UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

PG-BGA

CARACTERIZAÇÃO DE UM PRODUTO ODONTOLÓGICO À BASE DE PRÓPOLIS: AÇÕES ANTIBACTERIANA E IMUNOMODULADORA

KARINA BASSO SANTIAGO

Tese apresentada ao Instituto de Biociências, *Câmpus* de Botucatu, UNESP, para a obtenção do título de Doutor junto ao Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração: Biologia Celular Estrutural e Funcional.

Orientador: Prof. Dr. José Maurício Sforcin

BOTUCATU – SP
2015

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

PG-BGA

UNIVERSIDADE ESTADUAL PAULISTA

"Julio de Mesquita Filho"

INSTITUTO DE BIOCIÊNCIAS DE BOTUCATU

CARACTERIZAÇÃO DE UM PRODUTO ODONTOLÓGICO À BASE DE PRÓPOLIS: AÇÕES ANTIBACTERIANA E IMUNOMODULADORA

KARINA BASSO SANTIAGO

ORIENTADOR: PROF. DR. JOSÉ MAURÍCIO SFORCIN

Tese apresentada ao Instituto de Biociências, *Câmpus* de Botucatu, UNESP, para a obtenção do título de Doutor junto ao Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração: Biologia Celular Estrutural e Funcional.

Orientador: Prof. Dr. José Maurício Sforcin

BOTUCATU – SP
2015

FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: ROSEMEIRE APARECIDA VICENTE-CRB 8/5651

Santiago, Karina Basso.
Caracterização de um produto odontológico à base de própolis : ações antibacteriana e imunomoduladora / Karina Basso Santiago. - Botucatu, 2015

Tese (doutorado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Instituto de Biociências de Botucatu
Orientador: José Maurício Sforcin
Capes: 21100004

1. Própole. 2. Agentes antibacterianos. 3. Streptococcus mutans. 4. Monócitos. 5. Placas dentarias. 6. Citocinas. 7. Clorexidina.

Palavras-chave: Clorexidina; Monócito; Placa bacteriana; Própolis; Streptococcus mutans.

Á minha querída mãe

"Teus braços sempre se abrem quando preciso de um abraço. Teu coração sabe compreender quando preciso de uma amiga. Teus olhos sensíveis se endurecem quando preciso de uma lição. Tua força e teu amor me dirigiram pela vida e me deram as asas que precisava para voar."

Obridaga, te amo.

Autor desconhecido

Agradecimentos

Agradeço, inicialmente, à uma mulher especial, que, como uma rocha, me dá sustento, suporte e amor intenso: minha Mãe;

À meu amado marido André Luís, um homem especial, que por sua paciência e zelo, soube esperar por mim e apoiar-me quando mais precisei;

Aos meus irmãos Ricardo e Larissa, que sempre se mostraram fiéis amigos e verdadeiros companheiros nas horas mais críticas;

Ao meu estimado Orientador, Prof. Dr. José Maurício Sforcin, pelo qual tenho admiração imensa, pelo incansável trabalho, paciência, ajuda, incentivo, sabedoria e principalmente pela amizade;

Aos professores Ary Fernandes Junior, Marjorie de Assis Golim e Vera Lucia Mores Rall, e suas equipes, pela gentileza, dedicação e conhecimento, fundamentais para este trabalho;

Ao meu amigo e companheiro de trabalho Bruno José Conti, pelas experiências compartilhadas e grande ajuda nessa jornada, e aos colegas de laboratório Pedro, Abigail, Yahima, Ariane, Lívia, Lucas, Fernanda, Anthony, e em especial à Eliza de Oliveira Cardoso pela convivência, trabalho, ajuda, paciência e momentos agradáveis;

Aos professores e funcionários do Departamento de Microbiologia e Imunologia, por me receberem com tanto carinho e pelos momentos de descontração;

Aos funcionários da seção de Pós-Graduação pela assistência prestada;

À Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), pela bolsa de doutorado (2011/22112-0) e auxilio à pesquisa (2012/14614-8) para execução deste projeto;

E à Deus, que me deu forças para continuar sempre, mesmo com tantos obstáculos no caminho.

"A mente que se abre a uma nova ideia, jamais voltará ao seu tamanho original".

Albert Einstein

Lista de Abreviaturas e Siglas

ATCC: American Type Culture Collection

BHI: Brain-Heart Infusion

CFU: Colony-Forming Unit

CHX: Chlorhexidine

DMSO: Dimetilsulfóxido

ELISA: Enzyme-Linked Immunosorbent Assay

H_2O_2: Peróxido de Hidrogênio

IL: Interleucina

LPS: Lipopolissacarídeo

MTT: 3-(4,5-dimetiltiazol-2yl)-2,5-difenil brometo de tetrazolina

O. D.: Optical density

P: Própolis

PAMPs: Padrões Moleculares Associados ao Patógeno

PBMC: Peripheral Blood Mononuclear Cells

PBS: Phosphate Buffered Saline

P/CHX: Associação própolis/clorexidina

RPMI: Roswell Park Memorial Institute

S. mutans: *Streptococcus mutans*

TLR: *Toll-like* receptor

TNF-α: Fator de Necrose Tumoral alfa

UNESP: Universidade Estadual Paulista

Resumo

A cárie e a doença periodontal são doenças infecciosas que refletem falha no equilíbrio da microbiota bucal, e seus agentes podem ser transmissíveis. A clorexidina tem sido utilizada como agente antimicrobiano no combate à carie, porém seu uso é controverso devido à ocorrência de efeitos colaterais. Assim, tem sido incansável a busca de novos produtos com atividade antimicrobiana e com menor efeito colateral. A própolis é um produto resinoso, elaborado pelas abelhas a partir de diversas partes das plantas, e se destaca por suas propriedades biológicas e farmacológicas, e pela possibilidade de aplicação na indústria farmacêutica e alimentícia. Neste projeto, visamos comparar a própolis com um agente antimicrobiano (clorexidina), bem como a combinação própolis/clorexidina, diretamente sobre *Streptococcus mutans* ou sobre a ação de monócitos humanos contra esta bactéria. Para tal, monitoramos a qualidade do produto odontológico elaborado à base de própolis em relação à sua estabilidade e possível contaminação durante 120 dias. Avaliamos o efeito do produto sobre *S. mutans*, determinando a concentração inibitória mínima e a concentração bactericida mínima, bem como seu efeito sobre monócitos humanos, avaliando a expressão de receptores celulares, a via de sinalização do NF-kB, a produção de citocinas pró- e antiinflamatórias, e a atividade bactericida de monócitos contra este agente causador da cárie. Tomados em conjunto, os dados evidenciaram que o produto apresentou-se palatável e livre de contaminantes, além de inibir o crescimento de *S. mutans*. Este produto pode favorecer o reconhecimento de antígenos por monócitos, ativar a via de sinalização do NF-kB e aumentar a atividade bactericida de monócitos humanos contra *S. mutans*. Ademais, o produto também desempenhou papel anti-inflamatório quanto à produção de citocinas, o que pode trazer benefícios no tratamento de doenças periodontais. Esses achados abrem perspectivas para novas investigações, o que denota a aplicação prática desta pesquisa e suas implicações na indústria farmacêutica.

Palavras-chave: *Streptococcus mutans*, própolis, citocina, placa bacteriana, monócito, clorexidina.

Abstract

The caries and periodontal disease are infectious diseases, reflecting failure to balance the oral microbiota, whose agents can be transmitted. Chlorhexidine has been used as anti-caries agent, but its use is controversial due to the occurrence of side-effects. Thus, there is a need to search for new products with antimicrobial activity and fewer side-effects. Propolis is a resinous product produced by bees from different parts of the plants, and stands out for its biological and pharmacological properties, and the application possibility in the pharmaceutical and food industry. In this project, we aimed to investigate propolis, an antimicrobial agent (chlorhexidine) and the combination propolis/chlorhexidine, directly on S. mutans and the action of human monocytes against this bacteria. The quality of propolis-based odontological product was analysed with regard to its stability and possible contamination for 120 days. We evaluated the effect of this product on *S. mutans* determining the minimum inhibitory concentration and minimum bactericidal concentration, and its effect on human monocytes, evaluating cell receptors expression, the NF-kB signaling pathway, the production of pro- and anti-inflammatory cytokines, and the bactericidal activity of these cells against *S. mutans*. Together, data showed that propolis/chlorhexidine in combination was palatable and contaminant-free, besides inhibiting the growth of *S. mutans*. The product may favor the recognition of antigens by human monocytes, activate the NF-kB signaling pathway and increase the bactericidal activity of monocytes against *S. mutans*. Also, the combination played an anti-inflammatory role in cytokine production, what can be beneficial in the treatment of periodontal diseases. The findings open perspectives for further research, denoting the practical application of this research and its implications for the pharmaceutical industry.

Keywords: *Streptococcus mutans*; propolis; cytokines; bacterial plaque; monocyte; chlorhexidine.

Sumário

Introdução

1. Introdução

Própolis é um produto resinoso e balsâmico, coletado e elaborado pelas abelhas a partir de algumas partes de plantas como brotos, ramos, cascas de árvores, exsudatos resinosos e botões florais (BANKOVA, 2005), ao qual as abelhas adicionam secreções salivares, cera e pólen (ALMEIDA *et al.*, 2006). As abelhas utilizam a própolis para selar aberturas e controlar variações de temperatura no interior da colméia. A própolis também é utilizada para embalsamar insetos e outros invasores que morrem no interior da colméia, para evitar sua decomposição e contribuir para manter o ambiente interno asséptico, protegendo-o contra bactérias e vírus (SALATINO *et al.*, 2005).

A própolis é composta por 50% de resina, 30% de cera de abelha, 10% de óleos aromáticos e essenciais, 5% de pólen e 5% de outras substâncias variadas, além de impurezas (BURDOCK, 1998). A coloração da própolis pode variar entre amarelo claro, vermelho escuro, verde e até marrom, dependendo da sua origem botânica e estação sazonal (SALATINO *et al.*, 2005). Sua composição química é extremamente complexa, variando conforme a localização geográfica e a flora da região. As principais fontes de própolis no apiário da UNESP de Botucatu são *Baccharis dracunculifolia* DC (alecrim-do-campo), seguida de *Araucaria angustifolia* (Bert.) O. Kuntze e *Eucalyptus citriodora* Hook (BANKOVA *et al.*, 1999).

Os constituintes da própolis como materiais cerosos, bálsamos, óleos essenciais e derivados fenólicos, podem ser extraídos em solventes como etanol, éter, acetona, tolueno e tricloroetileno (CUNHA *et al.*, 2004). Foram identificados mais de 300 componentes presentes na própolis; destes, os principais componentes biologicamente ativos da própolis brasileira são os ácidos diterpênicos e ácidos *p*-cumáricos prenilados. Há também chalconas, ácido benzóico, benzoaldeído, álcoois, acetona, compostos fenólicos, ácido cinâmico, ácido cafeico e derivados, di- e triterpenos (DE CASTRO, 2001).

Os compostos presentes na própolis produzida no apiário da UNESP, Campus de Botucatu, foram analisados por cromatografia gasosa (GC), cromatografia gasosa associada à espectrometria de massa (GC-MS) e cromatografia em camada delgada (TLC), revelando que seus principais grupos são: compostos fenólicos (flavonóides, ácidos aromáticos, benzopiranos) di- e triterpenos, óleos essenciais, entre outros. Os flavonóides estão presentes em pequenas quantidades (4'-O-metil canferol, 5,6,7-trihidroxi-3,4'dimetoxiflavona, aromadendrina-4'-metil éter); ácido *p*-cumárico prenilado e dois benzopiranos: *E* e *Z* 2,2-

dimetil-6-carboxietenil-8-prenil-2H-benzopiranos); óleos essenciais (espatulenol, (2Z, 6E)-farnesol, benzoato de benzila e acetofenonas preniladas); ácidos aromáticos (ácido diidrocinâmico, ácido *p*-cumárico, ácido ferúlico, ácido cafeico, ácido 3,5-diprenil-*p*-cumarico, 2,2-dimetil-6-carboxi-etenil-8-prenil-2H-1-benzo-pirano) (BANKOVA *et al.*, 1998; SFORCIN, 2007).

Pesquisas evidenciaram que variações sazonais na composição da nossa amostra de própolis não são significativas, havendo concentrações dos compostos biologicamente ativos em todas as estações do ano (BOUDOUROVA-KRASTEVA *et al.*, 1997; SFORCIN, 2007). A própolis se destaca por suas propriedades biológicas e farmacológicas, e pela possibilidade de aplicação na indústria farmacêutica e alimentícia, embora seja utilizada na medicina desde a antiguidade (SALATINO *et al.*, 2005; CASTRO *et al.*, 2007).

Há inúmeros estudos que investigaram as propriedades biológicas da própolis, principalmente suas ações imunomoduladora, antimicrobiana, anti-inflamatória, anestésica, antitumoral e anticariogênica (CASTRO *et al.*, 2007; SFORCIN, 2007). Outros autores têm sugerido a ausência de efeitos colaterais após a administração de própolis a curto ou longo prazo em ratos (SFORCIN *et al.*, 1995; SFORCIN *et al.*, 2002; MANI *et al.*, 2006; MANI *et al.*, 2008).

A própolis tem despertado interesse de pesquisadores da área odontológica em diversas especialidades, com o intuito de minimizar quadros de inflamação decorrentes de procedimentos cirúrgicos e, principalmente, como agente antimicrobiano no controle da placa bacteriana, sem produzir efeitos colaterais que comprometam a saúde bucal.

A cavidade bucal é um sistema de crescimento aberto, onde nutrientes e micro-organismos são introduzidos e removidos facilmente. Os micro-organismos que são capazes de aderir às superfícies da cavidade bucal são os que se estabelecem nesse sistema (BARBIERI *et al.*, 2007). A cárie e a periodontite são doenças infecciosas, que refletem falha no equilíbrio da microbiota bucal, e cujos agentes podem ser transmissíveis. São infecções decorrentes de acúmulo de bactérias na superfície dentária, formando um biofilme, e sua progressão desencadeia a destruição de tecidos mineralizados dos dentes (MEALEY & OCAMPO, 2007). Entre os principais fatores para o estabelecimento da cárie estão: hospedeiro com dentes susceptíveis e colonizados por micro-organismos potencialmente cariogênicos, dieta com alto consumo de carboidratos e higiene bucal precária (SHANMUGAM *et al.*, 2013).

O biofilme dental se forma primeiramente com a aderência bacteriana à superfície da película adquirida, mediada por adesinas. Em seguida, ocorre a agregação de bactérias àquelas já aderidas, permitindo o desenvolvimento do biofilme dental (SILVA *et al.*, 2008). Com uma conduta contínua de agressão, o biofilme dental adquire, ao longo do seu desenvolvimento, novas espécies, como *Streptococcus mitis, Streptococcus mutans, Streptococcus sanguis, Streptococcus sobrinus* e *Lactobacillus casei,* que podem provocar danos ao esmalte e tecido gengival (PEREIRA *et al.*, 2006).

S. mutans são bactérias Gram positivas ovaladas, que agrupam-se aos pares ou em cadeias. São anaeróbios facultativos e sua temperatura ótima de crescimento é de 37°C, formam colônias pequenas, fortemente aderidas ao meio de cultura, e com bordas irregulares (BARBIERI *et al.*, 2007). Os estreptococos do grupo mutans que se encontram na cavidade bucal são considerados importantes agentes patogênicos, predominantemente associados com o início e a patogenia da cárie dental. Sabe-se que, em pacientes com maior incidência de cárie, a concentração de *S. mutans* é elevada na saliva. *S. mutans* e outros membros de estreptococos bucais do grupo mutans produzem glicosiltransferases, que hidrolizam a sacarose da dieta em glicose e frutose, e unem os resíduos de glicose entre si para formar glicanos insolúveis. Esses glicanos conferem aos micro-organismos a capacidade de aderir às superfícies lisas dos dentes e formar a matriz do biofilme dental (BARBIERI *et al.*, 2007). *S. mutans* também produzem maior quantidade de ácidos a partir de carboidratos do que outras bactérias bucais, por serem capazes de fermentar grande variedade de açúcares e serem mais resistentes aos ácidos do que outros estreptococos bucais. Esses micro-organismos também sintetizam polissacarídeos intracelulares que podem ser metabolizados para produzir ácidos na ausência de carboidratos fermentáveis exógenos (DRUMOND *et al.*, 2004).

