform fraction had lower MIC values ranging from <6.25 µg/mL (*S. sobrinus*) to 50-100 µg/mL (*S. mutans* and *S. aureus*), and MBC ranging from 25-50 µg/mL (*S. sobrinus*) to 100-200 µg/mL (*S. mutans* and *S. aureus*), when compared with values for the hexane fraction (Table 2).

Table 2: Results of antimicrobial tests (MIC and MBC) of ethanolic extract of propolis (EEP), chloroform, and hexane fraction, and isolated compounds.

Red Propolis	MIC (µg/mL)				MBC (µg/mL)			
	S. mutans	*S. sobrinus*	*S. aureus*	*A. naeslundii*	*S. mutans*	*S. sobrinus*	*S. aureus*	*A. naeslundii*
EEP	100-200	>6.25	100-200	25-50	200-400	50-100	200-400	50-100
Chlorof. Fract.	50-100	>6.25	50-100	25-50	100-200	25-50	100-200	50-100
Hex. Fract.	200-400	6.25-12.5	50-100	50-100	400-800	200-400	100-200	100-200
Comp. 1*	25-50	<6.25	25-50	25-50	50-100	25-50	50-100	50-100
Comp. 2*	50-100	25-50	50-100	50-100	100-200	200-400	100-200	>1600

*Compound 1: 2',4'-dihydroxy-7-methoxyisoflavan (neovestitol); Compound 2: vestitol (2',7-diidroxi- 4'metoxyisoflavan).

Chloroform fraction seems to be the major source of bioactive compounds of Brazilian red propolis. We observed that the two compounds showed strong activity against all tested bacterial strains (Table 2). Vestitol presented MIC of 31.2- 62.5 µg/mL, showing no distinction among the microorganisms assessed. Neovestitol showed MIC ranging from <6.25 µg/mL (*S. sobrinus*) to 25-50 µg/mL, thus being more potent than vestitol.

Regarding MBC, vestitol showed values from 62-125 (*S. aureus*) to >1600 µg/mL (*A. naeslundii*) while 2',4'-dihydroxy-7-methoxyisoflavan (neovestitol) showed results from 25-50 µg/mL (*S.sobrinus*) to 50-100 µg/mL (*S. mutans*, *A.naeslundii*, and *S. aureus*).

Piccinelli *et al.* (2011) isolated 20 compounds from Brazilian and Cuban red propolis and, among them, vestitol and neovestitol was also isolated. However, the authors did not study any pharmacological activity of these compounds. Therefore, this work went further and demonstrated its structures by RMN and studied their biological properties.

Righi *et al.* (2011) isolated 21 compounds from chloroform fraction, including vestitol (called 7-O-metilvestitol), and 7 other compounds from methanolic fraction of

Brazilian red propolis. The MIC of methanolic fraction against *Pseudomonas aeruginosa*, *Bacillus subtillis*, *Candida albicans*, *Salmonella typhimurium*, *Klebsiella pnemoniae*, *Enterococcus faecallis*, *Escherichia coli*, *Proteus mirabilis*, and *Streptococcus pyogenes* was determined, and the lowest value was 256 µg/mL.

Recently, Oldoni *et al.* (2011) reported antimicrobial activity of isolated compounds from Brazilian red propolis against the same microorganisms used in this work. However, compounds isolate by Oldoni are already known in the literature and none showed antimicrobial activity better than neovestitol. Also, antimicrobial activity of neovestitol is innovative and further investigations are necessary to elucidate its anti-biofilm and anti-caries properties, as well its mechanism of action.

3.3. Anti-inflammatory activity

EEP and neovestitol showed inhibitory activity against neutrophil migration at 10 mg/Kg dose while vestitol showed inhibitory activity without statistical difference at 1, 3, and 10 mg/Kg. So, both isolated compounds presented anti-inflammatory effects. Therefore, anti-inflammatory effect of red propolis may be due to the combined effect of both compounds isolated in this study. Also, it seems to be the first report of anti-inflammatory properties of compounds isolated from Brazilian red propolis. Although these compounds were already described in literature, none of the studies reported their anti-inflammatory properties before (Cuesta-Rubio *et al.,* 2007, Campo Fernández *et al.,* 2008).

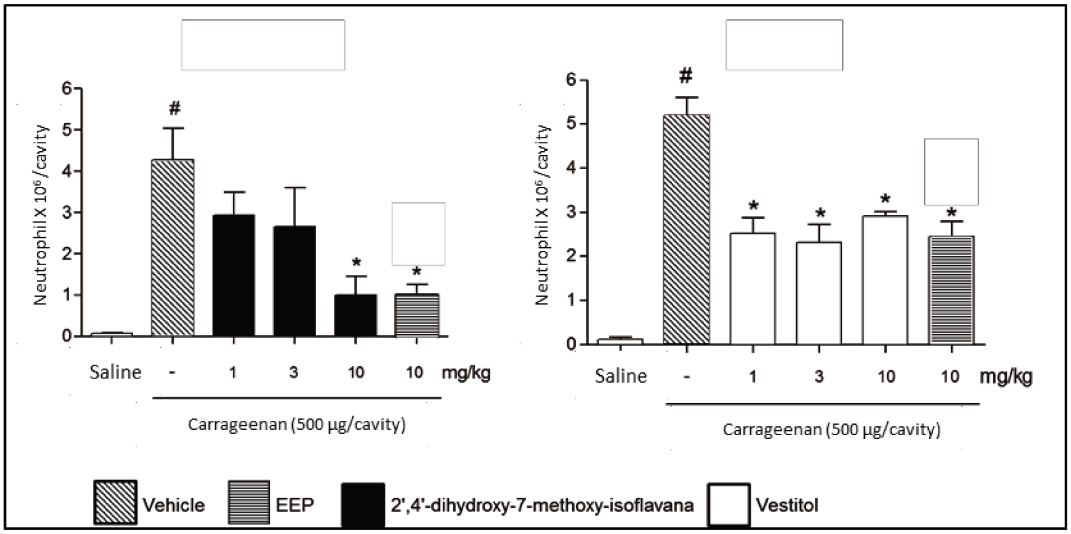

Figure 4: Recruitment of leukocytes into the peritoneal cavity induced by carrageenan. The neutrophil migration was determined 4 hours after injection of carrageenan 500 ug / cavity. Mice were previously treated with vehicle (saline), ethanolic extract of propolis (EEP), 2',4'-dihydroxy-7-methoxy-isoflavana (neovestitol), and vestitol, followed by carrageenan injection. The results are expressed as mean ± S.E.M., n = 5-6. The symbol (#) indicates statistical difference compared to saline group. The symbol (*) indicates statistical difference compared to carrageenan group (one-way ANOVA followed by Bonferroni test, $p < 0.05$).

4. Conclusions

The bioactive isoflavonoids neovestitol (2',4'-dihydroxy-7-methoxyisoflavan) and vestitol, both isolated from Brazilian red propolis, showed *in vitro* antimicrobial and anti-inflammatory properties. Therefore, further investigations are necessary to improve the production process, elucidate the mechanisms of action, and investigate other possible pharmacological properties.

5. Acknowledgements

The authors are grateful to Mr. Alessandro Esteves for providing the Brazilian red propolis samples. This research was supported by FAPESP (#2008/58492-8), CNPq (CNPq 200174/2009-6).

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5. CAPÍTULO 2

Anti-biofilm and anti-caries properties of purified fraction of the Brazilian red propolis

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Abstract

Brazilian red propolis (type 13) has shown inhibitory activity against microorganisms involved in the pathogenesis of dental caries, such as *Streptococcus mutans*. Red propolis has a complex chemical profile, and bioassay-guided fractionation identified an Active Purified Fraction from Propolis (APFP). The objective of this study was to chemically characterize and evaluate the effects of APFP on the formation, expression of virulence factors, and overall architecture of dental biofilms. Biofilms of *S. mutans* UA159 were formed on saliva-coated hydroxyapatite surfaces and their biochemical, molecular, and structural features were analyzed. Moreover, APFP was tested in a rodent model of caries to assess its potential efficacy *in vivo* study. A concentration of 800 μg/ml APFP was the most effective treatment, which significantly impaired *S. mutans* biofilm development by largely disrupting the synthesis and subsequent accumulation of insoluble polysaccharides and total protein. These biochemical changes were reflected in dramatic alterations to the 3D structural organization and total thickness of APFP-treated biofilms. Furthermore, APFP inhibited the activity of the glucosyltransferases B, C, and D enzymes.. Microarray analysis revealed that 58 genes were differentially expressed after topical treatments with APFP (vs. control); the expression profiles were confirmed via RT-qPCR. The majority of differentially regulated genes (52) were down-regulated, suggesting that APFP impairs

virulence by reducing expression of genes that may be essential for robust biofilm formation.. In support of this, APFP represses transcription of several genes involved in biofilm formation, extracellular-matrix assembly, and virulence, including *copY*, *copA*, *sloA*, *copZ*, *gtfD*, and *amyA*. Not one of the up-regulated genes are known to play a role in stress tolerance/adaptation, which indicates that APFP does not elicit a strong stress response in *S. mutans* biofilms. Of even greater impact, APFP effectively reduces the number and severity of caries lesions in our rodent mode. Furthermore, APFP was as effective as fluoride (the gold standard of caries prevention) in the *animal study*. Taken together, this data supports the hypothesis that APFP is a promising natural anti-biofilm agent which targets, at least in part, the ability of *S. mutans* to produce exopolysaccharides and express virulence traits and/or gene products essential for biofilm formation and subsequent caries development.

Keywords: red propolis, biofilm, *S. mutans*, caries

1. Introduction

Dental caries, a multifactorial and biofilm-dependent disease, occurs in the presence of acidogenic and aciduric bacteria (Hamada & Slade, 1980), which interact in a mixed community to facilitate the formation of a biofilm on susceptible tooth surfaces (Marsh, 2003). The detection of *Streptococcus mutans* in dental plaque-biofilms is often associated with the development of caries (Loesche, 1986), as this organism can metabolize a variety of dietary sugars to produce acids, while it specifically utilizes sucrose to produce glucan (Bowen and Koo, 2011). Glucan faciliates *S. mutans* adhesion and accumulation onto tooth surfaces; consequently, acids accumulate within the biofilm millieu. Acid production causes the demineralization of the tooth enamel and subsequent development of cavitations (the hallmark of caries), thus exposing dentin or pulp to oral environment. Caries is both a costly and progressive disease, which affects children and adults worldwide (Marsh, 1999; Bowen, 2002; Paes Leme *et al.,* 2006).

The canonical virulence factors of *S. mutans* have been intensively studied for their role in pathogenesis, manipulation of which may lead to improved therapies to treat and/or prevent caries. Among these, glucosyltransferases (Gtfs) have received significant attention, as the glucans produced by these enzymes represent a main structural component of dental plaque-biofilms. Thus, glucans, particularly insoluble glucans, have often been considered to be determining virulence factors, which facilitate the tight adherence and accumulation of cariogenic streptococci on the tooth surface (Schilling & Bowen, 1992). In addition, it has been shown that these glucans increase the porosity (Bowen 2002), thus making it even more cariogenic. Moreover, intracellular polysaccharides act as metabolic source to bacteria product acid during periods of carbohydrate limitation in the oral cavity (Tanzer *et al.,* 1976; Pandit *et al.,* 2011), this way collaborating to its virulence.

As a consequence, one of the primary strategies for caries prevention has been the development of therapeutic agents that target the aforementioned virulence factors, which can in turn impede biofilm formation (Koo *et al*., 1999, Duarte *et al*. 2003). Recently, natural products have been widely investigated as a potential source of novel and active therapeutic agents. As a result, there are a large number of natural agents currently under evaluation in clinical trials (Butler 2005) and roughly 74% of all approved therapeutic agents developed between 1981 and 2010 are of natural origin (Newman and Cragg, 2012). However, few reports in the literature describe the use of natural agents in caries prevention, and many of these studies lack essential elements, including but not limited to, chemical characterization of the agent(s) used, clinically relevant research designs, and proposed mechanisms of action (Jeon *et al.,*2011).

Among the natural products currently under investigation, Brazilian red propolis (type 13) Silva *et al.,* 2008), has been confirmed to be a potent antimicrobial and

antioxidant agent (Alencar *et al.,* 2007) and a potentially rich source of new bioactive compounds (Silva *et al.,* 2008; Righi *et al.,* 2011). However, prior to our work, there were no reports in the literature showing the Brazilian red propolis or its purified fractions could be effective in impairing S. mutans biofilm formation and/or caries *in vivo*.

