UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA
CAMPUS DE BOTUCATU

PRODUÇÃO DE CITOCINAS PRÓ- E ANTI-INFLAMATÓRIAS POR MACRÓFAGOS ESTIMULADOS *IN VITRO* COM PRÓPOLIS, ALECRIM-DO-CAMPO, CAPIM-LIMÃO E CRAVO-DA-ÍNDIA

Tatiana Fernanda Bachiega

Orientador: Prof. Adj. José Maurício Sforcin

Dissertação apresentada ao Programa de Pós-Graduação em Patologia da Faculdade de Medicina de Botucatu, Universidade Estadual Paulista - UNESP, para obtenção do título de Mestre em Patologia.

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FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO DE BIBLIOTECA E DOCUMENTAÇÃO - CAMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: *ROSEMEIRE APARECIDA VICENTE*

Bachiega, Tatiana Fernanda.
Produção de citocinas pró-e antiinflamatórias por macrófagos estimulados in vitro com própolis, alecrim-do-campo, capim-limão e cravo-da-índia / Tatiana Fernanda Bachiega. - Botucatu, 2011

Dissertação (mestrado) – Faculdade de Medicina de Botucatu, Universidade Estadual Paulista, 2011
Orientador: José Maurício Sforcin
Capes: 21102007

1. Produtos naturais. 2. Citocinas. 3. Imunologia celular.

Palavras-chave: Citocinas; Compostos isolados; Imunomodulação; Produtos naturais.

RESUMO

Nosso grupo tem se dedicado à investigação das ações biológicas da própolis, alecrim-do-campo, capim-limão e cravo-da-Índia. A própolis tem despertado a atenção dos pesquisadores em virtude de suas inúmeras propriedades biológicas, e o alecrim-do-campo é uma das principais fontes deste apiterápico em nossa região. Já o capim-limão e o cravo-da-Índia têm sido pouco avaliados no tocante à sua ação imunomoduladora. O objetivo deste trabalho foi avaliar o efeito imunomodulador do extrato e respectivos compostos isolados da: própolis (ácidos cumárico e cinâmico), do alecrim-do campo (ácido cafeico), do cravo-da-Índia (eugenol) e do capim-limão (citral) sobre a produção de citocinas (IL-1β, IL-6 e IL-10) por macrófagos peritoneais de camundongos BALB/c. Em protocolos com LPS, macrófagos foram incubados ora com os produtos naturais supracitados em diferentes concentrações e posteriormente desafiados com LPS; ora desafiados com LPS e posteriormente incubados com os produtos naturais. A dosagem das citocinas foi realizada através da técnica de ELISA. A própolis exerceu ação moduladora sobre a resposta imune e inflamatória, e os ácidos cinâmico e cumárico podem estar envolvidos em sua ação imunomoduladora. O alecrim-do-campo e o ácido cafeico também demonstraram efeito imunomodulador quanto à produção de citocinas. O capim-limão exerceu efeito inibitório sobre a produção de citocinas, sendo este efeito mais pronunciado em ensaios com o citral. Resultados semelhantes foram observados com o cravo-da-Índia e o eugenol. Nossos resultados sugerem que o potencial imunomodulador dos produtos naturais merece ser melhor explorado em futuras investigações, avaliando sua eficiência em doenças inflamatórias.

Palavras-chave: Imunomodulação, citocinas, produtos naturais, ELISA

ABSTRACT

Our group has been investigating the biological action of propolis, “alecrim-do-campo”, lemongrass and clove. Propolis has attracted the researchers’ attention due to its several biological properties, and “alecrim-do-campo” is its main vegetal source in our region. However, little is known concerning lemongrass and clove immunomodulatory action. The goal of this work was to evaluate the immunomodulatory effect of the following extracts and isolated compounds: propolis (coumaric and cinnamic acids), “alecrim-do-campo” (caffeic acid), clove (eugenol) and lemongrass (citral) on cytokines production (IL-1β, IL-6 and IL-10) by peritoneal macrophages of BALB/c mice. In LPS protocols, macrophages were incubated either with natural products in different concentrations and then challenged with LPS; or with LPS and then incubated with the natural products. Cytokine concentrations were measured by ELISA. Propolis exerted a modulatory action on the immune and inflammatory response and cinnamic and coumaric acids may be involved in its immunomodulatory action. “Alecrim-do-campo” and caffeic acid also exerted an immunomodulatory action on cytokines production. Lemongrass showed an inhibitory action on cytokines production, mainly in the assays with citral. Similar results were found using clove and eugenol. Our results suggest that the immunomodulatory potential of the natural products should be investigated in further studies, evaluating their efficacy in inflammatory diseases.

Keywords: Immunomodulation, cytokine, natural products, ELISA.

SUMÁRIO

Introdução

1. Introdução

Há um enorme legado histórico sobre o uso terapêutico de plantas em várias condições patológicas do homem e de animais. Com o avanço da pesquisa na área da Etnofarmacologia, suas atividades biológicas vêm sendo comprovadas e as plantas medicinais passaram a assumir um novo papel, como fontes inestimáveis para o desenvolvimento de novos fármacos (Kaileh *et al*., 2007), como o quimioterápico Paclitaxel® (Tamada *et al*., 2010) e o anti-inflamatório Acheflan® (Chaves *et al*., 2008).

Em relação ao sistema imune, há grande interesse em pesquisas etnofarmacológicas que relacionem o efeito imunomodulador de plantas medicinais a diversas condições fisiopatológicas, como por exemplo, a inflamação (Sforcin, 2007), a qual é benéfica quando aguda e regulada, porém passa a ser prejudicial quando crônica e exacerbada, situação esta em que a modulação exógena do sistema imune é desejável. Neste contexto, encaixam-se os produtos naturais e seus compostos isolados que possuem efeito imunomodulador (Gertsch *et al*., 2010), destacando-se sua ação na produção de citocinas – mediadores importantes da resposta imune e inflamatória (Spelman *et al.,* 2006).