A remoção do biofilme dentário constitui um fator importante na prevenção da cárie e doença periodontal (SILVA *et al.*, 2008). Devido às limitações dos métodos mecânicos de remoção, agentes antimicrobianos têm sido estudados no intuito de controlar a formação do biofilme, e diversas substâncias têm sido testadas como enxaguatório bucal, visando a redução dos micro-organismos cariogênicos (DRUMOND *et al.*, 2004).

Substâncias antimicrobianas como clorexidina, triclosan, timol e cloreto de cetilpiridínio têm sido utilizadas no combate à carie. O gluconato de clorexidina (CHX) é o mais estudado, por ser geralmente mais efetivo contra micro-organismos Gram positivos, como os *Streptococcus mutans* (LIBÉRIO *et al.*, 2009). CHX é adsorvida às superfícies oral e bacteriana, interferindo no equilíbrio osmótico bacteriano, na formação de película adquirida

e na adsorção bacteriana ao dente. Alguns estudos mostraram *in vivo* a capacidade da CHX em reduzir a formação de biofilme e inibir a síntese de polissacarídeos insolúveis da matriz intermicrobiana do biofilme dental (SILVA *et al.*, 2008).

Porém, o uso da CHX como agente anticárie ainda é controverso. A ocorrência de efeitos colaterais, como pigmentação dos dentes, da língua, de restaurações e próteses, além da descamação e ferimento na mucosa oral, distúrbios no paladar e gosto amargo, leva a restrições quanto ao tempo de uso. Assim, tem sido incansável a busca de novos produtos, com atividade antimicrobiana e menor efeito colateral, que possam ser utilizados no controle profilático e terapêutico de bactérias cariogênicas (LIBÉRIO *et al.*, 2009).

Alguns produtos naturais são utilizados desde a antiguidade como agentes químicos para diferentes propósitos. Estudos com própolis, tomilho, cacau e *Arnica montana,* abriram a possibilidade do uso de substâncias naturais como antimicrobianos orais (SILVA *et al.*, 2008), com a vantagem de não apresentarem efeitos colaterais relatados (DRUMOND *et al.*, 2004).

Avaliando a ação antimicrobiana da própolis, foi verificada sua eficiente ação inibidora, *in vitro*, sobre várias linhagens de bactérias Gram positivas e limitada ação contra bactérias Gram negativas (SFORCIN *et al.*, 2000). Castro *et al.* (2007) evidenciaram que a própolis proveniente das regiões Nordeste e Sudeste apresentam forte atividade antimicrobiana sobre a bactéria *Streptococcus mutans*. Inúmeros estudos indicam também o bom desempenho da própolis como agente antimicrobiano oral. Ensaios *in vitro e in situ* com própolis e *Streptococcus mutans* indicaram redução significativa destas bactérias (DRUMOND *et al.*, 2004; LIBÉRIO *et al.*, 2009). Também foram obtidos resultados positivos contra *S. mutans* com o uso da própolis em forma de bochecho (ALMEIDA *et al.*, 2006; DUAILIBE *et al.*, 2007). LIBÉRIO *et al.* (2009) não obtiveram efeitos tóxicos após tratamento tópico oral com um gel à base de geopropólis produzida por meliponíneos.

Além do problema da cárie, também há casos de gengivite e sangramento na cavidade bucal. Os monócitos advindos do sangramento gengival migram para o foco inflamatório; contudo, há poucos dados na literatura sobre a ação imunomoduladora da própolis em células humanas, mais especificamente sobre a ação de monócitos contra bactérias cariogênicas.

Com relação ao sistema imunológico, a própolis modula os eventos iniciais da resposta imune em camundongos, induzindo a expressão de *Toll-like receptor* (TLR)-2 e TLR-4 e produção de citocinas pró-inflamatórias (ORSATTI *et al.*, 2010), bem como a geração de espécies reativas do oxigênio (H_2O_2) por macrófagos peritoneais (ORSI *et al.*,

2000). A própolis também induz aumento na atividade fungicida de macrófagos contra *Paracoccidioides brasiliensis* (MURAD *et al.*, 2002) e na atividade bactericida contra *Samonella* Typhimurium, envolvendo a participação de intermediários reativos do oxigênio e do nitrogênio (ORSI *et al.*, 2005). Porém, como já mencionado acima, poucos são os trabalhos encontrados na literatura pertinente sobre seu efeito em células humanas envolvidas na resposta imunológica.

Monócitos/macrófagos são células do sistema fagocítico mononuclear, sendo de fundamental importância na imunidade inata do hospedeiro. Monócitos podem ser distinguidos dos macrófagos por vários critérios, além de sua localização, por estarem na circulação sanguínea, enquanto macrófagos estão nos tecidos (AUFFRAY *et al.,* 2009). Monócitos possuem citoplasma e núcleo menores, maior conteúdo de mieloperoxidase e níveis menores de esterases não específicas. Funcionalmente, são menos aderentes e possuem atividade fagocitária menor. Expressam menor número de antígenos de histocompatibilidade de classe II e de receptores para porção Fc de imunoglobulinas ou para o componente C3 do sistema complemento. No entanto, as diferenças entre monócitos e macrófagos são mais quantitativas do que qualitativas, sendo comumente difícil a diferenciação entre as duas células (WILTROUT & VARESIO, 1991).

O reconhecimento de antígenos através de receptores celulares, seu processamento e apresentação são eventos críticos para o desencadeamento de uma resposta imunológica, e os fagócitos elaboram respostas aos seus alvos de acordo com seu microambiente e presença de determinadas citocinas (DJALDETTI *et al*., 2002; FREEMAN & GRISTEIN, 2014).

TLRs reconhecem micro-organismos através da detecção de produtos ou estruturas microbianas conservadas, denominados de padrões moleculares patógeno-associados (PAMPs). Os PAMPs estão ausentes em células eucarióticas, mas podem estar presentes em micro-organismos patogênicos ou não, fazendo com que os TLRs sejam capazes de distinguir o próprio do não-próprio (MEDZHITOV, 2001). TLRs reconhecem um diverso, porém limitado, número de PAMPs. Como exemplo, TLR-2 reconhece uma grande quantidade de produtos microbianos, como peptidioglicanos, lipopolissacarídeo (LPS) de bactérias Gram-negativas, lipoproteínas, componentes de parede celular de micobactérias, dentre outros. Tal variedade de ligantes pode ser explicada, em parte, pela cooperação existente entre TLR-2 e TLR-1 ou TLR-2 e TLR-6, ocorrendo formação de heterodímeros. Já TLR-4 tem como agonistas LPS de bactérias Gram-negativas, ácido lipoteicóico e a proteína de fusão F do vírus respiratório sincicial (RSV). Ácidos nucléicos virais ou bacterianos são reconhecidos por

TLR-3/7/8 e 9 enquanto o TLR-5 tem como ligante a flagenina bacteriana (TAKEDA & AKIRA, 2005).

Após reconhecimento de micro-organismos, há ativação de vias de transdução de sinal, ativação de vários fatores de transcrição, como, por exemplo, NF-kB, relacionados à expressão gênica de TNF-α, IL-6 e IL-10. A via de sinalização do NF-kB é tipicamente pró-inflamatória, podendo ser ativada por inúmeros estímulos intra- ou extracelulares e levando à produção de citocinas como TNF-α. A IL-6 pode ser regulada por múltiplos fatores de transcrição, incluindo NF-kB e AP-1 (HUNGNESS *et al.*, 2000).

O NF-kB é um fator de transcrição com capacidade de realizar a transcrição gênica somente quando localizado no núcleo celular. No citoplasma, é incapaz de regular a transcrição, devido ao controle realizado pelo inibidor de kB (I-kB) que se liga ao NF-kB, inativando-o. Para o deslocamento do NF-kB para o núcleo, a proteína IKK precisa ser fosforilada e ativada pelo pAkt, sendo então capaz de fosforilar o I-kB, promovendo sua ubiquitinação e consequente degradação proteossômica. Na ausência do I-kB, o NF-kB vai até o núcleo onde é capaz de exercer sua função, induzindo a transcrição de vários genes (CHANG *et al.*, 2003; DOLCET *et al.*, 2005). Em células normais, o NF-kB só é ativado depois de receber o estímulo adequado que o leve a transcrever os genes-alvo.

Individualmente ou em combinação, as citocinas interagem com seus receptores específicos, modulando a função celular. Citocinas como TNF-α são importantes no processo de ativação de monócitos e macrófagos, enquanto que outras citocinas, como a IL-10, são consideradas fatores de desativação celular.

TNF-α é considerado uma citocina multifuncional, sendo um dos mediadores inflamatórios mais importantes. O TNF-α exerce funções diversas como indução da produção de outras citocinas e quimiocinas pelas células do sistema imunológico, além de induzir a expressão de moléculas de adesão e aumento da permeabilidade no endotélio, o que propicia o aumento do número de monócitos no local da inflamação (HEHLGANS & PFEFFER, 2005).

O papel do TNF-α no organismo não se restringe apenas à ativação de macrófagos/monócitos, e sua ação contra micro-organismos vai depender de outras atividades exercidas por esta citocina. O TNF-α está envolvido na necrose de tumores, no choque endotóxico, caquexia, febre, anorexia, produção de proteínas de fase aguda, entre outros. Assim, quando produzido e liberado em quantidades moderadas, tem efeito benéfico ao organismo, enquanto que, em quantidades elevadas, pode causar consequências indesejáveis.

Conforme já mencionado, a IL-10 apresenta ação inibitória sobre macrófagos ativados e células dendríticas, estando envolvida no controle de reações da imunidade inata e da imunidade mediada por células. A IL-10 inibe a expressão de moléculas co-estimulatórias e moléculas de histocompatibilidade de classe II, promove a resposta imune humoral e inibe a resposta imune celular, diminuindo a produção de citocinas do perfil Th1, a proliferação de células T antígeno-específicas, e os níveis de citocinas pró-inflamatórias como o TNF-α. Vários fatores de transcrição, incluindo STAT3, AP-1, Sp-1 e Sp-3 estão implicados na regulação da produção de IL-10 por monócitos e células T (NORKINA *et al.*, 2007).

As evidências indicam que a IL-6 exerce importante papel na transição da imunidade inata para a adquirida. Na imunidade inata, atua como estímulo para a produção de neutrófilos e síntese de proteínas de fase aguda. A inflamação aguda é caracterizada por uma infiltração inicial de neutrófilos, que é substituída por monócitos e células T após 24-48 horas, visando prevenir o dano tecidual causado pelo acúmulo de proteases secretadas por neutrófilos e espécies reativas do oxigênio no sítio inflamatório. Células endoteliais ativadas por TNF-α e IL-1β produzem IL-6 e várias quimiocinas, atraindo neutrófilos na fase inicial da inflamação. Além de seu papel quimioatrativo para monócitos, a IL-6 auxilia na diferenciação de monócitos para macrófagos. As células T helper, após ativação pelas células apresentadoras de antígeno, podem se diferenciar em Th1, Th2, Th17 e células T reguladoras. A IL-6 tem sido apontada como uma citocina capaz de favorecer a diferenciação da célula T em Th2 e Th17 (SCHELLER *et al.*, 2011). Ainda na imunidade adaptativa, IL-6 atua na diferenciação de linfócitos B em plasmócitos (YOON *et al.*, 2009).

Durante a inflamação, há níveis elevados de TNF-α e IL-1, os quais também atuam como pirógenos endógenos. A IL-6 está envolvida não somente na resposta imunológica, apresentando ações pró- e anti-inflamatórias, como também em processos regenerativos, na regulação do metabolismo, na manutenção óssea e funções neurais (SCHELLER *et al*., 2011).

Os ativadores de transcrição induzem também a expressão de genes envolvidos na resposta imune do organismo, como quimiocinas, moléculas de histocompatibilidade (MHC), moléculas co-estimulatórias importantes para ativação das células T, como CD80 (B7-1) que se liga ao CD28, e CD40, a qual atua como uma molécula transmembrânica sinalizadora, levando à ativação de fatores de transcrição, regulando um amplo espectro de processos moleculares e celulares, incluindo o início e progressão das respostas adaptativas celular e humoral (MEDZHITOV, 2001; HAN *et al*., 2003).

Com base nos dados expostos e na falta de informações sobre a associação da própolis a produtos odontológicos, este trabalho foi idealizado com o intuito de responder inúmeras questões, visto que há inúmeros micro-organismos na cavidade bucal. Assim, aventamos a hipótese de que a própolis, associada a uma menor concentração de clorexidina, poderia exercer um possível efeito sinérgico contra *S. mutans* e sobre a atividade bactericida de monócitos humanos. Para tal, foi avaliada a qualidade do produto elaborado à base de própolis/clorexidina em relação à sua estabilidade e possível contaminação de acordo com o tempo de prateleira. Outro aspecto investigado foi a ação antimicrobiana dos compostos diretamente sobre *S. mutans*, determinando a concentração inibitória mínima sobre este micro-organismo. Considerando que basicamente não há dados na literatura sobre o efeito da própolis e da associação própolis/clorexidina em células humanas, avaliamos sua possível toxicidade sobre monócitos humanos *in vitro*. O efeito modulador deste apiterápico e de sua associação com a clorexidina foi avaliado quanto à expressão de receptores celulares por citometria de fluxo, a via de sinalização do NF-kB, produção de citocinas pró- e anti-inflamatórias (TNF-α, IL-10 e IL-6) por monócitos, e atividade bactericida destas células sobre *Streptococcus mutans* – agente causador da cárie.

Esta tese encontra-se apresentada sob a forma de dois capítulos a serem submetidos à publicação. O Capítulo 1, relacionado à caracterização do produto odontológico e sua ação antibacteriana, será submetido à publicação junto a revista Clinical Oral Investigations, sendo intitulado: "*Characterization of a propolis-based odontological product: microbiological control and antibacterial action*".

Os dados obtidos sobre o efeito do produto em células humanas estão apresentados no Capítulo 2, intitulado **"***Immunomodulatory effects of a propolis-based odontological product on human monocytes*", a ser submetido à publicação junto a International Immunopharmacology.

Referências

2. Referências Bibliográficas*

ALMEIDA, R. V. D.; CASTRO, R. D.; PEREIRA, M. S. V.; PAULO, M. Q.; SANTOS, J. P.; PADILHA, W. W. N. Efeito clínico de solução anti-séptica a base de própolis em crianças cárie ativas. **Pesquisa Brasileira em Odontopediatria e Clínica Integrada** v. 6, p. 87-92, 2006.

AUFFRAY, C.; SIEWEKE, M. H.; GEISSMANN, F. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. **Annual Review of Immunology**. v. 27, p. 669-92, 2009.

BANKOVA, V. S. Recent trends and important developments in propolis research. **Evidence-based Complementary and Alternative Medicine**. v. 2, p. 29-32, 2005.

BANKOVA, V. S.; POPOV, S. S.; MAREKOV, N. L. On the chemical composition of some propolis fractions with antiviral action. **Acta Microbioliologica Bulgarica**. v. 23, p. 52-7, 1998.

BANKOVA, V. S.; BOUDOUROVA-KRASTEVA, G.; SFORCIN, J. M.; FRETE, X.; KUJUMGIEV, A.; MAIMONI-RODELLA, R.; POPOV, S. S. Phytochemical evidence for the plant origin of Brazilian propolis from São Paulo state. **Zeitschrift für Naturforschung**. v. 54, p. 401-5, 1999.

BARBIERI, D. S. V.; VICENTE, V. A.; FRAIZ, F. C.; LAVORANTI, O. J.; SVIDZINSKI, T. I. E.; PINHEIRO, R. L. Analysis of the *in vitro a*dherence of *Streptococcus mutans* and *Candida albicans.* **Brazilian Journal of Microbiology** v. 38, p. 624-31, 2007.

BOUDOUROVA-KRASTEVA, G.; BANKOVA, V. S.; SFORCIN, J. M.; NIKOLOVA, N.; POPOV, S. Phenolics from Brazilian propolis. **Zeitchrift fur Naturforschung**. v. 52c, p. 676-9, 1997.

