“UNIVERSIDADE ESTADUAL PAULISTA “JÚLIO DE MESQUITA FILHO”
FACULDADE DE MEDICINA
CAMPUS DE BOTUCATU

EFEITO DA PRÓPOLIS E DE COMPOSTOS ISOLADOS SOBRE A EXPRESSÃO DE RECEPTORES, PRODUÇÃO DE CITOCINAS E ATIVIDADE FUNGICIDA DE MONÓCITOS HUMANOS

MICHELLE CRISTIANE BUFALO

Tese apresentada ao Programa de Pós-Graduação em Patologia da Faculdade de Medicina - Universidade Estadual Paulista - UNESP, como parte dos requisitos para obtenção do título de Doutor em Patologia.

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Palavras-chave: Ácido cafeico; Citocinas; Fator de transcrição nuclear; Monócitos; Própolis.

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Revisão da Literatura

Revisão da Literatura

1. Própolis

1.1. Definição, origem e composição química

Própolis é um termo genérico utilizado para denominar o material resinoso e balsâmico, coletado e processado pelas abelhas a partir de diversas partes das plantas como brotos, ramos, cascas de árvores, exsudatos resinosos e botões florais (BANKOVA, 2005). A palavra própolis é proveniente do grego, sendo que o prefixo "pro" significa "em defesa", enquanto que o sufixo "polis" significa "cidade" (GHISALBERTI, 1979). As abelhas utilizam a própolis para a construção e manutenção da colméia, sendo empregada para selar aberturas e controlar variações de temperatura no interior da mesma, principalmente no inverno; também é utilizada para embalsamar insetos invasores que morrem no interior da colméia, evitando sua decomposição, além de contribuir para manter o ambiente interno asséptico, protegendo-o contra bactérias e vírus (SALATINO *et al.*, 2005).

Várias espécies são consideradas fontes vegetais da própolis. No Brasil, sugere-se que espécies dos gêneros *Araucaria*, *Clusia*, *Baccharis, Vernonia*, *Weinmania, Diclenia*, *Hyptis*, *Eucalyptus, Populus*, dentre outras, são visitadas pelas abelhas para a coleta da resina, dependendo da região geográfica do país (BANSKOTA *et al.,* 1998; PARK *et al*., 2002; SANTOS *et al*., 2003). Na região de Botucatu, sudeste brasileiro, *Araucaria angustifolia* (Bertolini) Otto Kuntze ("pinheiro-do-Paraná"), *Baccharis dracunculifolia* D.C. ("alecrim do campo" ou "vassourinha") e *Eucalyptus citriodora* Hook são importantes fontes vegetais da própolis (BANKOVA *et al*., 1999). De acordo com TEIXEIRA *et al*. (2005), as abelhas africanizadas (*Apis mellifera* L.) possuem preferência por *B. dracunculifolia* como fonte vegetal de própolis no sudeste do Brasil.

A composição química da própolis é considerada extremamente complexa e seus componentes químicos variam dependendo da origem vegetal. Em geral, a própolis é composta de 50% de resinas e bálsamos, 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 e vermelho escuro, passando pelo verde e marrom, dependendo de fatores, como a sua origem vegetal e composição química. Nesse sentido, a cor verde característica de algumas amostras de própolis brasileira é uma conseqüência de sua origem botânica e de relativo significado (SALATINO *et al.*, 2005).

Análises químicas indicam que este produto apícola possui mais de 300 componentes diferentes e, destes, os flavonóides são considerados os principais compostos responsáveis

pelas atividades antimicrobianas e demais propriedades farmacológicas da própolis européia, a qual tem, como origem botânica, principalmente as plantas do gênero *Populus*, enquanto que 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).

O método de preparo dos extratos de própolis também pode influenciar na separação de seus compostos químicos bem como sua atividade biológica, uma vez que os diferentes solventes solubilizam e extraem diferentes compostos. Os extratos mais utilizados experimentalmente são os etanólicos, em diferentes concentrações, metanólico e o aquoso. 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).

1.2. Propriedades biológicas da própolis

A própolis tem despertado o interesse da comunidade científica nos últimos anos, por apresentar inúmeras propriedades biológicas, tais como antimicrobriana, antitumoral, imunomoduladora, entre outras; bem como por seu potencial para o desenvolvimento de novas drogas (SFORCIN, 2007; SFORCIN & BANKOVA, 2011).

1.2.1. Atividade antimicrobiana

As atividades antimicrobianas da própolis tem sido documentadas através de ensaios que comprovam sua eficácia sobre diferentes bactérias (SFORCIN *et al*., 2000, MAVRI *et al*., 2012), fungos (SFORCIN *et al*., 2001, CIGUT *et al*., 2011), vírus (GEKKER *et al*., 2005; BUFALO *et al*., 2009b) e parasitas (FREITAS *et al*., 2006; GRESSLER *et al*., 2012). *In vitro*, a própolis pode atuar diretamente sobre os microrganismos, e *in vivo*, pode preferencialmente estimular o sistema imunológico, ativando os mecanismos efetores envolvidos na destruição de microrganismos (SFORCIN, 2007).

Estudos realizados em nosso laboratório confirmaram a eficiente ação inibidora da própolis, *in vitro*, sobre várias linhagens de bactérias Gram-positivas, como *Staphylococcus aureus* e limitada ação contra bactérias Gram-negativas, como *Escherichia coli* e *Pseudomonas aeruginosa* (SFORCIN *et al.*, 2000). Dados do nosso laboratório confirmaram também a atividade anti-parasitária da própolis sobre *Giardia duodenalis* (FREITAS *et al.*, 2006) e a ação antifúngica sobre *Candida albicans* e *Candida tropicalis* (SFORCIN *et al.*,

2001). Verificamos a eficiente ação da própolis e de extratos obtidos de *B. drancunculifolia* sobre a replicação do poliovírus tipo 1 (BÚFALO *et al.*, 2009b). Recentemente, outros estudos demonstraram a atividade anti-parasitária da própolis sobre *Trypanossoma cruzi*, agente etiológico da doença de Chagas, o qual apresentou-se sensível à ação deste produto apícola *in vitro* (SALOMÃO *et al*., 2011).

A própolis pode também apresentar efeitos sinérgicos com drogas antimicrobianas e sua associação a drogas comercialmente disponíveis é um campo de interesse para o desenvolvimento de novos produtos pela indústria farmacêutica. OKSUZ *et al*. (2005) verificaram atividade sinérgica entre ciprofloxacina e própolis no tratamento de ceratite experimental causada por *Staphylococcus aureus*. ORSI *et al*. (2012a) demonstraram que a própolis diminuiu a resistência da parede bacteriana a antibióticos (amoxicilina, ampicilina e cefalexina) e apresentou efeitos sinérgicos com antibióticos que atuam sobre o ribossomo (cloranfenicol, tetraciclina e neomicina); no entanto, não houve ação sinérgica da própolis com antibióticos que atuam sobre o DNA (ciprofloxacina e norfloxacina) e ácido fólico (cotrimoxazol) (ORSI *et al*., 2012b). Em conjunto, estes dados sugerem que a própolis pode potencializar o efeito de alguns antibióticos, particularmente aqueles com ação na parede e no ribossomo bacterianos.

Diversos trabalhos têm sugerido que os principais compostos responsáveis pela atividade antimicrobiana da própolis são os flavonóides e ésteres de ácidos fenólicos (SFORCIN *et al*., 2000; CASTALDO & CAPASSO, 2002; PIETTA *et al*., 2002). UZEL *et al*. (2005) verificaram que alguns flavonóides (pinocembrina e galangina) e o éster fenetílico do ácido cafeico (CAPE) induziram inibição da RNA polimerase bacteriana. Outros flavonóides e os ácidos caféico, benzóico e cinâmico atuam na membrana ou parede celular do microrganismo, causando danos funcionais e estruturais (SCAZZOCCHIO *et al*., 2005).

1.2.2. Atividade antitumoral

A própolis apresenta atividade contra diferentes células tumorais *in vitro* e *in vivo* (WATANABE *et al*., 2011), e os principais mecanismos pelos quais a própolis afeta estas células estão relacionados com a inibição do crescimento celular durante o ciclo celular, à apoptose e interferência nas vias metabólicas (SFORCIN, 2007; WATANABE *et al*., 2011; SAWICKA *et al*., 2012).

Trabalhos experimentais do nosso laboratório confirmaram a atividade antitumoral da própolis, *in vitro*, sobre células de tumor venéreo transmissível (TVT) canino, células de carcinoma de laringe humano (HEp-2) e células de osteossarcoma canino (OSA), de forma

tempo-concentração-dependente (BASSANI-SILVA *et al*., 2007; BÚFALO *et al*., 2009a, CINEGAGLIA *et al*. (no prelo). A atividade antiproliferativa de constituintes isolados da própolis sobre células tumorais também tem sido avaliada. Derivados do ácido cinâmico (bacarina e drupanina), flavonoides e outros compostos fenólicos foram avaliados sobre células de leucemia (HL-60), demonstrando seu efeito citotóxico *in vitro* (MORENO *et al*., 2005). Os flavonóides despertam a atenção dos pesquisadores por afetar a proliferação, diferenciação e induzir apoptose de células cancerígenas (HAVSTEEN, 2002).

ORSOLIC *et al*. (2005) relataram que a ação antitumoral da própolis poderia ser devida ao sinergismo de seus constituintes. Além disso, a ação da própolis poderia ser comparada com drogas anti-tumorais ou ser associada a elas, a fim de investigar uma possível ação sinérgica. Entretanto, a maioria dos trabalhos nesta área foi realizada utilizando ensaios *in vitro*, avaliando a ação direta sobre diferentes linhagens tumorais. É importante ressaltar que a administração de produtos apícolas a animais ou humanos é seguida de sua absorção, biotransformação e biodisponibilidade, e a ação antitumoral dos mesmos *in vivo* pode ocorrer devido à sua ação imunomoduladora, exercendo efeitos terapêuticos.

De acordo com ORSOLIC *et al*. (2006), a propriedade antitumoral da própolis e de alguns de seus constituintes pode ser associada com sua ação imunomoduladora *in vivo*, principalmente devido a ativação de macrófagos, que por sua vez poderiam produzir fatores solúveis e interferir diretamente nas células tumorais ou nas funções das células imunes.

A resposta imune celular mediada por linfócitos T ativados exerce um importante papel na eliminação de células tumorais, havendo aumento de linfócitos infiltrados em tumores associado à redução da progressão dos mesmos em pacientes com câncer colo-retal ou melanoma. Nos últimos anos, diferentes estratégias para estimular a imunidade antitumoral têm sido estudadas em humanos; entretanto, ainda não foi desenvolvida uma imunoterapia consistentemente efetiva contra nenhum tipo de malignidade. Ademais, há mecanismos de escape dos tumores frente à imunidade inata ou adaptativa (WATANABE *et al*., 2011). Este apiterápico, bem como seus compostos isolados, apresentam eficiente ação contra diferentes células tumorais *in vitro* e *in vivo*, sugerindo seu potencial para o desenvolvimento de novas drogas antitumorais (SFORCIN & BANKOVA, 2011).

1.2.3. Atividade imunomoduladora

A própolis tem sido bastante investigada quanto à sua ação nas respostas imune e inflamatória.

A inflamação é uma resposta biológica envolvendo muitos processos fisiopatológicos que ocorrem devido a injúria tecidual e como parte da defesa do hospedeiro contra microrganismos, caracterizada por alterações bioquímicas e vasculares, infiltração de diversos tipos de leucócitos e posterior remodelamento do tecido inflamado. A inflamação é classicamente dividida em dois tipos: aguda e crônica. A inflamação aguda é uma resposta a curto prazo e frequentemente resulta em cura devido à remoção do estímulo e reparação do tecido. Por outro lado, a inflamação crônica é uma resposta prolongada e persistente, que envolve destruição do tecido e pode ser associada a muitas doenças como por exemplo, doenças autoimunes (BRODSKY *et al*., 2010).

No processo inflamatório, os macrófagos têm importante papel na defesa contra patógenos invasores e, uma vez ativados por estímulos inflamatórios como, por exemplo, lipopolissacárideo (LPS), produzem uma grande variedade de mediadores pró-inflamatórios, incluindo prostaglandina E_2 (PGE_2), óxido nítrico (NO), citocinas como TNF-α e IL-1, entre muitos outros (GELLER & BILLIAR, 1998). A PGE_2 é sintetizada pela enzima ciclo-oxigenase (COX), enquanto que o NO é sintetizado pela enzima óxido nítrico sintase (NOS). Macrófagos ativados com LPS expressam a NOS induzível (iNOS), que produz altas quantidades de NO a partir da L-arginina. A produção de NO pela iNOS contribui para seu efeito antimicrobiano; por outro lado, quantidades aumentadas podem estar envolvidas na fisiopatologia de muitas doenças inflamatórias (GUZIK *et al*., 2003). A expressão de moléculas pró-inflamatórias é regulada por vários fatores de transcrição e vias de sinalização, e dentre estas vias, proteínas quinase ativadas por mitógenos (MAPKs) são moléculas sinalizadoras que possuem importante papel na regulação do crescimento, diferenciação, sobrevivência e apoptose celular, na resposta celular a citocinas e estresse. A via das MAPKs inclui p38 MAPK (HAN *et al*., 1994), c-Jun N-terminal kinase (JNK) e quinase regulada por sinal extracelular (ERK) (DAVIS, 1994) que estão envolvidos na expressão de COX_2 e iNOS em macrófagos ativados por LPS (TSATSANIS *et al*., 2006). MAPKs têm importante papel na ativação do fator de transcrição nuclear NF-κB, o qual por sua vez regula a expressão de genes envolvidos nas respostas imune e inflamatória (CARTER *et al*., 1999). O NF-κB é formado pelas proteínas da família Rel: p50, p52, p65 (Rel A), c-Rel e RelB, sendo que o heterodímero mais conhecido e mais estudado é composto pelas subunidades p50 e p65. Este composto está presente no citoplasma na forma inativa e associado à proteína inibitória κB (IκB). Muitos estímulos como LPS, citocinas e oxidantes ativam este complexo, induzindo a fosforilação e degradação de IκB e permitindo a translocação do NF-κB para o núcleo, onde

ativará a transcrição de genes alvos envolvidos na resposta imune e inflamatória, incluindo COX_2, iNOS e TNF-α, entre outros (GELLER & BILLIAR, 1998; BRODSKY *et al*., 2010). A inibição da atividade de fatores de transcrição sugere o possível desenvolvimento de novos agentes com propriedades anti-inflamatórias.