Dentre os produtos naturais e compostos isolados investigados nesta dissertação temos a própolis, o alecrim-do-campo, o capim-limão e o cravo-da-Índia, bem como os ácidos cumárico, cinâmico e cafeico, o citral e o eugenol.

1.1 Inflamação

A inflamação é uma resposta ativada por diversos estímulos e condições que interferem na homeostase do organismo. Esta resposta é complexa, envolvendo diversas células como neutrófilos, macrófagos e mastócitos, bem como a participação de diversos mediadores. Este processo pode ser agudo ou crônico, e se a resposta inflamatória for controlada, é tida como benéfica (nos casos de proteção contra infecção, por exemplo). Porém, se essa resposta for exacerbada, passa a assumir um caráter patológico como nos casos de asma, em que a inflamação descontrolada leva à fibrose dos pulmões e consequente comprometimento deste órgão (Medzhitov, 2008).

A resposta bem sucedida para uma injúria, independente de sua natureza, pode ser subdividida em três estágios: inflamação, proliferação e regeneração. Após o reconhecimento de um sinal de “alerta”, tem início uma série de alterações bioquímicas e vasculares, com infiltração de diversos tipos de células imunes efetoras, com subseqüente remodelamento do tecido inflamado. Neste contexto, os macrófagos assumem um importante papel, devido às suas várias funções, tais como: fagocitose, processamento e apresentação de antígenos aos linfócios T, remoção de células apoptóticas, indução de angiogênese, reparação e remodelamento de tecidos (Chawla *et al*., 2010).

Nos casos de infecções microbianas, receptores de células envolvidas na imunidade inata, tais como os *Toll-like receptors* (TLRs), reconhecem patógenos, levando à produção de uma variedade de mediadores inflamatórios tais como aminas vasoativas, componentes do sistema complemento, mediadores lipídicos, peptídeos vasoativos, enzimas proteolíticas, quimiocinas e citocinas (Medzhitov, 2008).

As aminas vasoativas (histamina e serotonina) aumentam a permeabilidade dos vasos e causam vasodilatação, porém, dependendo do contexto, podem também promover vasoconstrição, resultando em hipoperfusão cardíaca e cerebral, hipertensão, entre outras patologias de origem vascular (Callera *et al*., 2006). Os componentes do sistema complemento C3a, C4a e C5a (também conhecidos por anafilatoxinas) são produzidos através das vias de ativação deste sistema e promovem o recrutamento de monócitos e granulócitos, induzindo a degranulação de mastócitos e afetando também a permeabilidade dos vasos (Mc Geer *et al*., 2005).

Mediadores lipídicos (eicosanóides e fatores ativadores de plaquetas) são derivados de fosfolipídios. O ácido aracdônico é metabolizado para formação de eicosanóides tanto pelas ciclooxigenases (COX-1 e COX-2), que geram prostaglandinas e tromboxanos, ou por lipoxigenases, que geram leucotrienos e lipoxinas. As prostaglandinas (PGE) causam vasodilatação, hiperalgesia e febre, enquanto as lipoxinas inibem o processo inflamatório e reparam tecidos lesados. Outra classe de mediadores lipídicos são os fatores ativadores de plaquetas, gerados pela acilação de lisogliceril éter fosforilcolina,

um derivado da hidrólise de fosfolipídeos de membrana mediada por fosfolipase A_2, que induzem recrutamento de leucócitos, vasoconstrição, aumento da permeabilidade vascular e ativação de plaquetas (Barton *et al*., 2008). Já os peptídeos vasoativos são gerados pela proteólise do fator de Hageman, trombina ou plasmina, sendo responsáveis pela vasodilatação e aumento da permeabilidade vascular, tanto direta quanto indiretamente, por indução da secreção de histamina pelos mastócitos (Smalley *et al*., 2009).

As enzimas proteolíticas (elastina, catepsinas e metaloproteinases) possuem diversos papéis na inflamação, degradando a matriz extracelular, remodelando tecidos e favorecendo a migração de leucócitos (Silva-Lucca *et al*., 2010).

Citocinas são glicoproteínas produzidas por diversas células envolvidas ou não na resposta imune, e possuem uma infinidade de papéis na inflamação, que serão melhor discutidos posteriormente (Medzhitov *et al*., 2008). Quimiocinas são produzidas por diversos tipos celulares em resposta ao estado inflamatório, sendo responsáveis principalmente pela quimiotaxia em tecidos afetados (Medzhitov, 2008).

Considerando que há uma grande quantidade de mediadores produzidos após o desencadeamento da resposta inflamatória, o parâmetro da inflamação pesquisado por nós neste trabalho foi a produção de citocinas, e o modelo utilizado em nosso trabalho incluiu o desafio de macrófagos com lipopolissacarídeo (LPS) antes e após a incubação das células com os seguintes produtos naturais e compostos isolados: própolis e ácidos cinâmico e cumárico, *Baccharis dracunculifolia* e ácido cafeico, capim-limão e citral, cravo-da-Índia e eugenol (Shin *et al.,* 2006).

1.2. Citocinas

As citocinas são proteínas secretadas pelas células da imunidade inata e adaptativa em reposta a microrganismos e outros antígenos, estimulando o crescimento e diferenciação de linfócitos, ativação de diferentes células efetoras para eliminação de antígenos, e estimulando a produção de células hematopoiéticas. Macrófagos e linfócitos eram considerados as principais fontes de citocinas; entretanto, foi demonstrado que outras células do sistema

imune bem como células nucleadas podem secretar citocinas sob certas circunstâncias (TRAYHURN & WOOD, 2004).