BURDOCK, G. A. Review of the biological properties and toxicity of bee propolis (propolis). **Food and Chemical Toxicology**. v. 36, p. 347-63, 1998.

---

* UNIVERSIDADE ESTADUAL PAULISTA. Coordenadoria Geral de Bibliotecas, Editora UNESP. Normas para publicações da UNESP. São Paulo: Editora UNESP, 1994, v.2: Referências bibliográficas.

BIOSIS. Serial Sources for the BIOSIS' previews data base. Philadelphia, 1991. 451p.

CASTRO, M. L.; CURY, J. A.; ROSALEN, P. L.; ALENCAR, S. M.; IKEGAKI, M.; DUARTE, S.; KOO, H. Própolis do sudeste e nordeste do Brasil: influência da sazonalidade na atividade antibacteriana e composição fenólica. **Quimica Nova,** v. 30, p. 1512-1516, 2007.

CHANG, F.; LEE, J. T.; NAVOLANIC, P. M.; STEELMAN, L. S.; SHELTON, J. G.; BLALOCK, W. L. Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplasic transformation: a target for cancer chemotherapy. **Leukemia**, v. 17, p. 590-603, 2003.

CUNHA, I. B. S.; SAWAYA, A. C. H. F.; CAETANO, F. M.; SHIMIZU, M. T.; MARCUCCI, M. C.; DREZZA, G. S. P.; CARVALHO, P. O. Factors that influence the yield and composition of Brazilian propolis extracts. **Journal of the Brazilian Chemical Society.** v. 15, p. 964-70, 2004.

DE CASTRO, S. L. Propolis: biological and pharmacological activities. Therapeutic uses of this bee-product. **Annual Review of Biomedical Science**. v. 3, p. 49-83, 2001.

DJALDETTI, M.; SALMAN, H.; BERGMAN, M.; DJALDETTI, R.; BESSLER, H. Phagocytosis – the mighty weapon of the silent warriors. **Microscopy Research and Technique**, v. 57, p. 421–31, 2002.

DOLCET, X.; LIOBET, D.; PALLARES, J.; MATIAS-GUIU, X. NF-kβ in development and progress of human cancer. **Virchows Archiv**, v. 5, p. 475-82, 2005.

DRUMOND, M. R. S.; CASTRO, R. D.; ALMEIDA, R. V. D.; PEREIRA, M. S. V.; PADILHA, W. W. N. Estudo Comparativo in vitro da atividade antibacteriana de produtos fitoterápicos sobre bactérias cariogênicas**. Pesquisa Brasileira em Odontopediatria e Clínica Integrada,** v. 4, p. 33-38, 2004.

DUAILIBE, S. A.; GONÇALVES, A. G.; AHID, F. J. Effect of a propolis extract on Streptococcus mutans counts in vivo. **Journal of Applied Oral Science,** v. 15, p. 420-23, 2007.

FREEMAN, S. A.; GRISTEIN, S. Phagocytosis: receptors, signal integration, and the cytoskeleton. **Immunological Reviews**, v. 262, p. 193-215, 2014.

HAN, D. C.; HUANG, G .T. H.; LIN, L. M.; WARNER, N. A.; GIM, J. S.; JEWETT, A. Expression of MHC class II, CD70, CD80, CD86 and pro-inflammatory cytokines is differentially regulated in oral epithelial cells following bacterial challenge. **Oral Microbiology Immunology,** v. 18, p. 350-8, 2003.

HEHLGANS, T.; PFEFFER, K. The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games. **Immunology,** v. 115, p. 1-20, 2005.

HUNGNESS, E. S.; PRITTS, T. A.; LUO, G. J.; SUN, X.; PENNER, C. G.; HASSELGREN, P. O. The transcription factor activator protein-1 is activated and interleukin-6 production is increased in interleukin-1beta-stimulated human enterocytes. **Shock,** v. 14, p. 386-91, 2000.

LIBÉRIO, S. A.; PEREIRA, A. L.; ARAÚJO, M. J.; DUTRA, R. P.; NASCIMENTO, F. R.; MONTEIRO-NETO, V.; RIBEIRO, M. N.; GONÇALVES, A. G.; GUERRA, R. N. The potential use of propolis as a cariostatic agent and its actions on mutans group streptococci. **Journal of Ethnopharmacology.** v. 125, p. 1-9, 2009.

MANI, F.; DAMASCENO, H. C. R.; NOVELLI, E. L. B.; MARTINS, E. A.; SFORCIN, J. M. Propolis: effect of different concentrations, extracts and intake period on seric biochemical variables. **Journal of Ethnopharmacology.** v. 105, p. 95-8, 2006.

MANI, F.; DAMASCENO, H. C. R.; NOVELLI, E. L. B.; SFORCIN, J. M. Biochemical determinations of propolis-treated rats: effects of different concentrations, extracts and intake period. **Biosaúde.** v. 10, p. 3-16, 2008.

MEALEY, B. L.; OCAMPO, G. L. Diabetes mellitus and periodontal disease. **Periodontology 2000,** v. 44, p. 127–153, 2007.

MEDZHITOV, R. Review: Toll-like receptors and innate immunity. **Nature Reviews Immunology**, v. 1, p. 135-145, 2001.

MURAD, J. M.; CALVI, S. A.; SOARES, A. M. V. C.; BANKOVA, V.; SFORCIN, J. M. Effects of propolis from Brazil and Bulgaria on fungicidal activity of macrophages against *Paracoccidioides brasiliensis*. **Journal of Ethnopharmacology**, v. 79, p. 331-334, 2002.

NORKINA, O.; DOLGANIUC, A.; SHAPIRO, T.; KODYS, K.; MANDREKAR, P.; SZABO, G. Acute alcohol activates STAT3, AP-1, and Sp-1 transcription factors via the family of Src kinases to promote IL-10 production in human monocytes. **Journal of Leukocyte Biology**, v. 82, p. 752-762, 2007.

ORSATTI, C. L.; MISSIMA, F.; PAGLIARONE, A. C.; BACHIEGA, T. F.; BÚFALO, M. C.; ARAÚJO JR., J. P.; SFORCIN, J. M. Propolis immunomodulatory action *in vivo* on Toll-like receptors 2 and 4 expression and on pro-inflammatory cytokines production in mice. **Phytotherapy Research**, v. 24, p. 1141-1146, 2010.

ORSI, R. O.; FUNARI, S. R. C.; SOARES, A. M. V. C.; CALVI, S. A.; OLIVEIRA, S. L.; SFORCIN, J. M.; BANKOVA, V. S. Immunomodulatory action of propolis on macrophage activation. **Journal of Venomous Animals and Toxins**, v. 6, p. 205-219, 2000.

ORSI, R. O.; SFORCIN, J. M.; FUNARI, S. R. C.; BANKOVA, V. Effects of Brazilian and Bulgarian propolis on bactericidal activity of macrophages against *Salmonella* Typhimurium. **International Immunopharmacology**, v. 5, p. 359-368, 2005.

PEREIRA, J. V.; PEREIRA, M. S. V.; SAMPAIO, F. C.; SAMPAIO, M. C. C.; ALVES, P. M.; ARAÚJO, C. F. R.; HIGINO, J. S. Efeito antibacteriano e antiaderente *in vitro* do extrato da *Punica granatum* Linn. sobre microrganismos do biofilme dental. **Revista Brasileira de Farmacognosia** v. 16, p. 88-93, 2006.

SHANMUGAM, K. T.; MASTHAN, K. M. K.; BALACHANDER, N.; SUDHA, J.; SARANGARAJAN, R. Dental Caries Vaccine – A Possible Option? **Journal of Clinical and Diagnostic Research.** v. 7, p. 1250-1253, 2013.

SALATINO, A.; TEIXEIRA, E. W.; NEGRI, G.; MESSAGE, D. Origin and chemical variation of Brazilian propolis. **Evidence-based Complementary and Alternative Medicine.** v. 2, p. 33-8, 2005.

SCHELLER, J.; CHALARIS, A.; SCHMIDT-ARRAS, D.; ROSE-JOHN, S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. **Biochimica et Biophysica Acta**, v. 1813, p. 878-888, 2011.

SFORCIN, J. M.; FUNARI, S. R. C.; NOVELLI, E. L. B. Serum biochemical determinations of propolis-treated rats. **The Journal of Venomous Animals and Toxins**. v. 1, p. 31-7, 1995.

SFORCIN, J. M.; FERNANDES JR., A.; LOPES, C. A. M.; BANKOVA, V. S.; FUNARI, S. R. C. Seasonal effect on Brazilian propolis antibacterial activity. **Journal of Ethnopharmacology**, v. 73, p. 243-9, 2000.

SFORCIN, J. M.; KANENO, R.; FUNARI, S. R. C. Absence of seasonal effect on the immunomodulatory action of Brazilian propolis on natural killer activity. **Journal of Venomous Animals and Toxins.** v. 8, p. 19-29, 2002.

SFORCIN, J. M. Propolis and the immune system: a review. **Journal of Ethnopharmacology**. v. 113, p. 1-14, 2007.

SILVA, M. S. A.; SILVA, M. A. R.; HIGINO, J. S.; PEREIRA, M. S. V.; CARVALHO, A. A. T. Atividade antimicrobiana e antiaderente *in vitro* do extrato de *Rosmarinus offi cinalis* Linn. sobre bactérias orais planctônicas. **Revista Brasileira de Farmacognosia,** v. 18, p. 236-240, 2008.

TAKEDA, K.; AKIRA, S. Toll-like receptors in innate immunity. **International Immunology**, v. 17, p. 1-14, 2005.

YOON, S. B.; LEE, Y. J.; PARK, S. K.; KIM, H. C.; BAE, H.; KIM, H. M.; KO, S. G.; CHOI, Y.; OH, M. S.; PARK, W. Anti-inflammatory effects of *Scutellaria baicalensis* water extrat on LPS- activated RAW 264.7 macrophages. **Journal of Ethnopharmacology**, v. 125, p. 286-290, 2009.

WILTROUT, R. H.; VARESIO, L. Activation of macrophages for cytotoxic and suppressor effector functions. **In: OPPENHEINN, D. Immunophysiology**, p. 365-385, 1991.

Capítulo 1

Characterization of a propolis-based odontological product: microbiological control and antibacterial action

Karina Basso Santiago, Bruno José Conti, Eliza de Oliveira Cardoso, Gilce Maria Piana, Bruna Fernanda Murbach Teles Andrade, Mirella Rossitto Zanutto,Vera Mores Rall, Ary Fernandes Júnior, José Maurício Sforcin*

Department of Microbiology and Immunology, Biosciences Institute, UNESP, 18618-970, Botucatu, SP, Brazil.

* Corresponding author. Tel.: +55 14 38800445; fax: +55 14 38153744.
E-mail address: sforcin@ibb.unesp.br (J.M. Sforcin).

ABSTRACT

Dental caries is an infectious disease characterized by biofilm formation, demineralization and destruction of the tooth. *Streptococcus mutans* is the main bacterial species involved in the formation of the dental biofilm. Chlorhexidine (CHX), an antimicrobial agent used against cariogenic microbiota, has been used for dental caries prevention; however, side-effects restrict its use. Propolis is a resinous and balsamic product, collected by bees from vegetative sources. It is known for its significant biological properties and has been found to act as an antimicrobial agent in the control of bacterial plaque. Here, we investigated a propolis-based odontological product. Its organoleptic properties (color, smell, flavor and precipitates), microbial contamination (presence of thermotolerant coliforms, mesophilic bacteria, mold and yeast, *Salmonella, Shigella, Staphylococcus aureus*, and *Pseudomonas aeruginosa*) were evaluated, and its antibacterial action against *S. mutans* was determined by the minimum inhibitory and bactericidal concentrations. The odontological product was yellow, cloudy, free of microbial contamination and had a pleasant smell and taste of propolis. Both propolis and CHX exerted an inhibitory action on the growth of *S. mutans in vitro* in lower concentrations than that found in the product. No synergistic or antagonistic effects were seen when these antimicrobial agents were combined. The propolis-based odontological product itself exerted a bactericidal activity at all concentrations assayed in this study. This may be of interest for the pharmaceutical industry.

Keywords:
Propolis
Chlorhexidine
Odontological product
Antibacterial action

Introduction

Dental caries is an infectious disease characterized by demineralization and destruction of the tooth. Demineralization of tooth structure is caused by the organic acids produced in the dental plaque biofilm by the metabolic action of the cariogenic microorganisms on fermentable carbohydrates. Dental caries is a multifactorial disease caused by susceptible teeth of the host, cariogenic agents, and factors such as diet with a high intake of carbohydrates and precarious oral hygiene (Shanmugam *et al.*, 2013).

The dental biofilm is a biological process associated with the attachment and proliferation of oral bacteria to a protein pellicle in the tooth surface. The main bacteria that participate in the formation of the dental biofilm are *Streptococcus mutans* (the primary agent of this disease)*, Streptococcus mitis, Streptococcus sanguis, Streptococcus sobrinus* and *Lactobacillus casei.* The interaction between the bacterial adhesins and lectin in the dental pellicle favor the binding of *S. mutans* to the tooth. After adhesion, the secretion of glucosyl transferases helps in the accumulation of more *S. mutans* and other bacteria (Franca *et al.*, 2014).

The inhibition and removing the plaque biofilm is important to control and prevent dental caries and mechanical methods of reduction and removal of pathogenic flora are commonly used; however, a variety of antibiotics and antiseptics have been introduced as adjvants to the mechanical therapy (Greenstein, 2005).

Chlorhexidine (CHX), an antimicrobial agent against the cariogenic microbiota, has been used for dental caries prevention. CHX is a broad-spectrum antiseptic and antimicrobial agent that has been widely used on the skin and in the oral cavity. Clinically, CHX is the most extensively agent in mouth rinses and is typically used at concentrations of 0.12 and 0.2% (Ribeiro *et al.*, 2004). However, several studies have reported that CHX may cause side-effects such as staining the teeth, altered taste sensation, soreness and dryness of the oral

tissues, desquamative lesions, and ulcerations of the gingival mucosa (Franca *et al.*, 2014). Irrespective of these side-effects, CHX cannot be used over extended periods of time, owing to its genotoxicity and cytotoxicity (Li *et al.*, 2014). CHX is problematic, thus, new mouthwash agents are needed. Recent studies have shown that the antimicrobial activity of different natural products such as propolis can be used for the prophylactic and therapeutic control of dental caries (Libério *et al.*, 2009).

Propolis is a complex mixture of resinous and balsamic substances collected by bees from different parts of plants such as flower buds, shoots and exudates. Bees also add salivary secretions, wax and pollen, and some variation in its color, texture and consistency are expected. It is used by bees as an antiseptic material as well as to embalm natural enemies that died inside the beehive, avoiding decomposition.

Propolis displays an inhibitory action against various strains of Gram positive bacteria and a limited action against Gram-negative ones (Sforcin *et al.*, 2000). The efficacy of propolis as an oral antimicrobial agent was assessed *in vitro* against *S. mutans* (Libério *et al.*, 2009). Positive results were also obtained against *S. mutans* with the use of a mouthwash containing propolis (Duailibe *et al.*, 2007). Anticarie effects of propolis were shown to involve two different mechanisms: a direct activity against *Streptococcus mutans* and inhibition of glucosyl transferase activity, leading to inhibition of bacterial adhesion (Hayacibara *et al.*, 2005). Propolis has attracted attention of researchers from the dental field, in order to reduce inflammation resulting from surgical procedures and as an antimicrobial agent in controlling plaque biofilm, without side effects that compromise health.

We prepared a propolis-based odontological product and tested in humans (Piana, 2013). This product exhibited comparable antimicrobial activity to CHX and we have consequently deposited a patent (BR 10 2015 003982-4). Here, we assessed the organoleptic properties of this product regarding its color, smell, flavor and precipitates. We also assayed for microbial contamination, assessing for the presence of thermotolerant coliforms, mesophilic bacteria, mold and yeast, *Salmonella, Shigella, Staphylococcus aureus,* and *Pseudomonas aeruginosa* after storing the product for 120 days. The antibacterial action of this product against *S. mutans in vitro* was evaluated by determining its minimum inhibitory concentration and minimum bactericidal concentration.

