



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



**Efeito da própolis *in vivo* sobre a expressão de receptores semelhantes
a Toll (TLR-4 e TLR-2) e produção de citocinas por camundongos
BALB/c**

Cláudio Lera Orsatti

Dissertação apresentada ao Instituto de Biociências, UNESP, Campus de Botucatu, para obtenção do título de Mestre no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração: Biomoléculas – Estrutura e Função.

**BOTUCATU – SP
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*“Se eu vi mais longe, foi por estar
de pé sobre ombros de gigantes.”
(Isaac Newton)*

*“Penso noventa e nove vezes e
nada descubro; deixo de pensar,
mergulho em profundo silêncio -
e eis que a verdade se me revela.”
(Albert Einstein)*

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Resumo

A própolis tem sido motivo de intensa investigação científica e sua ação imunomoduladora vem sendo mencionada. A recente descoberta e caracterização da família dos receptores semelhantes a Toll (TLR) tem desencadeado grande interesse no campo da imunidade inata. Tal interesse deve-se ao fato de estes receptores proporcionarem um papel vital no reconhecimento microbiano e no desenvolvimento da resposta imune adaptativa. Tendo em vista que há muito a ser investigado no tocante à relação imunomodulação-TLR, o objetivo deste projeto foi avaliar o efeito da própolis na expressão dos mesmos, bem como determinar a produção de citocinas pró-inflamatórias (IL-1 β e IL-6) por macrófagos e esplenócitos de camundongos BALB/c. A expressão e a produção de citocinas do perfil Th1 (IL-2 e IFN- γ) e Th2 (IL-4 e IL-10) por esplenócitos também foram avaliadas. A própolis induziu aumento na produção basal de IL-1 β e na expressão de TLR-2 e TLR-4 em macrófagos peritoneais. A expressão de TLR-2 e TLR-4 e a produção de IL-1 β e IL-6 também apresentaram valores aumentados em esplenócitos de camundongos tratados com própolis. Estes dados sugerem que a administração de própolis a curto prazo a camundongos pode estimular os eventos iniciais da resposta imune. A própolis não afetou a expressão e produção de IL-2, IL-4 e IL-10; contudo, a produção basal e estimulada de IFN- γ foi inibida após a administração deste produto apícola, o que sugere seu efeito antiinflamatório in vivo.

Palavras chave: : Citocinas; Imunomodulação, Própolis; Real-time PCR.; Toll-like receptors.

Abstract

Propolis has been the subject of intense scientific research and its immunomodulatory action has been mentioned. The recent discovery and characterization of Toll like receptors (TLR) has triggered a great interest in the field of innate immunity. This interest is due to the fact that these receptors provide a vital role in microbial recognition and development of the adaptive immune response. Considering that there is much to be investigated regarding the relationship TLR-immunomodulation, the goal of this project was to evaluate propolis effect on TLR-2 and TLR-4 expression, and to determine the production of proinflammatory cytokines (IL-1 β and IL-6), in macrophages and spleen cells from BALB/c mice. Th1 (IL-2 and IFN- γ) and Th2 (IL-4 and IL-10) cytokines' expression and production were also assessed in spleen cells. IL-1 β basal production and TLR-2 and TLR-4 expression were increased in peritoneal macrophages of mice treated with propolis. TLR-2 and TLR-4 expression and IL-1 β and IL-6 production were also increased in spleen cells of propolis-treated mice. These data suggest that propolis administration over a short-term to mice stimulates the initial steps of the immune response. Propolis did not affect IL-2, IL-4 and IL-10 expression and production, but both basal and Con A-stimulated IFN- γ production were inhibited after propolis administration, what suggests its anti-inflammatory action in vivo.

Keywords: cytokine; Immunomodulatory; Propolis; Real-time PCR.; Toll-like receptors.

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1. Introdução

A utilização de plantas medicinais e produtos naturais acompanha o homem há centenas de anos, e a utilização destes produtos vem crescendo progressivamente em todo o mundo. Entretanto, a utilização destes produtos muitas vezes é acompanhada do desconhecimento de suas propriedades medicinais, devido à falta de informação sobre sua eficácia, posologia e segurança. Assim, faz-se necessária a investigação destes produtos para compreender melhor seus mecanismos e modos de atuações (Berman & Straus, 2004).

Vários ensaios tem sido realizados com o intuito de observar tais efeitos, principalmente com relação ao sistema imunológico (Sforcin, 2007; Mainardi *et al.*, 2009). A ação imunomoduladora de plantas medicinais, produtos naturais e seus componentes químicos despertaram interesse em várias áreas, principalmente em medicina humana e veterinária. O sucesso terapêutico obtido atualmente nos tratamentos clínicos é devido, em grande parte, ao conhecimento popular. A utilização de plantas e produtos naturais oferece um potencial de modalidade terapêutica para o tratamento de várias condições diferentes, incluindo efeitos sobre as citocinas (Spelman *et al.*, 2006).

1.1. *Própolis*

A própolis é um produto natural, elaborado pelas abelhas a partir de material coletado em botões, gemas e córtex vegetais, tendo sido empiricamente utilizada por apresentar inúmeras propriedades biológicas, dentre elas, a imunomoduladora (Sforcin, 2007).

Atualmente, a própolis tem despertado a atenção de pesquisadores interessados em elucidar cientificamente suas propriedades (Banskota *et al.*, 2001). Nesse sentido, o efeito da própolis tem sido estudado por nosso grupo, evidenciando-se suas ações: antibacteriana, antifúngica, antiparasitária, antiviral e imunomoduladora (Sforcin *et al.*, 2000; Murad *et al.*, 2002; Freitas *et al.*, 2006; Sforcin, 2007; Bufalo *et al.*, 2009).

1.2. *Composição química da própolis*

A composição química da própolis é muito complexa, sendo constituída basicamente por resinas e bálsamos (55%), ceras (35%), óleos vegetais (10%) e pólen

(5%). Investigando a estrutura química dos componentes da própolis, encontra-se, em sua composição, flavonóides, ácidos aromáticos, ésteres, aldeídos, terpenos, aminoácidos, polissacarídeos, hidrocarbonetos, alcoóis, hidroxibenzenos, dentre outros (Burdock, 1998).

A composição química da própolis por nós coletada ao longo das quatro estações na região de Botucatu, SP, foi analisada, verificando-se que diferenças sazonais não são significantes, mas predominantemente quantitativas. Os principais constituintes desta própolis foram isolados e identificados: flavonóides presentes em pequenas quantidades (4'-O-metil caempferol, 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, (2*Z*, 6*E*)-farnesol, benzoato de benzila e acetofenonas preniladas); ácidos aromáticos (ácido diidrocinâmico, ácido *p*-cumárico, ácido ferúlico, ácido cafeico, ácido 3,5-diprenil-*p*-cumárico, 2,2-dimetil-6-carboxietenil-8-prenil-2H-1-benzo-pirano); di- e triterpenos, entre outros (Boudourova-Krasteva *et al.*, 1997; Bankova *et al.*, 1998). Não houve efeito da sazonalidade sobre a composição química da própolis coletada em nosso apiário. Ademais, as fontes vegetais da própolis obtida no apiário de nossa Universidade foram investigadas, sendo *Baccharis dracunculifolia* a principal fonte, seguida de *Araucaria angustifolia* e *Eucalyptus citriodora* (Bankova *et al.*, 1999).

1.3. Efeitos biológicos da própolis

A própolis é um produto seguro, não havendo efeitos colaterais após sua administração. A administração de própolis a ratos, 2 vezes ao dia, durante 3 dias consecutivos, não induziu alterações na concentração sérica de proteínas totais, glicose, uréia, creatinina, triglicérides, colesterol, HDL-colesterol, bem como nas atividades específicas das transaminases (AST e ALT) e da desidrogenase láctica (LDH). Neste trabalho, foi observado também a ausência de efeito da sazonalidade sobre a atividade da própolis nas variáveis bioquímicas (Sforcin *et al.*, 2002). Dando continuidade a estes trabalhos, visando avaliar o efeito de diferentes concentrações de própolis administradas diariamente a ratos durante 30 dias; observou-se que não houve alterações significativas quanto à concentração sérica de lipídios totais, triglicérides, colesterol, HDL-colesterol, e nem na atividade específica da AST e LDH. Avaliando o perfil bioquímico de ratos tratados com própolis a longo prazo (30 90 e 150 dias), os resultados não evidenciaram diferenças entre o grupo controle e os grupos tratados com própolis, sugerindo a

ausência de efeitos colaterais após tratamento com este produto apícola (Mani *et al.*, 2006). Após suplementação com própolis de homens e mulheres saudáveis, observou-se a ausência de alterações sobre parâmetros bioquímicos em mulheres, possivelmente devido à ação antioxidante dos estrógenos (Jasprica *et al.*, 2007).

Com relação aos seus efeitos no sistema imune, foi evidenciado que a própolis induz elevação na geração de peróxido de hidrogênio (H_2O_2) por macrófagos peritoneais de camundongos e inibição na liberação de óxido nítrico (NO) por estas células, de forma dose-dependente (Orsi *et al.*, 2000). Em ensaios de quimioluminescência com neutrófilos de coelhos, foi observado efeito inibitório da própolis e de alguns dos seus componentes sobre a produção de ânion superóxido por estas células (Simoes *et al.*, 2004). Esses resultados são interessantes, pois a inibição da explosão respiratória poderia levar à persistência de antígenos no hospedeiro. Entretanto, o mecanismo de ação da própolis sobre a produção de radicais livres por macrófagos ainda não está elucidado (Cuesta *et al.*, 2005).

Lopes *et al.* (2003) avaliaram a ação de extratos das plantas fontes de própolis em nossa região sobre a produção de H_2O_2 e NO por macrófagos, verificando a ausência de efeito sobre a produção de tais metabólitos. Estes resultados sugeriram que a ação da própolis é uma consequência dos produtos derivados das plantas e adição de substâncias secretadas pelas abelhas, atuando possivelmente de forma sinérgica. Em 2007, analisamos o efeito do extrato total das partes aéreas, do lavado glandular, das folhas, das raízes e o óleo essencial de *B. dracunculifolia*, bem como de substâncias purificadas, sobre o estado de ativação de macrófagos. Os dados revelaram que o extrato do lavado glandular, das folhas e das raízes induziu a elevação na geração de H_2O_2 por macrófagos. Com relação aos compostos isolados, o óxido de *Baccharis* e o friedelanol estimularam a produção de H_2O_2 . Estes resultados sugerem uma ação ativadora de extratos e compostos isolados de *B. dracunculifolia* sobre macrófagos (Missima *et al.*, 2007).