Diversos pesquisadores têm observado o efeito imunomodulador da própolis ou de seus constituintes frente à produção de fatores envolvidos no processo inflamatório, como citocinas, prostaglandinas, quimiocinas, entre outros, em diferentes modelos experimentais (KHAYYAL *et al*, 1993; HU *et al.*, 2005; PAULINO *et al.*, 2006). A própolis apresentou efeito inibitório sobre a proliferação de esplenócitos, o que pode ser atribuído a presença dos flavonóides (SÁ-NUNES *et al.*, 2003). ANSORGE *et al.* (2003) verificaram que a própolis suprime a síntese de DNA de células mononucleares do sangue periférico (PBMC) e células T purificadas, e os efeitos foram mediados pelo CAPE, quercetina e hesperidina. O CAPE apresentou efeitos inibitórios sobre os fatores de transcrição-κB (NF-κB) e fator nuclear das células T ativadas (NFAT) (MÁRQUEZ *et al.*, 2004), e, como consequência, inibiu também a transcrição do gene da IL-2, seu receptor (IL-2R - CD25) e a proliferação de células T humanas, fornecendo novas informações sobre os mecanismos moleculares envolvidos nas atividades anti-inflamatória e imunomoduladora deste composto natural. Entretanto, IVANOVSKA *et al*. (1995) verificaram que esplenócitos de camundongos tratados com ácido cafeico – constituinte isolado da própolis, apresentaram capacidade proliferativa. Além disso, a concentração de IL-1β no soro dos animais tratados com ácido cinâmico apresentou-se elevada, sugerindo que este componente da própolis poderia ativar macrófagos, que por sua vez poderiam estimular linfócitos, desencadeando os eventos iniciais da resposta imune adaptativa.

HU *et al*. (2005) observaram que extratos de própolis diminuíram a intensidade da resposta inflamatória através da inibição da produção de prostaglandina e de NO. O efeito anti-inflamatório da própolis sobre a produção de NO foi observado por outros autores, em camundongos com edema de pata (TAN-NO *et al*., 2006). Outro mecanismo pelo o qual a própolis pode conter a inflamação seria através da diminuição da produção de citocinas pró-inflamatórias (por exemplo, IL-1β), conforme observado por ANSORGE *et al*. (2003) em culturas de células mononucleares do sangue periférico (PBMC) e de linfócitos T humanas, seguida por alta produção da citocina anti-inflamatória TGF-β.

A administração de própolis a curto prazo (3 dias) a camundongos inibiu a produção de IFN-γ por esplenócitos (ORSATTI *et al.*, 2010b). Além disso, camundongos C57BL/6

tratados com própolis durante 14 dias apresentaram inibição na produção de IL-1β, IL-6, IFN-γ e IL-2 por células esplênicas, sugerindo sua ação anti-inflamatória, uma vez que está bem estabelecido que as citocinas orquestram e perpetuam as doenças inflamatórias crônicas (MISSIMA *et al.*, 2009; MISSIMA *et al.*, 2010).

Os efeitos anti-inflamatórios de compostos isolados da própolis também têm sido investigados. O CAPE inibiu a produção de leucotrieno e de prostaglandina via inibição da ciclooxigenase (COX), além de impedir que o fator de transcrição nuclear NF-κB se ligue ao DNA, levando à supressão da expressão gênica de citocinas inflamatórias (NATARAJAN *et al*., 1996). SHIN *et al*. (2004) investigaram os efeitos anti-inflamatórios do ácido cafeico e do CAPE sobre a produção de PGE_2 e NO induzidas pelo LPS em linhagem celular de macrófagos murinos Raw 264.7. O CAPE inibiu a expressão de iNOS, COX-2 e TNF-α, afetando a ativação do fator de transcrição NF-κB induzida pelo LPS, demonstrando assim sua propriedade anti-inflamatória neste modelo experimental.

Embora a própolis apresente ação anti-inflamatória, dependendo do modelo experimental e das condições adotadas, este produto também apresenta ação estimuladora. Com relação à imunidade inata, a administração de extrato etanólico de própolis a camundongos durante 3 dias influenciou as etapas iniciais da resposta imune, estimulando a expressão de *Toll-like receptors* (TLR-2 e TLR-4) e a produção de citocinas pró-inflamatórias (IL-1β e IL-6) por macrófagos e células esplênicas, contribuindo assim para o reconhecimento de microrganismos e para a possível ativação de linfócitos pelas células apresentadoras de antígenos (ORSATTI *et al*., 2010a). A própolis também induziu aumento na geração de peróxido de hidrogênio (H_2O_2), favorecendo a eliminação de microrganismos (ORSI *et al*., 2000). O aumento da produção de H_2O_2 por macrófagos peritoneais também foi evidenciado quando estas células foram incubadas com extratos de *B. dracunculifolia* e alguns de seus compostos isolados, como óxido de *Baccharis* e friedelanol, sugerindo a ação ativadora de extratos e compostos isolados desta planta sobre macrófagos (MISSIMA *et al.*, 2007). Avaliamos também o efeito da própolis sobre a atividade microbicida de macrófagos, verificando que este apiterápico induziu 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).

A própolis pode exercer também importante ação na indução da resposta imune humoral. Ratos tratados com própolis apresentaram maior produção de anticorpos, não

havendo efeito de compostos isolados ou do extrato *B. dracunculifolia* neste ensaio (SFORCIN *et al.*, 2005). A administração de CAPE a camundongos BALB/c também induziu aumento da produção de anticorpos (PARK *et al*., 2004), e o efeito sinérgico de compostos pode ser responsável pelas diferentes atividades farmacológicas da própolis. Estes dados sugerem fortemente a capacidade adjuvante da própolis, em associação com as vacinas. Como exemplo, FISCHER *et al*. (2007) associaram a própolis à vacina contra herpesvírus Suid 1 (SuHV-1), verificando-se que os camundongos inoculados com SuHV-1, hidróxido de alumínio e própolis apresentaram títulos de anticorpos mais elevados em relação ao controle.

BACHIEGA *et al*. (2012) investigaram o efeito da própolis e de compostos isolados sobre a produção de citocinas pró- e anti-inflamatórias (IL-1β, IL-6 e IL-10) por macrófagos peritoneais de camundongos BALB/c, em protocolos de desafio com LPS. A própolis e os ácidos cinâmico e *p*-cumárico estimularam a produção de IL-1β, enquanto que, a produção de IL-6 e IL-10 foram inibidas, mesmo em presença do LPS, demonstrando que a própolis pode exercer ações pró- e anti-inflamatória, modulando a resposta imune e inflamatória dependendo da concentração utilizada.

Com base nestes achados, conclui-se que a própolis pode exercer tanto ação pró- como anti-inflamatória, dependendo do modelo experimental, da concentração utilizada, período de ingestão, entre outros fatores. O caráter pró-inflamatório da própolis poderia ser aproveitado na terapêutica vacinal, devido ao seu papel adjuvante e à sua reconhecida ação na resposta imune humoral, conferindo consequentemente maior produção de anticorpos. Por outro lado, o perfil anti-inflamatório da própolis poderia ser explorado no tratamento de doenças inflamatórias, minimizando reações indesejáveis e estabelecendo possíveis interações da própolis a outras terapias, visando sua aplicação em humanos ou animais.

2. Monócitos, macrófagos da linhagem Raw 264.7 e linhagem celular de carcinoma hepático humano (HepG-2)

Neste projeto, a célula escolhida para investigar os efeitos da própolis nos eventos iniciais da resposta imune em humanos foi o monócito, avaliando a expressão de marcadores de superfície celulares (TLR-2, TLR-4, HLA-DR e CD80), a produção de citocinas (TNF-α e IL-10) e a atividade fungicida contra *Candida albicans*.

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. 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). Estas células podem ser ativadas após interação com microrganismos ou seus produtos, como endotoxinas e componentes da parede celular, ou com receptores de superfície celular. Neste contexto, os receptores semelhantes a Toll (TLR) foram uma das descobertas recentes mais importantes no campo da Imunologia devido à função que exercem na resposta imune. Estes receptores têm a capacidade de reconhecer diferentes microrganismos patogênicos e, quando ativados por estes, desencadear vias de sinalização intracelular que resultam na indução da resposta imune inata através da produção de citocinas e ativação celular (TAKEDA *et al.*, 2003).

TLRs pertencem à família dos receptores transmembrânicos tipo I, caracterizados por um domínio extracelular e um domínio intracelular. O domínio extracelular é composto por uma cadeia rica em leucina (domínio LRR) que está relacionada ao reconhecimento do microrganismo e com a transdução do sinal, além de ser também necessária para a dimerização do receptor (KOBE & KAJAVA, 2001). Já o domínio intracelular, denominado Toll/receptor IL-1 (TIR), tem a função de mediar interações entre o TLR e proteínas intracelulares envolvidas na transdução do sinal (ARMANT & FENTON, 2002).

Estes receptores reconhecem microrganismos 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 tanto em microrganismos patogênicos ou não, fazendo com que os TLRs sejam capazes de distinguir o próprio do não-próprio (MEDZHITOV, 2001), embora este mecanismo ainda seja desconhecido. TLRs reconhecem um diverso, porém limitado, número de PAMPs. Como exemplo, TLR-2 reconhece uma grande quantidade de produtos microbianos, como peptidioglicanos, LPS de bactérias Gram-positivas, 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 o 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 flagelina bacteriana (TAKEDA & AKIRA, 2005).

Os TLRs são agrupados em uma mesma família segundo as suas características estruturais e similaridade sequencial. Há 10 TLR identificados em humanos e 12 em camundongos (MUZIO *et al*., 2012) e são expressos em diferentes locais da célula. TLR-1/2/4/5/6 e 10 encontram-se na superfície celular, enquanto os TLR-3/7/8 e 9 são expressos em membranas de compartimentos intracelulares (NICHOLAS *et al.*, 2006). Estes receptores estão presentes tanto em células imunes como macrófagos, células dendríticas, linfócitos T e B e células NK, como também em células endoteliais e epiteliais (SUTMULLER *et al.*, 2006).

Conforme já mencionado, tais receptores têm a capacidade de reconhecer microrganismos e ativar vias de transdução de sinal, como a proteína quinase ativada por mitógeno (MAPK), culminando na ativação de fatores de transcrição como o ativador proteico 1 (AP-1), NF-κB e NFAT, os quais podem ser responsáveis pela expressão de genes envolvidos na resposta imune, como citocinas, quimiocinas, moléculas de histocompatibilidade, como o HLA-DR, e moléculas co-estimulatórias, como CD80 (B7-1), que liga-se ao CD28, sendo importante para ativação das células T (CARTER *et al*., 1999; MEDZHITOV, 2001). Há receptores para um grande número de citocinas na superfície celular, estando a maioria deles expressa em baixa densidade, embora o número possa aumentar quando as células são ativadas. Individualmente ou em combinação, as citocinas interagem com seus receptores específicos, modulando a função celular. Citocinas como o 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.

Os TLRs reconhecem diversos microrganismos, dentre eles, a *Candida albicans*, patógeno mais comumente encontrado na microbiota normal das superfícies da mucosa oral, gastro-intestinal e genito-urinária de indivíduos saudáveis. Em condições normais, este fungo não causa doença, porém, quando as defesas imunes estão comprometidas, o balanço da microflora normal é desregulado e a *C. albicans* transforma-se em um patógeno oportunista. O mecanismo de defesa contra a candidíase sistêmica envolve principalmente o reconhecimento do fungo através dos receptores presentes na superfície celular, seguido da ingestão por células envolvidas na imunidade inata, como monócitos, macrófagos e neutrófilos. Após ativação destas células por este fungo, ocorre a liberação de citocinas como TNF-α, IL-1β e IL-6, bem como geração de intermediários reativos do oxigênio e do nitrogênio, eventos importantes que auxiliam na fagocitose. Ambos TLR-2 e TLR-4 têm sido

implicados na defesa do hospedeiro contra *C. albicans* através do reconhecimento de ligantes presentes na membrana celular deste fungo: fosfolipomanana é reconhecido pelo TLR-2, e o TLR-4 interage com resíduos ligados a O-manosil (GASPAROTO *et al*., 2010).

Além da realização dos ensaios com monócitos neste projeto, demos continuidade à esta linha de pesquisa avaliando os efeitos da própolis e do ácido cafeico em cultura de macrófagos da linhagem Raw 264.7 de camundongo, na Universidade de Coimbra (UC), Portugal, durante o estágio de doutoramento Sanduíche no Exterior. Estas células são derivadas de camundongos BALB/c e transformadas pelo vírus da leucemia murina de Abelson, utilizada como modelo celular no Centro de Neurociências e Biologia Celular da UC, para ensaios de avaliação das propriedades anti-inflamatórias de produtos naturais através da dosagem de NO e outros mediadores, e para o estudo das vias de sinalização intracelular. Inicialmente, foi avaliada a citotoxicidade da própolis e do ácido cafeico pelo método colorimétrico do brometo de 3-[4,5-dimetil-tiazol-2-il]-2,5-difeniltetrazólio (MTT), para exclusão de concentrações que afetassem a viabilidade celular. Em seguida foram avaliados os efeitos da própolis e do ácido cafeico sobre a produção do NO induzida pelo LPS. A partir destes dados foram escolhidas as concentrações que apresentassem o melhor efeito inibitório na produção do NO, para realização dos ensaios das vias de sinalização intracelulares relacionadas ao processo inflamatório (NF-κB e MAPKs).

Visando também uma maior aplicabilidade deste estudo, foi analisado o possível efeito citotóxico da própolis e de seu constituinte isolado sobre a linhagem celular de carcinoma hepático humano (HepG-2) - modelo utilizado nos ensaios *in vitro* para determinar hepatotoxicidade, uma vez que os produtos naturais são consumidos pela população. Ademais, no laboratório de Farmacognosia da Faculdade de Farmácia da UC foram realizados ensaios sobre a atividade antioxidante da própolis, bem como o estudo fitoquímico da composição química da mesma.

3. Justificativa do trabalho

Neste trabalho, investigamos inicialmente a hipótese de que a própolis poderia modular a resposta imunológica do hospedeiro contra doenças infecciosas, avaliando seu efeito sobre monócitos humanos, através da expressão de receptores importantes para o reconhecimento de patógenos bem como de receptores envolvidos na apresentação de antígenos e ativação de linfócitos T (TLR-2, TLR-4, HLA-DR, CD80). Em seguida, investigamos a produção de citocinas pró- e anti-inflamatórias (TNF-α e IL-10,

respectivamente), para compreender melhor a ação deste apiterápico nos eventos iniciais da resposta imune. Finalmente, analisamos a ação candidacida da própolis, visando correlacioná-la com os objetivos iniciais desta pesquisa. Para verificar o possível envolvimento de compostos responsáveis pela ação da própolis, foi investigado ácido cafeico nos mesmos ensaios.