Materials and methods

Propolis sample and chlorhexidine

Propolis was collected in the Beekeeping Section, UNESP, *Campus* of Botucatu. 30% ethanolic extracts of propolis were prepared (30 g of propolis, completing the volume to 100 mL with 70% ethanol), in the absence of bright light, at room temperature, with moderate shaking. After a week, extracts were filtered and the dry weight of the extracts was calculated (130 mg/mL) (Sforcin *et al*., 2005).

Chlorhexidine 0.12% (Swerts *et al.,* 2005) was purchased from a local pharmacy in Botucatu, SP, Brazil.

Preparation of a propolis-based odontological product and its organoleptic properties

Different concentrations of propolis (0.4, 1.3, 2.6 and 6.5%, in a final volume of 7.5 mL) and CHX (7.5 mL, only at 0.12%) totaling 15 mL were combined and put in an amber glass bottle (Table 1). Organoleptic tests were performed to check the color, smell, flavor and pellets of the manufactured product within 120 days (Piana, 2013).

Table 1. Concentration (% v/v) of the combinations propolis/chlorhexidine used for organoleptic analysis.

Combination	Propolis (%)	Chlorhexidine (%)
A	0.4	0.12
B	1.3	0.12
C	2.6	0.12
D	6.5	0.12

Microbiological control of the product

Thermotolerant coliforms, mesophilic bacteria, mold and yeast, as well as the presence of *Shigella*, *Staphylococcus aureus*, *Salmonella* and *Pseudomonas aeruginosa* were investigated according to a Brazilian Resolution (RDC n° 24/11) of the National Agency of Sanitary Surveillance (ANVISA) with regard to the quality control of specific drugs, searching and identifying microbial contaminants.

The microbiological quality of the product was assessed using 2.6% propolis and 0.12% CHX (combination C), since such combination showed acceptable sensorial properties in the organoleptic analysis. The microbiological control of propolis and CHX alone was investigated as well.

The various preparations of propolis and CHX were assayed at 0, 10, 20, 30, 60, 90 e 120 days after preparation for a possible microbial contamination. For such analyses, 10 mL of the samples were added into 90 mL of sterile peptone buffered water and homogenized during 2 minutes. From the initial dilution (10^{-1}), decimal dilutions (10^{-2} and 10^{-3}) were prepared in saline.

Most probable number (MPN) of thermotolerant coliforms

One milliliter of each solution was transferred to a test tube containing an inverted Durham tube and containing 10 mL of lauryl sulfate broth (Merck). The following tests were performed in triplicate. This was incubated at 35ºC for 24–48 h. In positive tubes, a gas production was observed in the Durham tube and three loopfuls of each positive tube were transferred to tubes with an inverted Durham tube containing 10 mL of EC (Merck), which were incubated at 45ºC for up to 48 h. After incubation, the presence of gas was observed in the inverted Durham tube and a MPN table was used to calculate thermotolerants coliforms/mL (Kornacki & Johnson., 2001).

Mesophilic bacteria count

The pour plate method was used to count the bacteria. One milliliter of each solution was dispensed in a dish and mixed with 15 ml of plate count agar (PCA). Colony-forming units (CFU) were counted after incubation at 35ºC for 24 h. In order to calculate the final concentration, the number of CFU was multiplied by the inverse dilution factor of the respective plate (Morton, 2001).

Mold and yeast count

The surface spread technique was used to count mold and yeast. Briefly, 0.1 mL of each of the various propolis/CHX solutions was deposited in the surface of potato dextrose

agar (Merck). The pH was adjusted to 3.5 by adding a sterile 10% tartaric acid solution (Sigma). After incubation at room temperature for 7 days, cell counts were performed by detecting 15–150 CFU. The result was corrected by the dilution and expressed as CFU/mL (Beuchat & Cousin, 2001).

Presence of Salmonella

For Salmonella detection, 10 mL of the product was homogenized in 90 mL of peptone buffered water, transferred to an erlenmeyer flask and incubated at 35ºC for 24 h. After incubation, 1 mL was added to a tube containing 10 mL of tetrathionate broth (Merck) and incubated at 35ºC, and Rappaport broth (Merck) was incubated with 0.1mL from the pre-enrichment at 42ºC for 24h. Afterwards, a loopful of each selective broth was plated onto Petri dishes containing xylose lysine desoxycholate agar (XLD-Merck) and *Salmonella–Shigella* agar (SS, Merck), and plates were incubated for 24 h at 37ºC. The subsequent procedures were not performed since no CFU was observed in previous steps (Andrews *et al.*, 2001).

Presence of Shigella

For *Shigella* detection, 10 mL of the sample was homogenized in 90 ml of selenite broth (Merck) in an erlenmeyer flask and incubated at 35ºC for 20 h. After this period, a loopful of this broth was plated onto Petri dishes containing XLD agar (Merck), MacConkey agar (Merck) and *Salmonella–Shigella* agar (SS, Merck) and plates were incubated for 48 h at 35ºC. The subsequent procedures were not performed since no CFU was observed in previous steps (Lampel, 2001).

Detection of Staphylococcus aureus

For *S. aureus* detection, 0.1 mL of each solution was plated on Baird Parker agar (Oxoid) with 5% egg yolk tellurite emulsion (Oxoid) and incubated at 35 °C for 48 h. The subsequent procedures were not performed since no CFU was observed in previous steps (Lancette & Bennett, 2001).

Presence of Pseudomonas aeruginosa

The surface spread technique was used to detect *P. aeruginosa*. Briefly, 0.1 mL of each solution was deposited on the surface of Cetrimide agar and incubated at 35ºC for 48 h. The subsequent procedures were not performed since no CFU was observed in previous steps (Murray, 2007).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Streptococcus mutans strains were isolated from patients of the Bauru School of Dentistry, USP, and bacterial strains were previously cultured (37ºC/24 h) in brain-heart infusion (BHI) broth. After, bacteria were standardized at 0.5 MacFarland scale in sterile saline solution. The sensitivity assays were performed by broth microdilution to determine the MIC values.

Initially, various concentrations of propolis (20-10000 µg/mL) and CHX (1-600 µg/mL) totaling 100 µL were incubated alone in 96-wells microplates containing BHI plus Tween 80 (0.5%). A control of propolis solvent (70% ethanol) was also included, in the same concentrations found in propolis (0.011–5.5%). To determine the MIC of the combinations propolis/CHX, the concentrations were used according to Table 2.

When the abovementioned plate was prepared, an inoculum (100 µL) of standardized bacterial suspensions containing approximately 1.5 x 10^6 CFU/mL was included in each test. The microplates were incubated (37ºC / 24 h) and the results were recorded after adding the indicator dye redox resazurin 0.01% (50 µL). Bacterial growth was indicated by color change from violet to pink and MIC was defined as the lowest concentration of the agents that inhibited the growth of microorganisms as indicated by resazurin with no color change (Osaka & Hefty, 2013).

Table 2. Concentration of propolis and chlorhexidine (μg/mL) used to analyse the minimum inhibitory concentration of the combinations.

Combination	Propolis	Chlorhexidine
1	1.325,00	2,50
2	663,00	2,50
3	663,00	1,25
4	1.325,00	1,25
5	1.325,00	0,83
6	663,00	0,83
7	2.650,00	5,00
8	2.650,00	2,50
9	2.650,00	1,25
10	2.000,00	5,00
11	2.000,00	2,50
12	2.000,00	1,25
13	1.800,00	5,00
14	1.800,00	2,50
15	1.800,00	1,25

To determine the minimum bactericidal concentration (MBC), an aliquot of each well containing the bacteria incubated with the MIC of the variables or with concentrations higher than the MIC was spread on BHI agar medium and incubated for 24 h / 37ºC. MBC was defined as the lowest concentration of the agents that resulted in no visible growth on the agar, confirming the absence of viable bacteria.

For the identification of a synergistic or antagonistic effect in the combinations, a Fractional Inhibitory Concentration Index (FICI) was calculated by determining the fractional inhibitory concentration (FIC) of each combination of the variables, defined by the relationship between the MIC of the substance used in combination and the MIC of the same substance alone. FICI is the sum of FICs (Hemaiswarya *et al.*, 2008; Palaniappan & Holley, 2010; Chung *et al.,* 2011). A classification for interaction between the variables was based on the index: FICI ≤ 0.5 indicated a synergistic effect; $0.5 < \text{FICI} < 4$ meant no effect and FICI ≥ 4 suggested an antagonistic effect (Odds, 2003).

Results

Organoleptic properties of the propolis-based odontological product

Ten subjects rated the odor and taste of the combinations, within 120 days of preparation. All combinations exhibited a pleasant odor of propolis. As to the flavor, the subjects reported that the combination A was tasteless, combinations B and C tasted only propolis and combination D had a bitter taste (Table 1).

Regarding the color and precipitate formation, the combination A was slightly cloudy with no precipitates (Figure 1A). The combinations B and C showed the same characteristics (yellow/cloudy) and few precipitates (Figures 1B and 1C, respectively). On the other hand, the combination D was cloudy with a strong precipitate formation (Figure 1D). Thus, the combination C was used throughout the experiments.

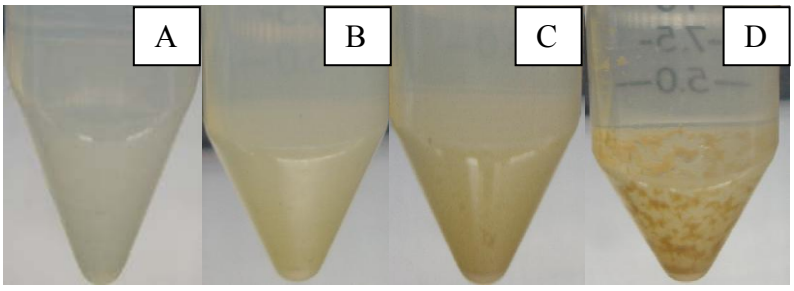


Figure 1. Color and precipitate formation of the combinations A, B, C and D after 120 days.

Microbiological control of the product

The presence of mesophilic bacteria, thermotolerant coliforms, *Staphylococcus aureus, Salmonella, Shigella, Pseudomonas aeruginosa,* molds and yeasts was not detected in the propolis-based odontological product or in its precursors alone, during all the experimental period (Table 3).

Table 3. Microbiological control of propolis, chlorhexidine and their combination after 0, 10, 20, 30, 60, 90 and 120 days.

Microorganism	Propolis	Chlorhexidine	Combination
Mesophilic bacteria	< 10 CFU/mL	< 10 CFU/mL	< 10 CFU/mL
Thermotolerant coliforms	< 3 MPN /mL	< 3 MPN/mL	< 3 MPN/mL
Staphylococcus aureus	< 100 CFU/mL	< 100 CFU/mL	< 100 CFU/mL
Salmonella spp	Absence/10 mL	Absence/10 mL	Absence/10 mL
Shigella spp	Absence/10 mL	Absence/10 mL	Absence/10 mL
Pseudomonas aeruginosa	< 100 CFU/mL	< 100 CFU/mL	< 100 CFU/mL
Mold and yeast	< 10 CFU/mL	< 10 CFU/mL	< 10 CFU/mL

CFU: Colony-forming units; MPN: most probable number.

Antibacterial action by determining the MIC and MBC

Bacterial growth was inhibited by 2650.0 µg/mL of propolis and 5.0 µg/ml of CHX *in vitro*. Regarding 70% ethanol (propolis solvent), it only showed an antibacterial action from 5.0% - a percentage equivalent to 9.000 µg/ml of propolis, what indicates no effect of the solvent in propolis action. Similar results were obtained for MIC and MBC for all variables, combined or not (Table 4).

Table 4. Minimum inhibitory concentration and minimum bactericidal concentration of propolis, chlorhexidine and 70% ethanol against *Streptococcus mutans*.

Propolis	Chlorhexidine	70% ethanol
2650.0 µg/mL	5.0 µg/mL	5.0%

As to the combinations propolis/CHX, only the numbers 7 to 10 and 13 (Table 2) exerted antibacterial effects. To verify a synergistic effect, indifference or antagonistic effect, the Fractional Inhibitory Concentration Index (FICI) of these combinations was calculated (Table 5). The combinations displayed an antibacterial action, without any synergistic or antagonistic effect, and presented a similar action to that exerted by propolis and CHX alone.

Table 5. Minimum inhibitory concentration of the combination propolis/chlorhexidine (μg/mL) against *Streptococcus mutans* and its respective FICI values.

Combination	Propolis + Chlorhexidine	FICI
7	2650.0 + 5.0	2.00 (I)
8	2650.0 + 2.5	1.50 (I)
9	2650.0 + 1.25	1.25 (I)
10	2000.0 + 5.0	1.75 (I)
13	1800.0 + 5.0	1.68 (I)

(I) = indifferent regarding antagonistic or synergistic effects.

After determing the MIC of propolis in combination or not with CHX *in vitro*, the antibacterial action of the propolis-based odontological product used in humans by our group (Piana, 2013) and assayed for microbial contamination was also determined by diluting serially propolis and CHX. As expected, all concentrations exerted a bactericidal action (data not shown).

Discussion

Microbial contamination of non-sterile pharmaceuticals has always been a major concern of manufacturers and regulatory agencies (Moniruzzaman *et al.*, 2012). The production of non-sterile pharmaceutical products is not fully aseptic and the presence of microbial contaminants is not uncommon. The incidence of microbial contaminants in pharmaceutical products is generally influenced by the origin of the ingredients (whether natural or synthetic) (Gamal *et al.*, 2011).

The presence of microbial contaminants in oral pharmaceuticals may lead to changes in their physical characteristics such as phase separation in emulsions, thinning of creams, presence of pellets and altered odor and color. The presence of microorganisms in pharmaceutical products affects their therapeutic activity as well and can be harmful to the patient health (Gamal *et al.*, 2011; Moniruzzaman *et al.*, 2012).

According to a Brazilian Resolution (RDC of ANVISA, n° 24/11), medications containing propolis alone or combined with other products are considered specific drugs, with prophylactic, curative or palliative applications. Thus, our trials were conducted according to this resolution and the storage time of the product was assessed, aiming to verify if it

remained viable for use concerning its organoleptic properties (odor, flavor, color and precipitate formation). One may verify that all combinations were acceptable in the organoleptic analysis and no change was found during the study. Moreover, there were no microbial contaminants and the combination C was used in the assays, which was previously used in humans by a dentist of our group in order to assess the control of dental plaque. The combination C was also tested in the microbiological control of the product.

In 1998, the World Health Organization (WHO) established procedures for quality control standards of herbal drugs and their products. In 2005, this guide was suitable for the detection and quantification of certain contaminants and residues in herbal preparations (WHO, 2005).

In Europe and in the United States, the acceptance criteria for microbiological quality were established in the European Pharmacopoeia (EP) and in the United States Pharmacopeia (USP), respectively. According to these pharmacopeias, tests shall be conducted using both raw materials and finished products and involve the microbial count of total aerobic microbial counts (TAMC) and total yeast and mold counts (TYMC), and tests for detecting *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Salmonella* spp (Wu *et al.*, 2014; Vu *et al.*, 2014). In Brazil, according to RDC n° 24/11, the microbiological control should research thermotolerant coliforms, mesophilic bacteria, mold and yeast, as well as the presence of *Shigella*, *Staphylococcus aureus*, *Salmonella* and *Pseudomonas aeruginosa*.

The microbiological control of natural products, particularly those from vegetable origin, should take into account that such products are in contact with environmental contaminants (Rocha *et al.*, 2004). Thus, natural products may be a risk, being necessary to identify appropriate measures to control their quality and ensure their safety since the collection until the final product. The high presence of fungi is a risk to the healty, since they may produce mycotoxins such as aflatoxin, which can be carcinogenic and mutagenic. They can also cause acute and chronic poisoning, allergies, diseases of the respiratory and digestive systems, and liver damage (Wu *et al.*, 2014; Gamal *et al.*, 2011). Fungi belonging to the genera *Penicillium*, *Aspergillus*, *Rhizopus*, *Mucor*, *Cladosporium* and *Aureobasidium* are often found associated with drugs of plant origin (Kneifel *et al.,* 2002).