Blonska *et al.* (2004), analisando o efeito do extrato etanólico da própolis e compostos derivados de flavona, observaram efeito inibitório a nível transcricional na expressão gênica da IL-1 β e iNOS de macrófagos J774A.1, relatando que os compostos testados contribuíram para a atividade antiinflamatória da própolis.

Com relação a sua ação contra microrganismos, trabalhos experimentais revelaram que macrófagos peritoneais de camundongos estimulados com própolis apresentaram aumento na atividade fungicida contra *Paracoccidioides brasiliensis*

(Murad *et al.*, 2002) e na atividade bactericida contra *Salmonella Typhimurium* (Orsi *et al.*, 2005). Em relação à própolis e parasitas, avaliando o efeito da própolis contra *Leishmania amazonensis* em culturas de macrófagos peritoneais, foi observado que extrato alcoólico de própolis ativou os macrófagos a destruírem estes microrganismos. A própolis não apresentou citotoxicidade aos macrófagos (Ayres *et al.*, 2007).

Recentemente, foi avaliada a ação da própolis em camundongos BALB/c submetidos a estresse, verificando que a administração desse produto apícola potencializou a geração de H₂O₂ por macrófagos peritoneais e impediu o aparecimento de centros germinativos no baço (Missima & Sforcin, 2008). A própolis induziu também aumento na imunidade celular de animais estressados e portadores de melanoma, sugerindo a possível utilização deste apiterápico em momentos de estresse (Missima *et al.*; submetido).

Quanto à resposta imune humoral, nosso grupo observou que o tratamento com própolis durante 3 dias induziu aumento na produção de anticorpos em ratos imunizados com albumina sérica bovina (Sforcin *et al.*, 2005). Estes achados reforçam a afirmação prévia de Scheller *et al.* (1988), os quais sugeriram que este apiterápico atuaria melhor sobre o sistema imune quando administrado a curto prazo, e que a atividade imunoestimulante da própolis poderia estar associada com a ativação de macrófagos e aumento de sua capacidade fagocítica. A própolis, quando utilizada como adjuvante em vacinas anti-SuHV-I, induziu aumento nos níveis de anticorpos e amplificou a resposta imune celular, apresentando uma maior porcentagem de animais protegidos contra a doença suína de Aujeszky (Fischer *et al.*, 2007).

Vários pesquisadores têm relatado a propriedade antitumoral da própolis. Camundongos inoculados com tumor mamário transplantável e apresentando metástases pulmonares foram tratados com compostos isolados da própolis (derivados do ácido cafeico e compostos flavonóides ativos), apresentando significativa diminuição no número de metástases e no crescimento tumoral, graças à atividade imunomoduladora da própolis, a qual induziu a produção de citocinas por macrófagos peritoneais (Orsolic & Basic, 2003). Trabalhos de nosso grupo evidenciaram que o extrato hidroalcoólico de própolis possui papel protetor no processo de carcinogênese do cólon, suprimindo o desenvolvimento de lesões pré-neoplásicas (Bazo *et al.*, 2002). Avaliando o efeito deste apiterápico sobre a ativação de células *natural killer* (NK) contra células tumorais, observamos também que a administração de própolis a ratos durante 3 dias induziu aumento na atividade lítica de células NK (Sforcin *et al.*, 2002).

1.4. Toll like receptors

O reconhecimento inicial de microrganismos patogênicos presentes no organismo pode se dar através de receptores expressos nas células envolvidas na imunidade inata. Dentre eles, podemos citar os receptores de superfície para manose, CD14, receptores para componentes do sistema complemento, e os recentemente estudados receptores semelhantes a Toll (*Toll-like receptors* = TLRs) (Anderson, 2000). Estes receptores podem ser divididos de acordo com a sua localização na célula: TLR-1/2/4/5/6/10 são expressos na superfície celular, enquanto que TLR-3/7/8/9 são expressos em compartimentos endossomais intracelulares.

Os TLRs são uma família de proteínas transmembrânicas caracterizadas por um domínio extracelular com repetições ricas em leucina (LRR) e um domínio intracelular Toll/IL-1R (TIR) (Medzhitov, 2001), sendo expressos por células imunológicas, como macrófagos, linfócitos B e T, células epiteliais e endoteliais (Sutmuller *et al.*, 2006; Kawai & Akira, 2006).

Em mamíferos, o domínio TIR também está associada a várias proteínas citoplasmáticas, incluindo a molécula adaptadora de sinalização MyD88 (Horng *et al.*, 2001; Yamamoto *et al.*, 2002), com função na transdução de sinal do receptor, resultando também no recrutamento de várias outras moléculas como TIRAP/Mal, IRAK-1, 2, 3, 4, TRAF-6, Trif e TRAM (Palm & Medzhitov, 2009; Takeda & Akira, 2004; Turvey & Hawn, 2006). A sinalização do TLR feita através destas proteínas adaptadoras resulta na translocação do fator de transcrição NF- κ B, com subsequente transcrição de genes para as citocinas pró-inflamatórias (Takeda & Akira, 2004),

Esses receptores reconhecem certos constituintes microbianos denominados padrões moleculares associados ao patógeno (PAMPs). Os PAMPs são produtos conservados pelos microrganismos e são essenciais para o seu metabolismo e sobrevivência (Medzhitov, 2001; Chen *et al.*, 2007). Os TLRs reconhecem, individualmente, um repertório distinto, mas limitado de PAMPs. Como exemplo de pares receptor-ligantes bem caracterizados, temos o TLR-4 e lipopolissacarídeos (LPS), TLR-5 e flagelina, TLRs-1/2/6 e lipoproteínas, zimosan, TLRs-3/7/8/9, lipopeptídeos de micobactérias e RNA e DNA virais (Turvey & Hawn, 2006).

O TLR das células da imunidade inata, uma vez ativado, resulta na indução de citocinas pró-inflamatórias, fagocitose e *burst* oxidativo. Como resultado desta ativação,

células dendríticas regulam positivamente moléculas co-estimulatórias, secretam citocinas imunomoduladoras como IL-12, aumentam o processamento de antígenos e a apresentação destes aos linfócitos T e B. Assim, os TLRs funcionam como uma ponte entre os sistemas inato e adaptativo (Pasare & Medzhitov, 2005)

O TLR-4 humano foi o primeiro TLR caracterizado em mamíferos, sendo expresso em macrófagos e células dendríticas (Medzhitov, 2001). O TLR-4 funciona como um receptor de transdução de sinal para LPS, o que foi confirmado em camundongos C3H/HeJ deficientes em TLR-4, os quais não apresentavam respostas ao estímulo por lipopolissacarídeo (Medzhitov, 2001). O reconhecimento do LPS pelo TLR-4 é complexo, e requer várias moléculas acessórias. Além do CD14, a proteína MD-2, expressa na superfície celular associada ao TLR-4, também é importante no reconhecimento ao LPS (Visintin *et al.*, 2006). Além do LPS, o TLR-4 reconhece também outros ligantes, como o ácido lipoteicoico (Medzhitov, 2001).

Em relação ao TLR-2, este receptor tem-se mostrado envolvido no reconhecimento de vários produtos microbianos, incluindo peptidoglicanos e lipoproteínas bacterianas, paredes celulares de fungos, dentre outros (Kawai & Akira, 2006). O TLR-2 pode exercer sua ação em cooperação com outros dois receptores: TLR-1 e TLR-6. Apesar disso, estes dois últimos são expressos em vários tipos celulares, enquanto que a expressão do TLR-2 parece ser restrita a células apresentadoras de antígeno e células endoteliais (MUZIO *et al.*, 2000).

Segundo Shishido *et al.* (2000), a ativação de TLR-2 tem papel central na regulação da inflamação vascular em camundongos, sugerindo que o TLR-2 induz aumento da produção de citocinas pró-inflamatórias (TNF- α , IL-1 β , e IL-6) e de espécies reativas de oxigênio. TLRs também podem ser ativados por moléculas de outros tecidos, como as do sistema cardiovascular, apresentando um elo entre o desenvolvimento de doenças cardiovasculares e o sistema imunológico, embora o papel individual dos membros da família TLR na fisiopatologia destas doenças e na prática clínica ainda mereça maiores investigações (Frantz *et al.*, 2007). No caso de pacientes com artrite reumatóide, estes apresentam alta expressão de TLR-2 e TLR-9 em fibroblastos e linfócitos. Estes receptores podem ser ativados por anticorpos, produtos de células necróticas e proteínas de choque térmico liberadas em altas quantidades pelos tecidos lesados (Andreakos *et al.*, 2004). Deste modo, a inibição da sinalização destes receptores tem sido estudada como uma opção terapêutica para a inflamação crônica.

A capacidade de sinalização dos TLRs pode ser modulada pelo IFN- γ , amplificando esta capacidade por regular positivamente as moléculas de sinalização dos receptores. Além disso, o IFN- γ aumenta a expressão do TLR, dos co-receptores e das moléculas acessórias como a MyD88, IRAK-1 e o TRAF-6, com conseqüente aumento da ativação do NF- κ B (Schroder *et al.*, 2006).

A relação entre expressão de TLR e produtos naturais despertou interesse dos pesquisadores e um recente trabalho investigou a ação do ginseng (*Panax ginseng* C.A. Mayer G 115), correlacionando a produção de citocinas por macrófagos peritoneais com a expressão de TLR-2 e TLR-4 em camundongos submetidos a estresse por natação (Pannacci *et al.*, 2006). Trabalhos recentes do nosso grupo de pesquisa relataram que administração de própolis a submetidos ao estresse induziu aumento na expressão dos TLR-2 e TLR-4, cuja expressão estava inibida nesses animais, evidenciando a ação imunorestauradora deste produto apícola (Pagliarone *et al.*, submetido).