Esta tese encontra-se apresentada sob a forma de capítulos a serem submetidos à publicação. O Capítulo 2 faz parte dos objetivos inicias da tese de doutorado e será submetido à publicação junto a revista *International Immunopharmacology*, sendo intitulado: **"The immunomodulatory effect of propolis and caffeic acid on receptors expression, cytokines production and fungicidal activity of human monocytes".**

Após a realização dos objetivos iniciais, fomos contemplados com a bolsa de Doutorado-Sanduíche no Exterior (PDSE-CAPES, processo nº: 5737-11-7) e permaneci 7 meses (fevereiro a agosto de 2012) nos laboratórios de Farmacognosia e no Centro de Neurociências e Biologia Celular da Universidade de Coimbra – Portugal, para o estudo da caracterização química da própolis por técnicas cromatográficas, e para avaliar o efeito da própolis e do ácido cafeico nas vias de sinalização intracelular, como quinases ativadas por agentes mitogênicos (MAPKs) e fator de transcrição nuclear (NF-κB) em células da linhagem Raw 264.7, na tentativa de compreender um possível mecanismo de ação da própolis bem como de um composto isolado.

Os dados obtidos após a realização deste estágio estão apresentados no Capítulo 3, intitulado **"Propolis and caffeic acid suppress LPS-stimulated pro-inflammatory response by blocking NF-κB and MAPK activation in macrophages",** a ser submetido à publicação junto ao *Journal of Ethnopharmacology*.

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Capítulo II

Artigo Científico

The immunomodulatory effect of propolis and caffeic acid on receptors expression, cytokines production and fungicidal activity of human monocytes

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Abstract

Propolis is a beehive product and its immunomodulatory action has been documented; however, little is known concerning its effect on human cells. The aim of this work was to investigate a possible role of propolis on the initial events of the immune response, assessing the expression of surface markers (TLR-2, TLR-4, HLA-DR and CD80), pro- and anti-inflammatory cytokines production (TNF-α and IL-10) and the candidacidal activity of human monocytes. The role of TLR-2 and TLR-4 on cytokines production and fungicidal activity induced by propolis was also investigated. Caffeic acid was investigated as a possible compound responsible for propolis action. Data showed that propolis upregulated TLR-4 and CD80 expression, whereas caffeic acid downregulated TLR-2 and HLA-DR expression. TNF-α and IL-10 production were stimulated by low concentrations of propolis and inhibited by higher ones. On the other hand, caffeic acid exerted an inhibitory effect on both cytokines production at all concentrations. The fungicidal activity against *Candida albicans* was increased using high concentrations of propolis and caffeic acid. The effect of propolis and caffeic acid on cytokine production was decreased by blocking TLR-4 whereas the fungicidal activity was affected by blocking TLR-2. In conclusion, propolis exerted an immunomodulatory action on human monocytes depending on concentration and TLR-2 and TLR-4 may be involved in propolis action. Caffeic acid seemed to be partially involved in propolis action.

1. Introduction

Propolis is a natural resinous product collected by bees from various parts of the plants such as buds, bark and resinous exudates [1]. More than 300 different components have been identified in propolis composition, what may vary according to the geographical regions and botanical sources [2]. Used for medicinal purposes since ancient times, this natural product has shown to possess many biological properties, including antimicrobial, antiviral, antifungal, anti-inflammatory, antitumoral and immunomodulatory, among others [3-9].

With regards to its immunomodulatory action, previous works from our laboratory have shown that propolis modulated the early events of immune response, inducing the expression of Toll-like receptors (TLR-2 and TLR-4) and the production of pro-inflammatory cytokines by peritoneal macrophages of mice [10]. Propolis also increased hydrogen peroxide (H_2O_2) generation [11] and cytokines production by macrophages [12]. *In vitro* assays demonstrated the modulatory action of propolis on murine peritoneal macrophages, increasing their microbicidal activity against *Paracoccidioides brasiliensis* [13] and *Salmonella* Typhimurium, involving oxygen and nitrogen intermediates [14]. In immunosupression models, administration of a water soluble derivative of propolis to mice prevented the cyclophosphamide effects and enhanced the survival rate of the animals, suggesting that propolis modulates the innate immunity via macrophage activation [15]. Herein, we proposed to evaluate the immunomodulatory action of propolis on the initial events of the immune response of human monocytes.

The immunomodulatory effect of propolis on human peripheral blood mononuclear cells (PBMC) may occur mainly due to its action on monocytes and its receptors that recognize pathogens [9,16]. The ability of the cells of innate immunity to recognize microbial products known as pathogen-associated molecular patterns (PAMPs) and to eliminate invading microbial pathogens has been largely attributed to Toll-like receptors (TLRs). TLR are expressed by antigen-presenting cells (APCs) such as monocytes/ macrophages, dendritic cells (DCs) and B cells, as well as by neutrophils, T cell and NK cells, and have an important role in the induction of adaptive immunity. The involvement of TLRs on fungi recognition has been described for *Candida albicans*, inducing the production of different types of cytokines [17]. TLR-2 recognizes phospholipomannan and stimulates the production of moderate amounts of pro-inflammatory cytokines and strong IL-10 and TGF-β responses [18], while TLR-4 recognizes mannan and induces mainly pro-inflammatory signals in APCs, leading to the activation of the immune response against the fungus [19].

After cellular activation, adapter proteins may activate the transcription nuclear factor κB (NF-κB) which in turn leads to the gene expression of cytokines, chemokines, antimicrobial peptides and co-stimulatory molecules [20]. After processing pathogens, APCs exhibit HLA-DR molecules, responsible for presenting peptides, as well as CD80 (B7-1) which acts as a co-stimulatory molecule for T cells activation, leading to acquired immune response [21].

This work evaluated propolis effects on TLR-2, TLR-4, HLA-DR and CD80 expression, pro- (TNF-α) and anti-inflammatory (IL-10) cytokine production, as well as the fungicidal activity of human monocytes against *Candida albicans*, in order to understand its effects on the initial events of the immune response. The involvement of TLR-2 and TLR-4 on propolis action was also investigated. Caffeic acid was studied as possible compound responsible for propolis action.

2. Material and Methods

2.1. Propolis sample and caffeic acid

Propolis was collected in the Beekeeping Section, UNESP. 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 (110 mg/mL) [22]. Propolis was diluted in RPMI 1640 culture medium (Gibco Laboratories, Grand Island, NY, USA) 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. Specific dilutions were prepared for each assay in order to achieve different propolis concentrations: 5, 10, 25, 50 and 100 μg.

Caffeic acid was purchased from Acros Organics (Morris Plains, NJ, USA), diluted in 100 μL of 70% ethanol and subsequently in medium RPMI. Dilutions were performed to obtain 5, 10, 25, 50 and 100 μg of caffeic acid. The same procedure was carried out with 70% ethanol (propolis and caffeic acid solvent).

2.2. Healthy blood donors and isolation of human monocytes

Ten healthy blood donors (aging 20-50 years) from the University Hospital, Botucatu Medical School, UNESP, were included in the present work, which was approved by the

Ethics Committee of Botucatu Medical School (CEP 3442-2010). 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-Hypaque (density=1.077) (Sigma Chemical Co., St Louis, MO, USA). Briefly, 20 mL of heparinized blood were added to an equal volume of RPMI 1640 culture medium containing 2 mM L-glutamine, 10% heat-inactivated fetal calf serum, 20 mM HEPES, and 40 mg/L gentamicin. Samples were added to 4 mL of Ficoll-Hypaque and centrifuged at 400 g for 30 min at room temperature. The interface layer of the PBMC was aspirated and washed twice with phosphate buffer saline (PBS) 0.1 M, pH=7 containing 0.05 mM ethylenediaminetetraacetic acid (PBS-EDTA) and once 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 $1x10^6$ monocytes/mL in RPMI medium supplemented with fetal calf serum.

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 Brasil, Ltda) colorimetric assay [23]. Monocytes ($1x10^6$ cells/mL) were incubated with different concentrations of propolis, caffeic acid (5, 10, 25, 50 and 100 µg) or 70% ethanol (in the same concentrations found in propolis) for 18 h at 37 ºC and 5% CO_2, in a final volume of 500 µL. Control cells were incubated only with culture medium. 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 aspirated and 200 µL of dimethyl sulfoxide (DMSO- Sigma-Aldrich, USA) 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-2, TLR-4, HLA-DR and CD80 expression by monocytes

The expression of TLR-2, TLR-4, HLA-DR and CD80 by human monocytes was assessed using a FACSCalibur flow cytometer and Cell Quest software (Becton Dickinson, FranklinLakes, NJ, USA). PBMC containing $1x10^6$ cells/mL 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 or caffeic acid into polystyrene tubes for cytometric analysis (BD Labware, San Jose,

CA, 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), 1 μg/500 μL of Phycoerythrin (PE)-labeled anti-TLR4 (clone HTA 125) and Fluorescein (FITC)-labeled anti-TLR2 (clone TLR2.1). 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 1 μg/500 μL PE-labeled anti-CD80 (clone 2D10). Antibodies were purchased from Biolegend (San Diego, CA, USA).

Stained cells were incubated for 20 min in the dark at 4 ºC, then washed and fixed with 5% paraformaldehyde in PBS. Background staining was determined from cells incubated with 5 μg/500 μL of PE/Cy7, FITC and PE-labeled control isotype antibodies. Cells samples were washed and then analysed by flow cytometry. Ten thousand monocytes events, defined as cells with respective side scatter (SSC) and CD14 staining characteristics and corresponding levels of fluorescence for TLR-2, TLR-4, HLA-DR and CD80, were obtained for the gated $CD14^+$ cell. Results were expressed as percentage of positive CD14 cells or mean fluorescence intensity (MFI) of gated events.

2.5. Cytokines determination by enzyme-linked immunosorbent assay (ELISA)

To evaluate cytokine production, monocytes ($1x10^6$ cells/mL) were distributed into 24-well flat-bottomed plates (Nunc, Life Tech. Inc., MD, USA) and incubated with different concentrations of propolis, caffeic acid, 70% ethanol or LPS (20 μg/mL) for 18 h at 37 ºC and 5% CO_2. Afterwards, the supernatants were harvested for TNF-α and IL-10 measurement by ELISA, according to manufacturer's instructions (R&D Systems-Minneapolis, MN, USA). Briefly, a 96-well flat bottom Maxisorp (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 [24].

2.6. Fungicidal activity of human monocytes

Monocytes ($2x10^5$ cells/mL) were stimulated with different concentrations of propolis or caffeic acid for 18 h at 37 ºC and 5% CO_2. After incubation, stimuli were removed and

cells were challenged with 100 μL of a *C. albicans* suspension (ATCC 5314) prepared in RPMI medium and 10% heat-inactivated fetal calf serum containing $1x10^6$ yeasts/mL (yeast/monocytes ratio = 5:1) for 2 h at 37 ºC in 5% CO_2. Control cultures contained only yeast cells without monocytes. Co-cultures were harvested by aspiration and sterile distilled water was used to lyse monocytes (final volume of 2 mL). Then 20 μL was plated on brain-heart infusion (BHI) agar medium (Difco Laboratories, Detroit, MI, USA). Inoculated plates, in triplicate of each culture, were incubated at 37 ºC in sealed plastic bags to prevent drying and the number of colony forming units (CFU) per plate was counted after 48 h. The fungicidal activity percentage was determined by the following formula:

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

2.7. Role of TLR-2 and TLR-4 on cytokines production and fungicidal activity by monocytes

Monocytes ($1x10^6$ cells/mL) were distributed into 24-well flat-bottomed plates and incubated at 37 °C for 2 h, in a final volume of 500 μL. After this period, cells were pre-incubated with MAbs against TLR-2 (clone TL2.1) or TLR-4 (clone HTA 125) at 2 μg/mL for 1 h. Antibodies were purchased from Biolegend (San Diego, CA, USA). After incubation, supernatants were removed and cells were treated with different concentrations of propolis or caffeic acid for 18 h at 37 ºC. Supernatants of cell cultures were used for cytokine determination by ELISA.

To assess the fungicidal activity, monocytes ($2x10^5$ cells/mL) were pre-incubated with MAbs against TLR-2 or TLR-4 and incubated as previously described. After 1 h, supernatants were removed and monocytes were incubated with propolis or caffeic acid for 18 h at 37 ºC. Afterwards, medium was removed and monocytes were challenged with *C. albicans* as described above.

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 test were used to flow cytometry analysis, and ANOVA and Tukey-Kramer method were employed for others parameters. A *p* value of less than 0.05 was considered significant.

3. Results

3.1. Effect of propolis and caffeic acid on cell viability

The effect of propolis and caffeic acid on human monocytes viability were expressed in comparison to control (non-treated cells). All concentrations of propolis and caffeic acid did not affect cell viability (>98%). 70% ethanol (in the same concentrations of propolis) had no cytotoxic effects on monocytes (data not shown).

3.2. Effect of propolis and caffeic acid on TLR-2, TLR-4, HLA-DR and CD80 expression by monocytes

Propolis had no effect on TLR-2 expression neither in percentage of expression (%) nor in mean fluorescence intensity (MFI) (Fig. 1A). On the other hand, caffeic acid downregulated TLR-2 expression (MIF) by human monocytes at all concentrations (Fig. 1B).

With regards to TLR-4, propolis (5 μg) upregulated its expression (Fig. 1C) while caffeic acid had no effects at all concentrations (Fig. 1D).

Propolis had no effect on HLA-DR expression, whereas the percentage of CD80 expression was upregulated using 100 μg (Fig. 1E and 1G, respectively). In contrast, HLA-DR expression was inhibited after monocytes treatment with 25, 50 and 100 μg of caffeic acid (% and MIF) (Fig. 1F), with no effect on CD80 expression (Fig. 1H).

3.3. Cytokines production and the role of TLR-2 and TLR-4

Propolis significantly stimulated TNF-α production by human monocytes using 5, 10, 25 and 50 μg. However, a significant inhibitory action was seen using 100 μg of propolis comparing to control (Fig. 2A). As to caffeic acid, TNF-α production was inhibited at all concentrations (Fig. 2B).

IL-10 production was stimulated using 5 and 10 μg of propolis and inhibited after incubation with 50 and 100 μg (Fig. 2C). On the other hand, caffeic acid, at all concentrations, significantly inhibited its production (Fig. 2D).