Therefore, we elected to test a purified fraction of Brazilian red propolis (APFP) using a more comprehensive design than some of the contemporary studies; we have chemically characterized this fraction and tested its effects both *in vitro* and *in vivo* using a clinically applicable treatment scheme. Furthermore, we offer plausible explanations for its effectiveness *in vivo* through the use of both biochemical and molecular techniques aimed at evaluating the impact on *S. mutans* within plaque-biofilms. As hypothesized, we show that APFP is effective at inhibiting the formation, virulence factor expression, and architecture of *S. mutans* biofilms, which likely accounts for a significant reduction in both the incidence and severity of caries in a rodent model.

2. Material and Methods

In order to reach a purified anti-biofilm bioactive fraction from the Brazilian red propolis, it was generated an experimental design based on bioassay-guided fractionation which was carried out by using MIC and MBC determinations (Minimal Inhibitory and Bactericidal Concentration). Each step of the study is showed in Figure 1.

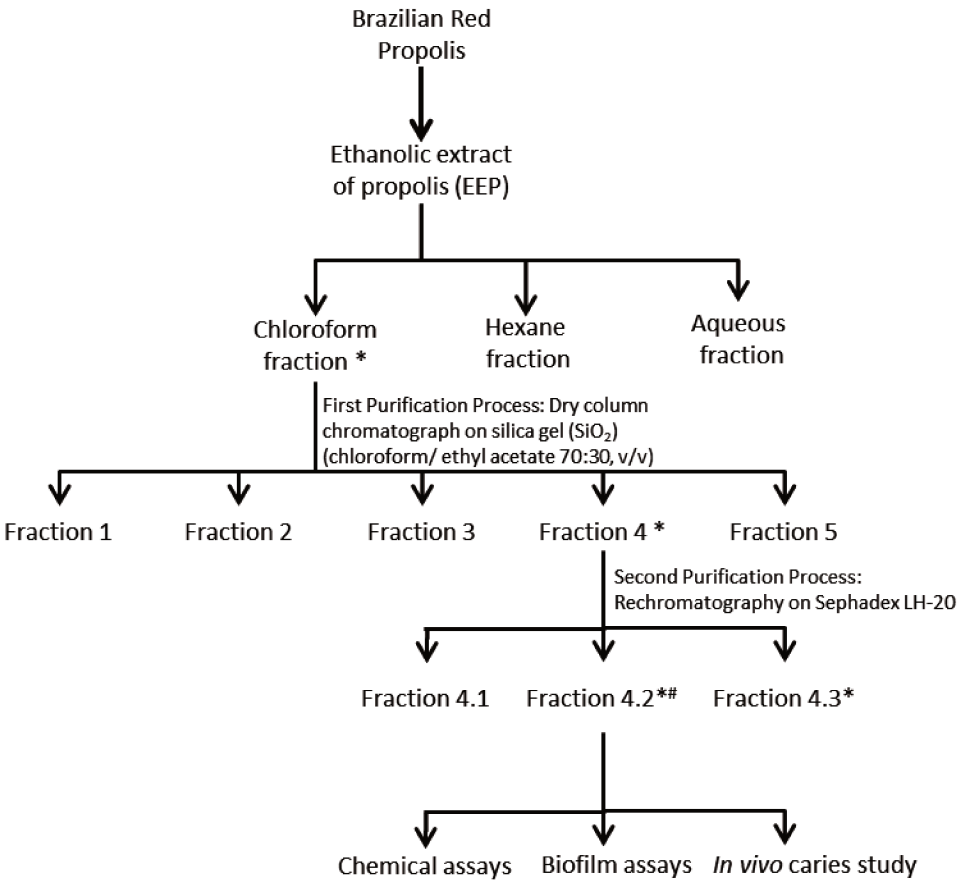

Figure 1: Experiment design of bioassay-guided fractionation of Brazilian red propolis in order to reach bioactive compounds. *Antimicrobial test revealed the best activity; #Easier to dissolve compared to others in the same level.

2.1. Extraction of Active Purified fraction (APFP) of red propolis

A sample of propolis was obtained from Maceio, Alagoas State, Northeast of Brazil SL 09.40 and WL 35.41. The propolis sample (64 g) was extracted with ethanol 80% (300 mL) in a water bath at 70ºC for 30 min, and after filtration, yielded the ethanolic extract of propolis (EEP). The EEP was further fractioned by liquid-liquid extraction with hexane and chloroform yielding 10 g of hexanic fraction (Hex-fr), 22 g of chloroform fraction (Chl-fr), and 1 g of aqueous fraction (Aqu-fr). The EEP, Hex-fr, Chl-fr and Aqu-fr were submitted to MIC and MBC bioguided assay in order to isolate the bioactive fraction. The active Chl-fr

(22 g) was subjected to open dry column chromatography on normal phase silica gel (particle size: 0.0063.0.2 mm; pore size: 60 Å; pore volume: ~0.8 cm^3/g; and specific surface area: 500 m^2/g) and eluted with a solvent mixture of chloroform/ethyl acetate (70:30, v/v) to create five major fractions. The fractions obtained were monitored by thin layer chromatography (TLC) using the anisaldehyde reagent (4-methoxy-benzaldehyde, acetic acid, sulphuric acid: 1.0:48.5:0.5), followed by incubation at 100ºC for 5 min. Fluorescent substances were visualized under ultra-violet (UV) light at the wavelengths of 254 nm and 366 nm (Alencar *et al.,* 2007). The 5 eluted fractions were also submitted to MIC and MBC bioassay-guided fractionation to select an active fraction. Fractions 1 and 5 showed no antimicrobial activity, and negligible activity was found in fractions 2 and 3. The most bioactive fraction (4) was chromatographed over a Sephadex LH-20 column (5x30 cm) using methanol to yield three fractions (4.1, 4.2, and 4.3). Fractions 4.2 and 4.3 were active, although, fraction 4.3 was rejected because it was impossible to dissolve it in the buffer solution used to perform biological assays (2.3; 2.4 and 2.5). Thus, fraction 4.2 was named the active purified fraction (APFP) and its chemical profile was analyzed by gas-chromatography coupled to MS. Also, it was tested in our biofilm model (biochemical, structural and molecular data), in glucosyltranferase activity assays, and in an *in vivo* study.

2.2. Chemical Analyses

2.2..1. Derivatization - formation of the trimethylsilyl derivatives (TMS)

Approximately 10 mg of APFP was placed in vials with 100 μL of MSTFA [N-methyl-N-(trimethylsilyl)-trifluoroacetamida] reagent. The vial was securely closed and placed in an incubator at 70°C for 30 minutes; this time was required for sample derivatization. After that time, the MSTFA was evaporated under nitrogen flow and the

product of derivatization (TMS-trimethylsilyl derivatives) was re-diluted in hexane (500 μL). The silanized sample was homogenized and used for injection into a gas chromatograph with mass spectrometry (GC-MS).

2.1.2.. Gas chromatography-mass spectrometry (GC-MS)

The GC-MS analyses were conducted on a Shimadzu gas chromatograph, model 2010 GC coupled to mass spectrometer Shimadzu, model QP 2010 Plus, equipped with a capillary column (30 m x RTX5MS 0.25 mm x 0.25 μm). The initial column temperature was 80°C for 1 min, which reached 250°C with rate of 20 °C/min remaining at this temperature for 1 min, 250 to 300°C with rate of 6°C/min for 5 min, 300 to 310°C the rate of 15°C/min for 5 min, 310 to 320°C the rate of 20°C/min for 10 min, adding 40 min of analysis. Helium was used as the carrier gas. The injector temperature was 280°C and the injection volume was 0.2 μl. The interface was maintained at a temperature of 280°C. The mass detector operated in scanning mode m/z 40 to 800. The integration was made in the software solution LabSolutions-GCMS and identification of detected compounds was performed by comparison with data from the mass spectra library Wiley 8TM and authentic standards injected under the same conditions of samples.

2.3. Biofilms

Figure 2 shows the experimental design of our biofilm assays. For biochemical data, biofilms were processed after 115 hours of formation, for architecture analyses with confocal laser microscope, after 43 and 67 hours, for molecular assays, after 45 hours.

Single-species biofilms of *S. mutans* strain UA159 were grown on 1.25 cm hydroxyapatite (HA) discs vertically suspended in 24-well plates using a wire disc holder, as described in an earlier publication (Koo *et al.,* 2010) Each disc was coated with clarified

and filter-sterilized human whole saliva as described previously (Koo *et al.,* 2005). The saliva was collected from a single donor according to protocols approved by the Ethics Committee in Research of the Piracicaba Dental School, University of Campinas (Protocol #033/2008). Also, each disc was inoculated with approximately 2 x 10^6 CFU/ml in ultra-filtered (10kDa cutoff, Millipore, Billerica, MA) yeast tryptone extract broth (UFYTE) containing 1% sucrose. At this point (19 h-old), the biofilms were treated twice daily (10 am and 4 pm) for a 60 s exposure until the end of experimental period with the following solutions: APFP (200, 400 or 800 µg/mL) or vehicle-control (20% ethanol, v/v), as shown in Figure 2.

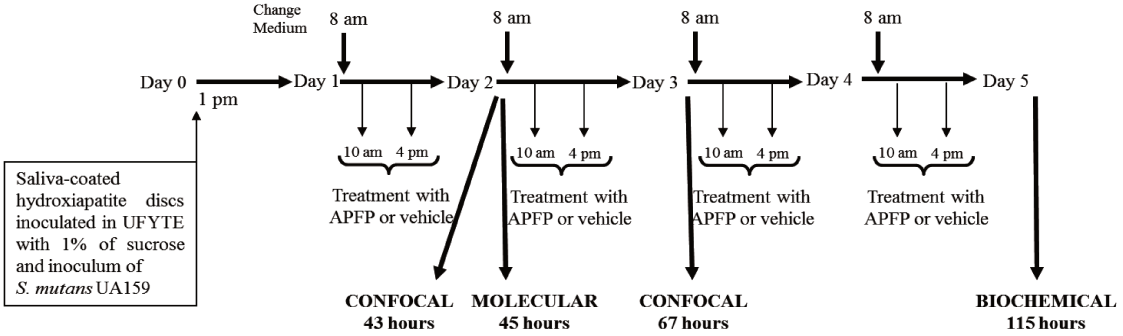

Figure 2: Single-species biofilm preparation and the experimental design of biofilm model treated with APFP for collection of biochemical, confocal, and molecular data.

2.3.1. Biochemical assays

At the end of the experimental period (115 h), the biofilms were removed and subjected to sonication using three 30 sec pulses at an output of 7 W (Branson Sonifier 150; Branson Ultrasonics, Danbury, Conn., USA) (Koo *et al.,* 2003); the sonication procedure provided the maximum recoverable viable counts. The homogenized suspension was analyzed for biomass (dry-weight), bacterial viability (CFU/mg of biofilm dry-weight), and polysaccharide composition. The extracellular water soluble (WSP) and insoluble polysaccharides (IP) and intracellular iodophilic polysaccharides (IPS) were extracted and

quantified by colorimetric assays as detailed by Koo *et al.* (2003) and Duarte *et al.* (2008); the exopolysaccharides were quantified by the phenolsulfuric method (Dubois *et al.* 1956) using glucose as the standard, whereas IPS was quantified using 0.2% I_2/2% KI solution and glycogen as the standard, as described by DiPersio *et al.* (1974).

2.3.2. Analyses of architecture and structural organization of the biofilms

The influence of the most effective concentration of APFP (determined from the results of biochemical data – 2.3.1) on the architecture and structural organization of the biofilms was examined by simultaneous *in situ* labeling of extracellular polysaccharides (EPS) and bacterial cells as described by Klein *et al*. (2009). Biofilms were formed during 43 and 67 hours as shown in the Figure 2 and described in the biofilms section (2.3).

Confocal imaging was performed using a Leica TCS SP1 confocal laser microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) equipped with argon ion and helium-neon lasers set at 488 and 633 nm, respectively (Klein *et al.,* 2009). Triple dichroic filters (488, 543 and 633nm) and emission filters (Chroma Technology Corp., Rockingham, VT) were used for detection of Alexa Fluor 647 and SYTO 9. Confocal images were acquired using a # 40, 0.8-numerical-aperture water immersion objective lens, which provided an optical section thickness of approximately 1 µm. Each biofilm was scanned at ten randomly selected positions, and z series were generated by optical sectioning at each of these positions (Xiao *et al.,* 2010).