1.5. Citocinas

Citocinas fazem parte de um grande grupo de proteínas solúveis, sendo mediadores da resposta imune, e controlando diferentes funções celulares que incluem proliferação, diferenciação e morte celular, por meio de ligações aos seus receptores na superfície celular (Romagnani, 2006). Classificadas em grupos (por exemplo, interleucinas, interferons, e quimiocinas), com base nas homologias estruturais de seus receptores, no passado acreditava-se, sobretudo, que eram antivirais ou agentes antineoplásicas (Oppenheim, 2001). Podem ser sintetizadas tanto por células do sistema imune como pelo endotélio vascular, adipócitos, neurônios, entre outras. Algumas citocinas podem ser classificadas como pró-inflamatórias, derivadas principalmente de células da resposta imune inata: fator de necrose tumoral alfa (TNF- α), interleucinas (IL)-1, IL-6 e IL-12, ou de células de padrão Th1: interferon-gama (IFN- γ), ou como citocinas anti-inflamatórias, sintetizadas principalmente por células do perfil Th2, por T regulatórias (Tregs): fator de transformação e crescimento beta (TGF- β), IL-4, IL-10, e IL-5 (Elenkov, 2004; Romagnani, 2006; Wan & Flavell, 2006).

Com relação às citocinas pró-inflamatórias, a IL-1 β é secretada principalmente por macrófagos, células endoteliais e fibroblastos e suas funções são semelhantes às do TNF- α , recrutando neutrófilos e induzindo a expressão de molécula de adesão endotelial, sendo este, um mecanismo essencial para a adesão de leucócitos na

superfície endotelial e posterior migração para os tecidos. A IL-1 β tem capacidade de induzir a síntese de outras citocinas, e apresenta função co-estimuladora de células T (Reyes *et al.*, 2003). Uma de suas principais funções na resposta inflamatória é induzir a síntese de proteínas plasmáticas de fase aguda (Krabbe *et al.*, 2004).

No que diz respeito a IL-6, atua tanto na imunidade inata quanto na adaptativa, sendo sintetizada por várias células (linfócitos, macrófagos, células endoteliais e fibroblastos, músculo-esqueléticas, dentre outras) e ativada pela via de sinalização JAK/STAT, em resposta a microrganismos e outras citocinas (IL-1 e TNF). É associada ao controle e coordenação de respostas do sistema imunológico, além de atuar nos sistemas hematopoiético, nervoso e endócrino e participar do metabolismo ósseo (Petersen & Pedersen, 2005). Na imunidade inata, estimula a síntese de proteínas da fase aguda pelo fígado, atuando na resposta de fase aguda. Entretanto, na imunidade adaptativa, IL-6 estimula o crescimento de células B, que se diferenciam em produtoras de anticorpos, e estimula a proliferação e ativação das células T (Forsey *et al.*, 2003). IL-6 apresenta um pico de meia vida curto (6 a 12 h), e sua elevação reflete e depende da ativação da resposta inflamatória. Indivíduos com quadros de sepse apresentam elevados níveis circulantes de IL-6 (Loisa *et al.*, 2003). Por sua vez, a produção muito elevada e constante de IL-1 e IL-6 pode levar a graves comprometimentos, acarretando colapso vascular, choque e morte (Oberholzer *et al.*, 2000).

Todavia, sugere-se que uma das principais funções da IL-6 seja a auto-limitação da resposta inflamatória. A IL-6 tem ação inibidora na expressão das citocinas pró-inflamatórias TNF- α e IL-1- β , ao aumentar a síntese do receptor antagonista da IL-1 (IL-1Ra). Assim, a IL-6 concomitantemente regula atividades pró- e anti-inflamatórias, contribuindo tanto para o desenvolvimento quanto para a resolução da resposta inflamatória (Petersen & Pedersen, 2005).

A diferenciação de células para Th1 é regulada por sinais ativadores e transdutores de transcrição (STATs), como STAT-4 e T-bet, e para a diferenciação das células Th2 são necessários os fatores STAT-6 e GATA-3 (Szabo *et al.*, 2003).

Dentre as citocinas do perfil Th1, destacamos o IFN- γ - citocina de “assinatura” do subgrupo Th1. Uma característica importante desta citocina é ser um potente ativador de fagócitos, sendo capaz de induzir o *burst* respiratório, auxiliando na fagocitose e destruição de antígenos, e inibindo a proliferação de células Th2.

Já a IL-2, é responsável pela expansão clonal após o reconhecimento antigênico, é um potente fator de crescimento de células T. Sua ativação resulta em expressão dos

genes de vias de transdução de sinais, incluindo JAK/STAT, principalmente JAK1, JAK3 e STAT5 (O'shea *et al.*, 2002). IL-2 tem sido utilizada clinicamente para reforçar a atividade de células T em pacientes com AIDS ou câncer, representando um importante ponto de controle para manipular o equilíbrio entre a regulação e a função de células T efectoras *in vivo*. A sua utilização vem crescendo constantemente, sendo uma molécula chave no controle das respostas das células T em pacientes com doenças malignas e infecciosas (Nelson, 2004). A presença da IL-4 inibe o desenvolvimento de células Th1.

Com relação às citocinas do padrão Th2, a IL-10 desempenha um papel fundamental no controle de respostas imunes e na manutenção da tolerância *in vivo* (Moore *et al.*, 2001). A sinalização da IL-10 compreende principalmente as vias STAT1 e STAT3 (Cavaillon, 2001). IL-10 é considerada principalmente uma citocina imunossupressora, embora diversos trabalhos demonstrem que a IL-10 aumenta a função das células natural killer, conduzindo facilmente à destruição do patógeno (Belardelli & Ferrantini, 2002; Blanco *et al.*, 2001; Mocellin *et al.*, 2003). Quanto à sua função imunoregulatória, exerce ação anti-inflamatória em determinadas circunstâncias, inibindo a produção de INF- γ e IL-12 e, conseqüentemente, inibindo a resposta de padrão Th1 (Moore *et al.*, 2001). Dentre outros eventos, a IL-10 está envolvida com a indução de produção de IL-4 e mudança de classe do anticorpo para IgG4, mostrando sua ligação e função com a modulação de doenças alérgicas em humanos. Além disso, a IL-10 limita a produção de outras citocinas, como por exemplo, citocinas pró-inflamatórias (IL-6, IL-18, IL-1, TNF) (Mocellin *et al.*, 2003).

No que diz respeito a IL-4, além de estimular a produção de anticorpos IgE, atua também como fator de desenvolvimento para as células Th2 a partir de células T CD4⁺ *naive*. É sinalizada pela via JAK/STAT (JAK3 e 4/STAT6). Estudos demonstraram que animais com deficiência em IL-4 possuíam menor quantidade de IgE, indicando sua importância na defesa contra infecções por helmintos, sendo essa a principal função das células Th2 (Kay, 2001). IL-4 apresenta efeito antagonista aos efeitos ativadores do IFN- γ principalmente sobre os macrófagos, inibindo reações mediadas por estas células (Tay *et al.*, 2009). Em animais estressados por imobilização, o tratamento com própolis a curto prazo (3 dias) exerceu ação restauradora sobre a produção de IL-4, a qual encontrava-se inibida nos animais estressados (Pagliarone *et al.*, submetido).

Considerando o fato de que a própolis apresenta ação imunomoduladora quando administrada a curto prazo, o objetivo desta dissertação foi avaliar seu possível efeito sobre a expressão de TLR-4 e TLR-2, bem como sobre a produção de citocinas pró-inflamatórias e de perfil Th1 e Th2 em camundongos BALB/c. Tal interesse deve-se ao fato de que não há dados na literatura pertinente sobre o efeito da própolis na expressão gênica de TLRs, e poucos autores investigaram sua ação sobre a produção de citocinas em camundongos. Desta forma, nosso trabalho propicia um maior entendimento sobre estes aspectos.

Com relação ao TNF- α , não foi possível a detecção desta citocina. Novos experimentos foram realizados com o intuito de avaliar sua produção em diferentes protocolos, utilizando o sobrenadante de culturas de macrófagos peritoneais e de células esplênicas, estimuladas ou não com LPS ou Con A em diferentes concentrações e em diferentes períodos de incubação, mas ainda assim a detecção de TNF- α não foi possível. Acreditamos que houve algum problema com o material para a dosagem da mesma.

Além dos objetivos propostos inicialmente, conseguimos avaliar também a expressão de TLR-2 e TLR-4 por esplenócitos totais, e analisamos a expressão gênica de citocinas de padrão Th1 (IL-2) e Th2 (IL-4 e IL-10) por esplenócitos totais, o que não fazia parte dos objetivos iniciais deste projeto.

Este projeto foi desenvolvido com o apoio da FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo), através da bolsa de Mestrado (06/58830-5) e do auxílio à pesquisa (07/02692-6) concedidos, sem os quais seria impossível a realização do mesmo, não só pelo apoio financeiro, mas também pelas críticas e sugestões apontadas pelos assessores.

Os resultados obtidos encontram-se apresentados em dois capítulos:

- ✓ Capítulo 1: **“Propolis immunomodulatory action *in vivo* on Toll-like receptors 2 and 4 expression and on pro-inflammatory cytokines production in mice”**, a ser submetido à revista **EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES**.
- ✓ Capítulo 2: **“Th1/Th2 cytokines’ expression and production by propolis-treated mice”**, a ser submetido à revista **FOOD CHEMISTRY**.

Manuscritos

Capítulo 1

Propolis immunomodulatory action *in vivo* on Toll-like receptors 2 and 4 expression and on pro-inflammatory cytokines production in mice

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ABSTRACT

Propolis is a bee product and its immunomodulatory action has been the subject of intense investigation lately. The recent discovery and characterization of the family of Toll-like receptors (TLR) have triggered a great interest in the field of innate immunity due to their crucial role in microbial recognition and development of the adaptive immune response. This work aimed to evaluate propolis effect on TLR2 and TLR4 expression and on the production of pro-inflammatory cytokines (IL-1 β and IL-6). Male BALB/c mice were treated with propolis (200 mg/kg) for three consecutive days, and TLR2 and TLR4 expression as well as IL-1 β and IL-6 production were assessed in peritoneal macrophages and spleen cells. Basal IL-1 β production and TLR2 and TLR4 expression were increased in peritoneal macrophages of propolis-treated mice. TLR2 and TLR4 expression and IL-1 β and IL-6 production were also upregulated in the spleen cells of propolis-treated mice. One may conclude that propolis activated the initial steps of the immune response by upregulating

TLRs expression and the production of pro-inflammatory cytokines in mice, modulating the mechanisms of the innate immunity.