To evaluate whether TLR-2 or TLR-4 could influence propolis or caffeic acid-induced TNF-α and IL-10 production, human monocytes were pre-incubated with anti-TLR-2 or anti-TLR-4 and then incubated with propolis and caffeic acid. TNF-α production did not change

by blocking TLR-2, however, a reduced production was seen by blocking TLR-4 and incubating the cells with propolis and caffeic acid at all concentrations (Fig. 3A, 3B).

Propolis-induced IL-10 production was not affected by blocking TLR-2, whereas the pre-incubation of monocytes with anti-TLR-4 prior to propolis resulted in lower levels of IL-10 production at all concentrations (Fig. 3C). As to caffeic acid, the inhibitory profile of IL-10 production was not altered when cells were pre-incubated with both anti-TLR-2 and anti-TLR-4 (data not shown).

3.4. Fungicidal activity and the role of TLR-2 and TLR-4

Non-stimulated cells presented a fungicidal activity against *C. albicans*, which was significantly higher using 50 and 100 μg of propolis and 100 μg of caffeic acid (Fig. 4A, 4B). Interestingly, this activity was not altered by blocking TLR-4; however, adding anti-TLR-2 to the culture cells, propolis and caffeic acid-induced fungicidal activity was inhibited at all concentrations (Fig. 4C, 4D).

4. Discussion

Propolis stimulated the expression of TLR-4 and CD80, which are important for microorganism recognition and stimulation of T cells, while caffeic acid inhibited TLR-2 and HLA-DR expression by human monocytes. Monocytes express about 80% of TLR-2, and 40-80% of TLR-4; furthermore, monocytes exhibit an increased expression of MHC-II in relation to CD80/86 [25]. These markers can be detected in immature dendritic cells and monocytes, what may be modulated in response to different stimuli such as propolis [26].

As to TNF-α and IL-10 production by human monocytes, both cytokines were stimulated by low concentrations of propolis and inhibited by higher ones. On the other hand, caffeic acid exerted an inhibitory effect on the production of both cytokines at all concentrations. Surprisingly, the fungicidal activity was increased using high concentrations of propolis and caffeic acid, what was not correlated with cytokine production, which occured using different concentrations. Higher concentrations of propolis contain higher concentrations of caffeic acid, what suggests its involvement in propolis activities. Since TNF-α is important to activate phagocytes while IL-10 may suppress these cells, one may speculate that the fungicidal activity of monocytes might have involved other effector mechanisms, such as nitrogen (NO) and oxygen (H_2O_2) reactive intermediates after interaction with the fungus. H_2O_2 production could be efficient in the first hours after phagocytosis, while NO could be important either in the beginning or late in the antimicrobial

activity of phagocytes [27]. Moreover, other mechanism of alternative fungicidal pathways can be also involved molecules non-oxidative during infection with *C. albicans*, such as hydrolytic enzymes found in lysosomes [28]. Although these mediators were not determined in our work, maybe the highest concentrations of propolis or caffeic acid could have induced H_2O_2, NO or lysosomal enzymes production, increasing the fungicidal activity of monocytes.

It is known that some phenolic compounds can interact with lipids in cell membrane unspecifically, based essentially in physical adsorption [29]. However, it is not known whether propolis compounds enter unspecifically or interact with cell receptors, exerting its biological activities. Although TLRs are able to recognize a broad spectrum of structurally ligands, there are few data in literature regarding the direct interaction of TLRs with natural products [30]. In order to investigate the mechanism of action of propolis compounds, we aimed at evaluating the role of TLR-2 and TLR-4 in cytokines production and fungicidal activity induced by propolis and caffeic acid, using monoclonal antibodies anti-TLR-2 or anti-TLR-4. The production of TNF-α and IL-10 induced by propolis and caffeic acid was inhibited by blocking TLR-4, whereas the fungicidal activity of monocytes was inhibited by blocking TLR-2, suggesting that propolis compounds may interact with TLR-4 and TLR-2 and trigger the activation of transcription factors such as NF-κB or the activator protein-1 (AP-1) which in turn induce the gene expression of cytokines and others mediators, in order to eliminate the invading pathogen.

Data from literature revealed the immunomodulatory effects of medicinal plants and their isolated components on the immune system in an attempt to understand its mechanisms of action on the initial events of the innate immunity. Propolis upregulated TLR-2 and TLR-4 expression and the production of pro-inflammatory cytokines such as IL-1β and IL-6 by murine macrophages and spleen cells [10]. *Panax ginseng* increased mRNA expression of both TLR-4 and TNF-α by macrophages, suggesting that it gradually stimulated these cells and isolated compounds such as ginsenosides were pointed out as the active components of ginseng that may act as agonists of TLR-4 [31]. Kwon et al [32] have evaluated *Cinnamomum cassia* (cinnamon) effect on macrophages cell line Raw 264.7, assessing the expression of MHC-II, CD80 and CD86. An inhibitory effect of this extract was observed on the expression of pro-inflammatory cytokines such as TNF-α and IL-1β and on surface molecules, suggesting that cinnamon had anti-inflammatory activity by inhibiting the activation and maturation of these cells in vitro.

Interestingly, besides microbial components, TLR-4 also recognizes plant-derived molecules such as taxol, a diterpene purified from *Taxus brevifolia* [33,34] and an isolated

fraction from the extract of *Ganoderma lucidum* [35]. To verify whether TLR-4 participates in the *Ganoderma lucidum* extract-mediated signal transductions, these authors used two genetically specific murine macrophage cell lines: HeNC2 (peritoneal macrophages from wild-type C3H/HeN mice with functional TLR-4) and GG2EE (peritoneal macrophages from C3H/HeJ mice lacking functional TLR-4). It has been reported that HeNC2 produced IL-1β after extract stimulation, suggesting that TLR-4 was involved in *Ganoderma lucidum* extract-regulated IL-1β expression [35]. Nakaya et al. [34] investigated a possible mechanism of action in response to the root extract of *Panax ginseng* in the same cell lines discussed above, verifying that the extract induced TNF-α production in C3H/HeN mice, demonstrating the importance of TLR-4 in the induction of pro-inflammatory cytokine stimulated by this extract. In our study, TLR-4 was involved in the TNF-α and IL-10 production induced by propolis and caffeic acid.

It is not known so far which component(s) of propolis would be responsible for its activities, although literature suggests the synergistic effect of its components [36]. In general, propolis upregulated cell receptors expression by monocytes, while caffeic acid downregulated them, suggesting that one isolated compound would not be responsible for propolis action but the some of them. Caffeic acid appeared to be a possible candidate partially responsible by the immunomodulatory activity of propolis, since its action was related to propolis action in cytokines production and fungicidal activity, in higher concentrations. Propolis chemical composition depends on the phytogeographic characteristics of the site of collection, since bees choose different plants as source of propolis in different environments [37]. This aspect difficults propolis standardization and it has been proposed that propolis biological properties should be linked to a detailed investigation of its chemical composition and to its botanical sources [1]. The main constituents of our propolis sample collected in the Beekeeping Section, UNESP, Campus of Botucatu, were isolated and identified: aromatic acids (caffeic acid, dihydrocinnamic acid, p-coumaric acid, ferulic acid, 3,5-diprenyl-p-coumaric acid, 2,2-dimethyl-6-carboxy-ethenyl-8-prenyl-2H-1-benzo-pyran); 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, (2Z,6E)-farnesol, benzyl benzoate and prenylated acetophenones); di- and triterpenes, among others. Moreover, the main vegetal source of propolis in Botucatu, São Paulo State, Brazil is *Baccharis*

dracunculifolia DC, followed by *Araucaria angustifolia* (Bert.) O. Kuntze and *Eucalyptus citriodora* Hook [38].

Propolis exerted immunomodulatory effects on cell receptors expression, cytokines production and fungicidal activity of human monocytes against *C. albicans*, without affecting cell viability and depending on concentration. Such actions involved the participation of TLR-2 and TLR-4, and caffeic acid was involved in propolis activity in some events. Further assays will provide a better comprehension of the mechanism of action of propolis isolated compounds.

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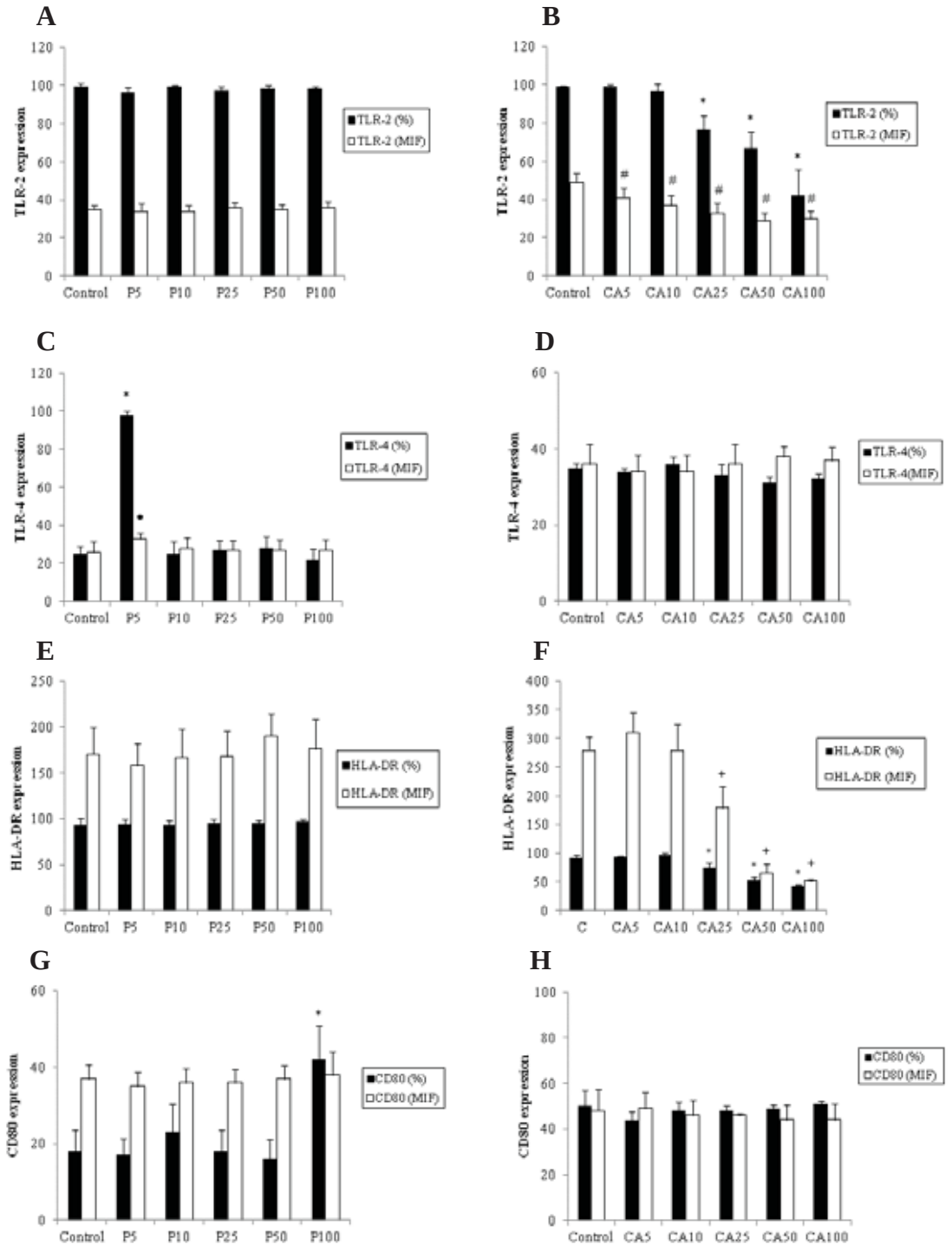

Fig. 1. Percentage (%) of TLR-2, TLR4, HLA-DR and CD80 expression and mean fluorescence intensity (MFI) of human monocytes incubated with 5, 10, 25, 50 and 100 µg of propolis (P) (A, C, E, G, respectively) or caffeic acid (CA) (B, D, F, H, respectively). Data represent mean and standard-deviation (n=10). *significantly different from respective control (%) (p<0.0001); #significantly different from respective control (MIF) (p<0.001); •significantly different from respective control (MIF) (p<0.05); +significantly different from respective control (MIF) (p<0.0001).

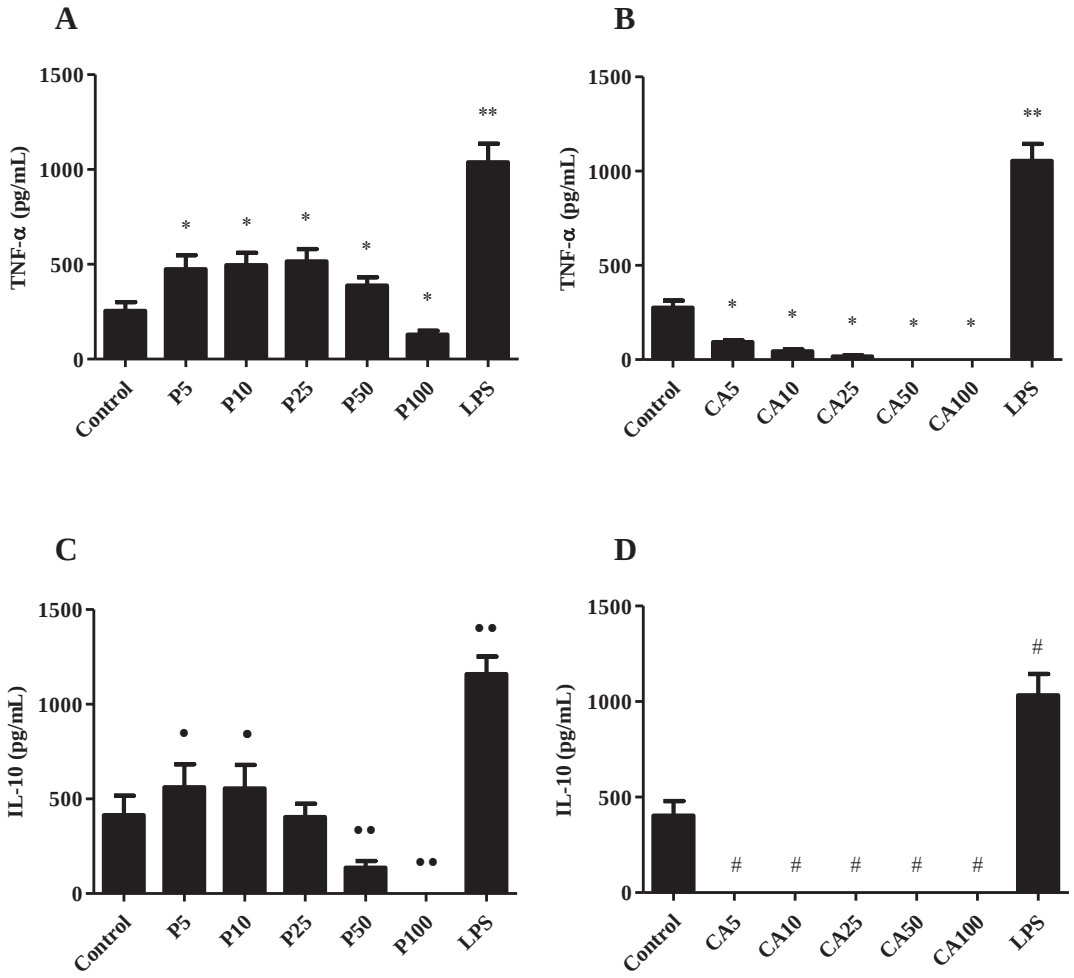

Fig. 2. TNF-α and IL-10 production (pg/mL) by human monocytes incubated with propolis (P) (A, C) or caffeic acid (CA) (B, D) at different concentrations (5, 10, 25, 50 and 100 µg) and LPS (20 µg/mL) for 18 h. Data represent mean and standard-deviation (n=10) *significantly different from control (p<0.001); •significantly different from control (p<0.01); **,••,#significantly different from control (p<0.0001).