Based in the microbiological control mentioned above, our propolis-based odontological product exerted a satisfactory performance according to the World Health Organization, which recommends that the presence of TAMC may be up to 5.0×10^7

CFU/mL in natural products used in teas and infusions, and up to 5.0 x 10^5 CFU/mL for internal use. Furthermore, the recommended value for TYMC is up to 5.0 x 10^4 CFU/mL for natural products used in the form of teas and infusions, and a maximum 5.0 x 10^3 CFU/mL for internal use. Our propolis-based odontological product also is in agreement with USP, which recommended a maximum of 10^2 TAMC, 10^1 TYMC and absence of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *E.coli* for non-sterile oral pharmaceuticals. Thus, our product meets national and international requirements, and may be used in the dental field.

Regarding the antibacterial action, both propolis and CHX exerted and inhibitory action alone *in vitro*. Moreover, no effect of the solvent (70% ethanol) was seen in propolis action. In order to verify a possible synergistic action between propolis and CHX on the growth of *S. mutans*, susceptibility tests were performed to determine the MIC for propolis in combination or not with CHX. Combinations 7-10 and 13 showed an antibacterial action, but no synergistic or antagonistic effects were observed according to the FICI, since the combinations displayed similar effects to those exerted by either propolis or CHX alone.

Our data reinforces previous findings reporting that propolis exhibits an efficient inhibitory activity against Gram-positive bacteria (SFORCIN *et al.,* 2000). Some authors attribute the antibacterial action of propolis to the presence of caffeic acid phenethyl ester (CAPE) and pinocembrin (Velázquez *et al.*, 2007; Chaillou & Nazareno, 2009). It has been also reported that some components present in propolis extracts such as flavonoids, cinnamic acid and caffeic acid may lead to structural and functional damage to the membrane or bacterial wall (Scazzocchio *et al.*, 2006). The MIC of a Tunisian propolis against oral streptococci ranged from 2-64 μg/mL, and was 8-32 μg/mL specifically for *S. mutans* (Kouidhi *et al.*, 2010).

Varnishes are preparations with different polymeric matrices, therapeutic agents (generally fluoride and CHX), and pharmaceutical additives used in dentistry to prevent caries. De Luca *et al.* (2014) assessed the MIC and MBC for a varnish containing propolis and a chitosan polymeric base, and reported values of 0.6-1.2 mg/mL against *S. mutans*. Here, the MIC of our propolis sample was 2.65 mg/mL. One should notice that the determination of MIC depends on the bacterial strain (inherent virulence and susceptibility), the methods employed and the solubility of the natural products in the medium, among other factors. Such details can vary significantly and different outcomes may be found (Dziedzic *et al.*., 2013).

After determining the MIC and MBC using a wide range of propolis and CHX concentrations lower than the ones found in the propolis-based odontological product, the

product itself was also evaluated. As expected, it exerted a bactericidal activity at all concentrations, because CHX concentrations were higher than the previously determined MIC. These findings have practical implications. The CHX concentration in most commercial mouth washes is 0.12% (1200 μg/mL). Our product contains 600 μg/mL of CHX and the combination showed an inhibitory activity against *S. mutans* even when diluted 64-fold to final CHX concentration as low as 9.5 μg/mL.

Taken together, data suggested that our odontological product was yellow, cloudy, free of microbial contamination and had a pleasant smell and taste of propolis. Propolis and CHX alone or in combination exerted an inhibitory action on the growth of *S. mutans in vitro*. No synergistic or antagonistic effects were seen. On the other hand, the propolis-based odontological product itself exerted a bactericidal activity at all concentrations, and this may be of interest for the pharmaceutical industry.

Acknowledgements

This work was supported by the State of São Paulo Research Foundation (FAPESP – 2012/14614-8 and 2011/22112-0). We are grateful to André Luis da Silva, Bauru School of Dentistry, USP, Brazil, for donating the *Streptococcus mutans* strains. We are also grateful to Dr. Anthony James Farlow for assisting with the preparation of the manuscript and English review.

The authors declare that no financial conflict of interest exists.

References

Andrews WH, Flowers RS, Silliker J, Bailey JS. *Salmonella*. In: Downes FP, Ito K, editors. Compendium of methods for the microbiological examination of foods. APHA Washington 2001; 357–80.

Beuchat LR, Cousin MA. Yeasts and molds. In: Downes FP, Ito K, editors. Compendium of methods for the microbiological examination of foods. APHA Washington 2001; 209–15.

Brasil. Resolução da Diretoria Colegiada - RDC no. 24/11, de 14 de junho de 2011. Regulamento Técnico do Registro dos Medicamentos Específicos. Diário Oficial da União, 7 jun 2011.

Chaillou L, Nazareno M. Chemical variability in propolis from Santiago del Estero, Argentina, related to the arboreal environment as the sources of resins. J Sci Food Agric 2009; 88: 978-83.

Chung PY, Navaratnam P, Chung LY. Synergistic antimicrobial activity between pentacyclic triterpenoids and antibiotics against S*taphylococcus aureus* strains. Ann Clin Microbiol Antimicrob 2011; 10: 1-6.

De Luca MP, Franca JR, Macedo FAFF, Grenho L, Cortes ME, Faraco AAG, Moreira AN, Santos VR. Propolis Varnish: Antimicrobial properties against cariogenic bacteria, cytotoxicity, and sustained-release profile BioMed Res Int 2014; 2014: 1-6.

Duailibe SA, Gonçalves AG, Ahid FJ. Effect of a propolis extract on Streptococcus mutans counts in vivo. J. Appl. Oral Sci 2007; 15: 420-3.

Dziedzic A, Kubina R, Wojtyczka RD, KabaBa-Dzik A, Tanasiewicz M, Morawiec T. The antibacterial effect of ethanol extract of Polish propolis on mutans streptococci and lactobacilli isolated from saliva. Evid. Based Complement. Alternat. Med. 2013; 2013: 1-12.

Franca JR, De Luca MP, Ribeiro TG, Castilho RO, Moreira AN, Santos VR and Faraco AAG Propolis - based chitosan varnish: drug delivery, controlled release and antimicrobial activity against oral pathogen bacteria. BMC Complement Altern Med 2014; 14: 1-11.

Gamal FMG, Reham AIA, Mohamed SEA. Microbial evaluation of some non-sterile pharmaceutical preparations commonly used in the egyptian market. Trop J Pharm Res 2011; 10: 437-45.

Greenstein G. Position paper: The role of supra-and subgingival irrigation in the treatment of periodontal diseases. J Periodontol 2005; 76: 2015–27.

Hayacibara MF, Koo H, Rosalen PL, Duarte S, Franco EM, Bowen WH, Ikegaki M, Cury JA. In vitro and in vivo effects of isolated fractions of Brazilian propolis on caries development. J Ethnopharmacol 2005; 101:110–5.

Hemaiswarya S, Kruthiventi AK, Doble M. Synergism between natural products and antibiotics against infectious diseases. Phytomedicine, 2008; 15: 639-52.

Kneifel W, Czech E, Kopp B. Microbial contamination of medicinal plants. A review. Planta Med 2002; 68: 5-15.

Kornacki JL, Johnson JL. Enterobacteriaceae, coliforms and *Escherichia coli* as quality and safety indicators. In: Downes FP, Ito K, editors. Compendium of methods for the microbiological examination of foods. APHA Washington 2001; 69–82.

Kouidhi B, Zmantar T, Bakhrouf A. Anti-cariogenic and anti-biofilms activity of Tunisian propolis extract and its potential protective effect against cancer cells proliferation. Anaerobe 2010; 16: 566-71.

Lampel KA. *Shigella*. In: Downes FP, Ito K, editors. Compendium of methods for the microbiological examination of foods. APHA Washington 2001; 381–5.

Lancette GA, Bennett RW. *Staphylococcus aureus* and Staphylococcal enterotoxins In: Downes FP, Ito K, editors. Compendium of methods for the microbiological examination of foods. APHA Washington 2001; 387–403.

Li YC, Kuan YH, Lee SS, Huang FM, Chang YC. Cytotoxicity and Genotoxicity of Chlorhexidine on Macrophages in vitro. Environ Toxicol 2014; 29: 452–8.

Libério SA, Pereira AL, Araújo MJ, Dutra RP, Nascimento FR, Monteiro-Neto V, Ribeiro MN, Gonçalves AG, Guerra RN. The potential use of propolis as a cariostatic agent and its actions on mutans group streptococci. J Ethnopharmacol 2009; 125: 1-9.

Moniruzzaman M, Ashrafi FF, Mia Z. Qualitative and quantitative microbiological studies of antacid and paracetamol suspensions from different drugstores of Dhaka Dhaka Univ. J. Biol. Sci. 2012; 21: 105-7.

Morton R.D. Aerobic plate count. In: Downes FP, Ito K, editors. Compendium of methods for the microbiological examination of foods. APHA Washington 2001; 63–67.

Murray PR, Baron EJ, Jorgensen JH, Landry ML, Pfaller MA. Manual of Clinical Microbiology. 9th edition, ASM Press, Washington, 2007; 1267.

Odds FC Synergy, antagonism, and what the chequerboard puts between them. J. Antimicrob Chemother 2003; 52: 1.

Osaka I, Hefty P.S. Simple resazurin-based microplate assay for measuring Chlamydia infections. AAC 2013; 57: 2838-40.

Palaniappan K, Holley, RA. Use of natural antimicrobials to increase antibiotic susceptibility of drug resistant bacteria. Int J Food Microbiol 2010; 140: 164-8.

Piana GM. Ação sinérgica da própolis e da clorexidina no controle da placa bacteriana bucal. 2013. 74p. Dissertação (Mestrado) – Programas de Pós-graduação em Biologia Geral e Aplicada, Instituto de Biociências-Unesp, 2013.

Ribeiro DA, Bazo AP, Silva CAF, Marques MEA, Salvadori DMF. Chlorhexidine induces DNA damage in rat peripheral leukocytes and oral mucosal cells. J Periodont Res 2004; 39: 358–61.

Rocha LO, Soares MMSR, Corrêa CL. Análise da contaminação fúngica em amostras de *Cassia acutifolia Delile* (sene) e *Peumus boldus* (Molina) *Lyons* (boldo-do-Chile) comercializadas na cidade de Campinas. Braz J Pharm Sci 2004; 40: 521-7.

Scazzocchio F, D'Auria FD, Alessandrini D, Pantanella F. Multifactorial aspects of antimicrobial activity of propolis. Microbiol Re 2006; 161: 327-33.

Sforcin JM, Fernandes-Junior A, Lopes CAM, Bankova VS, Funari SRC. Seasonal effect on Brazilian propolis antibacterial activity. J Ethnopharmacol 2000; 73: 243-9.

Sforcin JM, Orsi RO, Bankova V. Effect of propolis, some isolated compounds and its source plant on antibody production. J Ethnopharmacol 2005; 98: 301-5.

Shanmugam KT, Masthan KMK, Balachander N, Sudha J, Sarangarajan R. Dental Caries Vaccine – A Possible Option? JCDR 2013; 7: 1250-3.

Swerts MSO, Dias Costa AMD, Fiorini JE. Efeito da solução associada de clorexidina e própolis na inibição da aderência de *Streptococcus spp*. Rev Int Periodontia Clín 2005; 2: 10-16.

Velázquez C, Navarro M, Acosta A, Angulo A, Domínguez Z, Robles R, Robles-Zepeda R, Lugo E, Goycoolea FM, Velázquez EF, Astiazaran H, Hernández J. Antibacterial and free-radical scavenging activities of Sonoran propolis. J Appl Microbiol 2007; 103: 1747-56.

Vu N, Lou JR, Kupiec TC. Quality control analytical methods: Microbial Limit tests for nonsterile pharmaceuticals, Part 1 Int J Pharm Compd 2014; 18: 213-21.

WHO – World Health Organization. Quality control methods for medicinal plant materials. Revised Draft Update, 2005 - http://www.who.int/medicines/services/expertcommittees/pharmprep/QAS05_131Rev1_QCMethods_Med_PlantMaterialsUpdateSept0

Wu F, Groopman JD, Pestka JJ. Public health impacts of foodborne mycotoxins. Rev. Food Sci. Technol 2014; 5: 351–72.

Capítulo 2

Immunomodulatory effects of a propolis-based odontological product on human monocytes

Karina Basso Santiago [a], Bruno José Conti [a], Eliza de Oliveira Cardoso [a], Marjorie de Assis Golim [b], José Maurício Sforcin [a,*]

[a] *Department of Microbiology and Immunology, Biosciences Institute, UNESP, 18618-970, Botucatu, SP, Brazil.*
[b] *Laboratory of Flow Cytometry, Blood Center, School of Medicine, UNESP, 18618-970, Botucatu, SP, Brazil.*

* Corresponding author. Tel.: +55 14 38800445; fax: +55 14 38153744.
E-mail address: sforcin@ibb.unesp.br (J.M. Sforcin).

ABSTRACT

Periodontal diseases are characterized by chronic inflammation associated with the presence of specific bacteria in the periodontal pocket, which results in the destruction of soft tissue and the reabsorption of alveolar bone. Chlorhexidine is a antimicrobial agent and has been widely used as an antiseptic, but side-effects restrict its use. Propolis is a resinous and balsamic product, collected by bees from some parts of plants. It is known for its significant biological and pharmacological properties, attracting the attention of researchers from the odontological field. It has been found to reduce inflammation resulting from surgical procedures and as an antimicrobial agent in the control of bacterial plaque. Thus far, no side-effects that might compromise oral health have been observed. In this project, we aimed to investigate the effects of an odontological product containing propolis in combination with chlorhexidine in lower concentrations on human monocytes, evaluating cell markers expression, the NF-kB signaling pathway, the production of pro- and anti-inflammatory cytokines, and the bactericidal activity of these cells against *Streptococcus mutans.* Together, the data showed that the combination of propolis and chlorhexidine may favor the recognition of antigens by monocytes, activate the NF-kB signaling pathway and increase the bactericidal activity of human monocytes against *S. mutans*. Also, the combination played a role in anti-inflammatory cytokine production, which can be beneficial in the treatment of periodontal

diseases. These results may have implications for the development of odontological products with immunomodulatory action, and may have further reaching implications for the pharmaceutical industry.

Keywords:
Monocytes
Cytokine
Propolis
Chlorhexidine
Odontological product

1. Introduction

Propolis (P) is a resinous and balsamic product, collected and prepared by bees from some parts of plants as shoots, branches, bark, resinous exudates and flower buds, to which the bees add salivary secretions, wax and pollen [1]. The color of propolis may vary between yellow, red, brown, to green, depending on its botanical origin [2]. Its chemical composition is extremely complex, varying according to geographical location and the local flora. The main sources of propolis in the apiary of the University (UNESP, Campus of Botucatu) are *Baccharis dracunculifolia* DC, *Araucaria angustifolia* (Bert.) O. Kuntze and *Eucalyptus citriodora* Hook [3]. The composition of our propolis sample was analyzed, revealing that its main groups are: phenolics (flavonoids, aromatic acids, benzopyranes), di- and triterpenes, essential oils, among others [4].

There are numerous studies investigating the biological properties of propolis, especially their immunomodulatory, antimicrobial, anti-inflammatory, anti-tumor and anticariogenic actions [4,5]. Moreover, the absence of side-effects after short or long-term propolis administration to rats has been reported [6-8]. Propolis has attracted the attention of researchers from the odontological area, in order to reduce inflammation resulting from surgical procedures and as an antimicrobial agent in the control of bacterial plaque, showing no side-effects that could compromise the oral health.

Chlorhexidine (CHX) is a cationic antimicrobial agent and has been widely used as an antiseptic, being active against Gram-positive and Gram-negative bacteria, aerobic and facultative anaerobic, molds, yeast and viruses. Its action occurs by adsorption to the cell wall

of microorganisms, causing leakage of intracellular components, leading to cell death [9]. However, its use as an antimicrobial agent for oral use is still controversial due to the occurrence of side-effects, such as pigmentation of teeth, tongue, restorations and prostheses, desquamation and injury to the oral mucosa, and taste disorders. These side-effects restrict its pronloged use. Thus, the search for new products with significant antimicrobial activity and fewer side effects has increased, aiming the preventive or therapeutic control of bacteria that cause periodontal diseases [10].