Keywords:

Propolis

Toll-like receptors

Pro-inflammatory cytokines

Immunomodulation

1. Introduction

Recently, there has been a dramatic increase in the use of dietary supplements including natural and herbal products; however, information about the safety and efficacy of most natural products is vastly inadequate. In light of folk beliefs and the increased use of herbals, a rigorous investigation is needed to understand the mechanisms of action and efficacy of such products (Berman and Straus, 2004). Complementary and alternative medicines (CAMs) are used by more than 80% of the world's population, with more than 70% of the population using CAM at least once and annual spending in the U.S. reaching as much as \$34 billion (Mainardi *et al.*, 2009). Clinically and experimentally, there have been hundreds of trials looking at the effect of CAM on the immune system (Clarke and Mullin, 2008; Mainardi *et al.*, 2009; Sforcin, 2007).

There is a long history of propolis use by man, and today its use still continues in home remedies and personal products (Castaldo and Capasso, 2002; Sforcin, 2007). Propolis or bee glue is a resinous material collected by bees from several plant sources and used for construction and adaptation of their nests (Bankova, 2005b). Propolis chemical composition is very complex

and presents a great variability (Bankova, 2005a). Plants visited by bees in our apiary (UNESP, Campus of Botucatu) were identified and the main vegetal source of our propolis samples is *Baccharis dracunculifolia* DC., followed by *Eucalyptus citriodora* Hook and *Araucaria angustifolia* (Bert.) O. Kuntze (Bankova *et al.*, 1999). Propolis possesses several biological properties, such as antibacterial, antifungal, antiprotozoan, antiviral, antitumoral, anti-inflammatory, immunomodulatory, among others (Banskota *et al.*, 2001; Bazo *et al.*, 2002; Freitas *et al.*, 2006; Girgin *et al.*, 2009; Hu *et al.*, 2005; Murad *et al.*, 2002; Orsi *et al.*, 2005; Sforcin, 2007).

With regards to its immunomodulatory action, studies of our laboratory have shown that propolis increased hydrogen peroxide (H₂O₂) generation and inhibited nitric oxide (NO) production by peritoneal macrophages *in vitro* (Orsi *et al.*, 2000). Propolis also potentiated H₂O₂ generation by peritoneal macrophages in stressed mice (Missima and Sforcin, 2008). It has been reported that propolis stimulated the production of pro-inflammatory cytokines (TNF- α and IL-1 β) *in vitro* (Moriyasu *et al.*, 1994); nevertheless, there is no information concerning propolis action on immune cell receptors.

Toll-like receptors (TLRs) are widely expressed by various cells of the immune system (Barton and Medzhitov, 2003; Kawai and Akira, 2006). TLRs recognize conserved pathogen-associated molecular patterns shared by different microorganisms, such as bacterial lipopolysaccharide, bacterial or mycobacterial lipopeptides, viral RNA and DNA, among others, playing an essential role in the innate immune response and in the initiation of adaptive immune response (Hopkins and Sriskandan, 2005; Medzhitov, 2001). TLR2 recognizes, for example, components from Gram-positive bacteria and

zymozan, while TLR4 recognizes Gram-negative bacteria lipopolysaccharide (LPS). Signaling by the TLRs is initiated through the TLR transduction domains known as Toll/IL-1 receptor (TIRs domains), which interact with cytoplasmic adaptor proteins including MyD88, TIRAP/Mal, IRAK 1/2/3/4, TRAF-6, Trif and TRAM, resulting in the activation of the transcription factors NF- κ B and AP-1, with subsequent production of pro-inflammatory cytokines, chemokines, antimicrobial peptides and additional defense molecules (Barton and Medzhitov, 2003; Horng *et al.*, 2001; Palm and Medzhitov, 2009). TLRs stimulation leads to upregulation of inflammatory mediators such as the pro-inflammatory cytokines TNF- α , IL-1 β and IL-6 (Schnare *et al.*, 2001; Shishido *et al.*, 2006), increasing the processing and presentation of antigens to lymphocytes, and acting as a bridge between innate and adaptive immune responses (Pasare and Medzhitov, 2005).

Since there are no data regarding propolis effects on TLRs, this work evaluated TLR 2 and 4 expression by macrophages and spleen cells, as well as the production of pro-inflammatory cytokines (IL-1 β and IL-6) by these cells after propolis administration to mice over a short-term (3 days), in order to understand its effects on the initial events of the immune response.

2. Materials and methods

2.1 Propolis extract

Propolis, collected in the Beekeeping Section, UNESP, 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 (120 mg/ml) (Sforcin *et al.*, 2005). Propolis chemical composition was investigated using thin-layer chromatography (TLC), gas chromatography (GC), and gas chromatography-mass spectrometry (GC-MS) analysis. Specific dilutions were carried out to determine the concentration of propolis solution to be given to the animals (200 mg/Kg).

2.2 Animals and experimental groups

BALB/c male mice aged between 8 and 12 weeks were kept at 21-25 °C, with a 12 h /12 h light/dark cycle in the Department of Microbiology and Immunology, UNESP. Food and water were provided *ad libitum*.

Mice were divided into 2 groups (G1 and G2) of 10 animals each. G1 was considered as control, and received physiologic solution (NaCl 0.9%, 0.1 ml). G2 received propolis solution (200 mg/kg, 0.1 ml) for 3 consecutive days by gavage. Ethanol effects were also investigated, in order to observe a possible effect of propolis solvent. Immediately after the last treatment, animals were sacrificed using a CO₂ inhalation chamber. This work agreed with Ethical Principles in Animal Research adopted by Brazilian College of Animal Experimentation (COBEA) and was approved in May 5, 2007.

2.3 Peritoneal macrophages and spleen cells cultures

After sacrifice, peritoneal macrophages were obtained by inoculation of cold PBS into the abdominal cavity. After a soft abdominal massage, the peritoneal liquid was collected, transferred to sterile plastic tubes (Falcon) and centrifuged at 200 x g for 10 min. Cells were stained with neutral red (0.02%), incubated for 10 min at 37°C and counted in a hemacytometer. Cells were resuspended in cell culture medium (RPMI 1640 supplemented with 5% foetal calf serum, 2 mM L-glutamine, 20 mM HEPES, 2.5×10^{-5} M 2-mercaptoethanol – Sigma, USA), and cultured in a 96-welled flat-bottomed plate (Corning, USA) at a final concentration of 2×10^6 cells per well. Cells were incubated at 37 °C and, after 2 h, non-adherent cells were removed and macrophages were reincubated at 37 °C for 24 h and 5% CO₂ with or without LPS (5 µg/ml) (Missima and Sforcin, 2008).

After obtaining the peritoneal macrophages, spleens were aseptically removed and cells were suspended at a concentration of 5×10^6 /ml in RPMI 1640 as described above and cultured in flat-bottomed 24-well plates. Cells were cultured in triplicates (1 mL/well) and stimulated or not with LPS (5 µg/ml) for 24h at 37 °C and 5% CO₂.

2.4 Cytokine determinations

Supernatants of peritoneal macrophages and spleen cell cultures were collected and assayed for IL-1 β and IL-6 by enzyme-linked immunosorbent assay (ELISA), according to manufacturer's instructions (BD Biosciences,

USA). Briefly, a 96-well flat bottom Maxisorp microtiter plates (Nunc, USA) was coated with capture antibody specific for 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 captured 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 (Tan *et al.*, 2006).

2.5 RNA isolation and cDNA synthesis

Peritoneal macrophages were removed from the microplate by adding cold PBS to the wells. Afterwards, this material was kept in 250 µl of RNA Safer (Omega Bio-tek, Inc. USA). Spleens were removed and thirty mg were also kept in 250 µl of RNA Safer at –80 °C until RNA isolation procedures. Total RNA was extracted with RNeasy Mini RNA Isolation Kit (Qiagen – Healthcare, USA) following the manufacturer's instructions. After purification, RNA was treated with RQ1 RNase-free DNase (Promega, USA) for 30 min at 37 °C to avoid false-positive results due to amplification of contaminating genomic DNA. Concentrations of total RNA were determined from the absorbance value of the samples at 230 nm. cDNA was synthesized from 1 µg of total RNA and was reverse-transcribed with 2 µl of random primer (250 ng/µl) in a final volume of 10 µl and the mixture was incubated for 5 min at 70 °C. For each sample, the master mix was prepared with 8 µl of reaction buffer (Improm II 5x), 4.8 µl of MgCl₂ (25 mM), 2 µl of RNase Out (Invitrogen), 1 µl of dNTP mix (20 mM), 1 µl

Improm TR II (Promega, USA) and 13.2 µl of nuclease-free water. To each sample, 16 µl of master mix were added and the mixture was incubated for 5 min at 25 °C, 60 min at 42 °C and 15 min at 70 °C. Each cDNA sample was stored at –20 °C.