A IL-1β é um dos mediadores centrais de reações inflamatórias e apresenta função semelhante à do TNF-α, sendo importante na resposta do hospedeiro a infecções e outros estímulos inflamatórios, agindo em conjunto com o TNF-α na imunidade inata e na inflamação. Sua principal fonte celular são fagócitos mononucleares ativados, sendo também produzida por neutrófilos, células epiteliais e células endoteliais. Em baixa concentração, a IL-1β atua como mediadora de inflamação local, agindo nas células endoteliais, aumentando a expressão de moléculas de superfície que medeiam a adesão de leucócitos, como ligantes para integrinas. Quando secretada em maiores quantidades, exerce efeitos endócrinos, induzindo febre e síntese de proteínas de fase aguda pelo fígado (SIMS *et al*., 1994). Devido a padrões inflamatórios presentes de forma exacerbada na infecção por *Pseudomonas aeruginosa,* camundongos knockout para IL-1β exibiram aumento da resistência contra esta bactéria - principal agente etiológico da pneumonia, devido à concentração diminuída de citocinas e quimiocinas locais bem como o recrutamento de neutrófilos (SCHULTZ *et al*., 2002). Por outro lado, o pré-tratamento com IL-1β recombinante previniu danos na mucosa gástrica induzida por estresse em ratos, sugerindo o efeito protetor desta citocina nestas condições (HUANG *et al*., 1995).

A IL-6 pode ser secretada por vários tipos celulares, como macrófagos ativados, adipócitos e células musculares esqueléticas (FEBBRAIO & PEDERSEN, 2002), atuando tanto na imunidade inata quanto na adaptativa. A produção de IL-6 pode ser estimulada por citocinas como IL-1β e TNF-α. Na imunidade inata, atua como estímulo para a produção de neutrófilos e de proteínas de fase aguda; já na imunidade adaptativa, atua na diferenciação de linfócitos B em plasmócitos, sendo também fator de crescimento de plasmócitos neoplásicos (YOON *et al.,* 2009). Além disso, tem sido crescente o número de trabalhos que demonstram a ambigüidade da IL-6, por possuir tanto efeitos anti-inflamatórios quanto pró-inflamatórios. GABAY *et al.* (1997) demonstraram que a IL-6 pode ser considerada também uma citocina anti-inflamatória, inibindo a liberação de IL-1β e TNF-α e favorecendo a produção

do receptor de TNF solúvel. Por estas razões, a IL-6 tem sido considerada também uma citocina regulatória.

A IL-10 é uma citocina pluripotente, secretada por diversas populações celulares, dentre elas, os macrófagos (ASADULLAH *et al.,* 2003). Essa citocina pode inibir respostas imunes protetoras a infecções (MOORE *et al.*, 2001). A superexpressão de IL-10 tem sido relacionada com predisposição a complicações infecciosas após traumas, queimaduras e imunossupressão pós-cirúrgica (AYALA *et al*., 1994; WOICIECHOWSKY, 1998; KOBAYASHI *et al*., 2001). Por outro lado, a IL-10 tem efeito protetor devido à sua capacidade em prevenir uma resposta inflamatória exacerbada. Camundongos com produção deficiente dessa citocina exibiram resposta inflamatória prolongada após desafio com *Pseudomonas*, resultando em acúmulo de neutrófilos nos pulmões, condição favorável ao desenvolvimento de fibrose cística (CHMIEL *et al*., 2002). OBERHOLZER *et al.* (2002) demonstraram que a ausência de IL-10 favorece a suscetibilidade à toxina relacionada ao choque séptico. Estudos relatam também o papel protetor da IL-10 direto em casos de artrite experimental (PULUTI *et al*., 2002) e indireto em infecções por *Trichuris muris* (SCHOPF *et al*., 2002).

2. Produtos naturais e compostos isolados

2.1.Própolis, ácido cinâmico e ácido cumárico

A própolis é um produto natural, elaborado pelas abelhas a partir de material coletado das secreções de árvores, botões, gemas e córtex vegetais, resultando em um material resinoso, lipofílico e adesivo. A própolis é um conjunto complexo de substâncias, apresentando em sua composição aproximadamente 30% de ceras, 55% de resinas e bálsamos, 10% de óleos voláteis e 5% de pólen, além de impurezas (Burdock, 1998).

Pesquisas experimentais de nosso grupo revelaram que a própolis não apresenta efeitos colaterais, uma vez que variáveis séricas não foram alteradas após sua administração a curto, médio e longo prazo a ratos, tanto na forma de extrato aquoso quanto etanólico, em diferentes concentrações (Sforcin *et al*., 2002b; Mani *et al*., 2006).

A ação efetiva deste produto apícola contra diversos microrganismos também vem sendo relatada. Murad e colaboradores (2002) verificaram aumento da atividade fungicida de macrófagos peritoneais estimulados com própolis contra *Paracoccidioides brasiliensis.* Do mesmo modo, foi observado aumento na atividade antimicrobiana contra bactérias Gram-positivas e Gram-negativas (Orsi *et al*., 2005; Seidel *et al*., 2008). A atividade antiviral da própolis também foi demonstrada por Búfalo e colaboradores (2009).

Outras atividades biológicas como anti-neurodegenerativa (Chen *et al*., 2008), anti-hepatotóxica (Banskota *et al*., 2001) e anti-tumoral (Ahn *et al*., 2007) também foram atribuídas à própolis.

No tocante ao sistema imune, nosso grupo demonstrou que, em ratos tratados com própolis e imunizados com albumina sérica bovina, ocorreu aumento na produção de anticorpos (Sforcin *et al*., 2005). Resultado semelhante foi observado por Fischer e colaboradores (2007), utilizando a própolis como adjuvante em vacinas anti-SuHV-I (doença de Aujesky suína). Este produto apiterápico também promove ativação de macrófagos e aumento da sua capacidade fagocítica (Scheller *et al*., 1988), geração de peróxido de hidrogênio e óxido nítrico (Orsi *et al*., 2000) e potencialização da atividade lítica de células *natural killer* contra células tumorais (Sforcin *et al*., 2002a).