Periodontal diseases are characterized by chronic inflammation associated with the presence of specific bacteria in the periodontal pocket, which results in the destruction of soft tissue and the reabsorption of alveolar bone. The local inflammatory immune response is crucial for protecting periodontal tissues from various pathogens. Gingival epithelial cells play an important role in the innate immune response by producing antimicrobial peptides and chemokines that recruit neutrophils [11]. Monocytes from the gum bleeding come to the inflammatory site as well. Antigen-presenting cells (APCs) remove invading microorganisms such as *Streptococcus mutans* that cause caries by engulfing, processing and presenting them to T cells. Antigen-specific T-cell activation by T-cell receptor/MHC class II engagement requires a costimulatory signal which is induced by the binding between CD28 on T cells and B7-1 (CD80) and B7-2 (CD86) costimulatory molecules expressed by APCs, which produce inflammatory mediators that regulate the adaptive immune response [12].

Toll-like receptors (TLRs) may bind to products or conserved microbial structures, called pathogen-associated molecular patterns (PAMPs), which are absent in eukaryotic cells but may be present in both pathogenic and non-pathogenic microorganisms [13]. After recognition of microorganisms, several transcription factors are activated, such as NF-kB, leading to the gene expression of TNF-α, IL-6 and IL-10. Individually or in combination, cytokines interact with their specific receptors, modulating cellular function. Cytokines such as TNF-α are important in the activation of monocytes and macrophages process, whereas other cytokines, such as IL-10, are considered inactivating cell factors. IL-6 exerts both pro- and anti-inflammatory effects, and acts in regenerative processes, in regulating metabolism, maintaining bone and neural function [14].

Recently, our group produced a propolis-based odontological product whose patent was deposited in February 2015 (BR 10 2015 003982-4). This product was tested *in vivo* in humans, and here we wish to present its immunomodulatory effects *in vitro* on human monocytes. The effects of propolis in combination with lower concentrations of chlorhexidine

were assessed regarding the expression of cell markers (TLR-4, HLA-DR, CD80, CD86), NF-kB signaling pathway, pro- and anti-inflammatory cytokine (TNF-α, IL-10, IL-6) production by human monocyte and its bactericidal activity against *Streptococcus mutans* – one of the causative agents of dental caries.

2. Materials and methods

2.1. Propolis, chlorhexidine and the combination propolis/chlorhexidine

Propolis was collected in the Beekeeping Section, UNESP, Campus of Botucatu. Propolis was ground and 30% ethanolic extracts of propolis were prepared (30 g of propolis, completing the volume to 100 ml with 70% ethanol), in the absence of bright light, at room temperature, with moderate shaking. After a week, extracts were filtered and the dry weight of the extracts was calculated (130 mg/ml) [15]. Propolis was diluted in RPMI 1640 culture medium (Cultilab, Brazil) containing L-glutamine (0.1 g/l), sodium bicarbonate (2.2 g/l), nonessential amino acids (10 ml/l) and supplemented with 10% fetal calf serum (complete medium). Specific dilutions were prepared for each assay in order to achieve different propolis concentrations (P1: 0.2, P2: 1.0, P3: 2.0, P4: 5.0, P5: 10.0 and P6: 20.0 μg/ml). The same procedure was carried out with 70% ethanol (propolis solvent).

Chlorhexidine 0.12% [16] was purchased from a local pharmacy, and was diluted in RPMI 1640 culture medium to obtain CHX1: 1.2, CHX2: 6.0, CHX3: 12.0, CHX4: 30.0, CHX5: 60.0 and CHX6: 120.0 μg/ml.

For the combination propolis/chlorhexidine (P/CHX), the concentrations were P/CHX1: 0.2/1.2, P/CHX2: 1.0/6.0, P/CHX3: 2.0/12.0, P/CHX4: 5.0/30.0, P/CHX5: 10.0/60.0 and P/CHX6: 20.0/120.0 μg/ml.

2.2. Healthy blood donors and isolation of human monocytes

Ten healthy blood donors (aging 20–40 years) from the University Hospital, School of Medicine, UNESP, were included in the present work, which was approved by the Ethics Committee of the School of Medicine (CEP 4077-2011). An informed consent was signed by all blood donors. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized (50 U/ml heparin) venous blood using Ficoll-Paque (density = 1.077) (GE Healthcare Bio-

Sciences, Sweden). Briefly, 20 ml of heparinized blood were added to an equal volume of complete medium. Samples were added to 4 ml of Ficoll-Paque and centrifuged at 400 g for 30 min at room temperature. The interface layer of the PBMC was taken and washed three times with RPMI medium at 300 g for 10 min. Cell viability, as determined by neutral red (0.02%) staining, was > 95% in all experiments. Cells were resuspended at a final concentration of 1 x 10^6 monocytes/ml in complete medium. After 2 h, non-adherent cells were discarded, and monocytes were incubated with the stimuli for 18 h.

2.3. Cell viability by MTT assay

Cell viability was performed using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT – Sigma-Aldrich, USA) colorimetric assay [17]. Monocytes (1 x 10^6 cells/ml) were incubated with different concentrations of propolis, chlorhexidine and the combination P/CHX at 37 ºC and 5% CO_2, in a final volume of 500 µl. Control cells were incubated only with culture medium. After 18 h, culture medium was removed and 300 µl of MTT (1 mg/ml) in complete RPMI were added to the culture cells for 3 h. Afterwards, MTT was taken and 200 µl of dimethylsulfoxide (DMSO) was added to dissolve the formazan salt. Optical densities (O. D.) were read at 540 nm in an ELISA reader and the percentage of cell viability was calculated using the formula: [O. D. test / O. D. control] x 100. Assays were carried out in duplicate.

2.4. Flow-cytometry analysis of TLR-4, HLA-DR, CD86 and CD80 expression by monocytes

The expression of TLR-4, HLA-DR, CD86 and CD80 by human monocytes was assessed using a FACSCalibur flow cytometer and Cell Quest software (Becton Dickinson, USA). PBMC containing 5 x 10^5 cells/500 µl were incubated for 18 h at 37 ºC and 5% CO_2 with complete medium in the presence or absence of different concentrations of propolis, chlorhexidine and the combination P/CHX into polystyrene tubes for cytometric analysis (BD Labware, USA). Cells were washed and incubated with monoclonal antibodies (MAbs), according to the manufacturer's instructions: 0.25 µg/500 µl of Phycoerythrin-CY7 (PE/Cy7)-labeled anti-CD14 (clone M5E2), 0.50 µg/500 µl of Phycoerythrin (PE)-labeled anti-TLR4 (clone HTA125) and 1 µg/500 µl of ALEXA Fluor 488-labeled anti-CD86 (clone IT2.2). In other tubes, PBMC were incubated with 0.25 µg/500 µl of PE/Cy7-labeled anti-CD14, 0.25

µg/500 µl of FITC-labeled anti-HLA-DR (clone L243) and 0.50 µg/500 µl PE-labeled anti-CD80 (clone 2D10). Antibodies were purchased from Biolegend (USA).

Stained cells were incubated for 30 min in the dark at 4 ºC, then washed and fixed with 5% paraformaldehyde in PBS. Background staining was determined from cells incubated with 1 µg/500 µl of PE/Cy7, FITC and PE-labeled control isotype antibodies. Cell samples were washed with complete medium, then analysed by flow cytometry. Thirty thousand monocytes events, defined as cells with respective side scatter (SSC) and CD14 staining characteristics and corresponding levels of fluorescence for TLR-4, HLA-DR, CD86 and CD80, were obtained for the gated $CD14^+$ cell. Results were expressed as percentage of positive CD14 cells of gated events [18].

2.5. NF-kB signaling pathway

NF-kB signaling pathway was assessed in human monocytes according to the manufacturer's instructions (Cayman Chemical Company, USA). Monocytes (1 x 10^6 cells/ml) were distributed into 24-well flat-bottomed plates and incubated with different concentrations of propolis, chlorhexidine, combination P/CHX, lipopolysaccharide (LPS, 10 µg/ml) and medium alone as control for 45 min at 37 ºC and 5% CO_2 [19]. Afterwards, monocytes were placed in tubes (1.5 ml) in the presence of hypotonic buffer, which allow to disrupt the cell and nuclear membranes. The nuclear portion of NF-kB was detected by ELISA.

2.6. Bactericidal activity of human monocytes

Monocytes (2 x 10^5 cells/ml) were treated with different concentrations of propolis, chlorhexidine and combination P/CHX for 18 h at 37 ºC and 5% CO_2. After incubation, stimuli were removed and cells were challenged with 500 µl of a *Streptococcus mutans* suspension (ATCC 25175) in complete medium containing 1 x 10^6 bacteria/ml (monocytes/ bacteria ratio = 1:5) at 37 ºC in 5% CO_2. Control cultures contained only bacteria without monocytes. After 2 h, supernatants were collected and Triton X-100 (1%) was used to lyse monocytes, resulting in a final volume of 10 ml. Then, 10 µl was plated on brain-heart infusion (BHI) agar medium (Difco Laboratories, USA) in triplicate, and plates were

incubated at 37 ºC. The number of colony-forming units (CFU) per plate was counted after 48 h and the percentage (%) of bactericidal activity was determined by the formula:

% bactericidal activity = [1 - (mean CFU on experimental cultures / mean CFU on control plates)] x 100

2.7. Cytokine determination by enzyme-linked immunosorbent assay (ELISA)

To evaluate cytokine production, monocytes (1 x 10^6 cells/ml) were distributed into 24-well flat-bottomed plates (TPP Zellkultur und Labortechnologie, Switzerland) and incubated with different concentrations of propolis (0.2, 1.0 and 2.0 µg/ml), chlorhexidine (1.2, 6.0 and 12.0 µg/ml), combination P/CHX (0.2/1.2, 1.0/6.0 2.0/12.0 µg/ml), lipopolysaccharide (LPS, 10 µg/ml) and only culture medium as control at 37 ºC and 5% CO_2. Afterwards, the supernatants were harvested for TNF-α, IL-6 and IL-10 measurement by ELISA, according to the manufacturer's instructions (R&D Systems, USA). Briefly, a 96-well flat bottom Maxisorp (Thermo Scientific Nunc Maxisorp, USA) was coated with capture antibody specific to each cytokine. The plate was washed and blocked before 100 µl of the supernatants and serially diluted specific standards were added to the respective wells. Following a series of washing, the cytokine was detected using the specific conjugated detection antibody. The substrate reagent was added into each well and, after color development, the plate was read at 450 nm, using an ELISA plate reader.

2.8. Statistical analysis

Data were analyzed with Graph Pad statistical software (Graph Pad Software, Inc., San Diego, CA, USA). Analysis of variance (ANOVA) and Dunnett method were employed. Data were expressed as mean ± standard deviation of 10 individuals. A p value of less than 0.05 was considered significant.

3. Results

3.1. Monocyte viability

The effect of propolis, chlorhexidine and P/CHX on human monocytes viability was expressed in comparison to control (non-treated cells). Propolis in all concentrations (0.2, 1.0, 2.0, 5.0 10.0 and 20.0 µg/ml) did not affect cell viability (>98%) (Fig. 1A). 70% ethanol (in

the same concentrations found in propolis) had no cytotoxic effects on monocytes (data not shown).

After incubation with the highest concentrations of chlorhexidine alone (30.0, 60.0 and 120.0 μg/ml) (Fig. 1B) and combined with propolis P/CHX (5.0/30.0, 10.0/60.0 and 20.0/120.0 μg/ml) (Fig. 1C), a significant decrease in monocyte viability ($p < 0.0001$) was observed. Thus, the assays were carried out using only noncytotoxic concentrations.

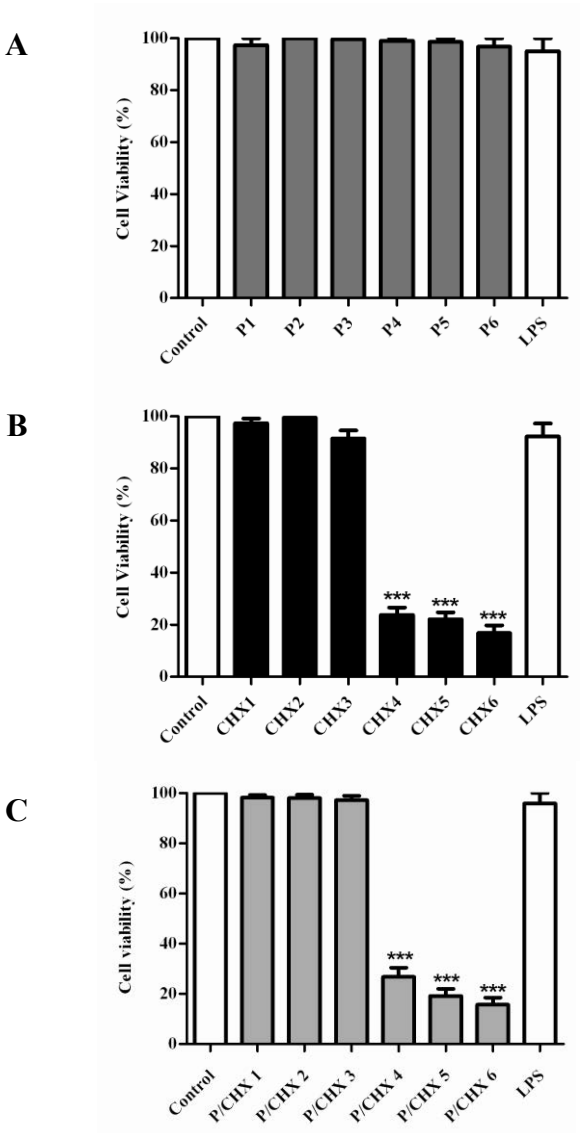


Fig. 1. Cell viability (%) of human monocytes incubated with (A) propolis P1 (0.2), P2 (1.0), P3 (2.0), P4 (5.0), P5 (10.0) and P6 (20.0 μg/ml), (B) chlorhexidine CHX1 (1.2), CHX2 (6.0), CHX3 (12.0), CHX4 (30.0), CHX5 (60.0) and CHX6 (120.0 μg/ml), (C) combination P/CHX1 (0.5/1.2), P/CHX2 (1.0/6.0), P/CHX3 (2.0/12.0), P/CHX4 (5.0/30.0), P/CHX5 (10.0/60.0) and P/CHX6 (20.0/120.0 μg/ml), and lipopolysaccharide LPS (10 μg/ml) for 18 h. ***$p < 0.0001$vs control (mean ± SD; n=10).

3.2. Effect of propolis, chlorhexidine and P/CHX on TLR-4, HLA-DR, CD86 and CD80 expression by monocytes

Propolis, chlorhexidine and P/CHX upregulated TLR-4 expression by human monocytes at all concentrations (Fig. 2A). On the other hand, the treatments had no effect on HLA-DR expression, since it was expressed in high percentage even in control (87.5%) (Fig. 2B).

With regard to the costimulatory molecules, propolis (at all concentrations) upregulated CD86 expression, whereas chlorhexidine and P/CHX had no effect on CD86 levels (Fig. 2C). Unexpectedly, chlorhexidine with or without propolis downregulated CD80 expression by human monocytes ($p < 0.01$), whereas propolis alone had no effects on its expression (Fig. 2D).