2.6 Quantitative Real Time PCR

Primers were designed based on sequences retrieved from GeneBank using the IDTSciTools (<http://www.idtdna.com>) software and synthesized by Applied Biosystems (USA). The sequences of specific primers were: 5'- CTT CCT GGT TCC CTG CTC GTT CTC - 3' (F) and 5'- CAA GAA CAA AGA AAA TGA GTC AAG - 3' (R) for murine TLR-2 (Gene Bank accession number: NM011905.2), and 5' - TGA CAG GAA ACC CTA TCC AGA GTT - 3' (F) and 5'- TCT CCA CAG CCA CCA GAT TCT -3' (R) for murine TLR-4 (Gene Bank accession number: NM021297.2). Primers for β-actin described in the literature (Yang and Glaser, 2002) were used: 5' – AAG TGT GAC GTT GAC ATC CGT AA – 3' (F) and 5' – TGC CTG GGT ACA TGG TGG TA – 3' (R). The PCR mixture consisted of 4 µl of cDNA, 0.4 µl of each primer (200 nM), 10 µl of 2X Power Sybr[®] Green PCR Master Mix (Applied Biosystems, USA) and 5.2 µl of sterile nuclease-free water for a final volume of 20 µl. The reaction conditions were as follows: 95°C/10 min for initial denaturation, amplification for 40 cycles (95 °C for 15 s for denaturation, 60 °C for 1 min for annealing and extension), and to confirm the PCR product and cycle of melting curve analysis at 95 °C for 15 s, 60 °C for 30 s and 95 °C for 15 s. Fluorescence data were collected during each annealing/extension step and threshold cycle numbers (C_T) were

determined using ABI PRISM[®] 7300 Sequence Detector (Applied Biosystems, USA) and Software SDS version 1.2.3 ("Sequence Detection Systems" 1.2.3 – 7300 Real Time PCR System – Applied Biosystems, USA). Reaction mixtures with no cDNA were used as negative controls and all PCR assays were performed in duplicate.

The standard-curve was generated by performing serial dilutions of cDNA. To the smallest dilution of cDNA standard it was given the relative value 100 and, the other 3 points were designated as 50, 25, and 12.5. Gene-specific expression values were normalized to expression values of β -actin (endogenous control) within each sample.

2.7 Statistics

Data were analyzed using Prism Graph Pad statistical software (Graph-Pad Software, Inc., USA). Significant differences between treatments were determined by ANOVA, followed by *t* test. Statistically significance was accepted when $P < 0, 05$.

3. Results

3.1 TLR-2 and 4 expression by macrophages cells

An increased TLR2 expression was observed in macrophages of propolis-treated mice ($P < 0.0002$) compared to the control group (Fig. 1A).

Likewise, an increased TLR4 expression was seen in these cells after propolis administration to mice ($P < 0.0001$) (Fig. 1B).

3.2 TLR-2 and 4 expression by spleen cells

Propolis administration to mice increased non-significantly TLR2 expression by spleen cells ($P > 0.05$) (Fig. 2A). On the other hand, TLR4 expression was significantly increased in propolis-treated mice ($P < 0.0048$) compared to the control group (Fig. 2B).

Ethanol (propolis solvent) did not affect propolis action on TLRs expression.

3.3 Pro-inflammatory cytokines production by peritoneal macrophages

With regards to peritoneal macrophages, propolis increased IL-1 β basal production in comparison to the control group ($P < 0.01$) (Fig. 3A). In LPS-stimulated cultures, no differences were seen between the groups (Fig. 3B). No differences were seen between groups concerning basal or LPS-stimulated IL-6 production (Fig. 4A and Fig. 4B).

3.4 Pro-inflammatory cytokines production by spleen cells

As to pro-inflammatory cytokine production by spleen cells, basal production of both IL-1 β and IL-6 was not detected in the experimental groups. In LPS-stimulated cultures, an increased IL-1 β ($P < 0.05$) and IL-6 ($P < 0.01$) production was seen in propolis-treated mice (Figs. 5 and 6, respectively).

4. Discussion

Ethanol (propolis solvent) did not influence propolis activity in any immunological assay.

The immunomodulatory action of medicinal plants, natural products and their chemical components raised a great interest in several areas, mainly in human and veterinary medicine. The therapeutic success achieved with current medical treatment is at least in part due to popular knowledge and the use of plants and natural products.

Regarding propolis composition, the main constituents of our sample, investigated by TLC, GC and GC-MS analysis, were: flavonoids (kaempferid, 5,6,7-trihydroxy-3,4'-dimethoxyflavone, aromadendrine-4'-methyl ether); a prenylated *p*-coumaric acid and two benzopyranes: *E* and *Z* 2,2-dimethyl-6-carboxyethenyl-8-prenyl-2H-benzopyranes); essential oils (spathulenol, (2*Z*,6*E*)-farnesol, benzyl benzoate and prenylated acetophenones); aromatic acids (dihydrocinnamic acid, *p*-coumaric acid, ferulic acid, caffeic acid, 3,5-diprenyl-*p*-coumaric acid, 2,2-dimethyl-6-carboxy-ethenyl-8-prenyl-2H-1-benzopyran); di- and triterpenes, among others (Bankova *et al.*, 1998).

Investigation of natural products effect on cytokines and their mechanism of action provided a potential therapeutic modality for the treatment of different conditions (Spelman *et al.*, 2006). Several studies have shown the immunomodulatory effects of medicinal plants and their components on cytokine production and other factors (Amirghofran *et al.*, 2000; Ganju *et al.*, 2003; Mainardi *et al.*, 2009; Spelman *et al.*, 2006); however, propolis effects on TLRs expression has not been investigated yet.

Propolis administration to mice increased the expression of TLR4 in peritoneal macrophages and spleen cells. Our results are in agreement with other authors who verified a higher TLR-4 expression in macrophages of mice treated with another natural product – ginseng (Pannacci *et al.*, 2006). Our data also showed an increased TLR-2 expression, indicating a differential regulation of the several members of the TLRs family. Ginseng was found to stimulate TNF- α and IL-1 β production by peritoneal macrophages, what was correlated to TLR4 expression in macrophages (Pannacci *et al.*, 2006). On the other hand, notoginseng inhibited both TNF- α and IL-1 β production and TLRs activation in dendritic cells. This natural product differentially affected the expression of coestimulatory molecules in dendritic cells, after activation of different TLRs by their ligands (Rhule *et al.*, 2008).

TLR4 signaling may occur through MyD88-dependent and MyD88-independent pathways, which mediate the activation of pro-inflammatory cytokine and Type I interferon genes (Lu *et al.*, 2008), whereas TLR-2 use MyD88 and TIRAP as essential adaptors for its activation. Thus, individual TLRs interact with different combinations of adaptor proteins and activate various transcription factors such as nuclear factor NF- κ B, AP-1 and interferon regulatory factors, driving a specific immune response (Kawai and Akira, 2005; 2006). Propolis seemed to have enabled both MyD88 and TIRAP/MAL pathways and possibly activated NF- κ B, since TLR2 and TLR4 expression was increased in peritoneal macrophages and spleen cells, with consequent increased production of pro-inflammatory cytokines, demonstrating for the first time a possible mechanism of action of this bee product in the initial steps of the immune response.

Our data revealed that TLRs expression after propolis administration to mice was followed by an increased IL-1 β and IL-6 production. These findings are in agreement with other authors, who verified an increased pro-inflammatory cytokines production by murine macrophages stimulated with propolis *in vitro* (Dimov *et al.*, 1991; Moriyasu *et al.*, 1994). After administration of capsules of propolis for 2 weeks to humans, the plasma concentration of TNF- β , IL-1 β , IL-6 and IL-8 had not changed, although the capacity for cytokine production by peripheral blood cells stimulated with LPS had increased significantly (Bratter *et al.*, 1999). It has been suggested that the increased IL-1 β production by macrophages from propolis-treated mice might be associated with enhanced T and B lymphocyte proliferation (Orsolic and Basic, 2003).

In our experimental model, propolis was administered to mice for 3 days, since it has been reported that propolis administration over a short-term leads to better results concerning the immune system, increasing the immunological response (Scheller *et al.*, 1988; Sforcin, 2007). Propolis treatment for 3 days increased the cytotoxic activity of natural killer cells against murine lymphoma (Sforcin *et al.*, 2002). On the other hand, works of our group verified that propolis administration for 14 days inhibited both IL-1 β and IL-6 production by C57BL/6 mice (Missima *et al.*, 2009).

Host cells possess multiple defensive mechanisms against infections, and TLRs play essential roles in innate immune responses by recognizing various pathogens-derived compounds. Propolis enhances the immune activity in various ways (Sforcin, 2007), and its use in vaccines as an adjuvant has been pointed out (Fischer *et al.*, 2007). The knowledge of propolis' mechanisms of action in the immune system has advanced in the last several

years and our data provides an additional contribution in the comprehension of murine cells activation by this bee product, culminating in TLRs expression and pro-inflammatory cytokines production.

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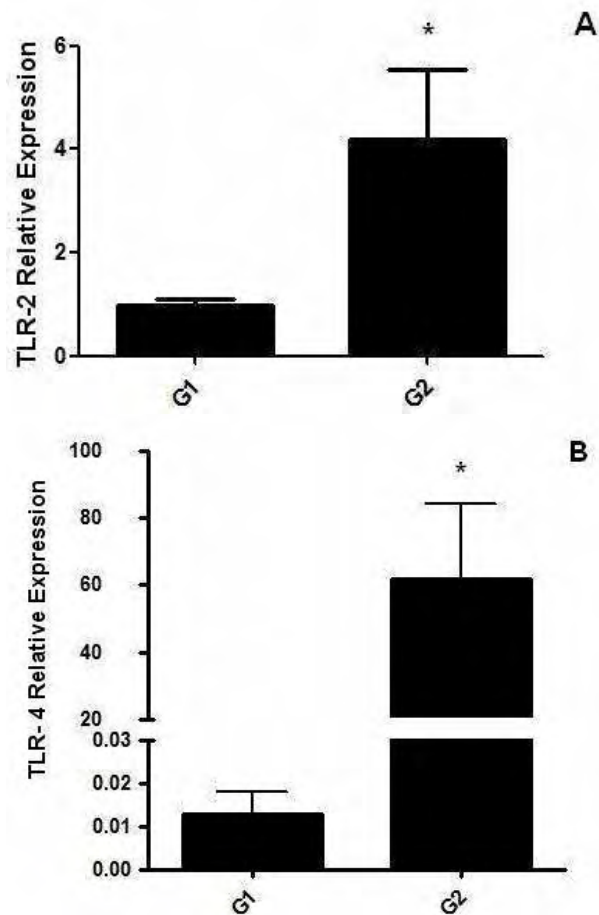


Fig. 1

Fig. 1. Relative quantification by real-time PCR to of TLR-2 (A) and TLR-4 (B) in peritoneal macrophages. All results, normalized to β -actin, were expressed as arbitrary units relative to the value of 1.0. G1: Control and G2: treated with propolis. * Significantly different from G1 (A) ($p < 0.0002$) and (B) ($p < 0.0001$).