Nosso grupo demonstrou também que este produto natural modula eventos iniciais da resposta imune em camundongos, tais como o aumento da expressão dos receptores TLR-2 e TLR-4 e da produção de lL1β e IL-6 por macrófagos e esplenócitos após administração da própolis a curto prazo (Orsatti *et al*., 2010). Avaliamos também a ação da própolis em camundongos BALB/c submetidos a estresse, verificando que sua administração potencializou a geração de peróxido de hidrogênio por macrófagos peritoneais e impediu o aparecimento de centros germinativos no baço (Missima & Sforcin, 2008). Em modelo de estresse agudo, a própolis apresentou papel imunorestaurador, recuperando os níveis de IL-4 bem como a expressão de TLR-4, mecanismos que auxiliam o hospedeiro a reconhecer microrganismos durante o estresse, favorecendo a resposta imune humoral (Pagliarone *et al*. 2009).

Sá-Nunes *et al*. (2003) verificaram que a própolis apresenta efeito inibitório sobre a proliferação de linfócitos *in vitro* e *in vivo*. Orsatti *et al*. (2010)

observaram inibição na produção de IFN-γ *in vivo*, por camundongos tratados com própolis. Estes achados de nosso laboratório, somados a outros da literatura pertinente, apontam para a possível ação anti-inflamatória deste produto apícola (Moura *et al*., 2009; Cole *et al.,* 2010).

Em relação aos compostos fenólicos, em que se enquadram os ácidos cinâmico e cumárico, este é um vasto grupo de substâncias com particular importância em vários aspectos da investigação científica, principalmente para a Etnofarmacologia e Fitoquímica. Plantas e produtos naturais contêm uma variedade de compostos fenólicos, como no caso da própolis por nós utilizada, em que a maior parte da sua composição é representada por tais compostos (Bankova *et al*., 1998). Estes compostos desempenham um importante papel antibacteriano, anti-mutagênico, anti-carcinogênico e antioxidante, além de contribuírem diretamente para a boa qualidade nutricional de frutas e derivados (Calixto, 2000; Aljadi *et al*., 2003; Ivanauskas, 2008). Nossa intenção, ao incluir no projeto os testes com os ácidos cumárico e cinâmico, foi comparar os efeitos da própolis sobre a produção de citocinas contando com o sinergismo de seus componentes, com o tratamento direto com os ácidos derivados deste produto apícola, a fim de avaliar o envolvimento de tais compostos na ação da própolis, bem como o possível efeito anti-inflamatório destas variáveis.

2.2. *Baccharis dracunculifolia* e ácido cafeico

As fontes vegetais da própolis obtida no apiário de nossa Universidade foram investigadas, sendo *Baccharis dracunculifolia* DC (popularmente conhecida como alecrim-do-campo) sua principal fonte (Bankova *et al*., 1999), razão pela qual incluímos o extrato deste vegetal em nossos ensaios. Avaliamos também o ácido caféico, encontrado tanto na própolis como em *B. dracunculifolia*, em nossos protocolos experimentais.

O gênero *Baccharis* pertence à família Asteraceae e possui cerca de 500 espécies distribuídas principalmente no Brasil, Argentina, Paraguai e Uruguai. Devido à enorme importância medicinal, comercial e biológica, inúmeras espécies de *Baccharis* têm atraído a atenção de muitos pesquisadores das áreas de Química, Farmacologia e Imunologia (Teixeira *et al*., 2005).

Em relação à suas atividades biológicas, foi demonstrado que o alecrim-do-campo possui ação antibacteriana (Leitão *et al*., 2004), anti-ulcerativa (Lemos *et al*., 2004), anti-carcinogênica (Búfalo *et al*., 2010), além de anti-protozoária (Da Silva Filho *et al*., 2009) e antiviral (Búfalo *et al*., 2009).

Trabalho de nosso grupo revelou que o extrato obtido a partir do alecrim-do-campo foi capaz de ativar macrófagos murinos *in vitro*, com consequente aumento na liberação de H_2O_2 (Missima *et al*., 2007), porém, quanto à resposta imune humoral, não foi detectado aumento na produção de anticorpos após imunização de ratos com albumina sérica bovina seguido de tratamento com este produto vegetal (Sforcin *et al*., 2005).

Um dos componentes isolados de *Baccharis dracunculifolia* é o ácido cafeico, um composto fenólico que em diversas pesquisas vem sendo relacionado à atividade anti-inflamatória, anti-oxidante e imunomoduladora (Fitzpatrick *et al.*, 2001; Song *et al.*, 2008). Diversos mecanismos de ação estão sendo atribuídos a este ácido, tais como modulação da sinalização intracelular e indução de apoptose de células tumorais (melanocíticas), alvos importantes para a prevenção e tratamento de diversas patologias, como o câncer de pele, fatos que justificam o crescente interesse de pesquisadores em demonstrar os efeitos benéficos deste ácido (Bose *et al*., 2009; Kudugunti *et al*., 2010).

2.3. Capim-limão e citral

O capim-limão (*Cymbopogon citratus)* é uma erva aromática muito utilizada no mundo todo, principalmente no Brasil para o tratamento de cólicas uterinas e distúrbios gastrintestinais. O chá de suas folhas também é usado como anti-séptico, antitérmico e calmante (Barbosa *et al*., 2008).