Image analyses using COMSTAT software: two independent biofilm experiments were performed, and 20 image stacks (512- by 512-pixel tagged image file format) were collected for each experiment. The confocal image stacks were analyzed by COMSTAT image-processing software (Heydorn *et al.,* 2000), as described by Klein *et al.*, 2009. This software was written as a script in Matlab 5.1 (The MathWorks, Natick, MA) equipped

with the Image Processing Toolbox, which generated several measurements for quantifying and characterizing the three-dimensional structure of biofilms (Heydorn *et al.,* 2000). In this study, the biomass of EPS and bacterial cells and the average thickness was calculated to determine the structural differences among the different biofilms. The three-dimensional architecture of the biofilms was visualized using Amira 5.0.2 (Mercury Computer Systems Inc., Chelmsford, MS). Confocal fluorescence data was imported into the software, and three-dimensional images of each of the components in the biofilms were created using voltex rendering (Klein *et al*. 2009, Koo *et al*., 2010).

2.3.3. Molecular assays with single species biofilms

2.3.3.1. Culture of *S. mutans* biofilms.

Biofilms were formed as described above (2.3) and in Figure 2. Four disc sets (eight discs) were treated with 800 μg/mL of APFP, and four disc sets were treated with the vehicle control. The biofilms were incubated in UFYTE with 1% sucrose at 37ºC and 5% CO_2 for 1 h following the final treatment at 46 h, and then the biofilms were collected for RNA extraction.

2.3.3.2. Purification of biofilm RNAs.

Following a 1 h incubation, each disc set was transferred to a RNAse-free conical tube containing 2.5 mL RNALater (Applied Biosystems/Ambion, Austin, TX), and the biofilm material was scrapped from the discs. The tubes were incubated overnight at 4ºC. Acid phenol:chloroform extractions were performed the following day to harvest crude RNAs, as previously described (Cury *et al.,* 2008). The RNAs were purified, and DNAse was treated on the column using the Qiagen RNeasy Micro kit (Qiagen, Valencia, CA). The RNAs were then subjected to a second DNaseI treatment with Turbo DNase (Applied

Biosystems/Ambion) and purified using the Qiagen RNeasy MinElute Cleanup kit (Qiagen). The RNAs were eluted in a final volume of 20 µL and quantified using the NanoDrop ND1000 Spectrophotometer (Thermo Scientific\NanoDrop, Wilmington, DE). The RNA quality was evaluated using an Agilent 2100 Bionalazyer (Agilent Technologies Inc., Santa Clara, CA), and all RNAs used to prepare cDNAs for microarray analysis were determined to have RIN values of 8.9 and above.

2.3.3.3. Microarray analysis. Whole genomic profiling was conducted using version 3 microarrays for *S. mutans* strain UA159 provided by the J. Craig Venter Institute (JCVI). A detailed description of these slides may be found at http://pfgrc.jcvi.org/index.php/microarray/array_description/streptococcus_mutans/version 3.html. Reference RNAs were prepared as previously described (Klein *et al.,* 2010), and cDNAs for both experimental and reference samples were synthesized following protocols provided by JCVI at http://pfgrc.jcvi.org/index.php/microarray/protocols.html. Experimental cDNAs were labeled with indocarbocyanine (Cy3)-dUTP, while reference cDNAs were labeled with indodicarbocyanine (Cy5)-dUTP (Amersham Biosciences, Piscataway, NJ). Hybridizations were performed using the MAUI hybridization system (BioMicro Systems, Salt Lake City, UT), and slides were then washed and scanned using a GenePix scanner (Axon Instruments Inc., Union City, CA) following JCVI protocols. After scanning, single-channel images were simultaneously uploaded into JCVI Spotfinder321 (http://www.tm4.org/spotfinder.html), creating an overlay image with both Cy5 and Cy3 channels. A Spotfinder grid file was downloaded from http://pfgrc.jcvi.org/index.php/microarray/array_description/streptococcus_mutans/version 3.html and adjusted to remove empty spots from the analysis. Spotfinder321 was used to identify spots and assign relative spot intensities in a .mev file for upload into JCVI

MIDAS (http://www.tm4.org/midas.html). MIDAS software was used to perform a lowess normalization and flag and remove spots with low intensity values (less than 1000). Statistical analysis was finally performed using BRB-Array Tools, which is available as freeware from http://linus.nci.nih.gov/BRB-ArrayTools.html. An unpaired class comparison was performed with a *P* value cutoff of 0.05 for four replicates of APFP-treated and four replicates of vehicle-treated samples (item 2.3.3.1).

2.3.3.4. Analysis and classification of microarray data.

MDV (available from LANL Oralgen http://www.oralgen.lanl.gov/) was used to assign gene names and functional classes to genes identified with BRB-Array Tools, as described previously (Klein *et al.,* 2010). Genes were then sorted in Microsoft Excel to identify those with an absolute fold-change of 1.5 or greater. These genes were then organized into the following functional categories: hypothetical, other (genes with functions that are not relevant to biofilm growth and/or infection), regulators, glycolytic pathway, stress (oxidative, osmotic, acid tolerance, or other), EPS, IPS and biofilm formation/adhesion using GOI (Gene of Interest) selection criteria as described in a previous publication (manuscript in preparation). This data was used to evaluate the impact of treatment by identifying the major themes affected.

2.3.3.5. Quantitative PCR (qRT-PCR) of selected genes for microarray data validation.

The same RNAs used to prepare cDNAs for microarray analysis also served as templates for cDNA synthesis using the Bio-Rad iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA) for qRT-PCR. Samples in which no reverse transcriptase was added to the reaction were used as negative controls to check for DNA contamination during qRT-PCR assays. cDNAs were amplified with specific primers using a MyiQ real-

time PCR detection system with iQ SYBR Green Supermix (Bio-Rad). The following genes were amplified in qRT-PCR assays with primer sets that were used in previous studies: *gtfBCD* (Koo *et al.,* 2006), *copY*, *sloA*, *msmK* and *sodA* (manuscript in preparation). Primers for *oppD* (5'- AATAAGGCAGACCGTGAC and 5'- CCAATCCGTTGACGCTGA) and *amyA* (5'- CCAAGCTGACAAGGAAGC and 5'- TGGTGTGGCTGTCATCATA) were designed using Beacon Designer 2.0 software (Premier Biosoft International, Palo Alto, CA). Standard curves were used to determine the relative number of cDNA molecules, which were normalized to the relative number of 16S rRNA cDNA molecules in each sample, as previously described (Koo *et al.,* 2006). These values were used to determine the fold of change between each treated sample and the vehicle control.

2.4. Glucosyltranferase (GTF) activity assays

The enzymes used were GTF B, C and D, which 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). Glucosyltransferase activity was measured by the incorporation of [^{14}C] glucose from labeled sucrose (NEN Research Products, Boston, MA) into glucans (Venkitaraman *et al*., 1995).

2.4.1. GTF in solution

Purified GTF B and C (1.0–1.5 U) were mixed with APFP (800 μg/mL) or vehicle control (20% ethanol, v/v) and incubated with a [^{14}C] glucose-sucrose substrate as described by Venkitaraman *et al.* (1995), except the incubation time that was reduced to 2 h.

2.4.2. GTFs adsorbed

To evaluate the activity of Gtf on the surface, hydroxyapatite beads were coated with clarified whole saliva free of Gtf activity in the presence or absence (control) of the test agent APFP (800 µg/mL), as described by Venkitaraman *et al*. (1995) and Wunder and Bowen (1999).

2.5. Animal Study

The animal experiment was reviewed and approved by the Ethical Committee on Animal Research at the University of Campinas, SP, Brazil – UNICAMP (Protocol # 1485-1) and was performed according to methods described previously (Bowen *et al.* 1988; Koo *et al*. 1999). A total of 39 SPF female pups free of *S. mutans* and SDA virus from 11 litters of SPF Wistar rats were provided by CEMIB (UNICAMP). At age 21 days, the pups were weaned and infected three successive days with S. *mutans* UA159, growing in 3 days as a biofilm, following the standard described in item 2.3 and Figure 2 (Murata *et al.,* 2010). Oral infection of the pups was confirmed at age 25 days by plating on MSB agar. At this age, the pups were randomly placed into three groups of 13 animals. From this point, the molar teeth of the animals were treated topically by means of a camel hair brush twice daily, as follows: APFP (800 µg/mL), Fluoride 250 ppm and vehicle control (20% ethanol, v/v). Each group of 13 animals was provided with diet 2000 (which contains 56% sucrose and no fluoride) and 5% sucrose water *ad libitum* (Bowen *et al.* 1988). The animals were weighed weekly, and their behavior and physical appearance was noted daily. The experiment proceeded for 5 weeks, at the end of which the animals were euthanized by CO_2 asphyxiation. The lower left jaw was aseptically dissected, suspended in 5.0 mL of sterile saline solution (0.9 %, w/v), and sonicated (three 10 sec pulses at 5 sec intervals, at 30 W, Vibracell, Sonics & Material Inc.); this procedure provides the maximum recoverable

viable counts. The suspension was plated on mitis salivarius agar plus bacitracin to estimate the *S. mutans* UA159 populations and on blood agar to determine the total cultivable microorganisms (Bowen *et al.* 1988). The smooth-surface and sulcal caries and their severity (Ds, dentin exposed; Dm, 3/4 of the dentin affected; Dx, all dentin affected) were evaluated according to Larson's modification of Keyes' system (Larson 1981). The determination of the caries score was blinded by codification of the jaws and performed by one calibrated examiner.

3. Results

Two major peaks (compounds 5 and 6) were found in the chemical profile of APFP (figure 3). Compound 5 was identified as 2',4'-dihydroxy-7-methoxyisoflavone (neovestitol), corresponding to 70% of relative percentage of APFP, while compound 6 was identified as vestitol (unpublished data), which has some activity against *S .mutans*, although neovestitol showed better inhibitory and bactericidal activity (data not shown).

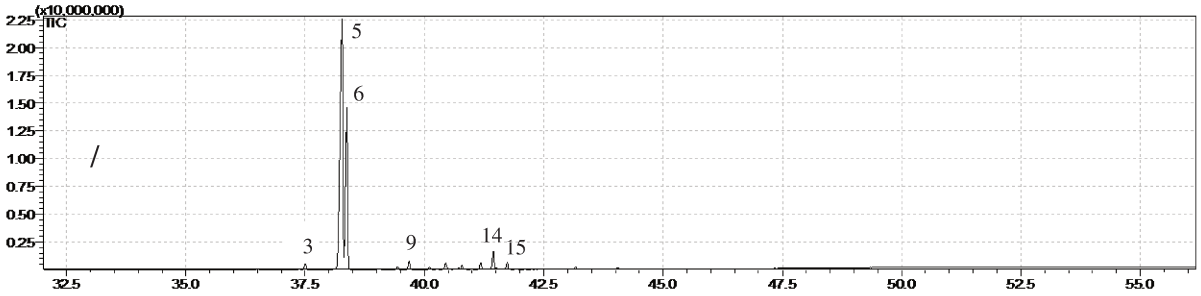

Figure 3: Chemical profile of red sub-fraction obtained through GC-MS. Compound number 5 was identified as Neovestitol (70 % of APFP) and compound number 6 was identified as Vestitol (29 % of APFP).

Table 1 shows results of biochemical data collected from single species biofilms treated with APFP concentrations of 800, 400, 200 µg/mL or with the vehicle control. The most effective concentration was determined to be 800 µg/ml, which inhibited 37% of

insoluble polysaccharide (IP) formation and 43% of protein formation. This concentration also reduced the biofilm dry-weight (DW) by approximately 20% and water soluble polyscharrides (WSP) by roughly 30% compared to the vehicle control. No statistical difference was observed for IPS (intracellular polysaccharide storage) or the total number of viable cells.

Table 1: Results of biochemical analysis [average (standard deviation)] in single species biofilms of *S. mutans* treated with APFP (800, 400, and 200 µg/mL) or vehicle.

APFP (µg/mL)	DW (mg)	IPS (µg)	IP (µg)	WSP (µg)	Protein (mg)	N. Cells (CFU/mL)
800	4.8 [a] (±0.2)	308.7 (±109.0)	1072.7 [a] (±377.9)	225.2 [a] (±225.26)	0.8 [a] (±0.2)	1.5E+08 (±7.3E+07)
400	5.3 [a,b] (±0.7)	371.7 (±169.6)	1427.6 [a,b] (±554.7)	287.0 [a,b] (±74.8)	1.2 [a,b] (±0.2)	2.1E+08 (±9.7E+07)
200	5.4 [a,b] (±0.9)	320.4 (±142.9)	1721,1 [a,b] (±394.1)	298.1 [a,b] (±73.2)	1.2 [a,b] (±0.3)	2.5E+08 (±4.6E+07)
Vehicle	6.0 [b] (±0.4)	386.8 (±167.5)	1712.3 [b] (±425.1)	319.2 [b] (±42.8)	1.4 [b] (±0.3)	1.7E+08 (±7.9E+07)

DW- dry-weight; IPS - intracellular polysaccharide; IP – insoluble polysaccharide; WSP – water soluble polysaccharide; N. Cells – number of viable cells. Vertically, results with no letters are not statistically different; different letters denote statistically different results, while same letters mean no statistical difference. Vehicle (control) = ethanol 80 % + AB buffer (1:4).