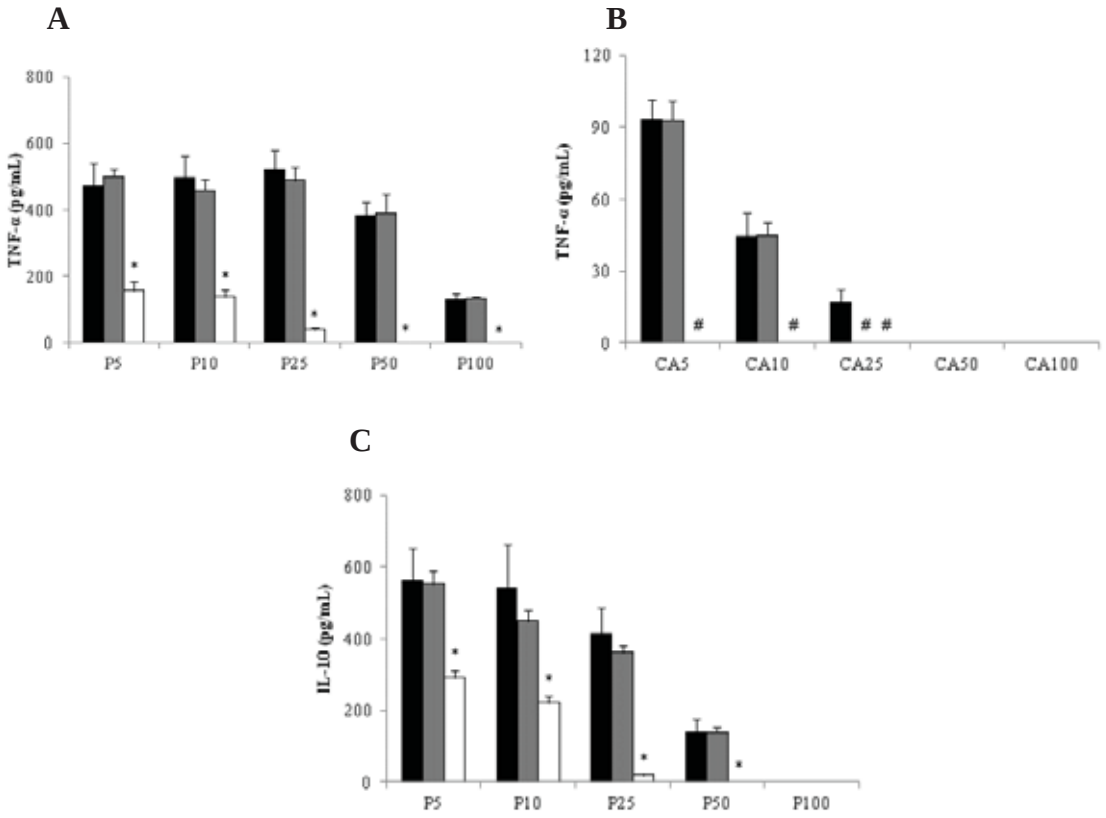

Fig. 3. TNF-α and IL-10 production (pg/mL) by human monocytes in the absence (black bar) or presence of anti-TLR-2 (gray bar) or anti-TLR-4 (white bar) for 1 h at 37 ºC. Afterwards, cells were treated with propolis (P) (A, C) or caffeic acid (CA) (B) at different concentrations (5, 10, 25, 50 and 100 μg) for 18 h. Data represent mean and standard-deviation (n=10). *significantly different from cells without blockade (p<0.001); #significantly different from cells without blockade (p<0.005).

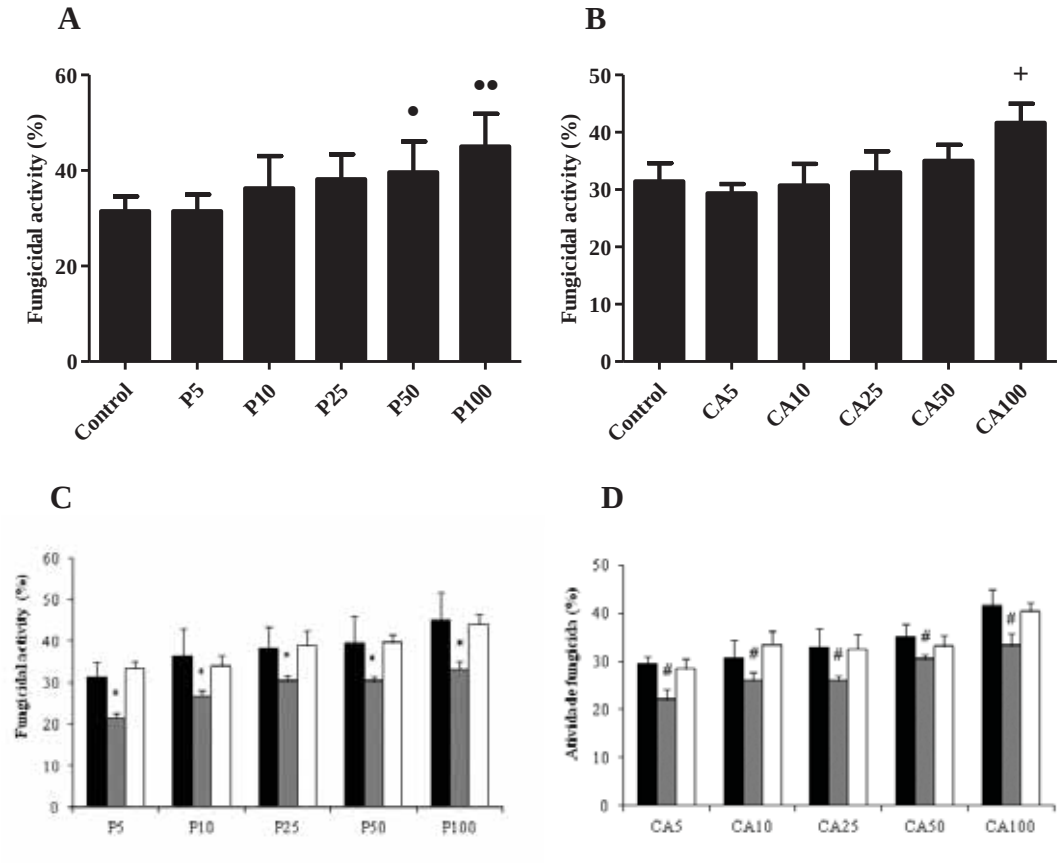

Fig. 4. Fungicidal activity (%) of human monocytes pre-incubated with propolis (A) or caffeic acid (B), in the absence (black bar) or presence of anti-TLR-2 (gray bar) or anti-TLR-4 (white bar) for 1 h at 37 ºC. Afterwards, cells were treated with propolis (P) (C) or caffeic acid (CA) (D) at different concentrations (5, 10, 25, 50 and 100 μg) for 18 h and challenged with *C. albicans* for 2 h. Data represent mean and standard-deviation (n=10). $^{\bullet}$significantly different from control (p<0.01); $^{\bullet\bullet,+}$significantly different from control (p<0.0001); *significantly different from cells without blockade (p<0.001); $^{\#}$significantly different from cells without blockade (p<0.005).

Capítulo III

Artigo Científico

Propolis and caffeic acid suppress LPS-stimulated pro-inflammatory response by blocking NF-κB and MAPK activation in macrophages

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Abstract

Ethnopharmacological relevance: Propolis is a bee product with numerous biological and pharmacological properties, such as immunomodulatory and anti-inflammatory activities. It has been used in folk medicine as a healthy drink and in food to improve health and prevent inflammatory diseases; however, little is known about its mechanism of action.

Aim of the study: The goal of this study was to explore the antioxidant and anti-inflammatory properties of propolis by addressing its intracellular mechanism of action. Caffeic acid was investigated as a possible compound responsible for propolis action.

Materials and Methods: The antioxidant properties of propolis and caffeic acid were evaluated by using the 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) scavenging method. To analyze the anti-inflammatory activity, Raw 264.7 macrophages were treated with different concentrations of propolis or caffeic acid and nitric oxide (NO) production was evaluated by Griess reaction. The concentrations of propolis and caffeic acid that inhibited NO production were evaluated on intracellular signaling pathways triggered during inflammation, named p38 mitogen-activated protein kinase (MAPK), c-jun NH_2-terminal kinase (JNK1/2), the transcription nuclear factor (NF)-κB and extracellular signal-regulated kinase (ERK1/2) by Western blot, using specific antibodies. A possible cytotoxic effect of propolis on hepatocytes was also evaluated, since this product can be used in human diets.

Results: Caffeic acid showed a higher antioxidant activity than propolis extract. Propolis and caffeic acid inhibited NO production by macrophages, at non-cytotoxic concentrations. Furthermore, both propolis and caffeic acid supressed LPS-induced signaling pathways (p38 MAPK, JNK1/2 and NF-κB). ERK1/2 was not affected by propolis extract or caffeic acid. In addition, propolis and caffeic acid did not induce hepatotoxicity at concentrations with strong anti-inflammatory potential.

Conclusions: Propolis exerted an antioxidant and anti-inflammatory action and caffeic acid may be involved in its inhibitory effects on NO production and intracellular signaling cascades, suggesting its use as a natural source of safe anti-inflammatory drugs.

Keywords: Propolis, Caffeic acid, Anti-inflammatory action, Nitric oxide, Mitogen-activated protein kinases, Nuclear factor-κB.

1. Introduction

Propolis is a resinous product used by bees to seal holes in the hive, smooth out the internal walls and protect the entrance against intruders (Bankova, 2005). This product has attracted researchers' interest in the last decade because of its numerous biological and pharmacological properties, such as antimicrobial (Sforcin et al., 2000; Freitas et al., 2006; Búfalo et al., 2009), antitumoral (Missima et al., 2010), immunomodulatory (Sforcin, 2007), anti-inflammatory (Silva et al., 2012), antioxidant (Kumazawa et al., 2010), among others. Chemical composition of propolis is complex and depends on the collection site and local flora. More than 300 different components have been identified so far, with phenolics compounds and flavonoids as major components (de Castro, 2001).

Propolis has been extensively used as a healthy drink and in food to improve health and prevent inflammatory diseases, and it is also used in folk medicine due to its hepatoprotective activity; however, little is known about their mechanisms of action (Kolankaya et al., 2012). The knowledge of phytochemicals molecular mechanisms became a good strategy in the search for new anti-inflammatory compounds.

Inflammation is the central feature of many physiopathological conditions in response to tissue injury and as a part of host defenses against microorganisms (Brodsky et al., 2010). In inflammatory processes, macrophages have a key role in providing an immediate defense against foreign agents. Upon activation with an inflammatory stimulus, such as lipopolisaccharyde (LPS), macrophages produce pro-inflammatory mediators, including nitric oxide (NO) (Geller and Billiar, 1998). NO is produced by inducible nitric oxide synthase (iNOS) in certain activated cells and acts as a host defense by damaging pathogenic DNA and as a regulatory molecule with homeostatic activities; however, excessive production of NO is pathogenic for host tissue itself because NO acts as a reactive radical directly damaging normal tissues. Thus, natural products inhibiting NO production induced by inflammatory stimuli may represent a beneficial therapeutic strategy (Nagaoka et al., 2003).

The expression of pro-inflammatory molecules is regulated by several transcription factors and signaling pathways. The functional nuclear transcription factor-κB (NF-κB) protein is a heterodimer composed mainly by p65 and p50 subunits (Urban et al., 1991). Under normal conditions, NF-κB is present in the cytoplasm in an inactive state, bound to an inhibitory protein κB (IκB). Activation by pro-inflammatory stimuli initiates an intracellular signaling cascade, resulting in the phosphorylation and subsequent degradation of IκB by the 26S-proteasome (Tanaka et al., 2001). IκB phosphorylation is mediated by the inhibitory

protein kappaB kinase (IKK) complex that contains two catalytic subunits named IKKα (IKK1) and IKKβ (IKK2) and a non-catalytic regulatory subunit named NF-κB essential modulator (NEMO) or IKKγ (Tang et al., 2003). The degradation of IκBα releases NF-κB, allowing it to translocate into the nucleus to induce the expression of cytokines, chemokines, anti-apoptotic and cell growth factors that are essential mediators of immune and inflammatory responses (Li and Verma, 2002). Thus, inhibition of NF-κB activation has attracted attention as a therapeutic approach for intervention in immune and inflammatory events (Lee et al., 2010).

Furthermore, LPS activates not only NF-κB, which is a pivotal transcription factor for inflammatory gene expression, but also induces phosphorylation of mitogen-activated protein kinases (MAPKs), which in turn, leads to the activation of NF-κB in macrophages (Jung et al., 2008; Ha et al., 2010). MAPKs are signaling molecules that play critical roles in the regulation of cell growth, differentiation, cell survival/apoptosis, cellular response to cytokines, stress and inflammation. Three major groups of MAP kinases have been identified: p38 MAP kinase family, extracellular signal-regulated kinase (ERK) family and c-Jun NH_2-terminal kinase (JNK) family (Platanias, 2003). MAPKs activity is also required for the expression of several macrophage functions, including NO production and can control the synthesis and release of pro-inflammatory mediators by LPS-activated Raw 264.7 cells (Cho et al., 2003).