Dados recentes da literatura apontam diversos efeitos biológicos deste vegetal, tais como: citoprotetor, antioxidante, anti-inflamatório, anticonvulsivante e anti-fúngico (Irkin *et al*., 2009; Figueirinha *et al*., 2010; Silva *et al*., 2010; Tiwari *et al*., 2010). Além destes efeitos, há relatos do uso deste produto vegetal como inseticida, principalmente contra insetos anofelinos, vetores da malária (Karunamoorth *et al*., 2010).

O citral (3,7-dimetil-2,6-octadienal) é um dos principais componentes de *C. citratus* e é amplamente utilizado na indústria farmacêutica como inibidor natural do crescimento de patógenos em perfumes e cosméticos (Guynot *et al*., 2003). Silva *et al*. (2008) demonstraram que o tratamento *in vitro* com citral é efetivo em combater várias espécies de *Candida*.

Trabalhos recentes de nosso laboratório evidenciaram a ação inibitória *in vivo* do extrato aquoso do capim-limão sobre a produção das citocinas pró-inflamatórias IL-1β e IL-6 por macrófagos murinos. Em ensaios *in vitro*, observamos que os óleos essenciais do capim-limão também inibiram a produção destas mesmas citocinas (Sforcin *et al*., 2009), fatos estes que nos incentivaram a continuar a pesquisa do potencial anti-inflamatório do capim-limão bem como de seu composto isolado, o citral.

2.4. Cravo-da-Índia e eugenol

Syzygium aromaticum, popularmente conhecido como cravo-da-Índia é uma das mais antigas e populares especiarias utilizadas no mundo todo, devido ao seu marcante aroma e sabor, sendo introduzida em várias partes do globo, não apenas como tempero em culinária, mas também devido às suas propriedades medicinais (Mazzafera *et al*., 2003).

Pesquisas científicas têm demonstrado tais propriedades medicinais pontualmente, atribuindo ao cravo-da-Índia atividades como: antibacteriana (Chaieb *et al*., 2007), anti-fúngica (Chami *et al*., 2005), anti-carcinogênica (Banerjee *et al*., 2006), anti-alérgica (Kim *et al*., 1998) e anti-mutagênica (Miyazawa & Hisama *et al*., 2001). Além disso, o tratamento com óleo essencial de cravo-da-Índia foi capaz de restaurar a imunidade celular e humoral de ratos imunossuprimidos com ciclofosfamida (Carrasco *et al*., 2009).

Verificamos que o tratamento de camundongos com o extrato aquoso do cravo-da-Índia inibiu a produção das citocinas pró-inflamatórias IL-1β e IL-6 por macrófagos peritoneais. Em ensaios *in vitro,* observamos que os óleos essenciais do cravo-da-Índia também inibiram a produção destas citocinas, além de caracterizarmos quimicamente nossa amostra de *S. aromaticum*, evidenciando o eugenol (4-alil-2-metoxifenol) como composto majoritário (Rodrigues *et al*., 2009).

O eugenol é um composto fenólico que vem sendo utilizado como anti-séptico e analgésico, principalmente nos países asiáticos (Morsy *et al*., 2008), porém outras atividades deste composto foram relatadas, como anti-inflamatória, anti-viral, anti-tumoral e antioxidante (Benecia *et al*., 2000; Ghosh *et al*., 2005; Lee *et al*., 2007). Devido às atividades relatadas e por ser o composto majoritário de nossa amostra de *S. aromaticum,* incluímos este composto isolado nos protocolos experimentais, a fim de avaliar seu potencial anti-inflamatório *in vitro*, comparando-o com o extrato do cravo-da-Índia.

3.Objetivos e apresentação da dissertação

Os objetivos desta dissertação foram:

- Avaliar a possível citotoxicidade dos seguintes produtos naturais e seus compostos isolados: própolis (ácidos cumárico e cinâmico), alecrim-do-campo (ácido cafeico), capim-limão (citral) e cravo-da-Índia (eugenol) sobre macrófagos peritoneais de camundongos BALB/c;
- Analisar o efeito *in vitro* destas variáveis sobre a produção de IL-1β, IL-6 e IL-10 por macrófagos murinos;
- Verificar a eficiência dos extratos e compostos isolados sobre a produção de citocinas por macrófagos desafiados com LPS antes ou após a incubação com os produtos naturais.

A presente dissertação encontra-se apresentada sob a forma de 4 capítulos que serão submetidos à publicação junto à revista Journal of Ethnopharmacology após a defesa, sendo intitulados:

1- **“The effects of propolis and its isolated compounds on cytokines production by murine macrophages”**

2- **“The effects of *Baccharis dracunculifolia* and caffeic acid on cytokines production by murine macrophages”**

3- **“Lemongrass and citral effect on cytokines production by murine macrophages”**

4- ***“In vitro* effects of clove and eugenol on cytokines production by murine macrophages”**

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Capítulo I

The effects of propolis and its isolated compounds on cytokines producion by murine macrophages

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ABSTRACT

Aim of the study: Propolis is an adhesive substance produced by honeybees from the bud and exudates of certain trees and plants. Since propolis and phenolic compounds, such as cinnamic and coumaric acids, have several biological properties, the goal of this work was to investigate the immunomodulatory effect of propolis and such compounds on cytokines production (IL-1β, IL-6 and IL-10) by peritoneal macrophages *in vitro* in different protocols with LPS challenge.

Material and methods: Peritoneal macrophages from BALB/c mice were incubated with propolis, coumaric and cinnamic acids in different concentrations for 24h. The concentrations that inhibited cytokines production were tested before or after macrophages challenge with LPS, in order to evaluate a possible immunomodulatory action. Supernatants of cell cultures were used for cytokines determination by ELISA.