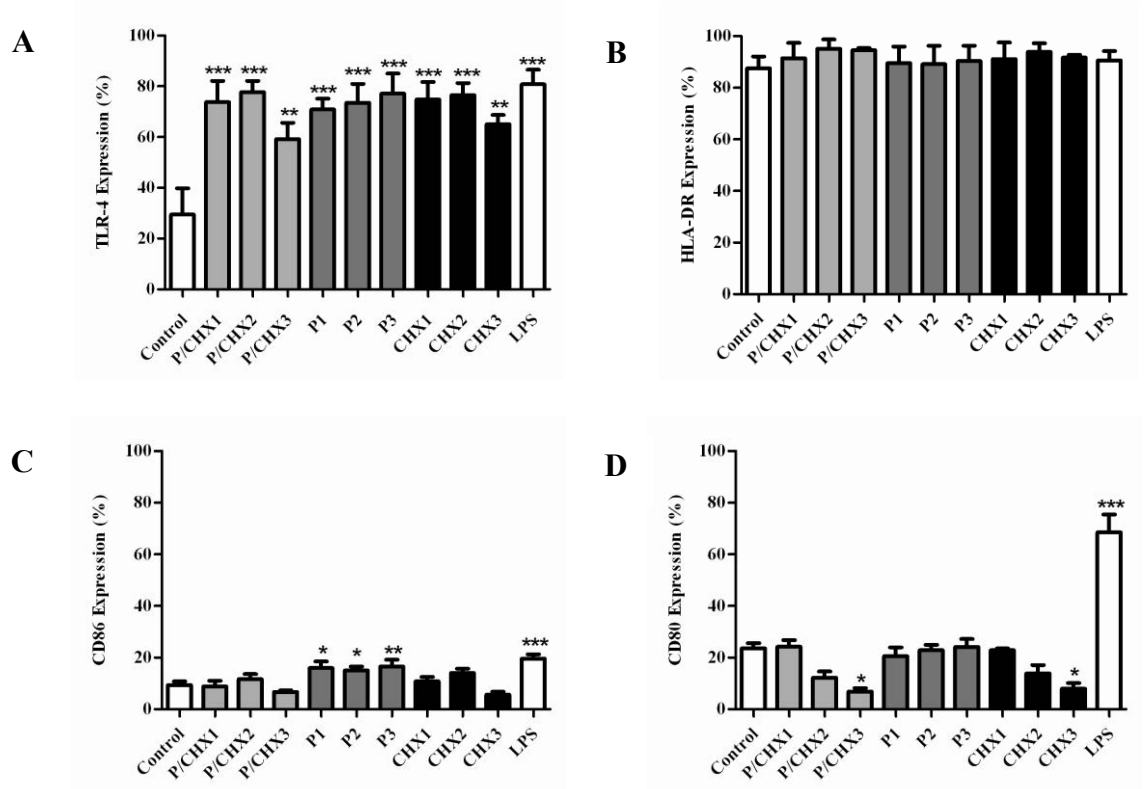


Fig. 2. Percentage (%) of (A) TLR4, (B) HLA-DR, (C) CD86 and (D) CD80 expression of human monocytes incubated with propolis P1 (0.2), P2 (1.0) and P3 (2.0 μg/ml), chlorhexidine CHX1 (1.2), CHX2 (6.0) and CHX3 (12.0 μg/ml) and combination P/CHX1 (0.2/1.2), P/CHX2 (1.0/6.0) and P/CHX3 (2.0/12.0 μg/ml) and lipopolysaccharide LPS (10 μg/ml) for 18 h. *$p < 0.01$; **$p < 0.001$; ***$p < 0.0001$ vs control (mean ± SD; n=10).

3.3. NF-kB signaling pathway

LPS upregulated the NF-kB activation, as expected. Compared to the control (medium alone), the incubation of human monocytes with propolis (0.2 and 2.0 μg/ml), chlorhexidine (6.0 and 12.0 μg/ml) and P/CHX (0.2/1.2 μg/ml) also increased the activation of this transcription factor ($p < 0.01$) (Fig. 3).

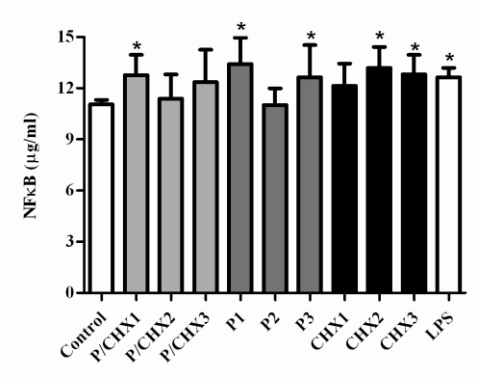


Fig. 3. NFκB quantification (μg/ml) by human monocytes incubated with propolis P1 (0.2), P2 (1.0) and P3 (2.0 μg/ml), chlorhexidine CHX1 (1.2), CHX2 (6.0) and CHX3 (12.0 μg/ml) and combination P/CHX1 (0.2/1.2), P/CHX2 (1.0/6.0) and P/CHX3 (2.0/12.0 μg/ml) and lipopolysaccharide LPS (10 μg/ml) for 18 h. *$p < 0.01$ vs control (mean ± SD; n=10).

3.4. Bactericidal activity

Non-stimulated monocytes exhibited a bactericidal activity (24 %) against *S. mutans*. This activity was increased significantly using propolis, chlorhexidine and the combination P/CHX association at all concentrations (Fig. 4).

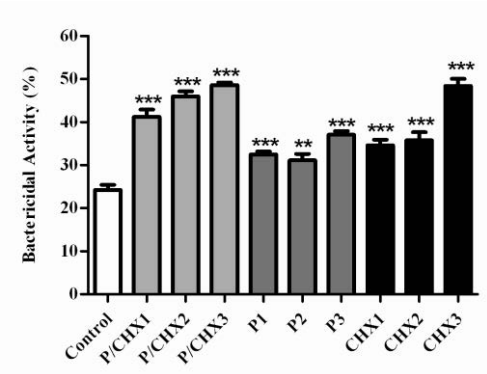


Fig. 4. Percentage (%) of bactericidal activity of human monocytes incubated with propolis P1 (0.2), P2 (1.0) and P3 (2.0 μg/ml), chlorhexidine CHX1 (1.2), CHX2 (6.0) and CHX3 (12.0 μg/ml) and combination P/CHX1 (0.2/1.2), P/CHX2 (1.0/6.0) and P/CHX3 (2.0/12.0 μg/ml) and lipopolysaccharide LPS (10 μg/ml) for 18 h and challenged with *Streptococcus mutans* for 2 h. **$p < 0.001$; ***$p < 0.0001$ vs control (mean ± SD; n=10).

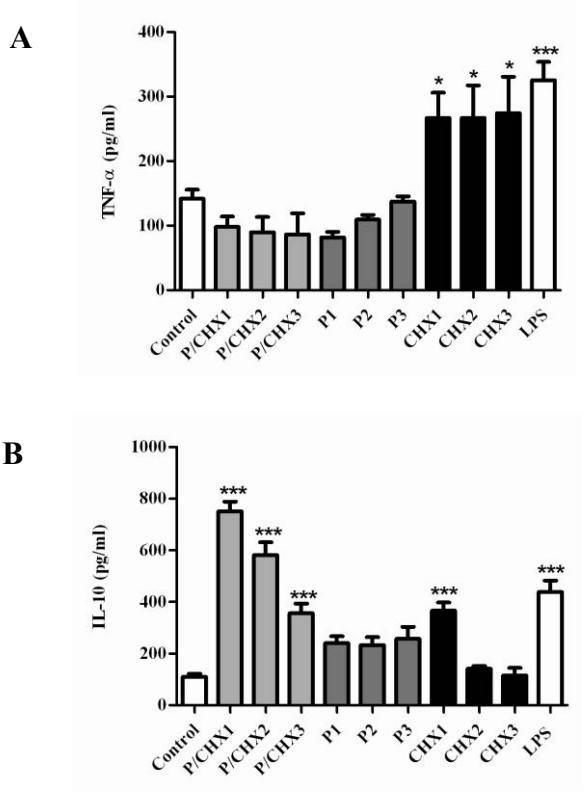
3.5. Cytokine production

Chlorhexidine (at all concentrations) increased significantly TNF-α production comparing to control ($p < 0.01$). Propolis had no effect on TNF-α production; however, its combination with chlorhexidine prevented the increase induced by chlorhexidine (Fig. 5A).

Regarding IL-10 production, although the propolis did not affect the release of this cytokine, an increased IL-10 production was seen after incubation with chlorhexidine alone (1.2 μg/ml) or associated with propolis (0.2/1.2, 1.0/6.0 and 2.0/12.0 μg/ml) compared to control ($p < 0.0001$) (Fig. 5B).

IL-6 levels were not altered after incubation with propolis (Fig. 5C), and its production was similar to control ($p > 0.05$). On the other hand, chlorhexidine associated or not with propolis significantly inhibited the production of IL-6.

LPS (positive control) stimulated all cytokine production by human monocytes ($p < 0.0001$).



C

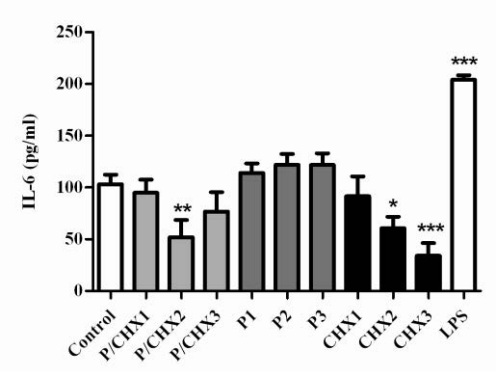


Fig. 5. (A) TNF-α, (B) IL-10 and (C) IL-6 production (pg/ml) by human monocytes incubated with propolis P1 (0.2), P2 (1.0) and P3 (2.0 μg/ml), chlorhexidine CHX1 (1.2), CHX2 (6.0) and CHX3 (12.0 μg/ml), the combination P/CHX1 (0.2/1.2), P/CHX2 (1.0/6.0) and P/CHX3 (2.0/12.0 μg/ml) and lipopolysaccharide LPS (10 μg/ml) for 18 h. *$p < 0.01$; **$p < 0.001$; ***$p < 0.0001$ vs control (mean ± SD; n=10).

4. Discussion

A propolis-based odontological product was investigated concerning its immunomodulatory action. First, propolis alone did not affect cell viability. These findings are in agreement with other works from our group evaluating the effects of propolis and its constituents on the viability of human monocytes [20-22]. Likewise, no cytotoxic effect was seen on peritoneal macrophages from BALB/c and C57BL/6 mice [23-25]. Regarding chlorhexidine effects, its highest concentrations affected cell viability (24%, 22% and 17%) even associated with propolis, leading to 27%, 19% and 16% viability. In an attempt to verify whether the cell viability affected by chlorhexidine concentrations could be due to the presence of distilled water, its effect alone was investigated on monocytes, and the viability remained unaltered (data not shown). Thus, one may conclude that the low viability of monocytes was exclusively due to chlorhexidine.

Several studies have reported that chlorhexidine may be both cytotoxic and genotoxic [26,27]. BONACORSI *et al.* [28] observed a reduced viability of macrophages exposed to chlorhexidine after 1 h (using concentrations higher than 20 μg/ml) and 24 h (higher than 5 μg/ml). LI *et al.* [29] observed a cytotoxic effect on RAW 264.7 cells after 1 h incubation with chlorhexidine 0.001%, with a maximum cytotoxic effect after 2 h, in a time-dose dependent manner. Thus, our experiments were carried out using only noncytotoxic concentrations of the combination P/CHX.

During the triggering of the immune response, there is an increased expression of certain genes such as cytokines, chemokines, histocompatibility molecules, and important co-

stimulatory molecules for T cell activation such as CD80 (B7-1) and CD86 (B7-2) [16]. Here, the combination P/CHX enhanced TLR-4 expression by human monocytes and propolis may be involved in this effect, since previous works from our group reported an increased TLR-4 expression by propolis-stimulated monocytes [21]. Propolis-based product inhibited CD80 expression without affecting CD86 and HLA-DR expression. These results suggested that the combination can facilitate antigen recognition by monocytes, but not the action of costimulatory molecules. A possible explanation could be the fact that the combination P/CHX increased IL-10 production, which inhibits the expression of co-stimulatory molecules and class II histocompatibility molecules [30].

The activation of TLRs can initiate multiple signaling pathways such as the transcription factor NF-kB, which controls the gene expression of several cytokines related to physiological or pathological processes [31]. The combination P/CHX induced the activation of NF-kB signaling pathway, indicating its activator profile, although its quantification was already high in control cells and similar to LPS-induced one. Thus, although a significant increase was seen in treated cells, one may speculate that this increase was biologically discreet.

The bactericidal activity against *S. mutans* increased after incubation of monocytes with the combination P/CHX, although propolis and chlorhexidine alone induced it as well. Recent studies of our group with human monocytes revealed that propolis, as well as the isolates cinnamic acid and caffeic acid, increased the fungicidal activity of monocytes against *Candida albicans* [20-22]. Our data is important, since there is no data in literature investigating the combination P/CHX effects on human monocytes. Although the mechanisms by which our treatments increased the microbicidal activity of monocytes were not investigated, one may speculate that the combination P/CHX might upregulate the production of reactive oxygen species (ROS) by human monocytes. Propolis increased peroxide hydrogen (H_2O_2) production by murine peritoneal macrophages [32], while no effect was seen incubating these cells with chlorhexidine [28]. However, both monocytes and macrophages can also destroy micro-organisms by non-oxidative mechanisms, such as acidification of phagosomes, increased lysosomal content and interaction with the complement system components [33]. Thus, our findings open the possibility of further investigation in this research field, since the presence of propolis could exert a compensatory effect in combination with chlorhexidine most probably inducing ROS generation, what is desirable

since this product increased the bactericidal effect of human monocytes using lower concentrations of chlorhexidine.

Our results showed that the combination P/CHX prevented the increase of TNF-α production induced by chlorhexidine in monocytes, suggesting a possible anti-inflammatory effect. TNF-α is a multifunctional cytokine, inducing other cytokines and chemokines production, the expression of adhesion molecules and the increased permeability of the endothelium, favoring monocytes to arrive into the inflammation site [34].

Although propolis did not affect the release of IL-10, there was an increase in the concentration of this cytokine when it was co-administered with chlorhexidine. IL-10 displays inhibitory effects on activated macrophages and dendritic cells, controlling innate and cell-mediated immunity. IL-10 inhibits the expression of co-stimulatory molecules and class II histocompatibility molecules, promotes the humoral immune response and inhibits cellular immune response, decreasing the cytokines production of Th1 profile, the proliferation of antigen-specific T cells, and the levels of pro-inflammatory cytokines such as TNF-α [30]. Since IL-10 exerts an anti-inflammatory action, our findings suggest that P/CHX may display an anti-inflammatory role, which may be beneficial in the treatment of periodontal diseases.

Propolis did not affect IL-6 production, but chlorhexidine inhibited the production of this cytokine, irrespective of the presence of propolis. IL-6 plays an important role in the transition from innate to acquired immunity. In innate immunity, it acts as a stimulus for neutrophils production and synthesis of acute phase proteins. In addition to its chemoattractant role for monocytes, IL-6 supports the differentiation of monocytes to macrophages. After activation by APCs, T helper cells can differentiate into Th1, Th2, Th17 and regulatory T cells. IL-6 has been identified as a cytokine able to promote the differentiation of T cells in Th2 and Th17 [14]. Moreover, IL-6 acts on the differentiation of B lymphocytes into plasma cells [35]. There has been an increasing number of works that demonstrate the ambiguity of IL-6 to possess both anti-inflammatory and proinflammatory effects. IL-6 is not involved only in inflammatory and infectious processes, but also in the regulation of metabolic and regenerative processes [14].

Taken together, data indicates that the combination P/CHX inhibits TNF-α and IL-6 production and stimulates IL-10 production by human monocytes, suggesting its anti-inflammatory role, reinforcing its beneficial application as an odontological product to be used in periodontal disease.

Correlating NF-kB activation to cytokine production, one may verify that the increased NF-kB activity induced by combination P/CHX was inversely proportional to TNF-α and IL-6 production. NF-kB is a transcription factor classically involved with the pro-inflammatory cytokines production; however, studies with knockout mice demonstrated that NF-kB may also exert an anti-inflammatory action [36]. NF-kB belongs to NFkB/REL transcription factor family, which is composed by five genes (NFkB1, NFkB2, RELA, c-REL and RELB) that originate seven proteins: p100, p105, p50, p52, RELA (known as p65), RELB and c-REL. NF-kB may be a homo or heterodimer and the composition of the dimer is important to regulate this pathway. Only the proteins p65, c-REL and REL-B possess transactivation domains and may activate the gene transcription directly, whereas homodimers with p50 and/or p52 subunits may suppress the transcriptional activity due to the absence of TAD domains, inhibiting the target genes of NF-kB and subsequently the inflammatory response [37]. Thus, these findings suggested that the anti-inflammatory role of NF-kB can occur by inhibition of proinflammatory genes and by interfering with the expression and activity of anti-inflammatory cytokines such as IL-10 [36]. These observations may support our results, and although other transcription factors are involved in IL-10 production, the combination P/CHX increased IL-10 production and decreased TNF-α and IL-6 concentration. One may speculate that NF-kB may be involved in the mechanism of action by which the odontological product modulated the production of these cytokines.