Since a reduction in the amount of insoluble polysaccharide was detected during biochemical analysis, the effect of APFP on the enzymes responsible for production of glucan (glucosyltransferases) was also assessed. Table 2 shows the results of the glucosyltransferases activity assays (for GtfB, C, and D) in solution and adhered to hydroxyapatite following treatment with either APFP or vehicle. We found that the activity of enzymes in solution treated with APFP was reduced by 65%, 63%, and 67% for Gtf B, C, and D, respectively (compared to vehicle control). The activity of Gtf B and D adhered to saliva-coated hydroxyapatite (sHA) was reduced by 18% and 45%, respectively

following APFP treatment. Therefore, APFP was effective in reducing the activity of enzymes in solution and bound to sHA, with exception of surface bound GtfC.

Table 3: Results of Glucosyltransferase activity assays

APFP (µg/mL)	GTF in solution (CPM)			GTF adhered to hydroxyapatite (CPM)		
	B	C	D	B	C	D
800	807.1 [a] (±135.7)	1201.0 [a] (±648.1)	744.7 [a] (±253.0)	2738.9 [a] (±154.1)	2355.1 (±1874.3)	1370.1 [a] (±101.3)
Vehicle	1905.4 [b] (±195.7)	3312.0 [b] (±600.5)	2088.5 [b] (±624.1)	3362.0 [b] (±310.0)	3338.6 (±2738.7)	2717.1 [b] (±217.8)

Statistical analysis was performed using a Tukey-Kramer test. Vertically, different letters mean statistically different results.

Due to a sharp reduction in the activity of the Gtf enzymes (the functions of which are critical for biofilm formation), we decided to evaluate the three-dimensional (3D) structure of *S. mutans* biofilms following APFP treatment. The 3D structure of single species biofilms of *S. mutans* was analyzed at two time-points: 43 and 67 hours, which corresponds to either two or four treatments with APFP, respectively. Using COMSTAT, we quantified the biomass and thickness of the bacteria cells (bacteria) and polysaccharides (EPS) within these biofilms.

During initial biofilm formation (at 43 hours), there was no statistical difference between the biomass of bacterial cells treated with APFP and the biomass of bacterial cells treated with the vehicle control. However, the thickness of bacterial cells and both the biomass and thickness of extracellular polysaccharides on biofilms was significantly reduced by 32.6 and 32%, respectively ($p \leq 0.05$ – Table 3) following APFP treatment.

At 67 h, or the mid-stage of biofilm formation, we observed a biomass and thickness profile that was similar to the profile observed at 43 h. However, at 67 h, the thickness of the bacteria cells in APFP treated biofilms was also reduced (by 49%) compared to vehicle treated biofilms. The defect in the biomass and thickness of polysaccharides produced by

APFP treated biofilms was more greatly reduced in 67 h biofilms: a 77.41% reduction in biomass and a 67.72% reduction in thickness (Table 3).

Table 3: Results of three-dimensional computerized analysis [mean (SD)] obtained through software Comstat from single species biofilm, formed during 43 and 67 hours, treated 2 and 4 times, respectively, with APFP.

Time point	**APFP (µg/mL)**	Biomass ($\mu m^3/\mu m^2$)		Thickness (µm)	
		Bacteria	EPS	Bacteria	EPS
43 hours	800	28.2 (±7.1)	83.4 [a] (±14.2)	23.6 [a] (±13.6)	54.4 [a] (±18.1)
	Vehicle	26.1 (±7.8)	123.8 [b] (±23.4)	104.8 [b] (±28.3)	168.7 [b] (±42.5)
67 hours	800	15.9 (±7.2)	53.9 [a] (±15,3)	97.4 [a] (±47.7)	133.9 [a] (±59.1)
	Vehicle	20.7 (±3,2)	105.5 [b] (±13,9)	122.5 [b] (±39.4)	197.0 [b] (±63.9)

Statistical Tukey-Kramer test performed separately for the different time points (43 or 67 hours). Vertically, results with no letters are not statistically different; different letters mean statistically different results, while same letters mean no statistical difference.

Figure 4 shows representative three-dimensional images of biofilms treated with APFP and vehicle at 43 (Figure 4A) and 67 hours (Figure 4B) of biofilm formation.

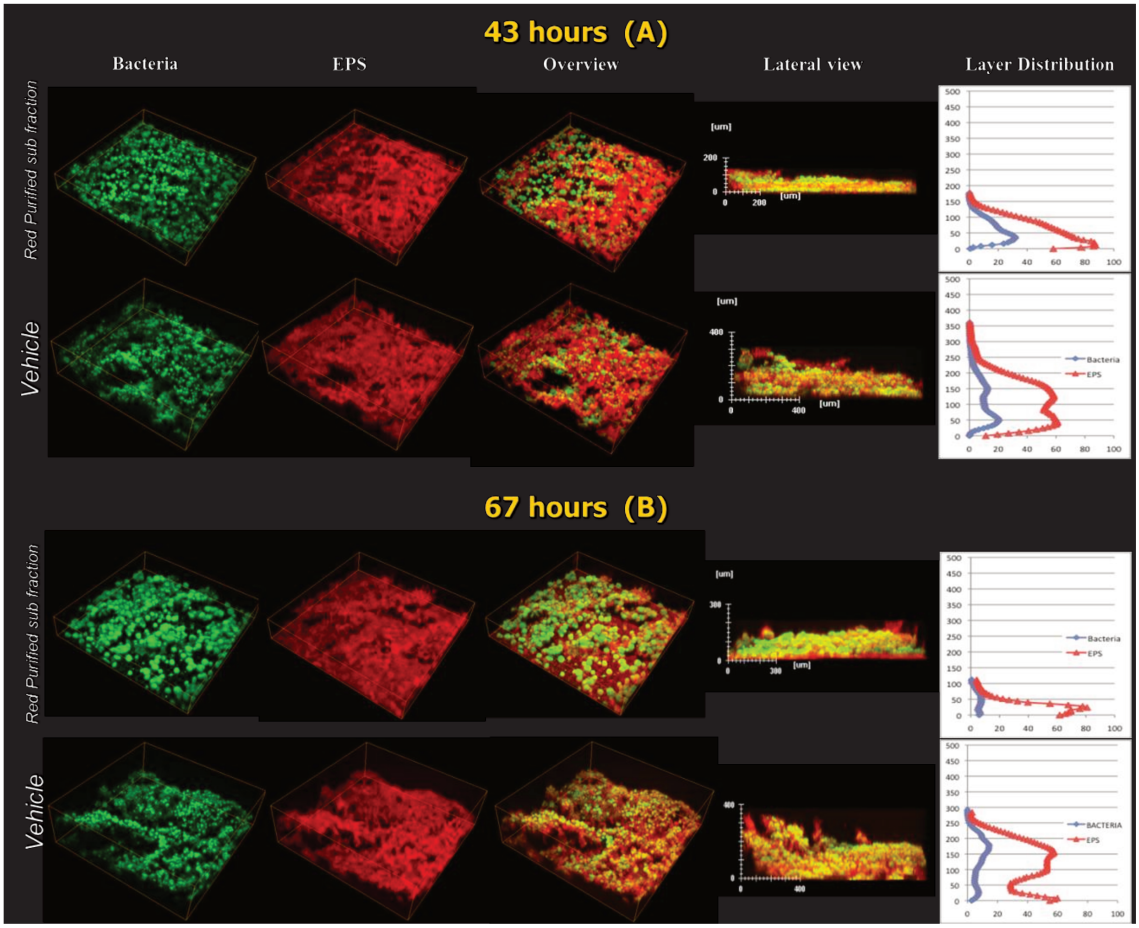

Figure 4: Representative three-dimensional images of single species biofilm, treated with APFP and vehicle, formed during: A: 43 (first time point) and B: 67 hours (second time point), acquired by confocal laser microscope and rendered by Amira software. Green and red represent bacterial cells and extracellular polysaccharides (EPS), respectively. Overview represents the entire biofilm (bacteria + polysaccharides overlaid); lateral view, the maximum thickness of biofilm and layer distribution, percentage of hydroxyapatite disc area covered with biofilm in each layer.

To better understand the mechanism(s) by which AFPF alters biofilm formation, we compared the transcriptional profiles of AFPF-treated biofilms to vehicle-treated biofilms. We subsequently found that 59 genes were differentially regulated in response to treatment (Figure 4). The majority of the genes identified were down-regulated compared to the vehicle control (52 genes), although a small number were induced in response to treatment (7 genes). We previously developed a classification system to organize microarray data (manuscript in preparation), which was used to categorize these genes into the following

functional groups: EPS, glycolytic pathway, stress (oxidative), regulators, hypothetical, other (genes with functions that are not relevant to biofilm growth and/or infection), and no gene was related to IPS and biofilm formation/adhesion categories (Figure 5).

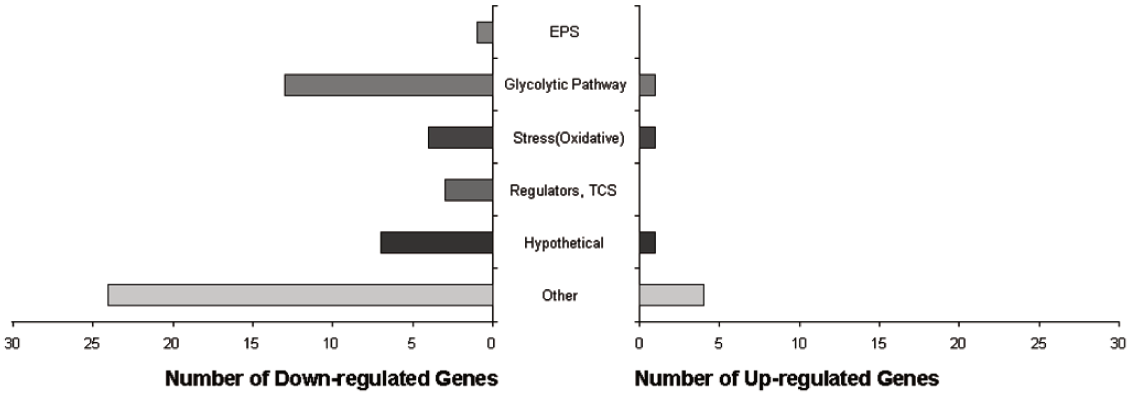

Figure 5: Microarray data, in which genes were organized in functional groups (EPS, glycolytic pathway, stress, regulators, hypothetical and other), from single species biofilm treated with APFP and vehicle.

Several genes were selected for validation with qRT-PCR, including: *gtfBCD*, *copY*, *copA*, copZ, *sloA*, *gtfD*, *amy A*, *msmK*, *oppD* and *sodA*. We have shown that our microarrays have low validation confidence in detecting mRNA levels of the *gtf* genes, especially when they are repressed, which further reduces the signal of already low abundance transcripts (Klein *et al.,* 2010; Koo *et al.,* 2010; Xiao *et al.,* 2010). Thus, some genes, such as *gtfBCD*, were not identified via microarray. However, due to their role in EPS synthesis (a key structural component of *S. mutans* biofilms), we evaluated these transcriptional profiles using qRT-PCR. In alignment with the microarray findings, although not the best method of detection of the gtf transcripts, there was no significant difference in *gtfBC* expression in AFPF treated biofilm (versus vehicle control). We were also unable to validate *oppD* or *msmK* expression. However, this was not surprising, as we relaxed our selection criteria in order to identify a greater number of differentially regulated genes (*P*-value cut-off of 0.05 versus 0.001). The remaining targets (*copYAZ*, *sloA*, *gtfD*,

and *amyA*) were significantly repressed in response to APFP treatment in 46 h biofilms (Figure 6).