Results: Propolis, coumaric and cinnamic acids stimulated IL-1β production. However, IL-6 production was significantly inhibited after propolis (5, 50 and 100 µg/well), coumaric and cinnamic acids (50 and 100 µg/well) incubation for 24 h. In LPS-challenge protocols, the addition of inhibitory concentrations of cinnamic and coumaric acids after LPS incubation prevented efficiently its effects on IL-6 production, whereas propolis inhibited LPS effects both before and after its addition. Propolis, coumaric and cinnamic acids (50 and 100 µg/well) inhibited IL-10 production as well. Both acids showed a similar inhibitory activity on IL-10 production when added after LPS challenge, while propolis counteracted significantly LPS action when added before and after LPS incubation.

*Conclusion*s: Propolis modulated the immune/inflammatory response, depending on concentration. Propolis efficiency may occur due to the synergistic effect of its several compounds, and one may speculate that cinnamic and coumaric acids may be involved in propolis action on cytokines production.

Keywords:

Propolis

Immunomodulation

Cytokines

Cinnamic acid
Coumaric acid

1. Introduction

Propolis is an adhesive substance produced by honeybees from the bud and exudates of certain trees and plants and stored inside their hives to protect from rain or bacterial invasion (Bankova et al., 2000). The main vegetal source of propolis in Botucatu, São Paulo State, Brazil, is *Baccharis dracunculifolia* D.C., followed by *Eucalyptus citriodora* Hook and *Araucaria angustifolia* (Bert.) O. Kuntze (Bankova et al., 1999).

Propolis has a long history as a general tonic promoting health, due to its several biological properties, such as anti-inflammatory (Hu et al., 2005), antibacterial (Sforcin et al., 2000), antifungal (Silica et al., 2005), antitumoral (Ahn et al., 2007), antioxidant (Simões et al., 2004), immunomodulatory (Sforcin, 2007), antihepatotoxic (Banskota et al., 2001), anti-viral (Búfalo et al., 2009), and antineurodegenerative (Chen et al., 2008). More than 300 constituents have been identified in different types of propolis (Bankova et al., 2000), and phenolics (e.g. cinnamic and coumaric acids) compounds (Popova et al., 2004) have been pointed out for the antioxidant, antimutagenic and anti-inflammatory effects of medicinal herbs and dietary plants (Huang et al., 2010).

Inflammation (either acute or chronic) is an important part of immuno-pathogenesis. During inflammatory diseases, macrophages produce an excessive amount of mediators such as inflammatory cytokines (Jung et al., 2007). Interleukin (IL)-1β is a pleiotropic pro-inflammatory cytokine inducing systemic and local responses to infection. It induces the expression of adhesion molecules on endothelial cells and chemokines, leading to the infiltration of inflammatory and immunocompetent cells (Dinarello, 2009). In addition, IL-1β causes fever, vasodilatation, hypotension and enhances pain sensitivity. On the other hand, the anti-inflammatory cytokine IL-10 decreases the production of pro-inflammatory cytokines, including TNF-α and IL-1β (O'Shea and Murray, 2008). IL-6 is a multifunctional regulator of immune response, hematopoiesis, and acute phase reactions. (Badache and Hynes, 2001).

Macrophage activation by lipopolysaccharides (LPS) results in the release of several inflammatory mediators including IL-1β, IL-6 and IL-10 (Parker and Schimer, 2001). These cytokines are produced and secreted by a variety of cell types and play a major role in the induction and regulation of inflammation, hematopoiesis, and immune reactions (Goldsby et al., 2005).

This work aimed to investigate the immunomodulatory effect of propolis on cytokines production (IL-6, IL-1β and IL-10) by peritoneal macrophages *in vitro.* Cinnamic and coumaric acids were investigated as possible compounds responsible for propolis action. The concentrations of propolis, cinnamic and coumaric acids that inhibited cytokines production were tested before or after macrophages challenge with LPS, in order to evaluate a possible anti-inflammatory action.

2. Material and methods

2.1. Propolis sample

Propolis was produced by *Apis mellifera* L. bees in the apiary located in the Lageado Farm, UNESP, Campus of Botucatu, using plastic nets. Propolis was ground and a 30% ethanolic extract was prepared in sterile conditions (30 g of propolis added to a 70% ethanol solution totaling 100 mL), in the absence of bright light, at room temperature and shaken moderately. After a week, extracts were filtered and final concentrations were calculated, obtaining the dry weight of the solutions (120 mg/mL) (Sforcin et al., 2008).

Propolis was frozen and has been used up to now, and its chemical composition was analysed previously (BANKOVA et al., 1998) using thin-layer chromatography (TLC), gas chromatography (GC), and gas chromatography–mass spectrometry (GC–MS) analysis.

2.2. Cinnamic and coumaric acids

Cinnamic and coumaric acids were purchased from Acros Organics (Morris Plains, NJ, USA).

2.3. Animals and peritoneal macrophages

Male BALB/c mice weighing 25–30 g and aged between 8 and 12 weeks were used (n = 15). Mice were kept in rooms at 21–25 ºC and 50% relative humidity, with a 12 h light/dark cycle. Food and water were provided *ad libitum*.

Peritoneal macrophages were obtained by inoculation of 3–5 mL of cold PBS in abdominal cavity. After a soft abdominal massage for 30 s, the peritoneal liquid was collected and put in sterile plastic tubes (Falcon). This procedure was repeated 3 or 4 times for each animal and cells were pooled. Tubes were centrifuged at 200 g for 10 min. Cells were stained with neutral red (0.02%), incubated for 10 min at 37 °C and counted in a haematocytometer to obtain a final concentration of 2 x 10^6 cells/mL. Cells were resuspended in cell culture medium (RPMI 1640, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 20 mM HEPES – Sigma, USA) and cultured in a 96-well flat-bottomed plate (Corning, USA) at a final concentration of 2 x 10^5 cells per well. Cells were incubated at 37 °C and, after 2 h, non-adherent cells were removed (Sforcin et al., 2007).

This work agrees with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation, and was approved in August 8, 2008 (protocol n°46/08).