Since the modulatory effect of natural products on macrophages/monocytes-triggered inflammatory processes may contribute to the establishment of new therapeutic alternatives for the treatment of pathologies with a strong inflammatory component, this study aimed to explore the antioxidant and anti-inflammatory properties of propolis by addressing its molecular mechanism of action, evaluating its effect in NO production, activation of MAPKs and NF-κB signaling pathways *in vitro*. A possible effect of propolis on human hepatic carcinoma cell line (HepG-2) was investigated, since it is an useful model *in vitro* to determine hepatotoxicity, considering that this product can be used by humans. Caffeic acid was studied as possible compound responsible for propolis action. Propolis composition was investigated as well.

2. Material and Methods

2.1. Propolis chemical composition and caffeic acid

Propolis was produced in the Beekeeping Section, UNESP. After ground, 30% ethanolic extracts of propolis were prepared (30 g of propolis, completing the volume to 100 mL with 70% ethanol), using ambar flasks, at room temperature, with moderate shaking. After a week, extracts were filtered and the dry weight of the extracts was calculated (110 mg/mL) (Sforcin et al., 2005).

Propolis was diluted in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich Química, Madrid, Spain), supplemented with 10% fetal bovine serum (Gibco, Paisley, UK), penicillin (100 U/mL) and streptomycin (100 µg/mL) and specific dilutions of this solution were prepared for each assay in order to achieve different propolis concentrations: 5, 10, 25, 50 and 100 µg.

Propolis chemical composition was determined using high performance liquid chromatography coupled to photodiode-array detector (HPLC-PDA). Propolis extract (5 mg/mL) was solubilized in 25% methanol (1 mL) and analysed by comparing retention times and spectra with chemical standards: caffeic, *p*-coumaric, chlorogenic and ferulic acids (Sigma Chemical Co., St. Louis, USA).

Caffeic acid was purchased from Acros Organics (Morris Plains, NJ, USA) and was diluted in 100 µL of 70% ethanol and DMEM media (1 mg/mL), preparing different concentrations: 5, 10, 25, 50 and 100 µg. The same procedure was carried out with 70% ethanol (propolis and caffeic acid solvent).

2.2. Cell lines

Raw 264.7 cells, a mouse leukaemic monocyte macrophage cell line (ATCC TIB-71), were cultured in DMEM media supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 µg/mL), at 37 ºC in a humidified atmosphere of 95% air and 5% CO_2.

Human hepatic carcinoma cell line (HepG-2 – ATCC HB-8065) cells were grown in DMEM supplemented with 1 g/L of glucose and pyruvate, 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 1.5 g/L of sodium bicarbonate, at 37 ºC and 5% CO_2. Both cultures of cells were monitored by microscopic observation in order to detect any morphological change, throughout the experiments.

2.3. 2.2-Diphenyl-1-picrylhydrazyl (DPPH) – antioxidant assay

DPPH was purchased from Sigma-Aldrich (St Louis, USA). Free radical-scavenging activity was evaluated according to Blois (1958). Aliquots of 100 µL were assessed by their reactivity with methanolic solution of 500 µM DPPH (500 µL) in the presence of 0.2 M acetate buffer, pH 6.0 (1 mL). Reaction mixtures (3 mL) were kept for 30 min at room temperature in the dark, in triplicates. The decreases in the absorbance were measured at 517 nm, in a Hitachi U-2000 (Tokyo, Japan) spectrophotometer. The DPPH• concentration (µg/mL) in the medium was calculated from the calibration curve determined by linear regression. The determination of the effective concentration (EC_{50}) value expresses the amount of sample necessary to decrease the absorbance of DPPH by 50% (Antolovich et al., 2002). Butylhydroxytoluene (BHT-Sigma Chemical Co., St. Louis, USA) was used as standard.

2.4. Cell viability by MTT assay

Assessment of metabolically active cells was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma Aldrich, St. Louis, USA) reduction colorimetric assay (Francisco et al., 2011). Raw 264.7 cells (0.3×10^6 cells/well) or HepG-2 cells (0.2×10^6 cells/well) were plated and allowed to stabilize for 12 h. Afterwards, cells were either maintained in culture medium (control) or pre-incubated with different concentrations of propolis, caffeic acid or ethanol 70% for 1 h, and later activated with 1 µg/mL LPS (from *E. coli* - serotype 026:B6, Sigma Chemical Co., St. Louis, USA) for 24 h. After treatments, MTT solution (5 mg/mL in phosphate buffered saline) was added and cells were incubated at 37 ºC for 15 min. Supernatants were removed and dark blue crystals of formazan were solubilized with 300 µL acid isopropanol (0.04N HCl in isopropanol). Quantification of formazan was performed using an ELISA automatic microplate reader (SLT, Austria) at 570 nm, with a reference wavelength of 620 nm.

2.5. Nitrite production by Griess method

NO production was measured by nitrite accumulation in the culture supernatants, using a colorimetric reaction with Griess reagent (Green et al., 1982). Briefly, 170 µL of culture supernatants were mixed with an equal volume of Griess reagent [0.1% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride and 1% (w/v) sulphanilamide containing 5% (w/v) H_3PO_4] and maintained during 30 min in the dark. Nitrite concentration was determined

by measuring the absorbance at 550 nm using a micro-plate reader (SLT, Austria). All measurements were performed in duplicate and expressed as NO micromolar concentrations.

2.6. Preparation of total protein extracts and Western blot analysis

Raw 264.7 cells (1.2 x 10^6 cells/well) were plated and, after stabilizing for 12 h, cells were either maintained in culture medium (control), or pre-incubated with 10 μg of propolis, or 10 μg of caffeic acid – noncytotoxic concentrations that inhibited NO generation, for 1 h. Then 1 μg/mL LPS was added for 30, 15 or 10 minutes, to verify the exact period of the phosphorylation of proteins. Cells were lysed with RIPA buffer (50 mM Tris–HCl, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 2 mM ethylenediamine tetraacetic acid) freshly supplemented with 1 mM dithiothreitol, protease and phosphatase inhibitor cocktails and sonicated (four times for 4 s at 40 μm peak to peak) in Vibra Cell sonicator (Sonics & Material INC.) to decrease viscosity. The nuclei and the insoluble cell debris were removed by centrifugation at 4 ºC, at 12.000 x *g* for 10 min. The postnuclear extracts were collected and used as total cell lysates. Protein concentration was determined by the bicinchoninic acid protein assay and cell lysates were denaturated in sample buffer (0.125 mM Tris pH 6.8, 2% (w/v) sodium dodecyl sulfate, 100 mM dithiothreitol, 10% glycerol and bromophenol blue). Western blot analysis was performed to evaluate the levels the activation of MAPKs and NF-κB signaling pathways. Briefly, equivalent amounts of protein (30 μg) were separated by 10% (v/v) SDS-PAGE followed by Western blot. To examine the different proteins, the blots were incubated overnight at 4 ºC with the respective primary antibodies: phospho-p38 MAPK (1:1000), phospho-JNK1/2 (1:1000), phospho-ERK1/2 (1:1000), phospho-IκB and total IκB (1:1000), obtained from Cell Signaling Technologies (Danvers, MA, USA). Protein detection was performed using the enhanced chemifluorescence system and the membranes were scanned for blue excited fluorescence on the Storm 860 (GE Healthcare). The generated signals were analyzed using the software TotalLab®. To demonstrate equivalent protein loading, membranes were stripped and reprobed with antibodies against the total form of MAPKs or with anti-β-tubulin antibody (Sigma Aldrich, St. Louis, USA).

2.7. Statistical analysis

Data were expressed as mean ± standard deviation (SD) of 3 similar experiments. For cell viability and NO production, statistical analysis was perfomed using one-way Anova followed by Dunnett's test. For the results of Western blot, two-sided unpaired t-test was

used. The statistical tests were applied using GraphPad Prism, version 5.02 (GraphPad Software, San Diego, CA, USA). A *P* value < 0.05 was considered statistically significant.

3. Results

3.1. Propolis analysis by HPLC-PDA

Propolis chemical composition of the sample was investigated by HPLC-PDA, showing that main compounds were caffeic acid and *p*-coumaric acid derivatives, condensed tannins and flavonoids in small quantities (Fig.1).

3.2. Antioxidant activity

The EC_{50} was 4.67 µg/mL for BHT, 18.51 µg/mL for propolis and 2.5 µg/mL for caffeic acid, demonstrating that caffeic acid showed a higher antioxidant activity than propolis extract and BHT (data not shown).

3.3. Cell viability

The *in vitro* cytotoxic effect of propolis and caffeic acid on Raw 264.7 macrophages viability was represented as percentage of control (non-treated cells), as shown in Fig. 2. Cell viability was higher than 80% at concentrations lower than 25 µg. Using 25, 50 and 100 µg, propolis and caffeic acid, exerted a cytotoxic effect. 70% ethanol had no effects on cells viability (data not shown).

As to HepG-2 cells, 5 and 10 µg showed no cytotoxicity (Fig. 3). These preliminary data suggest that propolis does not induce hepatotoxic effects.

3.4. NO production

As shown in Fig. 4, untreated macrophages Raw 264.7 produced low levels of nitrites (1.3 ± 0.19 µM). After macrophages activation with LPS for 24 h, nitrite production increased to 13.9 ± 0.90 µM. On the other hand, macrophages pre-treatment with propolis or caffeic acid decresead the LPS-induced nitrite production in all non-cytotoxic concentrations (10.4 ± 1.3 and 5.5 ± 1.541 for 5 and 10 µg/well of propolis, respectively; 10.2 ± 1.90 and 5.2 ± 0.78 for 5 µg/well and 10 µg/well of caffeic acid, respectively).

The concentration of propolis and caffeic acid used in the assays of intracellular signaling pathways by Western blot was chosen based on the absence of cytotoxicity, but

inhibiting more than 50% of NO production (10 μg/well of propolis and 10 μg/well of caffeic acid).

3.5. Effect of propolis and caffeic acid in the NF-κB intracellular signaling pathways

Since propolis and caffeic acid inhibited NO production triggered by the strong pro-inflammatory stimulus LPS, we next evaluated whether these two compounds could block LPS-induced NF-κB translocation, preventing IκBα degradation. Raw 264.7 cells were pretreated with propolis or caffeic acid (10 μg) for 1 h and incubated with LPS for 30, 15 and 10 min. As shown in Fig. 5 and Fig. 6, treatment with LPS for 10 min started the phosphorylation and subsequent degradation of IκBα, and at 15 min there was almost a complete degradation of cytosolic IκBα, indicating an increase in the NF-κB activity and consequently an increase in the production of inflammatory mediators, such as iNOS expression and NO production. After 30 min, this effect on degradation of IκBα remained.

Pretreatment with propolis (Fig. 5A) and caffeic acid (Fig. 6A) inhibited LPS-induced phosphorylation of IκB and partially prevented IκBα degradation (Fig. 5B and Fig. 6B, respectively) indicating an inhibitory effect in the LPS-induced NF-κB activity (at 10 min).

3.6. Effect of propolis and caffeic acid in the activation of MAPKs

The effect of propolis and caffeic acid on the activation of ERK1/2, JNK1/2 and p38 MAP kinases pathways was examined by detecting their phosphorylated forms by Western blot using specific antibodies. As shown in the Fig. 7 and Fig. 8, LPS stimulation for 30, 15 and 10 min induced phosphoryaltion of all MAPKs. On the other hand, pre-treatment with propolis and caffeic acid inhibited the LPS-induced phosphorylation of JNK1/2 (Fig. 7A and Fig. 8A, respectively) and p38 MAPK (Fig. 7B and Fig. 8B, respectively) at a short time (10 min), but had no effect in the ERK1/2 pathways (Fig. 7C and Fig. 8C, respectively). Propolis and caffeic acid alone added to control cells did not stimulate MAPKs signaling pathways. To demonstrate equivalent protein loading, membranes were incubated with antibodies against the total form of MAPKs, and there was no change in the proteins expression in our assays conditions (data not shown).

4. Discussion

The chromatographic profile of propolis extract by HPLC-PDA, showed that its main compounds were caffeic acid and *p*-coumaric acid derivatives, condensed tannins and

flavonoids in small quantities. Caffeic acid was identified in our propolis sample, showing strong antioxidant properties. It has been reported that cinnamic acid derivatives from Brazilian propolis showed potent antioxidant activity as well (Banskota et al., 2000).

Higher concentrations (25, 50 and 100 μg) of propolis and caffeic acid exerted a cytotoxic action on Raw 264.7 cells. The antiproliferative and antitumoral activities of propolis has been well documented on mostly transformed cell lines or tumor cells, but not on normal cells (MRC-5, human fibroblast-like fetal lung cell line) (Kouidhi et al., 2010).

Changes in cell growth could be associated with changes in signal transduction pathways (Ansorge et al., 2003). These authors showed the effect of isolated constituents from propolis on ERK-2 mRNA on human peripheral blood mononuclear cells (PBMC), observing a decreased ERK-2 expression when pythemagglutinin (PHA)-activated PBMC were exposed to quercetin, hesperidin, or caffeic acid phenethyl ester (CAPE), suggesting that propolis constituents modulated the MAP kinase ERK-2. This finding does not exclude additional signal pathways and transcription factors targeted by caffeic acid derivatives on the growth regulating. However, we did not find significant differences in the ERK1/2 signaling pathway after Raw 264.7 cells incubation with propolis or caffeic acid and subsequently stimulated by LPS in different periods of time.

Using noncytotoxic concentrations of propolis and its isolated compound, their effects on NO production were evaluated, and in an attempt to identify possible mechanisms for their inhibitory effect on NO production we further analyzed signaling pathways by Western blot, such as the activation of MAPKs and the transcription factor NF-κB. Data showed that propolis and caffeic acid inhibit LPS-induced NO production by macrophages in a concentration-dependent manner, acting at the transcriptional level and suggesting that their anti-inflammatory effect were mediated by the down-regulation of NF-κB, p38 MAP kinase and JNK1/2, but not ERK1/2.

NF-κB activation may be responsible, in part, for increased inflammatory mediators production, including NO (Wang et al., 2010). According to Jung et al. (2008), activation of MAPKs can regulate NO production by controlling the activation of NF-κB. Thus, various intracellular signaling pathways are involved in the modulation of NF-κB and inflammatory mediators' expression. MAPKs are a group of signaling molecules that play an important role in inflammatory processes (Jung et al., 2008) and it has been previously demonstrated that LPS regulates NO production through MAPK signaling pathways activation. LPS treatment results in the phosphorylation of p38 MAPK, ERK1/2 and JNK1/2, leading to activation of

NF-κB in macrophages (Cario et al., 2000). Thus, we investigated the effect of propolis and caffeic acid on the LPS-stimulated activation of NF-κB and MAPKs in Raw 264.7 macrophages. Interestingly, only the phosphorylation of p38 MAPK and JNK1/2 in response to LPS was decresead by propolis and caffeic acid treatments; no significant changes were observed in LPS-induced phosphorylation of ERK1/2 in response to both treatments. These results suggested that p38 MAPK and JNK1/2 but not ERK1/2 were involved in the inhibitory effect of propolis and caffeic acid on LPS-induced NF-κB activation and NO production. Accordingly, previous studies demonstrated that JNK1/2 (Zhou et al., 2008) and p38 MAPK, but not ERK1/2 (Chen and Wang, 1999) modulated NO production in LPS-stimulated Raw 264.7 macrophages.