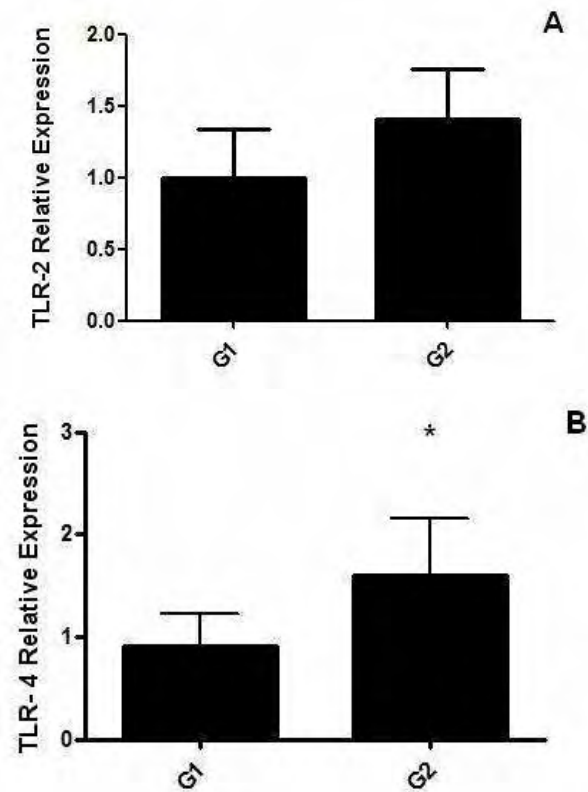


Fig. 2

Fig. 2. Relative quantification by real-time PCR to estimate the expression of TLR-2 (A) and TLR-4 (B) in spleen cells. All results, normalized to β -actin, were expressed as arbitrary units relative to the value of 1.0. G1: Control and G2: treated with propolis. * Significantly different from G1 ($p < 0.0048$).

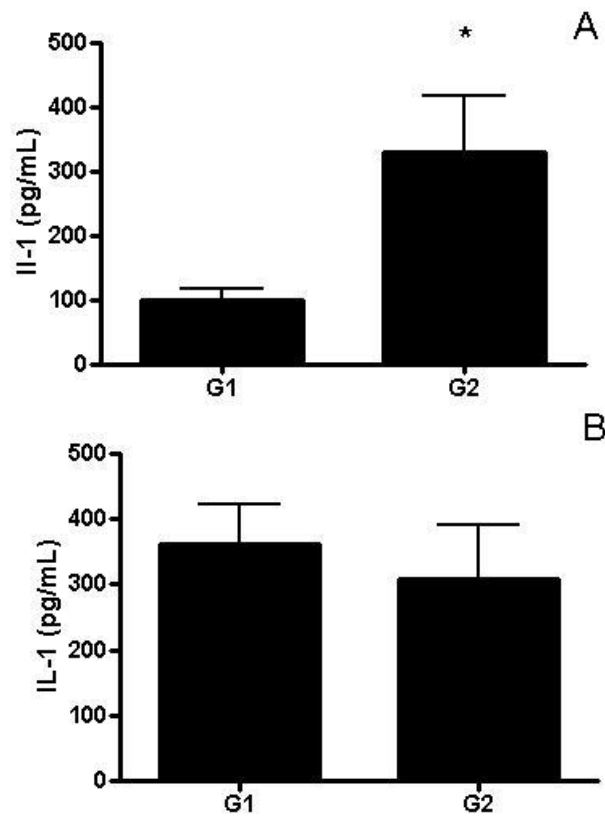


Fig. 3

Fig. 3. IL-1 β basal production (pg/mL) by peritoneal macrophages (A) or stimulated with LPS (5 μ g/mL) for 24 h (B). G1: Control and G2: treated with propolis. Data represent means and standard-deviation of 10 animals. * Significantly different from G1 ($p < 0.01$).

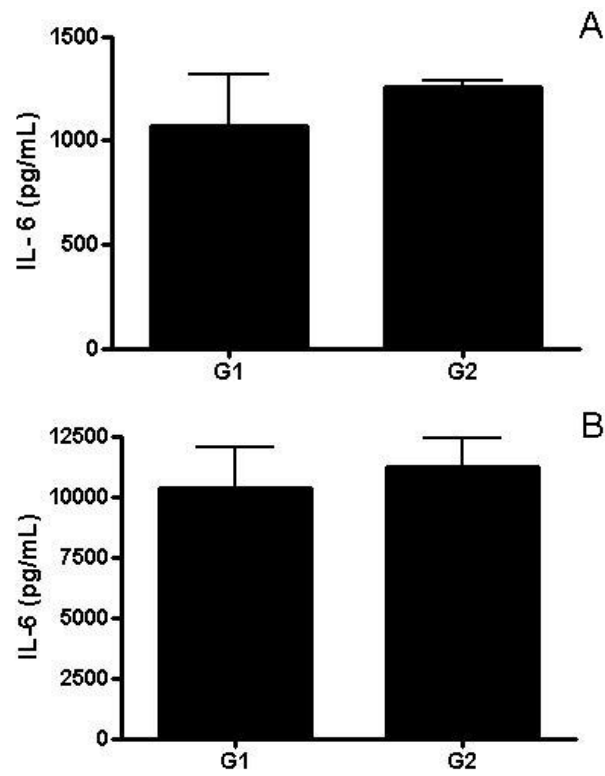


Fig. 4

Fig. 4. IL-6 basal production (pg/mL) by peritoneal macrophages (A) or stimulated with LPS (5 μ g/mL) for 24 h (B). G1: Control and G2: treated with propolis. Data represent means and standard-deviation of 10 animals.

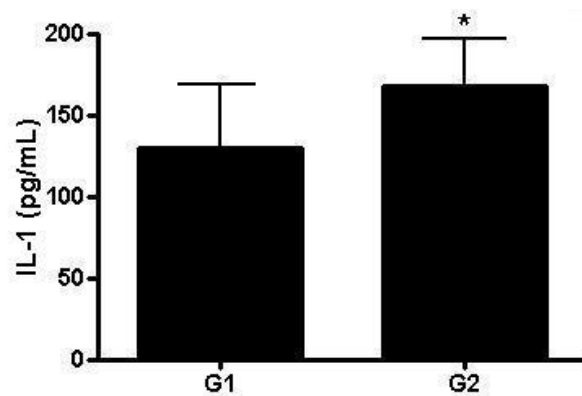


Fig. 5

Fig. 5. IL-1 β production (pg/mL) by spleen cells stimulated with LPS (5 μ g/mL) for 24 h. G1: Control and G2: treated with propolis. Data represent means and standard-deviation of 10 animals. * Significantly different from G1 (p<0.05).

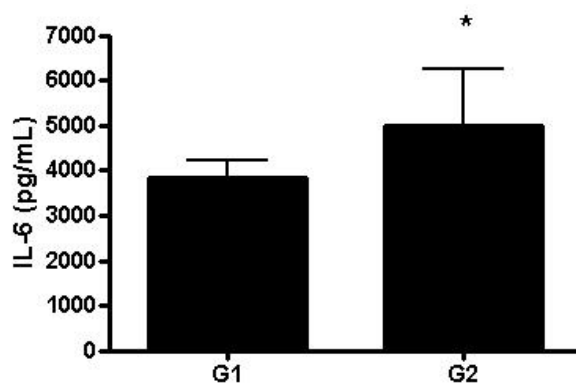


Fig. 6

Fig. 6. IL-6 production (pg/mL) by spleen cells stimulated with LPS (5 μ g/mL) for 24 h. G1: Control and G2: treated with propolis. Data represent means and standard-deviation of 10 animals. * Significantly different from G1 (p<0.01).

Capítulo 2

Th1/Th2 cytokines' expression and production by propolis-treated mice

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Abstract

Propolis is a natural product extensively used in food and beverages to improve health and prevent diseases, showing several biological properties, such as immunomodulatory. The goal of this work was to evaluate the effect of propolis administration over a short-term to mice on Th1 (IL-2 and IFN- γ) and Th2 (IL-4 and IL-10) cytokines' expression and production. Mice were treated with propolis by gavage for 3 days, spleens were removed and RNA was extracted to assess cytokine expression by real time-PCR. Supernatants of spleen cell cultures were used for cytokines determination by ELISA. Propolis did not affect IL-2, IL-4 and IL-10 expression and production, but both basal and Con A-stimulated IFN- γ production were inhibited after its administration. Since IFN- γ is a pro-inflammatory cytokine, our data suggest that propolis administration over a short-term to mice may be associated with an anti-inflammatory effect *in vivo*.

Keywords:

Propolis

Cytokine

Immunomodulation

Real Time-PCR

1. Introduction

Propolis has gained popularity in alternative medicine, and it is used in food and beverages to improve health and prevent diseases. Propolis is a chemically complex bee product, containing material collected from buds or exudates of plants, volatile substances and wax (Bankova, 2005). *In natura* it is composed of 30% wax, 50% resins and vegetable balsams, 10% essential and aromatic oils, 5% pollen, and other substances (Burdock, 1998). Its color varies from green, red to dark brown. Propolis has a characteristic smell and shows adhesive properties because it strongly interacts with oils and proteins of the skin.

Plants visited by bees in our apiary were identified and the main vegetal source of our propolis samples is *Baccharis dracunculifolia* DC., followed by *Eucalyptus citriodora* Hook and *Araucaria angustifolia* (Bert.) O. Kuntze (Bankova *et al.*, 1999). Propolis possesses several biological properties, such as antibacterial, antifungal, antitumoral, anti-inflammatory, immunomodulatory, among others (Freitas, Shinohara, Sforcin & Guimarães, 2006; Girgin *et al.*,

2009; Hu, Hepburn, Li, Chen, Radloff & Daya, 2005; Murad, Calvi, Soares, Bankova & Sforzin, 2002; Orsi, Sforzin, Funari & Bankova, 2005; Sforzin, 2007)

Cytokines are glycoproteins involved in innate and adaptive responses, as well as in cell growth and differentiation, angiogenesis, and repair processes regarding homeostasis (Oppenheim, 2001). Effectors T cells are divided into T-helper type 1 and type 2 (Th1 and Th2 cells), according to their cytokines pattern: Th1 cells secrete predominantly interferon-gamma (IFN- γ) and interleukin (IL)-2, whereas Th2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 (Romagnani, 2006; Wan & Flavell, 2006). Th1 cells differentiation is regulated by the transcription factors STAT-4 and T-bet, which are different and antagonistic to those controlling the Th2 cells development: STAT-6 and GATA-3 (Szabo, Sullivan, Peng & Glimcher, 2003).