2.4. Cytotoxicity assay

Crystal violet method was used to test the cytotoxicity of propolis, cinnamic and coumaric acids toward macrophages (Ait-Mbarek et al., 2007). Briefly, macrophages were seeded at a density of 2 x 10^5/well in 96-well plates and incubated with propolis or its acids (5, 10, 25, 50 and 100 µg/well). After 24 h, supernatants were removed and 100 µL 0.5% crystal violet solution was added to the cells. After 10-min incubation at room temperature, the plates were washed and viable crystal violet-stained cells were lysed with 1% sodium dodecyl sulphate. Optical densities (OD) were read at 492 nm in an ELISA

reader, and the percentage of cell viability was calculated using the formula: [OD test/OD control] x 100. Assays were carried out in triplicate.

2.5. In vitro assays

Peritoneal macrophages (2 x 10^5/well) were treated with propolis, cinnamic and coumaric acids at different concentrations (5, 10, 25, 50 and 100 µg/well) for 24 h at 37 °C and 5% CO_2. Afterwards, the supernatants were harvested for cytokines (IL-1β, IL-6 and IL-10) measurement.

Concentrations that inhibited cytokines production were used in others protocols, challenging the cells with LPS, as follows.

2.5.1. Propolis, cinnamic and coumaric acids incubation before LPS challenge

Macrophages were pre-treated with propolis (5, 50 and 100 µg/well), cinnamic (50 and 100 µg/well) or coumaric acid (50 and 100 µg/well) at the concentrations that inhibited IL-1β, IL-6 and IL-10 production for 2 h and then incubated with LPS (5 µg/mL) for 22 h. After this period, the culture supernatants were harvested and stored at -70ºC for cytokines measurement (Shin et al., 2006).

2.5.2. Propolis, cinnamic and coumaric acids incubation after LPS challenge

In another protocol, macrophages were stimulated with LPS (5 µg/mL) for 2 h and then incubated with propolis (5, 50 and 100 µg/well) or cinnamic acid or coumaric acid (50 and 100 µg/well) for 22 h. Afterwards, supernatants were collected and stored at -70ºC for cytokines determination (Shin et al., 2006).

Dexamethasone (DEX, 10^{-4} mol/L) (Zhuo et al., 2010) and LPS (5 µg/mL) were used as a negative and positive control, in order to inhibit and stimulate cytokine production, respectively.

2.6. Determination of cytokine production

IL-1β, IL-6 and IL-10 production was measured by enzyme-linked immunosorbent assay (ELISA), according to manufacturer's instructions (BD Biosciences, USA). Briefly, a 96-well flat bottom Nunc Maxisorp (Nunc/Apogent, USA) was coated with a 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 captured cytokine was detected using the specific conjugated detection antibody. The substrate reagent was added into each well and, after color development, the plate was read at 450 nm, using an ELISA plate reader (Tan et al., 2006).

2.7. Statistical analysis

Data were expressed as means and stardad-deviation of 5-7 similar experiments. Analysis of variance (ANOVA) and Dunnet's multiple comparison method were used. A probability (*P*) of 0.05 was chosen as the significance level (Zar, 1999).

3. Results

3.1. Cytotoxicity assay

Propolis, cinnamic and coumaric acid did not affect cell viability as determined by crystal-violet test (data not shown).

3.2. Cytokines production

3.2.1. IL-1β production

An increased IL-1β production was observed in macrophages treated with propolis, cinnamic and coumaric acids at all concentrations (5, 10, 25, 50 and 100 µg/well) (Fig. 1).

3.2.2. IL-6 production

IL-6 production decreased after macrophages incubation with propolis (5, 50 and 100 µg/well, $P < 0.0001$), cinnamic (50 µg/well, $P < 0.0001$ and 100 µg/well, $P < 0.001$) and coumaric acid (50 and 100 µg/well, $P < 0.0001$)(Fig. 2A).

The inhibitory concentrations of propolis (5, 50 and 100 µg/well) and isolated compounds (50 and 100 µg/well), were analyzed in the next protocol. Fig. 2B shows that the treatment with natural products prevented LPS action both before and after its challenge, and IL-6 production was lower than that induced by LPS alone ($P < 0.0001$).

3.2.3. IL-10 production

As to IL-10, propolis (50 and 100 µg/well) inhibited significantly its production, as well as coumaric acid (5 µg/well, $P < 0.001$; 10, 25, 50 and 100 µg/well, $P < 0.0001$). Cinnamic acid did not inhibit significantly IL-10 production, but the concentrations of 50 and 100 µg/well were tested in the LPS-challenge protocol, because they inhibited IL-6 production (Fig. 3A).

Propolis (50 and 100 µg/well) inhibited IL-10 ($P < 0.0001$) production either before or after macrophages incubation with LPS, whereas cinnamic and coumaric acid (50 and 100 µg/well) counteracted significantly LPS action only when added after LPS incubation ($P < 0.0001$) (Fig. 3B).