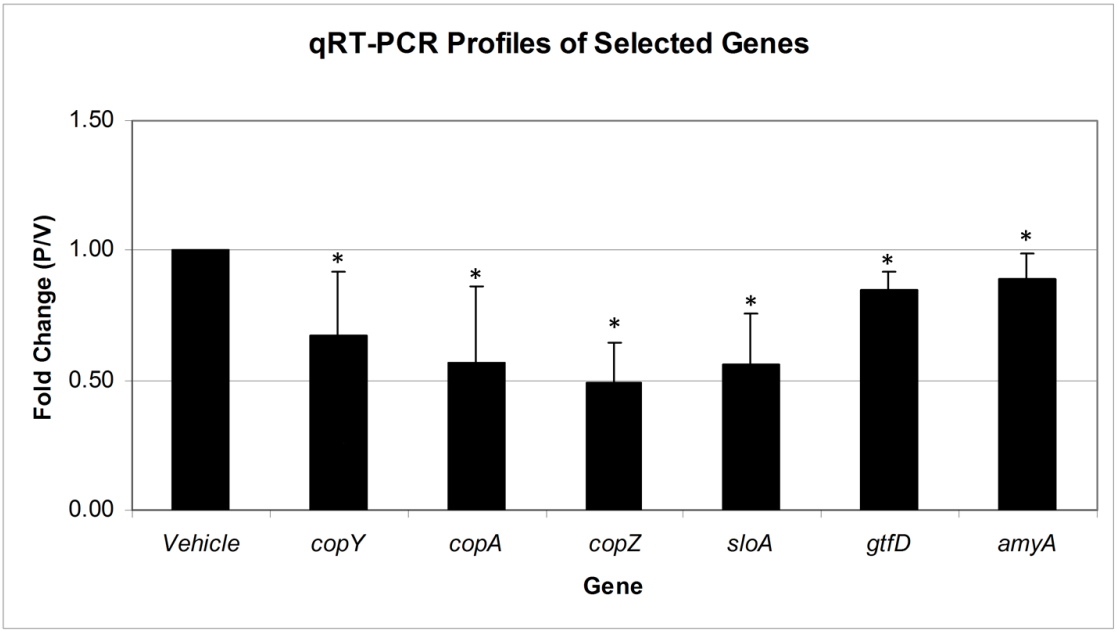

Figure 6: RT-qPCR analysis (microarray validation) of *gtfBCD*, *copY*, *sloA*, *msmK*, *oppD*, *amyA* and *sodA*. genes expression of biofilms treated with APFP and vehicle. These values were compared to those from vehicle-treated biofilms (corresponding to an arbitrary value of 1) to determine the change (n-fold) in gene expression. Data are expressed as means ± standard deviations of triplicates from at least three separate experiments. Values marked with an asterisk are significantly different from the value for the vehicle-treated biofilms ($P<0.05$, Tukey's test).

In order to assess the potential effectiveness of the APFP treatment, we evaluated its impact in an *in vivo* model of dental caries. During the course of the animal caries study, the rats remained in good health, exhibited normal behavior, and gained weight as expected. The average weight gains for individual groups of rats (3 groups: APFP, fluoride and vehicle) were not significantly different from one another ($p > 0.05$, data not shown). Table 5 shows the total cultivable microflora recovered from the oral cavity of infected animals, as well as the population of *S. mutans* individually (plated on selective media).. We determined that there was no significant difference in the total populations of bacterial

cells or the *S. mutans* population among the treatment groups (F, APFP, V). Table 5 also shows the effects of the treatments on the incidence and severity of smooth-surface and sulcal caries. The animals treated with APFP (800 μg/mL) or fluoride (250 ppm) displayed significantly less total smooth-surface and sulcal carious lesions than those treated with vehicle control ($p < 0.05$). Furthermore, APFP or fluoride treatment remarkably reduced the severity of both smooth-surface and sulcal caries when compared to the vehicle control group in all levels of dentin demineralization: Ds (slight dentin), Dm (moderate dentin) and Dx (extensive dentinal caries). There was a 50 to 80% of reduction at Dm and Dx levels, $p < 0.05$, for both APFP- and fluoride-treated groups. However, there was no statistically significant difference was observed between the values for APFP- and fluoride-treated groups related to any caries score.

Table 5: Results of *in vivo* study of the rat caries model (microorganism recovery, smooth-surface and sulcal caries) treated with APFP (800 μg/mL), Fluoride (250 ppm) and vehicle.

Treatments	Microorganisms		Smooth-surface caries				Sulcal Caries			
	Total#	*S. mutans*#	E	Ds	Dm	Dx#	E	Ds	Dm	Dx#
APFP	0.5E+05 (±0.7E+05)	0.2E+05 (±0.4E+05)	38.8[b] (±7.9)	29.9[b] (±8.4)	7.3[b] (±5.3)	0.8 (±0.6)	40.5 (±4.4)	29.8[b] (±5.1)	10.0[b] (±6.0)	2.3[b] (±2.7)
Fluoride	0.7E+05 (±1.1E+05)	0.2E+05 (±0.4E+05)	38.6[b] (±8.8)	29.5[b] (±8.0)	6.9[b] (±5.5)	1.6 (±2.5)	40.7 (±4.2)	30.3[a,b] (±5.5)	8.0[b] (±4.1)	1.3[b] (±1.9)
Vehicle	3.6E+05 (±3.7E+05)	2.9E+05 (±3.0E+05)	49.0[a] (±12.9)	41.1[a] (±14.2)	14.1[a] (±8.6)	3.2 (±3.2)	44.6 (±5.5)	35.8[a] (±6.1)	18.4[a] (±7.3)	6.8[a] (±5.4)

E, enamel caries; Ds, slight dentinal caries; Dm, moderate dentinal caries; Dx, extensive dentinal caries. Values followed by the same letters are not significantly different from each other ($P < 0.05$). ANOVA, using Tukey—Kramer without any transformation or (#) transformed with LOG_{10}

5. Discussion

. Crude extracts of natural products are typically composed of several different compounds. For this reason, fractionation is often performed on crude extracts using some method of chromatographic separation. The resulting fractionated samples may be selected

by testing their activity; further isolation of compounds in active fractions may be performed to yield individual active purified agents. However, because of the time and cost required to isolate and identify the various compounds from a crude mixture, collections of semi-purified chemical compounds from natural product extracts have been tested for (antimicrobial) activity more recently (Baker *et al.,* 2007).

This work demonstrates the anti-biofilm and anti-caries properties of APFP, a natural combination that is mainly compromised of the following two compounds: neovestitol and vestitol. Neovestitol is worthy of study, because it was recently isolated from Brazilian red propolis, which has anti-caries/antimicrobial effects. To the best of our knowledge, this is the first study that further characterizes the specific anti-biofilm and anti-caries properties of this compound (neovestitol), while simultaneously demonstrating its effectiveness *in vivo* using a rodent caries model. Vestitol, on the other hand, compromise a significantly smaller amount of the APFP active fraction.

APFP was able to inhibit biofilm formation on hydroxyapatite discs, apparently without altering the number of viable cells. Thus, we suggest that the mechanism of action for APFP is to reduce polysaccharide production, mainly extracellular polysaccharides, and to also decrease protein content. To better characterize this mechanism, we performed a combination of GTF activity tests, analyses of the architectural and structural organization of biofilms, and molecular assays (microarrays and RT-qPCR).

Results from the GTFs assays support the results of biochemical tests that show APFP reduces polysaccharide formation, although it is unable to inhibit the activity of surface-bound GTF C. In this situation, it is important to note that the different levels of inhibition for the two forms of the enzyme (in solution and surface-attached) may occur due to conformational changes in enzyme structure after adsorption (Venkitaraman *et al.,*

1995). Moreover, GTF C has the greatest affinity for hydroxyapatite when compared to other enzyme types (Jeon *et al.,* 2010) and very few agents have been demonstrated to inhibit its activity when absorbed or even in solution form (Bowen & Koo, 2011). These observations might explain why APFP was not able to inhibit GTF C activity for the surface-bound enzyme.

We also examined the three-dimensional structures of single-species biofilms, which confirmed that there was a reduction in the polysaccharide content following AFPF treatment. Thus, we have confirmed that treatment reduces EPS accumulation in biofilms, a critical structural component of virulent plaque biofilms, using several independent techniques (biochemical analysis, GTF activity assays, and structural analysis). Upon structural examination of APFP-treated biofilms, we noted that polysaccharides were present only over the base of the biofilm, forming "a carpet," while polysaccharides were present throughout the depth of vehicle-treated biofilms. This demonstrates that some changes alter the structure of these biofilms by altering or destabilizing the EPS-rich matrix. Based on these observations, APFP could act as an adjuvant in the process of destabilization of EPS-matrix, facilitating the penetration of other antimicrobial agents, by reducing the amount of extracellular matrix and exposing the bacterial cells that was protected by the matrix before, but after application of APFP, become exposed to the other antimicrobial agents. The APFP induced reduction of biofilm polysaccharides may occur via either one or both of the following mechanisms: 1) a decrease in glucosiltransferase enzyme activity and/or 2) a decrease in the expression of specific genes involved in the production or accumulation of EPS. Since there is no apparent alteration in the expression profiles of *gtf*B and *gtf*C following treatment and AFPF does reduce GTF enzymatic activity in vitro, the first option may be more plausible. However, APFP does decrease the

expression of *gtfD* and genes belonging to c*opYAZ* operon (e.g. *copY*), which encodes a copper transporter (Vats & Lee, 2001); *gtf* expression is induced in the presence of copper ions (Chen *et al.,* 2006). Reducing *gtfD* expression would likely in turn reduce the soluble glucans produced by GTF D, which can also be used as primer for insoluble glucan synthesis by GTF B (Yamashita *et al,* 1993; Bowen & Koo, 2011).

Genes from operon *CopYAZ* are part of the 59 genes that are differentially regulated in *S. mutans* biofilms in response to treatment with APFP. This number represents approximately 3% of the *S. mutans* UA159 genome, which indicates that there is a global, albeit modest, response to treatment. Therefore, reduction in protein content observed in the biochemical data (Table 1) was also found in the microarrays.

Genes were selected for validation based on their putative functions in the *S. mutans* stress response, EPS-rich matrix production and some novel functions. *SloA* is an ATP-binding protein that is translated from the *sloABCR* operon, which encodes a manganese and iron transport system required for endocarditis virulence (Paik *et al.,* 2003). Furthermore, *SloA* plays an important role in oxidative stress tolerance in *S. mutans* (Rolerson *et al.,* 2006). *CopY* is a small 147 amino acid protein with a heavy metal binding motif, which is translated from the *copYAZ* operon, that plays a role in biofilm detachment in *S. gordonii* (Mitrakul *et al.,* 2004). *GtfD* synthesizes soluble glucan (α1,6 linked glucose) and its function is critical for matrix production and normal biofilm development in *S. mutans* (Koo *et al.,* 2010; Xiao *et al.,* 2010; Bowen & Koo, 2011). *AmyA* is a unique protein that has been shown to degrade intracellular polysaccharides (IPS) (Simpson & Russel, 1998) and has been implicated in host-pathogen interactions for streptococcal species (Shelburne *et al.,* 2009). *OppD* has been implicated in *S. mutans* competence

(Mashburn-Warren *et al.,* 2010), although we did not confirm the expression profiles of this target.

The greatest number of genes identified in this study belonged to the other category (Figure 5) and are subsequently less attractive candidates for study, as they lack identified roles in biofilm formation and/or cariogenesis. However, this does eliminate the possibility that they could have novel functions in biofilm formation that remain to be elucidated. The next most abundant and relevant category was stress, specifically the oxidative stress-responsive genes and glycolytic pathway genes. The ability of APFP to repress genes involved in the adaptive stress response and glycolysis suggests that treatment would effectively impair the ability of the organism to survive *in vivo* where stresses are abundant and periods of both feast and famine are experienced. This may correlate with findings that show that APFP treatment reduces the occurrence and severity of caries disease in a rodent model of caries, which was also observed in this study (Duarte *et al.,* 2006; Hayacibara *et al.,* 2005).

The rodent model of caries may simulate more accurately the mechanical and chemical events that occur in the oral cavity, such as exposure to hydrodynamic forces, such as salivary flow and the abrasive forces *of toothbrushing (Koo el al., 2010).* Therefore, we selected this model to test of the effects of APFP were *in vivo and to* determine if these correlate with our previous *in vitro* findings.. Moreover, this model represents an intense cariogenic challenge because the animals are permitted to freely consume fermentable carbohydrates (*ad libitun* in diet and water). A reduction in the incidence and/or severity of caries under conditions that strongly favor cariogenicity is a good indicator of efficacy. Using this experimental setup (e.g. brief topical exposures in a

rodent model), facilitates initial assessment of agents that may one day be used in clinical models (Jeon *et al.,* 2010).