Th1 cells are crucial for cell-mediated immunity, whereas Th2 cells are involved in humoral responses. IFN- γ is a potent activator of mononuclear phagocytes, inducing the respiratory burst and enhancing microbe phagocytosis. IFN- γ promotes the differentiation of naïve CD4⁺ T cells to the Th1 subset and inhibits the proliferation of Th2 cells (Romagnani, 2006; Schinkel, 2003). IL-2 is responsible for the clonal expansion of T cells after antigen recognition, and the presence of IL-4 inhibits the development of Th1 cells (Skapenko, Niedobitek, Kalden, Lipsky & Schulze-Koops, 2004). IL-4-dominated immune responses may reflect the protective influence of Th2 cells during infection by gastrointestinal parasites (Choi & Reiser, 1998). IL-10 is an anti-inflammatory cytokine with downregulatory effects on macrophages. In a parasitic disorder, a paradigm was established linking a subpopulation of CD4⁺

T cells producing mainly IFN- γ to resistance, and CD4⁺ T cells producing mainly IL-4 and IL-10 to susceptibility (Scumpia & Moldawer, 2005).

The effect of natural products on cytokines and their mechanism of action provided a potential therapeutic modality for the treatment of different conditions (Spelman, Burns, Nichols, Winters, Ottersberg & Tenborg, 2006). Research on various herbs and natural products possessing immunomodulatory properties may be useful in reducing the risk of various diseases and cancers (Huang, Lin, Liao, Young & Yang, 2008). However, propolis' mechanisms of action are not fully elucidated and remain to be investigated.

Previous works of our laboratory showed that propolis administration over a short-term to animals led to better results concerning the immune system (Sforcin, 2007). Thus, this work aimed to investigate the expression and production of Th1 (IL-2 and IFN- γ) and Th2 (IL-4 and IL-10) cytokines after propolis administration to mice for three days.

2. Material and methods

2.1. Propolis extract

Propolis, collected in the Beekeeping Section, UNESP, 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 (120 mg/ml) (Sforcin, Orsi & Bankova, 2005). Propolis chemical composition was investigated using thin-

layer chromatography (TLC), gas chromatography (GC), and gas chromatography-mass spectrometry (GC-MS) analysis.

2.2. Animals and experimental groups

Male BALB/c mice aged between 8 and 12 weeks were kept in the Department of Microbiology and Immunology, UNESP, at 21-25 °C, with a 12 h /12 h light/dark cycle. Food and water were provided *ad libitum*.

Mice were divided into 2 groups (G1 and G2) of 10 animals each. G1 was considered as control, and received physiologic solution (NaCl 0.9%, 0.1 ml). G2 received ethanolic extract of propolis (200 mg/kg, 0.1 ml) for 3 consecutive days by gavage. Ethanol effects were also investigated, in order to observe a possible effect of propolis solvent. Immediately after the last treatment, animals were sacrificed using a CO₂ inhalation chamber. This work agreed with Ethical Principles in Animal Research adopted by Brazilian College of Animal Experimentation (COBEA) and was approved in May 5, 2007.

2.3. Th1/Th2 cytokines expression by real time-PCR

2.3.1. RNA isolation and cDNA synthesis

Spleens were removed and thirty mg were kept in 250 µl of RNA Safer (Omega Bio-tek, Inc. USA) at –80 °C until RNA isolation procedures. Total RNA was extracted with RNeasy Mini RNA Isolation Kit (GE – Healthcare, USA) following the manufacturer's instructions. After purification, RNA was treated with RQ1 Rnase-free Dnase (Promega, USA) for 30 min at 37 °C to avoid false-

positive results due to amplification of contaminating genomic DNA. Concentrations of total RNA were determined from the absorbance value of the samples at 230 nm. cDNA was synthesized from 1 µg of total RNA and was reverse-transcribed with 2 µl of random primer (250 ng/µl) in a final volume of 10 µl, and the mixture was incubated for 5 min at 70 °C. For each sample, the master mix was prepared with 8 µl of reaction buffer (Improm II 5x), 4.8 µl of MgCl₂ (25 mM), 2 µl of RNase Out (Invitrogen), 1µl of dNTP mix (20 mM), 1µl Improm TR II (Promega, USA) and 13.2 µl of nuclease-free water. To each sample, 16 µl of master mix were added and the mixture was incubated for 5 min at 25 °C, 60 min at 42 °C and 15 min at 70 °C. Each cDNA sample was stored at -20 °C.

2.3.2 Quantitative Real Time PCR

Primers were designed based on sequences retrieved from GeneBank using the IDTSciTools (<http://www.idtdna.com>) software and synthesized by Applied Biosystems (USA). The sequences of specific primers are listed in Table 1.

Primers for β-actin described in the literature (Yang & Glaser, 2002) were used: 5' – AAG TGT GAC GTT GAC ATC CGT AA – 3' (F) and 5' – TGC CTG GGT ACA TGG TGG TA – 3' (R). The PCR mixture consisted of 4 µl of cDNA, 0.4 µl of each primer (200 nM), 10 µl of 2X Power Sybr[®] Green PCR Master Mix (Applied Biosystems, USA) and 5.2 µl of sterile nuclease-free water for a final volume of 20 µl. The reaction conditions were as follows: 95 °C/10 min for initial denaturation, amplification for 40 cycles (95 °C for 15 s for denaturation, 60 °C for 1 min for annealing and extension), and to confirm the PCR product and

cycle of melting curve analysis at 95 °C for 15 s, 60 °C for 30 s and 95 °C for 15 s. Fluorescence data were collected during each annealing/extension step and threshold cycle numbers (C_T) were determined using ABI PRISM[®] 7300 Sequence Detector (Applied Biosystems, USA) and Software SDS version 1.2.3 ("Sequence Detection Systems" 1.2.3 – 7300 Real Time PCR System – Applied Biosystems, USA). Reaction mixtures with no cDNA were used as negative controls and all PCR assays were performed in duplicate.

The standard-curve was generated by performing serial dilutions of cDNA. To the smallest dilution of cDNA standard it was given the relative value 100 and the other 3 points were designated as 50, 25, and 12.5. Gene specific expression values were normalized to the expression values of β -actin (endogenous control) within each sample.

2.4. Th1/Th2 cytokines determination

2.4.1. Splenocyte cultures

After sacrifice, spleens were aseptically removed and cells were suspended at a concentration of 5×10^6 /ml in RPMI 1640 (Cultilab, Brazil) supplemented with 10% foetal calf serum, 1% L-glutamine, and cultured in flat-bottomed 24-well plates. Cells were cultured in triplicates (1 ml/well) and stimulated or not with concanavalin A (Con A - 10 μ g/ml) for 48h at 37 °C and 5% CO₂.

2.4.2 Cytokine determination

Supernatants of spleen cell cultures were collected and assayed for Th1 (IL-2 and IFN- γ) and Th2 (IL-4 and IL-10) cytokines by enzyme-linked immunosorbent assay (ELISA), according to manufacturer's instructions (BD Biosciences, USA). Briefly, a 96-well flat bottom Maxisorp microtiter plate (Nunc, USA) was coated with capture antibody specific for each cytokine. The plate was washed and blocked before 100 μ l of the supernatants and serially diluted specific standards were added to the respective wells. Following a series of washing, the captured 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 (Tan, Selvaratnam, Kananathan & Sam, 2006).

2.5. Statistical Analysis

Data were analyzed using Prism Graph Pad statistical software (Graph-Pad Software, Inc., USA). Significant differences between treatments were determined by Analysis of variance (ANOVA), followed by *t* test. Statistic significances were accepted when $p < 0, 05$.

3. Results

3.1. Propolis chemical composition

The main constituents of the sample collected in our apiary (UNESP, Campus of Botucatu) and investigated by TLC, GC and GC-MS analysis were:

flavonoids (kaempferid, 5,6,7-trihydroxy-3,4'-dimethoxyflavone, aromadendrine-4'-methyl ether); a prenylated *p*-coumaric acid and two benzopyranes: *E* and *Z* 2,2-dimethyl-6-carboxyethenyl-8-prenyl-2H-benzopyranes); essential oils (spathulenol, (2*Z*,6*E*)-farnesol, benzyl benzoate and prenylated acetophenones); aromatic acids (dihydrocinnamic acid, *p*-coumaric acid, ferulic acid, caffeic acid, 3,5-diprenyl-*p*-coumaric acid, 2,2-dimethyl-6-carboxy-ethenyl-8-prenyl-2H-1-benzo-pyran); di- and triterpenes, among others (Bankova, Boudourova-Krasteva, Popov, Sforcin & Funari, 1998).

3.2. *Th1 cytokines' expression and production*

Propolis administration to mice did not affect IL-2 expression and production by spleen cells ($p > 0.05$) (Fig. 1A; Table 1). Curiously, mRNA IFN- γ expression was not to detect in the experimental groups. IFN- γ basal production was significantly inhibited in propolis-treated mice ($p < 0.01$). In Con A-stimulated cultures, a decreased IFN- γ production was also seen in propolis-treated mice ($p < 0.01$) (Table 1).

Ethanol (propolis solvent) did not affect propolis action on Th1 cytokine expression and production.

3.3. *Th2 cytokines' expression and production*

No significant differences were seen in IL-4 and IL-10 expression after propolis administration to mice ($p > 0.05$) (Fig. 1B and 1C). With regards to Th2 cytokines production, IL-4 basal production was not detected in the experimental groups, and in Con A-stimulated cultures no significant differences were seen between propolis-treated mice and control group ($p > 0.05$) (Table

1). As to IL-10 production, no differences were seen in basal or Con A-stimulated production (Table 1).