In summary, propolis and chlorhexidine in combination may enhance the recognition of antigens by monocytes, activate the transcription factor NF-kB and increase the bactericidal activity of human monocytes against *S. mutans*. The alterations in cytokine concentrations are accordant with an anti-inflammatory action, and this may be useful to treat periodontal diseases. These findings open new perspectives for further research, what indicates the practical application of our work and its possible use by the pharmaceutical industry.

Acknowledgements

This work was supported by the State of São Paulo Research Foundation (FAPESP – 2012/14614-8 and 2011/22112-0). We are grateful to Dr. Cristiane Duque, School of Dentistry, UNESP, Araçatuba, Brazil, for donating the *Streptococcus mutans* strains. We are

also grateful to Dr. Anthony James Farlow for assisting with the preparation of the manuscript and English review.

The authors declare that no financial conflict of interest exists.

References

[1] Bankova VS. Recent trends and important developments in propolis research. Evid Based Complement Alternat Med 2005; 2: 29-32.

[2] Salatino A, Teixeira EW, Negri G, Message D. Origin and chemical variation of Brazilian propolis. Evid Based Complement Alternat Med 2005; 2: 33-8.

[3] Bankova VS, Boudourova-Krasteva G, Sforcin JM, Frete X, Kujumgiev A, Maimoni-Rodella R, Popov SS. Phytochemical evidence for the plant origin of Brazilian propolis from São Paulo state. Z Naturforsch 1999; 54: 401-5.

[4] Sforcin JM. Propolis and the immune system: a review. J Ethnopharmacol 2007; 113: 1-14.

[5] Búfalo MC, Figueiredo AS, Sousa JPB, Candeias JMG, Bastos JK, Sforcin JM. Anti-poliovirus activity of *Baccharis dracunculifolia* and propolis by cell viability determination and real-time PCR. J Appl Microbiol 2009; 107: 1669-80.

[6] Sforcin JM, Funari SRC, Novelli ELB. Serum biochemical determinations of propolis-treated rats. J venom Anim Toxins 1995; 1: 31-7.

[7] Mani F, Damasceno HCR., Novelli ELB, Martins EA, Sforcin JM. Propolis: effect of different concentrations, extracts and intake period on seric biochemical variables. J Ethnopharmacol 2006; 105: 95-8.

[8] Mani F, Damasceno HCR, Novelli ELB, Sforcin JM. Biochemical determinations of propolis-treated rats: effects of different concentrations, extracts and intake period. Biosaúde 2008; 10: 3-16.

[9] Hernandez EP, Botero TM, Mantellini MG, Mcdonald NJ, Nör JE. Effect of ProRoot® MTA mixed with chlorhexidine on apoptosis and cell cycle of fibroblasts and macrophages in vitro. Int Endod J 2005; 38: 137–143.

[10] Libério SA, Pereira AL, Araújo MJ, Dutra RP, Nascimento FR, Monteiro-Neto V, Ribeiro MN, Gonçalves AG, Guerra RN. The potential use of propolis as a cariostatic agent and its actions on mutans group streptococci. J Ethnopharmacol 2009; 125: 1-9.

[11] Matsuyama T, Kawai T, Izumi Y, Taubman MA. Expression of major histocompatibility complex class II and CD80 by gingival epithelial cells induces activation of CD4 T cells in response to bacterial challenge. Infect Immun 2005; 73: 1044-51.

[12] Konermann A, Deschner J, Allam JP, Novak N, Winter J, Baader SL, Jepsen S, Jäger A. Antigen-presenting cell marker expression and phagocytotic activity in periodontal ligament cells. J Oral Pathol Med 2012; 41: 340–7.

[13] Medzhitov R. Review: Toll-like receptors and innate immunity. Nat Rev Immunol 2001; 1: 135-145.

[14] Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochim Biophys Acta 2011; 1813: 878-888.

[15] Sforcin JM, Orsi RO, Bankova V. Effect of propolis, some isolated compounds and its source plant on antibody production. J Ethnopharmacol 2005; 98: 301-5.

[16] Swerts MSO, Costa AMD, Fiorini JE. Efeito da solução associada de clorexidina e própolis na inibição da aderência de *Streptococcus* spp. Rev Int Periodontia Clin 2005; 2: 10-16.

[17] Najafi MF, Vahedy F, Seyyedin M, Jomehzahed HR, Bozary K. Effect of water extracts of propolis on stimulation and inhibition of different cells. Cytotechnology 2007; 54:49–56.

[18] Nakaira-Takahagi E, Golim MA, Bannwart CF, Puccia R, Peraçoli MT. Interactions between TLR2, TLR4 and mannose receptors with gp43 from Paracoccidioides brasiliensis induce cytokine production by human monocytes. Med Mycol 2011; 49: 694-703.

[19] Wang LC, Lin YL, Liang YC, Yang YH, Lee JH, Yu HH, Wu WM, Chiang BL. The effect of caffeic acid phenethyl ester on the functions of human monocyte-derived dendritic cells. BMC Immunol 2009; 10: 1-13.

[20] Conti BJ, Búfalo MC, Golim MA, Bankova V, Sforcin JM. Cinnamic acid is partially involved in propolis immunomodulatory action on human monocytes. Evid Based Complement Alternat Med, 2013; 2013: 1-7.

[21] Búfalo MC, Graciani APB, Conti BJ, Golim MA, Sforcin JM. The immunomodulatory effect of propolis and caffeic acid on receptors expression, cytokine production and fungicidal activity of human monocytes, J Pharm Pharmacol 2014; 66: 1497-1504.

[22] Búfalo MC, Sforcin JM. The modulatory effects of caffeic acid on human monocytes and its involvement in propolis action. J Pharm Pharmacol, in press.

[23] Pagliarone AC, Missima F, Orsatti CL, Bachiega TF, Sforcin JM. Propolis effects on Th1/Th2 cytokines production by acutely stressed mice. J Ethnopharmacol 2009; 125: 230-233.

[24] Missima F, Pagliarone AC, Orsatti CL, Sforcin JM. The effect of propolis on pro-inflammatory cytokines produced by melanoma-bearing mice submitted to chronic stress. J ApiProd ApiMed Sci 2009; 1: 11-15.

[25] Orsatti CL, Missima F, Pagliarone AC, Bachiega TF, Búfalo MC, Araújo Jr JP, Sforcin JM. Propolis immunomodulatory action in vivo on Toll-like receptors 2 and 4 expression and on pro-inflammatory cytokines production in mice. Phytother Res 2010; 24: 1141-1146.

[26] Grassi TF, Camargo EA, Salvadori DM, Marques ME, Ribeiro DA. DNA damage in multiple organs after exposure to chlorhexidine in Wistar rats. Int J Hyg Environ Health 2007; 210: 163–167.

[27] Lee TH, Hu CC, Lee SS, Chou MY, Chang YC. Cytotoxicity of chlorhexidine on human osteoblastic cells is related to intracellular glutathione levels. Int Endod J 2010; 43: 430–435.

[28] Bonacorsi C, Raddi, MSG, Carlos IZ. Cytotoxicity of chlorhexidine digluconate to murine macrophages and its effect on hydrogen peroxide and nitric oxide induction. Brazilian J Med Biol Res 2004; 37: 207-212.

[29] Li Y, Kuan Y, Lee S, Huang F, Chang Y. Cytotoxicity and genotoxicity of chlorhexidine on macrophages in vitro. Environ Toxicol 2014; 29: 452-58.

[30] Norkina O, Dolganiuc A, Shapiro T, Kodys K, Mandrekar P, Szabo G. Acute alcohol activates STAT3, AP-1, and Sp-1 transcription factors via the family of Src kinases to promote IL-10 production in human monocytes. J Leukoc Biol 2007; 82: 752-762.

[31] Kawai T, Akira S. Signaling to NF-kappaB by Toll-like receptors. Trends Mol Med 2007; 13: 460-469.

[32] Orsi RO, Funari SRC, Soares AMVC, Calvi SA, Oliveira SL, Sforcin JM, Bankova VS. Immunomodulatory action of propolis on macrophage activation. J venom Anim Toxins 2000; 6: 205-219.

[33] Moonis M, Ahmad I, Bochhowot BW. Macrophages in host defense – an overview. Indian J Biochem Biophys 1992; 29: 115-122.

[34] Hehlgans T, Pfeffer K. The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games. Immunology 2005; 115: 1-20.

[35] Yoon SB, Lee YJ, Park SK, Kim HC, Bae H, Kim HM, Ko SG, Choi Y, Oh MS, Park W. Anti-inflammatory effects of *Scutellaria baicalensis* water extrat on LPS- activated RAW 264.7 macrophages. J Ethnopharmacol 2009; 125: 286-290.

[36] Lawrence, T. The nuclear factor NF-κB pathway in inflammation. Cold Spring Harb Perspect Biol 2009; 6: 1-10.

[37] Cruz-Machado, S. S. Lipopolissacarídeo (LPS): ativador e regulador da transcrição gênica via fator de transcrição NFKB. Rev Biol 2010; 4: 40-43.

Conclusões

3. Conclusões

Os resultados obtidos quanto à associação própolis/clorexidina permitiram obter as seguintes conclusões:

- ✓ A associação apresentou características sensoriais aceitáveis, o que pode ser vantajoso, pois reduziria a concentração de clorexidina utilizada e incluiria um produto natural que exerce inúmeras propriedades farmacológicas, sem apresentar efeitos adversos;

- ✓ Não foi detectada a presença de mesófilos, coliformes termotolerantes, *Staphylococcus aureus, Salmonella, Shigella, Pseudomonas aeruginosa,* bolores e leveduras na associação própolis/clorexidina, nem em seus precursores isoladamente, durante todo o período analisado. Ademais, nosso produto está de acordo com as resoluções nacional e internacional para controle de qualidade, estando apto para utilização na área odontológica;

- ✓ As concentrações de própolis e clorexidina utilizadas no enxaguatório bucal utilizado *in vivo* pela cirurgiã dentista apresentaram ação bactericida, e tais resultados têm implicações práticas, pois a quantidade de clorexidina utilizada comercialmente é 0,12% (1.200 µg/mL). Nosso produto elaborado para uso oral contém 600 µg/mL e, ao adicionarmos própolis, mesmo diluindo 1:64 vezes (9,5 µg/mL), a associação ainda apresentou ação inibitória contra *S. mutans* – agente causador da cárie;

- ✓ A própolis não afetou a viabilidade de monócitos humanos; entretanto, as concentrações mais elevadas de clorexidina afetaram a viabilidade celular, mesmo quando associadas à própolis. O efeito citotóxico da clorexidina ocorreu independente da porcentagem de água destilada em sua composição;

- ✓ A associação induziu aumento na expressão de TLR-4, não afetou a expressão de CD86, mas inibiu a de CD80. Não houve efeito dos tratamentos sobre HLA-DR. Estes resultados indicam que a associação pode favorecer o reconhecimento de antígenos por monócitos, mas não a ação das moléculas co-estimulatórias importantes para ativação da célula T;

- ✓ A incubação de monócitos humanos com a associação própolis/clorexidina induziu aumento na atividade do NF-kB, indicando o perfil ativador sobre este fator de transcrição;

- ✓ A atividade bactericida de monócitos humanos contra *Streptococcus mutans* apresentou-se aumentada após incubação com a associação;

- ✓ A associação própolis/clorexidina inibiu a produção de TNF-α e IL-6, além de induzir aumento na concentração de IL-10 por monócitos. Estes achados sugerem que a associação própolis/clorexidina desempenha papel anti-inflamatório, o que pode trazer benefícios no tratamento de doenças periodontais.

Anexos

UNESP UNIVERSIDADE ESTADUAL PAULISTA

CAMPUS DE BOTUCATU

INSTITUTO DE BIOCIÊNCIAS

DEPARTAMENTO DE MICROBIOLOGIA E IMUNOLOGIA

BOTUCATU- SP-CEP-18.618-000 -FONE: (14) 3811-6058 -FAX: (14) 3811-6058236

CONSENTIMENTO LIVRE E ESCLARECIDO PARA PARTICIPAÇÃO EM PESQUISA

DE INDIVÍDUOS SAUDÁVEIS

Título da pesquisa: "ASSOCIAÇÃO DA PROPOLIS E PRODUTOS ODONTOLÓGICOS SOBRE A ATIVIDADE DE MONÓCITOS HUMANOS"

Você está sendo convidado(a) a participar, como voluntário(a), de uma pesquisa que tem como objetivo avaliar células mononucleares do sangue periférico, após incubação com a própolis e clorexidina, para compreender sua ação sobre estas células.

Para poder participar, o Senhor será questionado sobre uso de medicamentos ou drogas, bem como doenças, pois estes critérios impedem a participação. O único e possível desconforto é a retirada do sangue, e após a coleta de 40 mL de sangue, o mesmo será utilizado nos laboratórios da disciplina de Imunologia, onde será desenvolvido o estudo. Para responder quaisquer perguntas ou dúvidas, você poderá entrar em contato com os responsáveis por este trabalho. Para qualquer dúvida adicional, entrar em contato com o Comitê de Ética em Pesquisa, através do fone: (14) 3811-6143.

Informamos que o Sr. possui total liberdade de recusar ou de retirar o seu consentimento de participação neste estudo, sendo que esta ação não causará qualquer transtorno à sua pessoa. Informamos também que todas as informações obtidas neste estudo serão identificadas somente por número, sendo que seu nome não aparecerá quando os resultados do estudo forem apresentados.

Após aprovação do Comitê de Ética em Pesquisa, este documento será elaborado em 2 vias, sendo uma para o participante da pesquisa e outra para o arquivo do pesquisador.

O sujeito da pesquisa foi devidamente informado e esclarecido pelo pesquisador e/ou representante sobre a pesquisa, os procedimentos nela envolvidos, bem como os possíveis riscos decorrentes da participação.

Local e data: _____

Nome e assinatura do sujeito de pesquisa:_____

Prof. Adj. José Maurício Sforcin
Orientador
Depto. Microbiologia e Imunologia
IB – UNESP – Campus de Botucatu
Fone: 14-3811-6058 / sforcin@ibb.unesp.br

Karina Basso Santiago
Responsável pelo Projeto
Depto. Microbiologia e Imunologia
IB – UNESP – Campus de Botucatu
Fone: 14-3811-6058 karinabtu@yahoo.com.br

unesp
Universidade Estadual Paulista
Faculdade de Medicina de Botucatu

Distrito Rubião Junior, s/nº - Botucatu – S.P.
CEP: 18.618-970
Fone/Fax: (0xx14) 3811-6143
e-mail secretaria: capellup@fmb.unesp.br
e-mail coordenadoria: tsarden@fmb.unesp.br

Ética
Comitê de Ética em Pesquisa

Registrado no Ministério da Saúde
em 30 de abril de 1997

Botucatu, 05 de dezembro de 2011. Of. 539/2011

Ilustríssimo Senhor
Prof. Dr. José Maurício Sforcin
Departamento de Microbiologia e Imunologia do
Instituto de Biociências de Botucatu

Prezado Prof. José,

Informo que o Projeto de Pesquisa **(Protocolo CEP 4077-2011)** **Associação da própolis e produtos odontológicos sobre a atividade de monócitos humanos**, a ser conduzido por Karina Basso Santiago, orientada por Vossa Senhoria, com a colaboração de Gilce Maria Piana recebeu do relator parecer favorável, aprovado em reunião de 05/12/2011.

Situação do Projeto: **APROVADO**. Os pesquisadores deverão apresentar ao CEP ao final da execução do Projeto o **"Relatório Final de Atividades"**.

Atenciosamente,

Prof. Dr. Trajano Sardenberg
Coordenador do CEP