Remarkably, the detected *in vitro* effects of APFP were also observed *in vivo*, which highlights APFP from the others anti-caries agents since just a few of them were as effective as fluoride in reducing number and severity of smooth and sulcal caries as APFP did (Koo *et al*., 2002, 2003, 2005, 2010; Duarte *et al*., 2006; Gregoire *et al*., 2007

Fluoride has been used at 250 ppm in most of mouth rinses available for the population (Zero, 2006), and this concentration is commonly used as a positive control in caries study in rodent models (Branco-de-Almeida *et al.,* 2010). Although it is the gold standard and effective agent in caries prevention, fluoride does not completely protect against caries and it may not adequately address the infectious elements of the disease (Jeon *et al.,* 2011), as the best established role for fluoride is to physiochemically interfere with caries development by changing the demineralization and remineralization rates of dental enamel, favoring remineralization (Dawes & ten Cate, 1990).

Strategies to enhance fluoride activity are desirable, because fluoride is both effective and cost efficient, although not able to completely eradicate caries on its own. APFP may be a good candidate for a combination therapy, because its mechanism of action shares similarities to fluoride and it is as effective *in vivo* as fluoride, as demonstrated in this study. Thus, it is plausible that APFP could complement or enhance effects of fluoride. This hypothesis is based on results of association of other natural compounds with fluoride, such as apigenin and *tt*-franesol. The anti-caries effects of such combination therapies *in vivo* are greater than the natural compounds or fluoride alone (Koo *et al.,* 2005). Hence, future studies should test APFP or neovestitol (major compound of APFP) in combination with fluoride, since it is recognized that the fluoride used in association with another anti-

caries agent could present a greater effect (NIH, 2001) and the combination therapy is the newest approach to the prevention dental caries, as related by Koo *et al.,* 2008.

6. Conclusion

APFP is a promising anti-caries agent that does inhibit caries development *in vivo* in a rodent model, which could potentially be used in future clinical trials as a mouth rinse formulation, perhaps in combination to fluoride. However, it is first necessary to further characterize APFP by carrying out toxicology and cytotoxicity studies to ensure its safe use in such studies.

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6. CAPÍTULO 3

Vestitol and neovestitol isolated from Brazilian red propolis decreased neutrophils migration on inflammatory process

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Abstract

The purpose of this study is to evaluate the effect of Brazilian red propolis (crude extract) and isolated active compounds vestitol and neovestitol on the modulation of neutrophils migration during the inflammatory process, as well as their mechanisms of actions. To reach this objective, ethanolic extract of Brazilian red propolis (EEP) was submitted to a

bio-guided fractionation that originated 2 purified compounds isolated from it (vestitol and neovestitol). During the fractionation process, all the bioactive fractions were chemically monitorated by GC-MS. Also, EEP, vestitol and neovestitol were tested in order to check its effects on neutrophil migration and in levels of cytokines pro-inflammatory (TNF-α and IL-1β) and anti-inflammatory (IL-10). EEP was effective in preventing neutrophil migration to peritoneal cavity at a dose of 10 mg/kg ($p<0.05$). Regarding to cytokines, EEP inhibited the production of TNF-α ($p<0.05$) but no IL-1β levels ($p>0.05$). Furthermore, EEP increased the levels of anti-inflammatory IL-10 ($p<0.05$). Vestitol inhibits neutrophil migration induced by carrageenan at a dose of 1, 3 and 10 mg/kg with statistical difference ($p<0.05$). About cytokines, vestitol has no effects on production of IL-1 β and TNF- α ($p>0.05$). On the other hand, this compound increased the levels of the cytokine IL-10 ($p<0.05$). Neovestitol inhibited neutrophil migration induced by carrageenan at a dose of 10 mg/ kg ($p<0.05$). Concerning cytokines, IL-1 β and TNF- α had its levels decreased by neovestitol ($p <0.05$). In contrast, administration of neovestitol did not increase the levels of the cytokine IL-10 ($p>0.05$). In conclusion, vestitol and neovestitol seem to be a promising anti-inflammatory agent.
Keywords: Brazilian red propolis, vestitol, neovestitol, neutrophil, cytokines.

1. Introduction

The inflammatory process is a group of events resulting from participation of several chemical mediators that promote vascular events, edema and migration of leukocytes (Mackay, 2008). During the inflammatory process, production of different pro-inflammatory cytokines exerts chemotactic effects over the leukocytes, triggering their rolling and adhesion to endothelial cells and transmigration into inflammatory focus (Jones *et al.,* 1991).

Neutrophils are the main leukocyte sub-type involved in body response. Its movement from blood vessels to the injured tissue is a vital mechanism of the human-body's defense against infections. Although it has a protective effect, the intense neutrophil

migration is related to tissue damage in several inflammatory diseases (Malech & Gallin, 1987).

Thus, the investigation for new drugs that can block the neutrophils infiltration in different models of inflammation is intense in the literature (Hebert, 2000, Alencar *et al.,* 1999; Assreuy *et al.,* 1999, Napimoga *et al.,* 2007). Natural products from different sources have been widely used, and recently several of these are being scientifically investigated and have its anti-inflammatory effects confirmed (Newman *et al.,* 2003; Napimoga & Yatsuda, 2010). One example is the Brazilian propolis, which has already demonstrated its anti-inflammatory potential (Paulino *et al.,* 2002; Sforcin & Bankova, 2011).

Among the Brazilian propolis, the red one, or type 13 (Silva *et al.,* 2008), has previously demonstrated to have a potent antimicrobial and antioxidant activity (Alencar *et al.,* 2007) and is a rich source of new bioactive compounds (Silva *et al.,* 2008; Righi *et al.,* 2011). However, there are no reports in the literature about the use of this type of propolis or a purified compound from red propolis as a possible anti-inflammatory agent. A previous study headed by our research group showed that the ethanolic extract of red propolis can interfere with the modulation of neutrophils migration. In addition, we isolated and identified two isoflavones, vestitol and neovestitol with antimicrobial activity (manuscript in preparation).

Therefore, the purpose of this study was to evaluate the effect of Brazilian red propolis (crude extract) and isolated active compounds, vestitol e neovestitol, on the modulation of neutrophils migration during the inflammatory process, as well as their mechanisms of actions.

2. Material and methods

In order to reach purified potential anti-inflammatory compounds from the Brazilian red propolis, it was generated an experimental design based on bioassay-guided fractionation which was carried out by using evaluation of neutrophil migration. Each step of the study is showed in Figure 1.

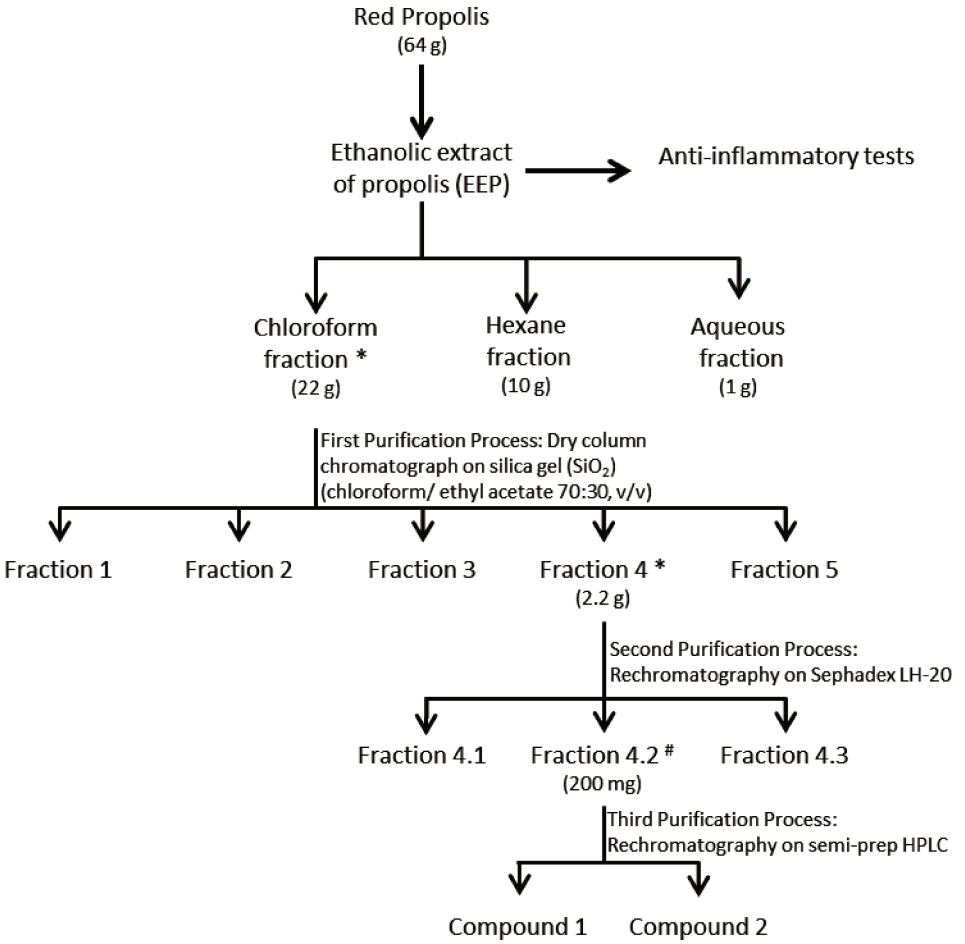

Figure 1: Experiment design of bioassay-guided fractionation in order to reach bioactive compounds with potential anti-inflammatory activity. Anti-inflammatory test: evaluation of neutrophil migration. * Best anti-inflammatory activities when compared with others from same line; # Best yield when compared with others from the same line.

2.2 Extraction and isolation of bioactive compounds

A sample of propolis was obtained from Marechal Deodoro, a city in the vicinity of Maceio, Alagoas State in the Northeast of Brazil (SL 09.40 and WL 35.41). The propolis sample (64 g) was extracted with ethanol 80% (300 mL) in water bath at 70 ºC for 30 min and after filtration yielded the ethanolic extract of propolis (EEP). The EEP was further fractioned by liquid-liquid extraction with hexane and chloroform yielding 10 g of hexanic fraction (Hex-fr), 22 g of chloroform fraction (Chl-fr) and 1 g of aqueous fraction (Aqu-fr). The EEP, Hex-fr, Chl-fr and Aqu-fr were tested regarding its potential anti-inflammatory activity by using the neutrophil migration assay (as described below). This test was used as a bioguided assay in order to reveal the bioactive fraction with anti-inflammatory activity. The active Chl-fr (22 g) was subjected to open dry column chromatography on normal phase silica gel (particle size: 0.0063.0.2mm; pore size: 60 Å; pore volume: ~0.8cm^3/g; and specific surface area: 500m^2/g) and eluted with a solvent mixture of chloroform/ethyl acetate (70:30, v/v) to afford five major fractions (Figure 1). The fractions obtained were monitored by thin layer chromatography (TLC) using the anisaldehyde reagent (4-methoxy-benzaldehyde, acetic acid, sulphuric acid: 1.0:48.5:0.5), followed by incubation at 100 ºC for 5 min. Fluorescent substances were visualized under ultra-violet (UV) light at the wavelengths of 254 nm and 366 nm (Alencar *et al.,* 2007). The five eluted fractions were also submitted to neutrophil migration evaluation bioassay-guided in order to select an active fraction. Fractions 1 and 5 showed no anti-inflammatory activity and negligible activity was found in fractions 2 and 3. The most bioactive fraction (4) was chromatographed over a Sephadex LH-20 column (5X30cm) using methanol to yield three fractions (4.1; 4.2 and 4.3). Fractions 4.1 and 4.2 were actives; however, fraction 4.1 was rejected because of its lower yield (0.0009 %). Thus, sub fraction 4.2 (Figure 1) was

purified by semi-preparative reverse-phase HPLC [Shimadzu PREP-ODS (H) 250X20mm column eluted with a gradient starting with CH3OH:H2O (65:35) to CH3OH:H2O(95:5) in 35 min, flow rate 3 mL/min] and yielded two active compounds: vestitol and neovestitol (manuscript in preparation).

2.3 Chemical Analysis

2.3.1. Derivatization - formation of the trimethylsilyl derivatives (TMS)

Approximately 10 mg of EEP, Chloroform fraction and fraction 4.2 were placed in vials with 100 μL of reagent MSTFA (N-methyl-N-(trimethylsilyl)-trifluoroacetamida). The vial was securely closed and placed in an incubator at 70 °C during 30 minutes, time required for sample derivatization. After that time, the MSTFA was evaporated under nitrogen flow and the product of derivatization (TMS-trimethylsilyl derivatives) was re-diluted in hexane (500 μL). The silanized sample was homogenized and used for injection into a gas chromatography with mass spectrometry (GC-MS).