Ethanol-treated mice also showed cytokines' expression and production similar to control.

4. Discussion

An efficient host defense against invading pathogenic microorganisms is achieved through the coordination of complex signaling networks that link the innate and adaptive responses. Upon interaction with antigen presenting cells such as dendritic cells, CD4⁺ T cells can differentiate into a variety of effector subsets: Th1 and Th2 cells, the more recently defined Th17 cells, and regulatory T (Treg) cells. Each T helper cell subset expresses its lineage-specific transcription factors and exclusive cytokines (Zhou, Chong & Littman, 2009). Th1 activation contributes to cell-mediated immunity whereas Th2 activation favors the humoral immune response (Das, Chen, Yang, Cohn, Ray & Ray, 2001). Th1/Th2 balance is a prerequisite for the functionality of immune system against infections.

Propolis immunomodulatory action has been widely investigated lately, both *in vitro* and *in vivo* (Sforcin, 2007). In the present study, IFN- γ gene expression was not detected, and one may speculate that it might have occurred under the threshold detection or previously to spleen removal. IFN- γ production was inhibited in propolis-treated mice. Propolis administration to mice for three days did not affect IL-2 expression and production. It has been reported that propolis inhibited human IL-2 production by T cells (Ansorge, Reinhold & Lendeckel, 2003). In a previous work of our group, propolis

administration for 3 days in the same concentration to stressed mice also inhibited both basal and Con A-stimulated IFN- γ production (Pagliarone, Missima, Orsatti, Bachiega & Sforcin, submitted). Likewise, propolis administration for 14 days to C57BL/6 mice inhibited IFN- γ and IL-2 production by spleen cells (Missima, Pagliarone, Orsatti, Araújo & Sforcin, submitted). It has been related that propolis decreases splenocyte proliferation both in the absence or presence of Con A (Sá-Nunes, Faccioli & Sforcin, 2003). Caffeic acid phenethyl ester is an active phenolic compound present in propolis, inhibiting human T-cell activation by targeting both NFAT and NF- κ B transcription factors, and inhibiting both IL-2 gene transcription and synthesis in stimulated T cells (Marquez, Sancho, Macho, Calzado, Fiebich & Munoz, 2004).

Taken together, these data point out to propolis anti-inflammatory activity. Flavonoids have an immunosuppressor effect on the lymphoproliferative response (You, Son, Chang, Kang & Kim, 1998) and on inflammatory cytokine production (Hodge, Hodge & Han, 2002). Since our propolis sample contains flavonoids in its chemical composition (Bankova *et al.*, 1998), they could be involved in the mechanisms by which propolis exerted its anti-inflammatory action, inhibiting IFN- γ production.

In this same model, we verified previously that propolis administration to mice for 3 days increased pro-inflammatory (IL-1 β and IL-6) cytokines production and stimulated the expression of Toll-like receptors (TLR-2 and TLR-4), suggesting that propolis may activate the initial steps of the immune response (Orsatti *et al.*, submitted). One may speculate that maybe some constituents of propolis could activate the cells of innate immunity, while others, e.g. flavonoids, could inhibit Th1 cells. Moreover, propolis compounds could

interact with different receptors in different cells, and this aspect deserves further investigation.

Propolis-treated mice showed no alteration in IL-4 expression and production. In contrast, it was observed that propolis inhibited IL-4 production by peripheral blood mononuclear cells and human T cells (Ansorge *et al.*, 2003). With respect to IL-10, currently considered a cytokine produced by Treg cells, there were no changes in its basal or Con A-stimulated production by propolis-treated mice. Propolis inhibited IL-10 production by peripheral blood mononuclear cells and human T cells (Ansorge *et al.*, 2003). Propolis also inhibited IL-10 production by spleen cells of BALB/c mice in an experimental model of asthma (Sy, Wu, Chiang, Wang & Wu, 2006).

Propolis and some of its constituents were shown to be capable of regulating lymphocyte growth via the Erk-2 signal pathway, suppressing pro-inflammatory and Th1 as well Th2 derived cytokines (Ansorge *et al.*, 2003). Our data reinforces these findings, and propolis administration over a short-term to mice affected IFN- γ production, what may be related to its anti-inflammatory properties. Since it is well established that cytokines orchestrate and perpetuate the chronic inflammatory features of several diseases, further investigation is still needed to understand the involvement of isolated compounds, synergistically or not, responsible for propolis anti-inflammatory action, as well as to check propolis efficiency in inflammatory diseases.

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Primers	Sequences 5' – 3'	Accession number
IL-2 Forward	CCC AAG CAG GCC ACA GAA TTG AAA	<u>X01772</u>
IL-2 Reverse	AGT CAA ATC CAG AAC ATG CCG CAG	<u>X01772</u>
IFN- γ Forward	AGA GGA TGG TTT GCA TCT GGG TCA	<u>M28381</u>
IFN- γ Reverse	ACA ACG CTA TGC AGC TTG TTC GTG	<u>M28381</u>
IL-4 Forward	AGA TGG ATG TGC CAA ACG TCC TCA	<u>MN_021283</u>
IL-4 Reverse	AAT ATG CGA AGC ACC TTG GAA GCC	<u>MN_021283</u>
IL-10 Forward	GGA CAA CAT ACT GCT AAC CGA C	<u>M37897</u>
IL-10 Reverse	TGG ATC ATT TCC GAT AAG GCT TG	<u>M37897</u>

Table 1. Sequences of primers for Th1 (IL-2 and IFN- γ) and Th2 (IL-4 and IL-10) cytokines.

Cytokines	Basal		Con A	
	G1	G2	G1	G2
IL-2	84.36	71.62	2175.00	2134.00
	± 18.36	± 20.61	± 91.33	± 119.30
IFN- γ	124.10	82.51 *	14870.00	10760.00 *
	± 36.37	± 19.85	± 3471.00	± 3056.00
IL-4	ND	ND	262.70	306.70
			± 79.17	± 83.41
IL-10	133.40	147.30	2674.00	2692.00
	± 32.39	± 38.64	± 937.10	± 571.30

Table 2. Th1 (IL-2 and IFN- γ) and Th2 (IL-4 and IL-10) basal production (pg/ml) by spleen cells or stimulated with Con A (10 μ g/ml) for 48 h. G1 = control; G2 = propolis. Data represent means and standard deviation of 10 animals. ND = not determined. * Significantly different from control ($p < 0.01$).

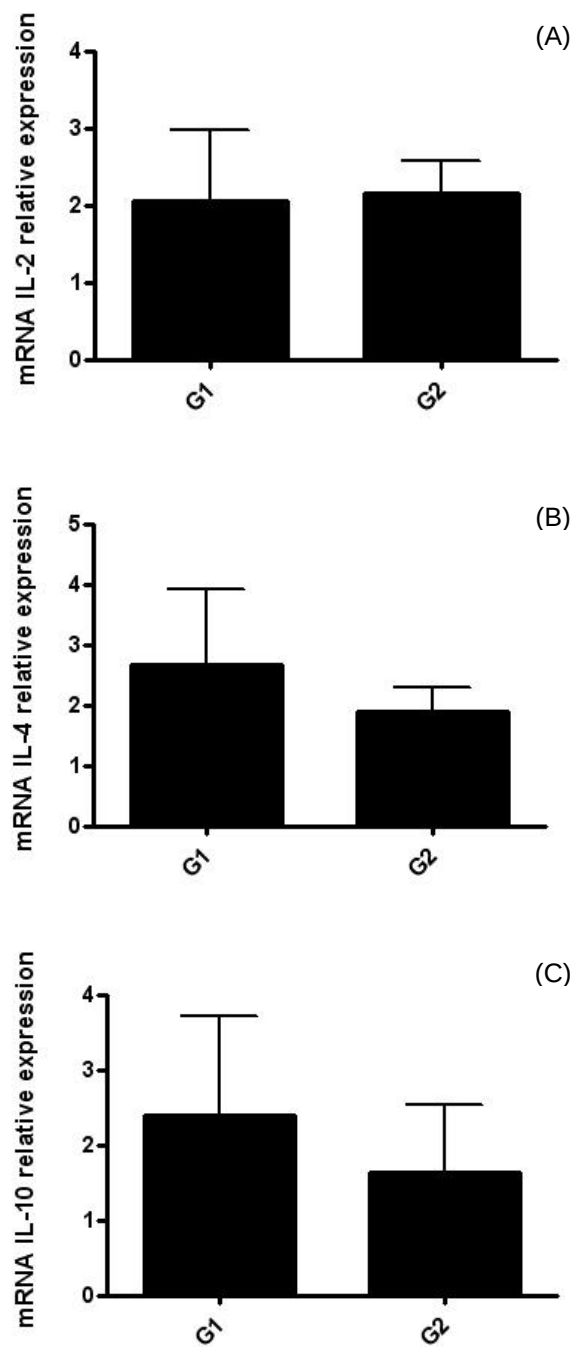


Fig. 1

Fig. 1. Relative quantification of mRNA IL-2 (A), IL-4 (B) and IL-10 (C) by real-time PCR in spleen cells. All results, normalized to β -actin, were expressed as arbitrary units relative to the value of 1.0. G1 = control; G2 = propolis.

Conclusões

A expressão dos receptores Toll-like pelos animais tratados com própolis esteve aumentada significativamente, sugerindo que a administração deste produto natural ativa esta importante via do sistema imune inato, tanto em macrófagos peritoneais como esplenócitos totais.

Outra evidência que reforça esta hipótese é o aumento da produção de das citocinas pró-inflamatórias (IL-1 β e IL-6), demonstrando indiretamente a possível ativação do fator de transcrição NF- κ B, e sugerindo a ação da própolis nas etapas iniciais da resposta imune inata.

Por fim, embora a administração de própolis a curto prazo a camundongos não afetou a expressão e produção de IL-2, IL-4 e IL-10, a produção basal e estimulada por Con A de IFN- γ foram inibidas após a administração deste produto apícola, sugerindo seu efeito antiinflamatório *in vivo*.

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