Among propolis constituents, CAPE has been one of the most extensively studied. Caffeic acid derivatives showed inhibitory effects on LPS-induced NO production in Raw 264.7 cells (Uwai et al., 2008). It has been reported that CAPE has anti-inflammatory properties and also is a potent and specific inhibitor of NF-κB activation and protein kinase B (Aκt) phosphorylation in cells T, but has no effect on p38 MAPK phosphorylation (Wang et al., 2010). It likely seems that CAPE inhibits the generation of reactive oxygen species by several mechanisms (Wadsworth and Koop, 1999). According to Jung et al. (2008), CAPE treatment inhibited LPS-induced NO production by Raw 267.4 cells, and this effect was mediated through the inhibition of IκB degradation and inhibition of both p38 MAPK and ERK phosphorylation, whereas the phosphorylation of JNK was not affected. It has been also reported that CAPE inhibited IκB phosphorylation, subsequent IκB degradation and further NF-κB activation and nuclear translocation, but did not affect MAPK family phosphorylation in human monocyte-derived dendritic cells (MoDCs) (Lee et al., 2010).

Quercetin, another active component of propolis, inhibited not only LPS-stimulated NF-κB activation in Raw 267.4 macrophages but also LPS-induced IκBα phosphorylation in bone marrow-derived macrophage (Nair et al., 2006). *In vitro* experiments demonstrated that another phenolic compound found in propolis, artepillin C (3,5-*bis*(3-methyl-2-butenyl)-*p*-coumaric acid), decreased NO level in the supernatant of Raw 264.7 cells culture and also inhibited NF-κB activity (Paulino et al., 2008).

Taken together, our data are in agreement with those found in literature, since propolis and caffeic acid inhibited NF-κB pathway activation and NO production by Raw 264.7 cells. Propolis and caffeic acid also inhibited the phosphorylation of MAPKs. So far, our data showed that propolis and caffeic acid exerted a potent *in vitro* anti-inflammatory effect which

could be mediated through down-regulation of NF-κB and MAPK pathways and inhibition of NO accumulation. In addition, propolis and caffeic acid showed no hepatotoxicity using the same anti-inflammatory concentrations. This is a crucial information, since propolis is extensively used in food, beverage and in folk medicine for treating various ailments (Banskota et al., 2001). It is also suggested that both propolis and caffeic acid may act as potential molecules for the development of new anti-inflammatory drugs.

In conclusion, propolis exerted an antioxidant and anti-inflammatory activities, through inhibition of NO production and signaling pathways NF-κB, p38 MAPK and JNK1/2. Furthermore, caffeic acid was partially responsible for propolis anti-inflammatory action.

Acknowledgements

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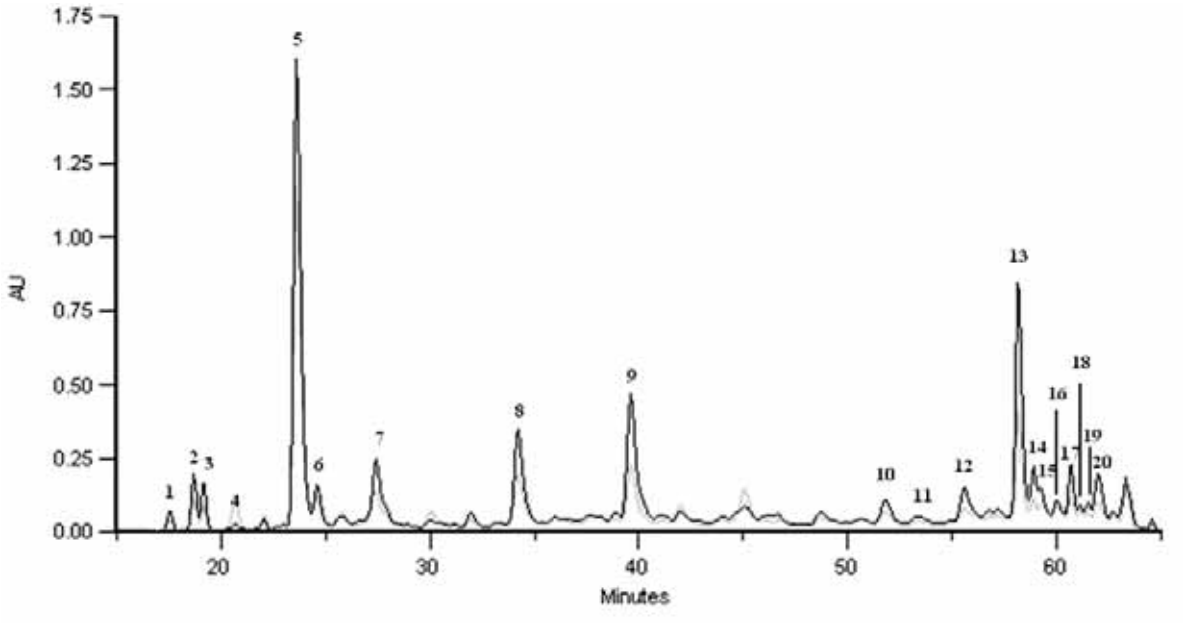

Fig. 1. HPLC profile of propolis extract. 1, 2, 3, 7, 8, 9, 10, 11: caffeic acid derivatives; 4: condensed tannin; 5, 12, 13, 14, 15, 17, 18, 19, 20: *p*-coumaric acid derivatives; 6, 16: flavonoids. Chromotograms were recorded at 280 nm and 320 nm.

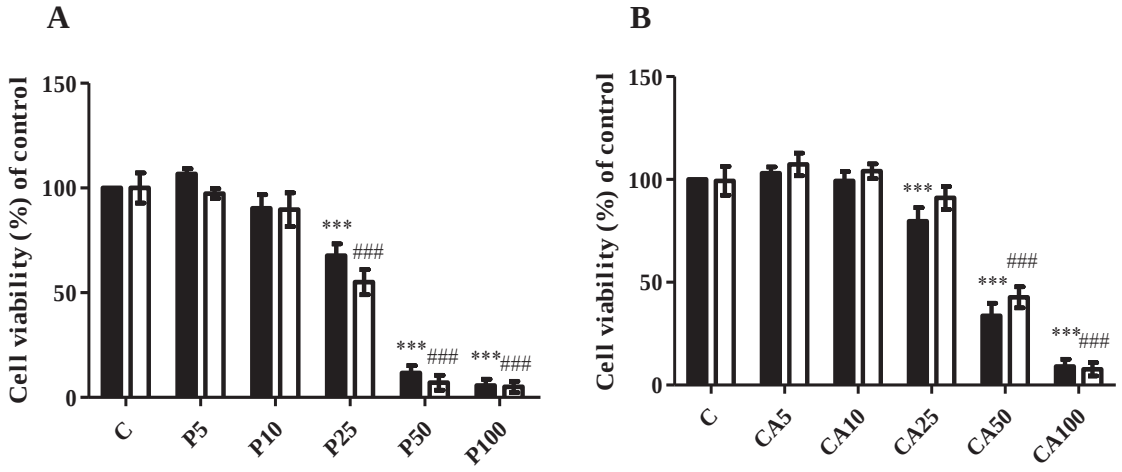

Fig. 2. Cell viability (% of the control) of Raw 264.7 cells after incubation with different concentrations of (A) propolis (P) or (B) caffeic acid (CA) (5, 10, 25, 50 and 100 μg) for 1 h, followed incubation with LPS (white bar) or without LPS (black bar) for 24 h. Data represent mean ± SD of 3 independent assays. $^{***}P < 0.001$, compared to control without LPS; $^{###}P < 0.001$, compared to control with LPS.

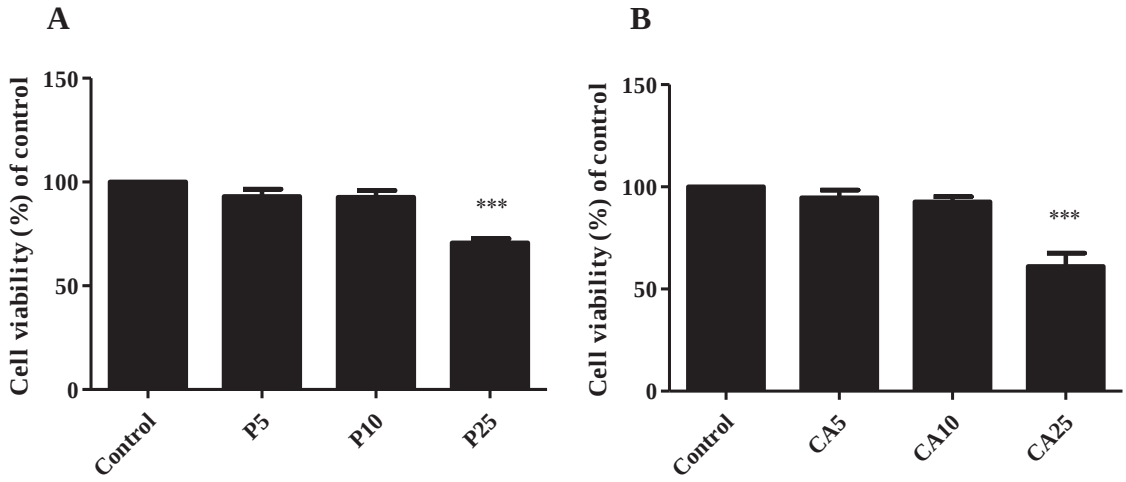

Fig. 3. Cell viability (% of the control) of HepG-2 cells after incubation with different concentrations of (A) propolis (P) or (B) caffeic acid (CA) (5, 10, 25 µg) for 24 h. Data represent mean ± SD of 3 independent assays. $^{***}P < 0.001$, compared to control.

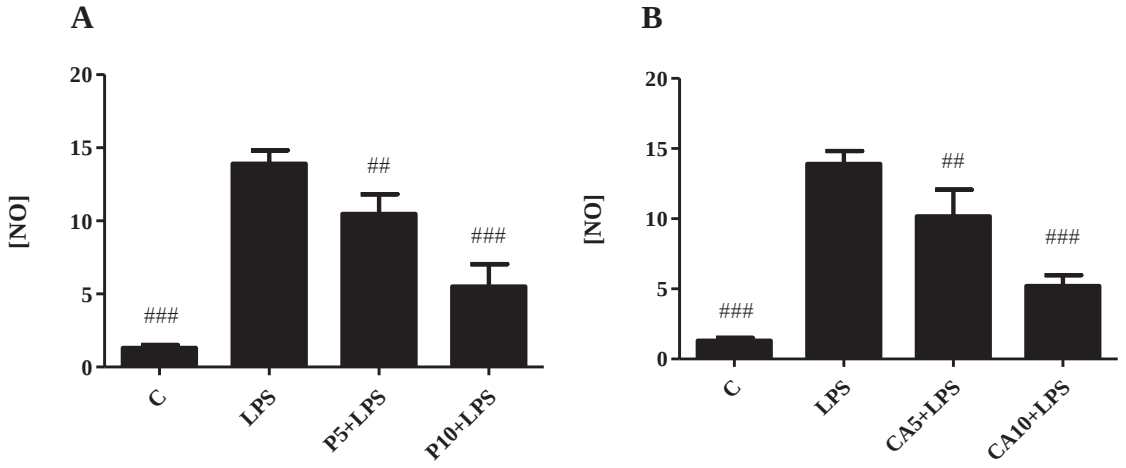

Fig. 4. Effect of (A) propolis (P) or (B) caffeic acid (CA) on nitrites production. Raw 264.7 cells were maintained in culture medium (control) or pre-incubated with different concentrations of propolis or caffeic acid (5 and 10 µg) for 1 h, and then stimulated with 1 µg/mL LPS for 24 h. Data represent mean ± SD of 3 experiments. $^{##}P < 0.01$; $^{###}P < 0.001$, compared to LPS.

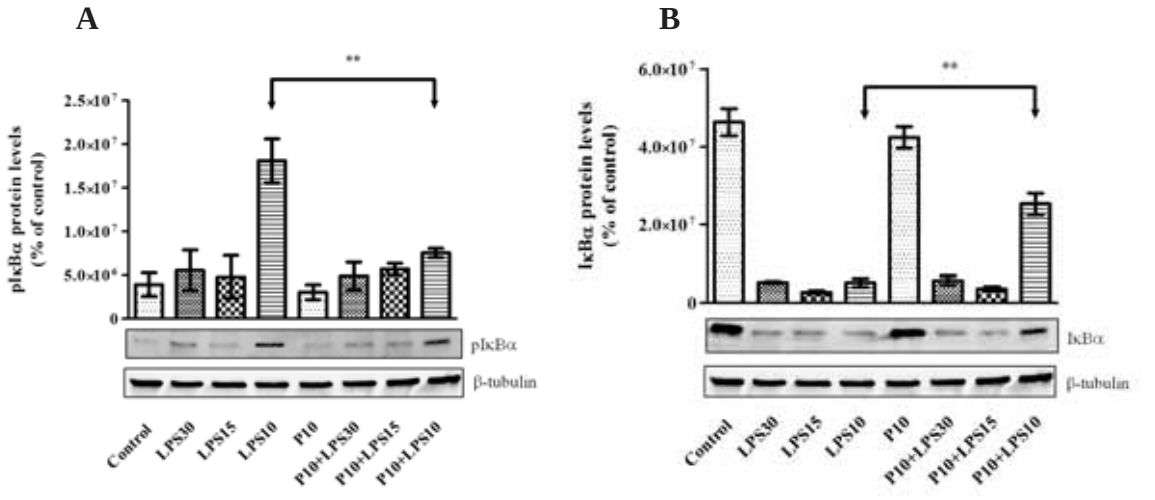

Fig. 5. Effect of propolis (P) on the phosphorylation of IκB (A) and total IκB (B) in LPS-stimulated Raw 264.7 cells. Cells were pre-treated with propolis (P-10 μg) for 1 h and then incubated with LPS (1 μg/mL) for 30, 15 and 10 min. An anti-β tubulin antibody was used to confirm equal protein loading. The blot is representative of 3 similar blots. $^{**}P < 0.01$, compared to LPS10.