2.3.2. Gas chromatography-mass spectrometry (GC-MS)

The GC-MS analysis were conducted on a Shimadzu gas chromatograph, model 2010 GC coupled to mass spectrometer Shimadzu, model QP 2010 Plus, equipped with a capillary column (30 m x RTX5MS 0.25 mm x 0.25 μm). The initial column temperature was 80 °C for 1 min, and reached 250 °C with rate of 20 °C/min remaining at this temperature for 1 min, 250 to 300 °C with rate of 6 °C/min, for 5 min, 300 to 310 °C the rate of 15 °C/min for 5 min, 310 to 320 °C the rate of 20 °C/min for 10 min, adding 40 min of analysis. Helium was used as carrier gas. The injector temperature was 280 °C and the injection volume was 0.2 μL. The interface was maintained at a temperature of 280 °C. The mass detector operated in scanning mode m/z 40 to 800. The integration was made in the

software solution LabSolutions-GCMS and identification of detected compounds was performed by comparison with data from the mass spectra library Wiley 8TM and authentic standards injected under the same conditions of samples.

2.4 Anti-inflammatory analysis

2.4.1 Animals

Male SPF (specific-pathogen free) Balb/c mice weighing 20-25 g were housed in temperature (22-25 °C), 12 h light/12 h dark and humidity (40-60 %) with access to water and food *ad libitum*. All experiments were conducted in accordance with the approval of the Institutional Committee for Ethics in Animal Research (CEUA/UNICAMP protocol number: 1484-1).

2.4.2 Experimental procedure to evaluate neutrophil migration

The animals were used only in a single experimental group. For the determination of neutrophil migration to peritoneal cavity EEP, fractions and isolated compounds (1, 3, 10 or 30 mg/kg) were administered by subcutaneous (s.c.) injection, 15 min before the administration of inflammatory stimuli by intraperitoneal (i.p.) injection of carrageenan at 500 µg/cavity in naive mice. The vehicle (DMSO 1 %) was used as negative control. Mice were killed 4 h after the challenge (carrageenan) administration and the peritoneal cavity cells were harvested by washing the cavity with 3 mL of phosphate buffered saline (PBS) containing EDTA 1 mM. The volumes recovered were similar in all experimental groups and equated to approximately 95% of the injected volume.

In order to count the total number of cells, a newbauer chamber was used. Smears was prepared using a cytocentrifuge, stained with fast panoctic kit and the different cells were counted (until 100 cells) using an optical microscope (1000x). Results were expressed as the number of neutrophils per cavity.

2.4.3 Cytokine assays

Based on previous test (2.4.2) the EEP, vestitol and neovestitol were selected for the quantification of pro-inflammatory (TNF-α and IL-1β) and anti-inflammatory (IL-10) cytokines. The mice were treated with EEP, vestitol or neovestitol (10 mg/kg, i.p.) 15 minutes prior the injection of carrageenan (500 µg/cavity). Vehicle was used as the negative control. After 3 hours, the animals were sacrificed and the peritoneal fluid was collected as described previously. The levels of TNF-α, IL-1β and IL-10 were determined by ELISA using the protocols as supplied by the manufacturers (Peprotech® Inc.). The results are expressed as picograms/mL.

2.4.4. Statistical analysis

Data were expressed as mean ± S.E.M., statistical comparisons between groups were made using analyses of variance (ANOVA) followed by Bonferroni test. Significance was accepted when the p value was ≤ 0.05.

3. Results

Figure 2 shows chromatograms of EEP and its bioactive fractions selected in this bioguided study. It was observed a decreasing number of peaks according to further fractionation. Moreover two peaks are identified in the chromatogram from the fraction 4.2: neovestitol (peak 5, molecular weight 416 g/mol.) and vestitol (peak 6, molecular weight 272 g/mol) (figure 2C). The finding represents compound 1 and 2 (Figure 1) such as vestitol and neovestitol, respectively.

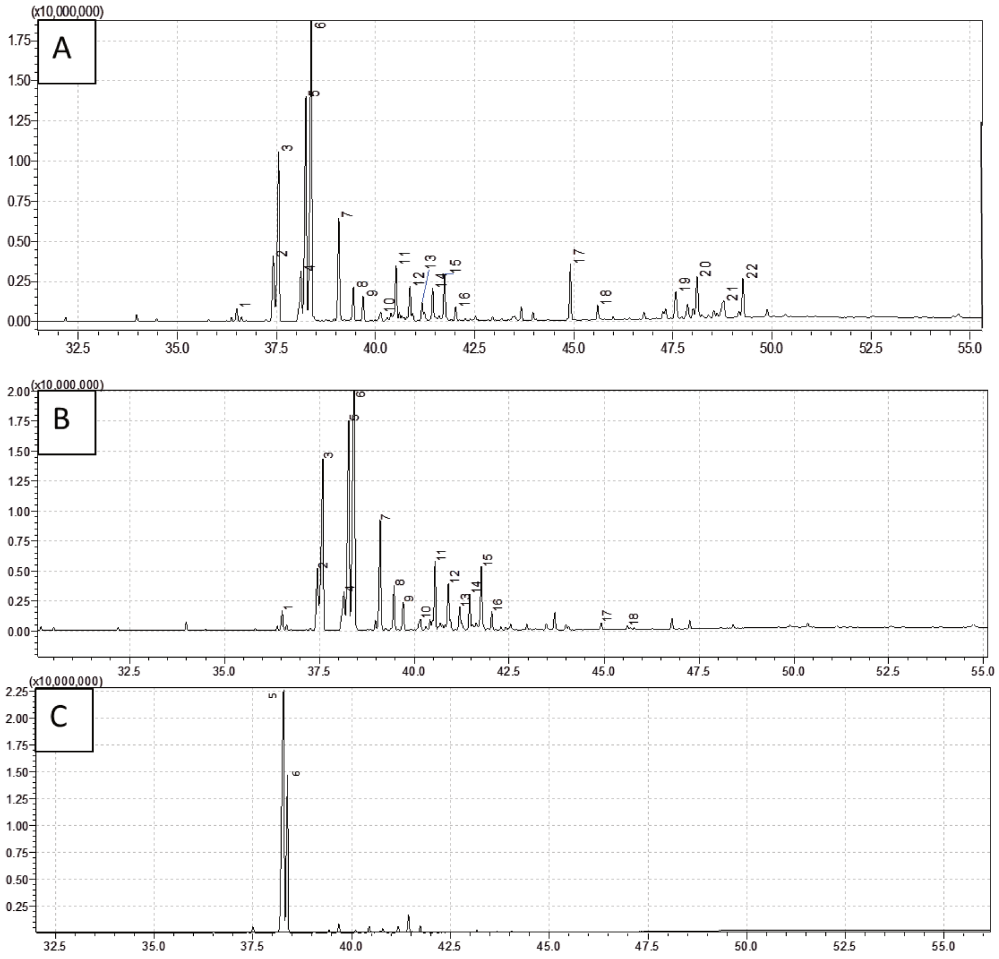

Figure 2: Chemical profile obtained by GC-MS technique of: EEP (2**A**); Chloroform fraction (2**B**), and fraction 4.2 (2**C**).

Figure 3 shows the results of EEP and its bioactive fractions selected in this bioguided study using the inhibition of neutrophils recruitment into the peritoneal cavity assay. We noticed that the administration of EEP and chloroform fraction at a dose of 10 mg/kg decreased the influx of neutrophils into the peritoneal cavity ($p < 0.05$) when compared to the carrageenan group (Figure 3A and 3B). The fraction 4.2 (Figure 3C) showed inhibitory activity on neutrophil migration at doses of 1, 3 and 10 mg/kg ($p < 0.05$).

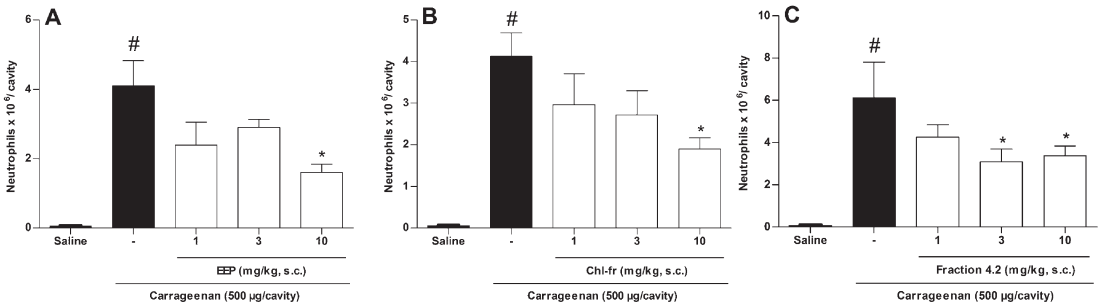

Figure 3: Recruitment of leukocytes into the peritoneal cavity induced by carrageenan. The neutrophil migration was determined 4 hours after injection of carrageenan 500 µg / cavity. Mice was previously treated with vehicle (saline), ethanolic extract of propolis (EEP, **A**), chloroform fraction (Chl-fr, **B**), and fraction 4.2 (Subfr, D) followed by carrageenan injection. The results are expressed as mean ± S.E.M., n = 5-6. The symbol (#) indicates statistical difference compared to saline group. The symbol (*) indicates statistical difference compared to carrageenan group (one-way ANOVA followed by Bonferroni test, p <0.05).

Two compounds were isolated after bioguided fractionation from the fraction 4.2: vestitol and neovestitol (Figure 4).

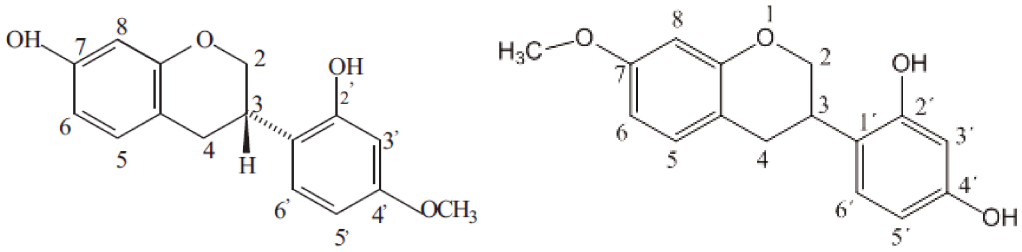

Figure 4. Chemical structure of bioactive compounds isolated from Brazilian red propolis – vestitol and neovestitol – with potential to inhibited neutrophils migration.

The vestitol demonstrated an inhibitory effect when administrated at lower doses (1, 3, 10 mg/kg, Figure 5A) whereas neovestitol showed inhibitory activity at dose of 10 mg/kg (Figure 5B) on the neutrophils migration induced by carrageenan.

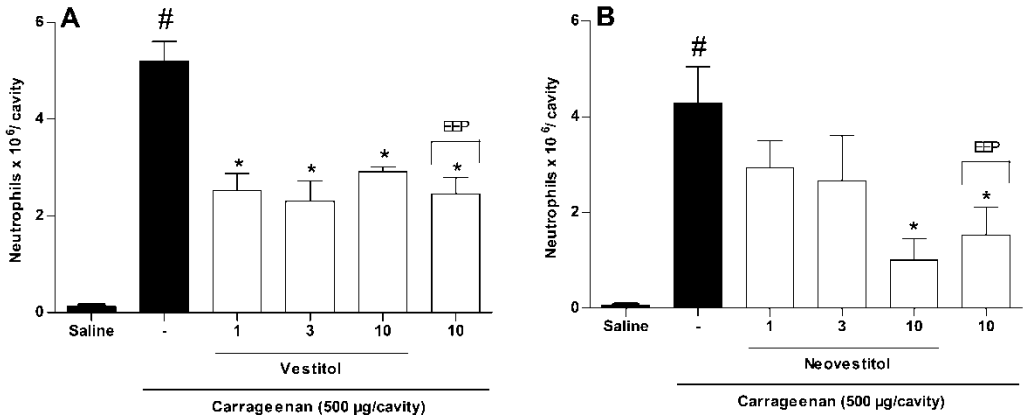

Figure 5: Inhibitory effect of vestitol and neovestitol on the neutrophils migration into the peritoneal cavity induced by carrageenan. The neutrophil migration was determined 4 h after the injection of carrageenan 500 µg/cavity. Mice previously treated with vehicle (saline and carrageenan), vestitol (**A**), neovestitol (**B**), or ethanolic extract of propolis (EEP). The data are expressed by mean ± SEM, n = 6. Symbols indicate statistical difference ($p < 0.05$, Bonferroni test). # compared to the saline group; * compared to the carrageenan group.

The administration of vestitol showed increased levels of IL-10 (Figure 6C, $p < 0.05$) during the inflammatory process. However, pretreatment with vestitol did not change levels of IL-1 β and TNF-α (Figure 6A and B, $p > 0.05$).

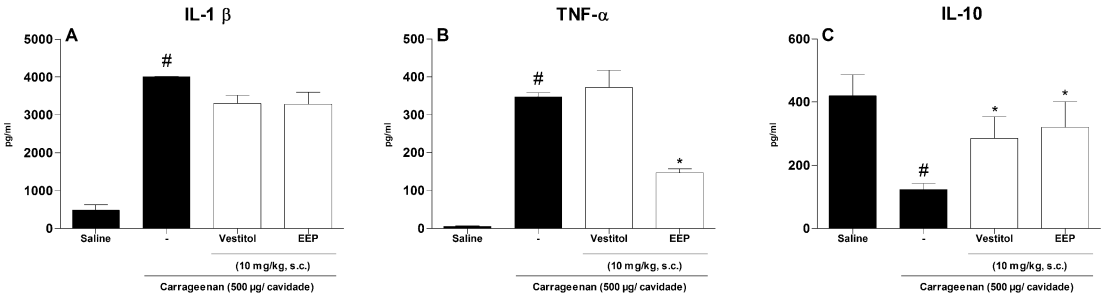

Figure 6: Quantification of IL-1β (**A**), TNF-α (**B**) and IL-10 (**C**) in mice subjected to intraperitoneal injection of carrageenan. Mice were previously treated with vehicle (saline), vestitol at 10 mg/kg and ethanolic extract of propolis (EEP) at 10 mg/kg followed by carrageenan injection. The results are expressed as mean ± S.E.M., n = 5-6. The symbol (#) indicates statistical difference compared to saline group. The symbol (*) indicates statistical difference compared to carrageenan group (one-way ANOVA followed by Bonferroni test, $p < 0.05$).

In the present study it was observed that the neovestitol inhibited both IL-1β and TNF- α (Figure 7A and B, p <0.05). On the other hand, administration of neovestitol did not influence the levels of IL-10 (Figure 7C, p> 0.05).

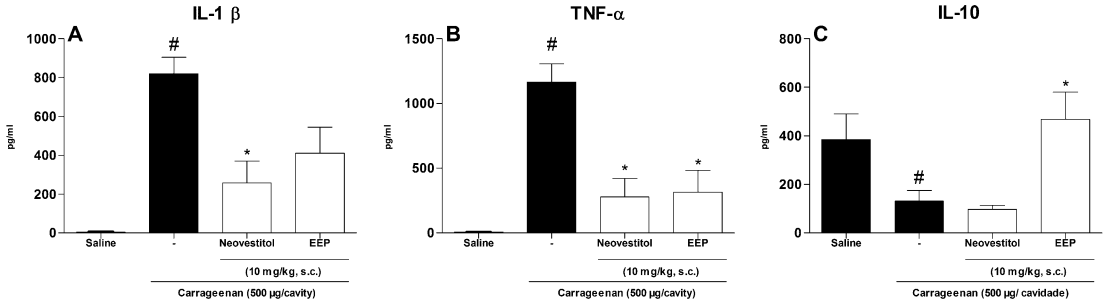

Figure 7: Quantification of IL-1β (**A**), TNF-α (**B**) and IL-10 (**C**) in mice subjected to intraperitoneal injection of carrageenan. Mice were previously treated with vehicle (saline), neovestitol at 10 mg/kg and ethanolic extract of propolis (EEP) at 10 mg/kg followed by carrageenan injection. The results are expressed as mean ± S.E.M., n = 5-6. The symbol (#) indicates statistical difference compared to saline group. The symbol (*) indicates statistical difference compared to carrageenan group (one-way ANOVA followed by Bonferroni test, p <0.05).

4. Discussion

The test for the recruitment of neutrophils into the peritoneal cavity induced by carrageenan in mice, is a method which stimulates different inflammatory mediators, including cytokines IL-1β and TNF-α. Once released, these cytokines unleash an immune and non-immune inflammatory response. Although these cytokines alone did not show chemotactic effect for leukocytes, they can stimulate different substances that can induce the recruitment of leukocytes, such as platelet activating factor (PAF), leukotriene B4 (LTB4) and chemokines. In addition, these cytokines act on endothelial cells stimulating the expression of selectins and up-regulation of adhesion molecules ICAMs (Smith, 1993, Dinarello, 2000). In contrast, IL-10 is an anti-inflammatory cytokine and inhibits activity of macrophages. Moreover, IL-10 decreases the production of pro-inflammatory cytokines such as TNF-α and IL-12 (Gately *et al.,* 1998). Therefore, IL-10 makes the defense system

or organism return to homeostasis, reducing the negative effects of inflammatory process (Baggiolini, 1998).

Many of the new drugs developed aim to interfere with the migration of neutrophils by antagonizing cytokines, chemokines, integrins and selectins involved in inflammatory process or stimulating chemical mediators that inhibit neutrophils migration (Mackay *et al.,* 2008; Nunes *et al.,* 2009; Napimoga *et al.,* 2008).

This study demonstrated that the ethanolic extract of propolis (EEP) inhibited the migration of neutrophils to the inflammatory focus. This effect may occur due to the decrease of pro-inflammatory cytokines such as IL-1β and TNF-α, and the increase of anti-inflammatory cytokine IL-10. Interestingly, neovestitol seems to be the compound responsible for reduction of TNF-α and IL-1β levels while vestitol seems to be responsible for increasing of IL-10 levels. So far, these 2 compounds seem to be the main actives of red propolis. Therefore, anti-inflammatory effect of red propolis may be due to a synergetic effect of these two compounds isolated in this study. Also, it appears to be the first report in the literature of anti-inflammatory properties of Brazilian red propolis and its isolated compounds. Vestitol and neovestitol were described before in the literature (Cuesta-Rubio *et al.,* 2007, Campo Fernández *et al.,* 2008) but this study is the first report of their anti-inflammatory properties involving the study of pro-inflammatory (IL-1 β and TNF- α) and anti-inflammatory (IL-10) cytokines.

This work corroborates several others that isolated many different bioactive compounds such as apigenin, *tt*-farnesol (Koo *et al.,* 2003), CAPE (Su *et al.,* 1994) artepelin C (Kimoto *et al.,* 1998) from Brazilian propolis. In this way, the value of Brazilian propolis is well-recognized in the literature and this natural product already

demonstrated its potential to be a promising source of new bioactive molecules with potential anti-inflammatory activity.

5. Conclusion

EEP of Brazilian red propolis and its 2 isolated compounds, vestitol and neovestitol, were effective in preventing neutrophil migration and modulate levels of some cytokines involved in inflammation such as IL-1β, TNF- α and IL-10 . Therefore, these 2 compounds isolated from Brazilian red propolis, seem to be a promising anti-inflammatory agents.

6. Aknowledgments

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7. CONCLUSÕES

O processo de fracionamento bioguiado revelou-se um método eficaz para obtenção de compostos bioativos, sendo possível isolar e identificar 2 compostos: vestitol e neovestitol (recentemente identificado).

A fração bioativa (4.2) contendo a associação natural de vestitol e neovestitol foi eficaz na inibição da formação do biofilme oral em níveis bioquímicos, moleculares e estruturais. A fração também inibiu o desenvolvimento de cárie em modelo animal, revelando uma promissioridade para futuros estudos clínicos.

O extrato bruto da própolis vermelha e seus compostos isolados (vestitol e neovestitol) inibiram a migração dos neutrófilos durante processo inflamatório, provavelmente devido a inibição de citocinas pró-inflamatórias. No entanto, é necessária a realização de novos estudos mais específicos para confirmar essa hipótese.

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6. ANEXOS

Anexo I: Resolução 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 nível (mestrado ou doutorado) e à Unidade de defesa, fazendo referência à Universidade Estadual de Campinas, sendo o projeto gráfico das capas definido pela PRPG.
II. Primeira folha interna dando visibilidade à Universidade, a Unidade de defesa, ao nome do autor, ao título do trabalho, ao número de volumes (quando houver mais de um), ao nível (mestrado ou doutorado), a área de concentração, ao nome do orientador e co-orientador, ao local (cidade) e ao ano de depósito. No seu verso deve constar a ficha catalográfica.
III. Folha de aprovação, dando visibilidade à Comissão Julgadora com as respectivas assinaturas.
IV. Resumo em português e em inglês (ambos com no máximo 500 palavras).
V. Sumário.
VI. Corpo da dissertação ou tese dividido em tópicos estruturados de modo característico à área de conhecimento.
VII. Referências, formatadas segundo normas de referenciamento definidas pela CPG da Unidade ou por critério do orientador.
VIII. Todas as páginas deverão, obrigatoriamente, ser numeradas, inclusive páginas iniciais, divisões de capítulos, encartes, anexos, etc... As páginas iniciais poderão ser numeradas utilizando-se algarismos romanos em sua forma minúscula.
IX. Todas as páginas com numeração "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 retirados pelas Unidades em no máximo, cinco dias úteis para impressão preto e branco e 10 dias úteis para coloridas.

§ 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

Anexo II: Certificado de aprovação do Comitê de ética em humanos

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

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "**Avaliação das atividades antibacteriana, anticárie e antinflamatória do(s) novo(s) composto(s) isolado(s) da própolis vermelha**", protocolo nº **033/2008**, dos pesquisadores **BRUNO BUENO SILVA e PEDRO LUIZ ROSALEN**, satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 02/04/2008.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project "**Evaluation of antibacteria, anticaries and antinflamatory activities of new isolated compounds of red propolis**", register number **033/2008**, of **BRUNO BUENO SILVA and PEDRO LUIZ ROSALEN**, comply with the recommendations of the National Health Council – Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 02/04/2008.

Prof. Pablo Agustin Vargas
Secretário
CEP/FOP/UNICAMP

Prof. Jacks Jorge Júnior
Coordenador
CEP/FOP/UNICAMP

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

Anexo III: Certificado de aprovação do Comitê de Ética Animal (estudo em ratos)

UNICAMP

CEEA/Unicamp

Comissão de Ética na Experimentação Animal
CEEA/Unicamp

CERTIFICADO

Certificamos que o Protocolo nº **1485-1**, sobre **"Avaliação das atividades antibacteriana, anticárie e antiinflamatória do(s) novo(s) composto(s) isolado(s) da própolis vermelhas (estudo em ratos)"**, sob a responsabilidade de **Prof. Dr. Severino Matias Alencar / Bruno Bueno Silva**, 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/Unicamp em **31 de março de 2008**.

CERTIFICATE

We certify that the protocol nº **1485-1**, entitled **"Evaluation of antibacteria, anticaries, and antiinflamatory activities of new compounds isolated of red propolis (study in rats)"**, 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 31, 2008**.

Campinas, 31 de março de 2008.

Profa. Dra. Ana Aparecida Guaraldo
Presidente

Fátima Alonso
Secretária Executiva

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

Telefone: (19) 3521-6359
E-mail: comisib@unicamp.br
http://www.ib.unicamp.br/ceea/

Anexo IV: Certificado de aprovação do Comitê de Ética Animal (estudo em camudongos)

UNICAMP

CEEA/Unicamp

Comissão de Ética na Experimentação Animal
CEEA/Unicamp

CERTIFICADO

Certificamos que o Protocolo nº **1484-1**, sobre **"Avaliação das atividades antibacteriana, anticárie e antiinflamatória do(s) novo(s) composto(s) isolado(s) da própolis vermelhas (estudo em camundongos)"**, sob a responsabilidade de **Prof. Dr. Severino Matias Alencar / Bruno Bueno Silva**, 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/Unicamp em **31 de março de 2008**.

CERTIFICATE

We certify that the protocol nº **1484-1**, entitled **"Evaluation of antibacteria, anticaries, and antiinflamatory activities of new compounds isolated of red propolis (study in mice)"**, 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 31, 2008**.

Campinas, 31 de março de 2008.

Profa. Dra. Ana Aparecida Guaraldo
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Anexo V: Comprovante de submissão de artigo

Assunto: Inflammation Research - Manuscript ID IR-2012-0066
De: iredit@springer.com
Data: Qua, Fevereiro 22, 2012 14:07
Para: bruno@fop.unicamp.br
Prioridade: Normal
Opções: Ver cabeçalho completo | Ver Versão para Impressão | Baixar como um arquivo

22-Feb-2012

Dear Dr. Bueno-Silva:

Your manuscript entitled "Vestitol and neovestitol isolated from Brazilian red
propolis decreased neutrophils migration on inflammatory process" has been
successfully submitted online and is presently being given full consideration for
publication in Inflammation Research.

Your manuscript ID is IR-2012-0066.

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