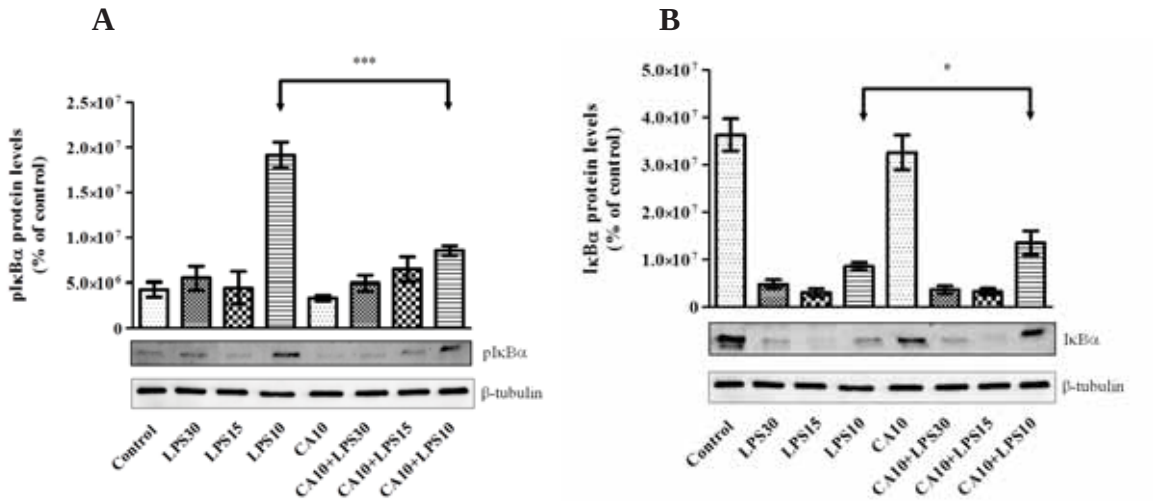

Fig. 6. Effect of caffeic acid (CA) on the phosphorylation of IκB (A) and total IκB (B) in LPS-stimulated Raw 264.7 cells. Cells were pre-treated with caffeic acid (P-10 μg) for 1 h and then incubated with LPS (1 μg/mL) for 30, 15 and 10 min. An anti-β tubulin antibody was used to confirm equal protein loading. The blot is representative of 3 similar blots. $^{***}P < 0.001$; $^{*}P < 0.05$, compared to LPS10.

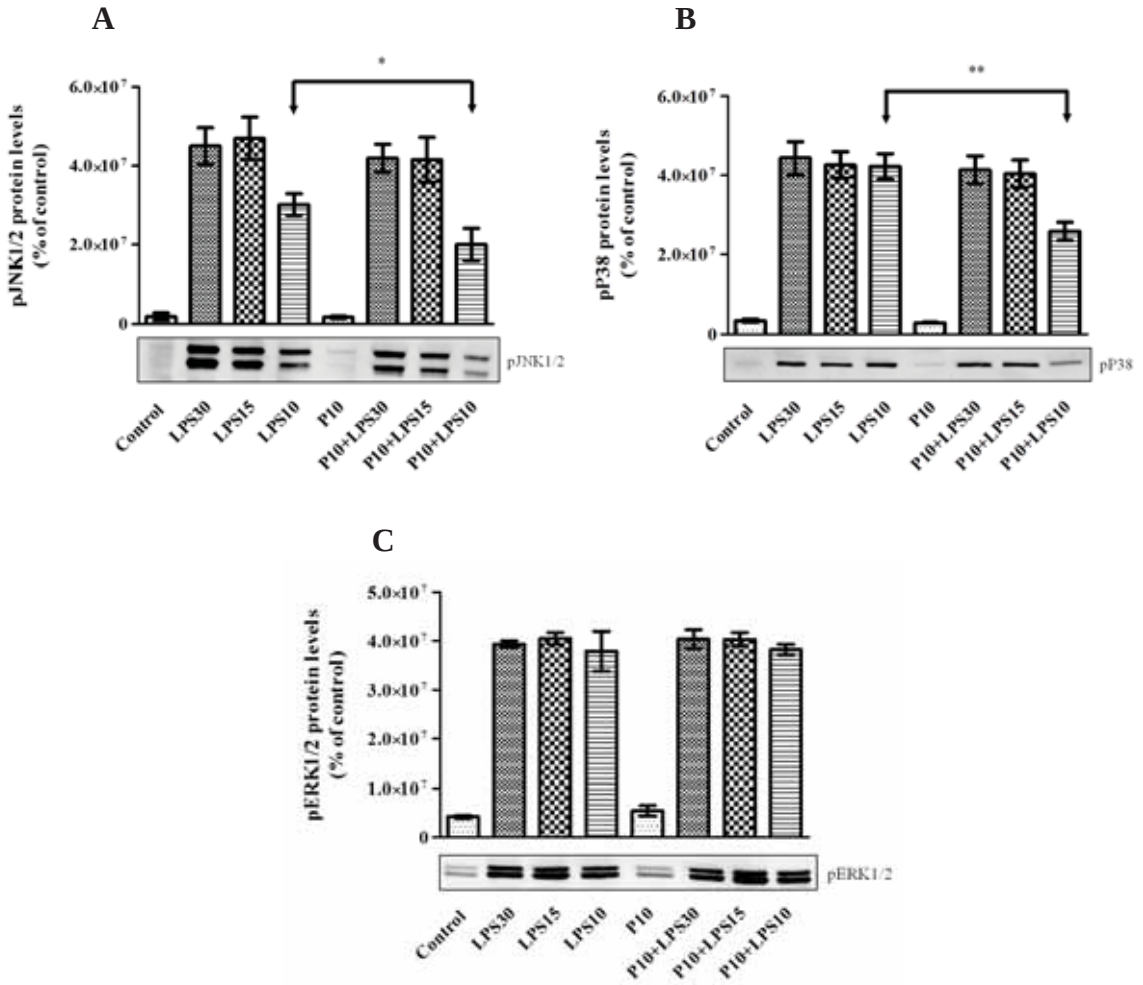

Fig. 7. Effect of propolis on pJNK1/2 (A), pP38 MAPkinase (B) and pERK1/2 (C) activation in LPS-stimulated Raw 264.7 cells. Cells were pre-treated with propolis (P-10 μg) for 1 h and then incubated with LPS (1 μg/mL) for three time points (30, 15 and 10 min). The blot is representative of 3 similar blots. $^{*}P < 0.05$; $^{**}P < 0.01$, compared to LPS10.

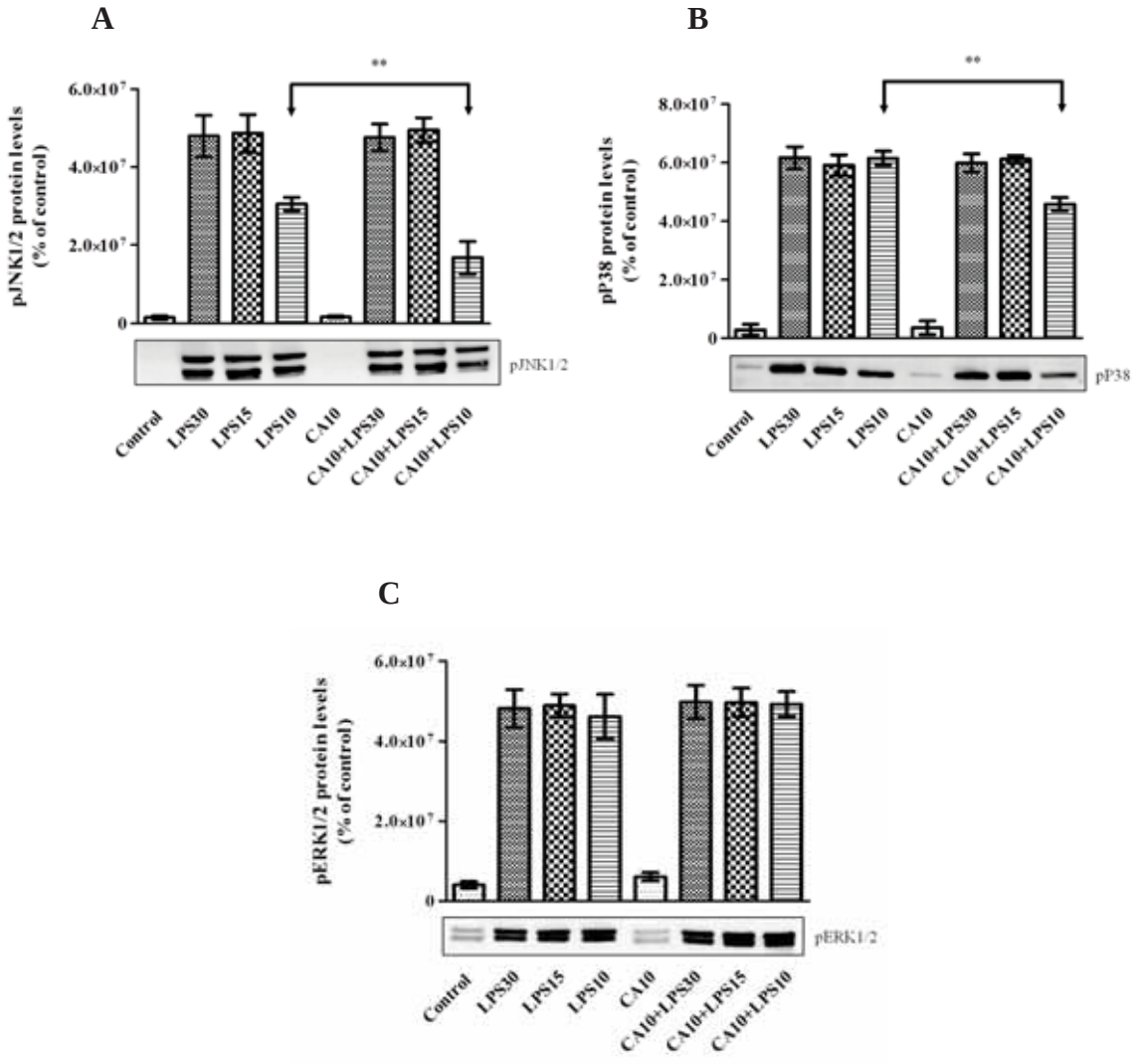

Fig. 8. Effect of caffeic acid on pJNK1/2 (A), pP38 MAPkinase (B) and pERK1/2 (C) activation in LPS-stimulated Raw 264.7 cells. Cells were pre-treated with caffeic acid (CA-10 μg) for 1 h and then incubated with LPS (1 μg/mL) for three time points (30, 15 and 10 min). The blot is representative of 3 similar blots. $^{**}P < 0.01$, compared to LPS10.

Conclusões

Conclusões

- A própolis, em baixas concentrações, modulou a ativação de monócitos humanos, induzindo aumento na expressão de TLR-4 e na produção de TNF-α e IL-10, além de modular também a atividade fungicida destas células e a expressão de CD80, porém em concentrações mais elevadas. Assim, a imunomodulação observada foi devida a diferentes concentrações deste apiterápico;
- A ação da própolis pareceu ser parcialmente devida aos receptores TLR-2 e TLR-4: o bloqueio de TLR-4 mostrou-se importante para a ação da própolis quanto à produção de citocinas (TNF-α e IL-10), enquanto o bloqueio de TLR-2 afetou a atividade fungicida de monócitos após a incubação com este apiterápico. No entanto, outros receptores que não foram estudados também poderiam estar envolvidos nos mecanismos de ação da própolis;
- O ácido cafeico inibiu eventos importantes para o desencadeamento da resposta imune: houve inibição na expressão de TLR-2, HLA-DR, e na produção de citocinas, importantes para o reconhecimento de antígenos e posterior apresentação dos mesmos para linfócitos T. Por outro lado, o ácido cafeico induziu aumento na atividade fungicida de monócitos;
- Assim como para a própolis, o bloqueio do TLR-4 inibiu a ação do ácido cafeico na produção de citocinas por monócitos, e o bloqueio de TLR-2 prejudicou a atividade fungicida, o que sugere a possível participação deste componente na ação da própolis;
- A própolis estimulou a expressão de alguns receptores celulares, a produção de citocinas e a atividade fungicida de monócitos, embora em concentrações diferentes. Os dois receptores estudados (TLR-2 e TLR-4) parecem ser alvos de ligação de componentes da própolis, embora estes dados preliminares mereçam ser melhor estudados;
- Tanto a própolis quanto o ácido cafeico exerceram ação antioxidante e anti-inflamatória sobre células Raw 264.7, dependendo da concentração. As menores concentrações inibiram a produção de NO induzida pelo LPS nestas células, atuando a nível transcricional e inibindo as vias NF-κB, p38 MAP kinase e JNK1/2;
- Própolis, bem como o ácido cafeico, em concentrações anti-inflamatórias, não apresentaram efeito citotóxico sobre as células HepG-2, sendo este dado importante, uma vez que a própolis tem sido utilizada pela população para diferentes fins;
- O ácido cafeico pode estar envolvido na ação da própolis em alguns eventos, embora nossos dados não permitam correlacionar efetivamente sua ação à da própolis. Ademais,

outros constituintes que não foram avaliados neste projeto também poderiam ter envolvimento na ação da própolis, isolada ou sinergicamente;

- Os dados obtidos com monócitos humanos revelaram o perfil ativador da própolis nos eventos da resposta imune, enquanto aqueles obtidos com a linhagem de células Raw 264.7 apontaram seu efeito anti-inflamatório. Estes achados sugerem que as diferentes ações da própolis dependem não só da concentração utilizada, como também do modelo experimental adotado e da população celular avaliada.

Anexo

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Ética

Registrado no Ministério da Saúde
em 30 de abril de 1997

Botucatu, 01 de março de 2.010

OF. 048/2010-CEP

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

Prezado Dr. José Maurício,

De ordem do Senhor Coordenador deste CEP, informo que Projeto de Pesquisa **(Protocolo CEP 3442-2010)** **"Ação da própolis e de compostos isolados sobre a expressão de receptores, produção de citocinas e atividade fungicida de monócitos humanos"**, a ser conduzido por Michelle Cristiane Búfalo, orientada por Vossa Senhoria, recebeu do relator parecer favorável, aprovado em reunião de 01 de março de 2.010.

Situação do Projeto: APROVADO. Ao final da execução deste Projeto, apresentar ao CEP **"Relatório Final de Atividades"**.

Atenciosamente,

Alberto Santos Capelluppi
Secretário do CEP