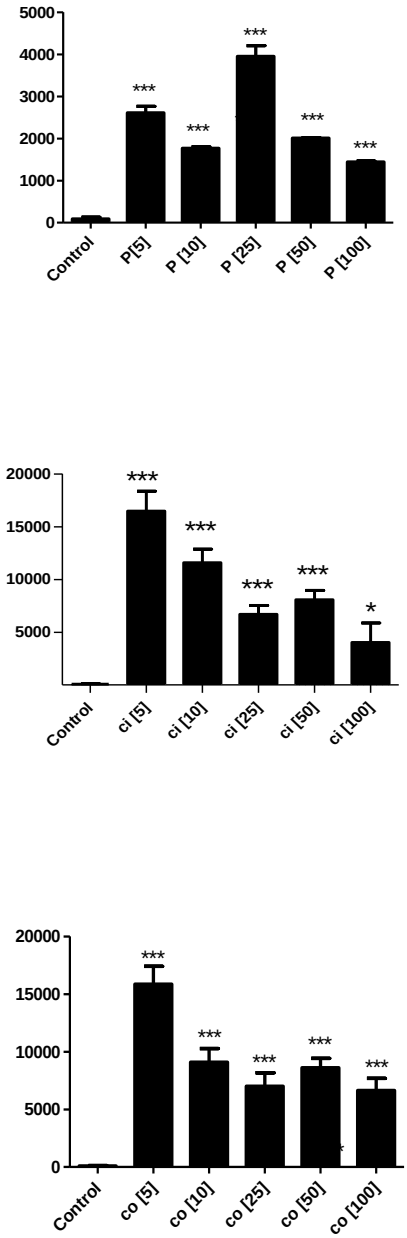

Fig. 1. IL-1β production by peritoneal macrophages incubated with propolis (P), cinnamic (ci) and coumaric (co) acids at different concentrations (5, 10, 25, 50 and 100 μg/well) for 24 h at 37ºC. Data are expressed as means ± standard-deviation Of 5-7 similar assays * significantly different from control ($P < 0.01$); *** significantly different from control ($P < 0.0001$).

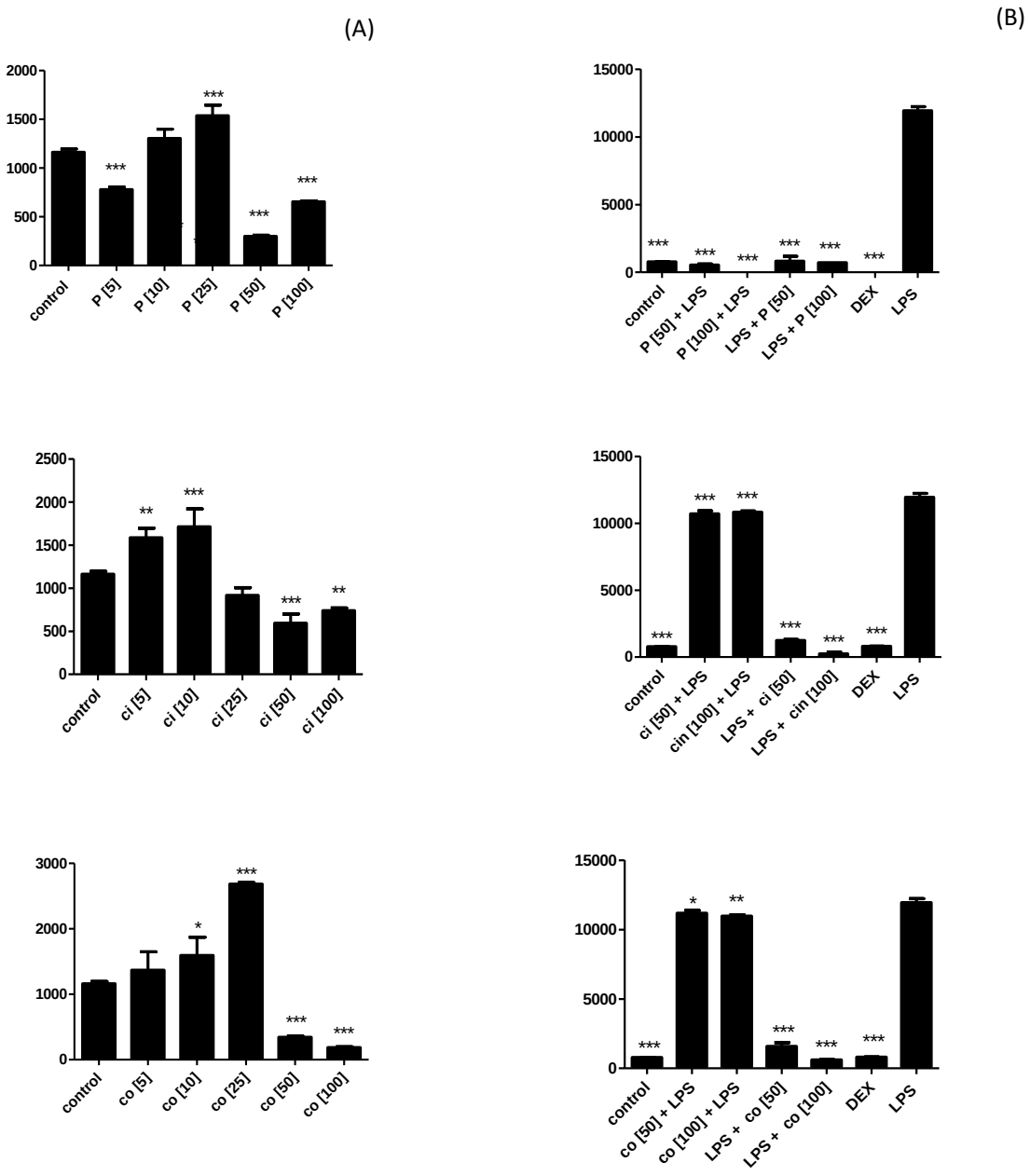

Fig. 2. (A) IL-6 production by peritoneal macrophages incubated with propolis (P), cinnamic (ci) and coumaric (co) acids at different concentrations (5, 10, 25, 50 and 100μg/well) for 24 h at 37°C. **(B)** IL-6 production by peritoneal macrophages stimulated with LPS 2 h before or after incubation with P, ci and co acids for 22 h. DEX (10^{-4} mol/L) and LPS (5 μg/well) were used as negative and positive control, respectively. Data are expressed as means ± standard-deviation of 5-7 similar assays. * significantly different from control ($P < 0.01$); ** significantly different from control or LPS ($P < 0.001$); *** significantly different from control or LPS ($P < 0.0001$).

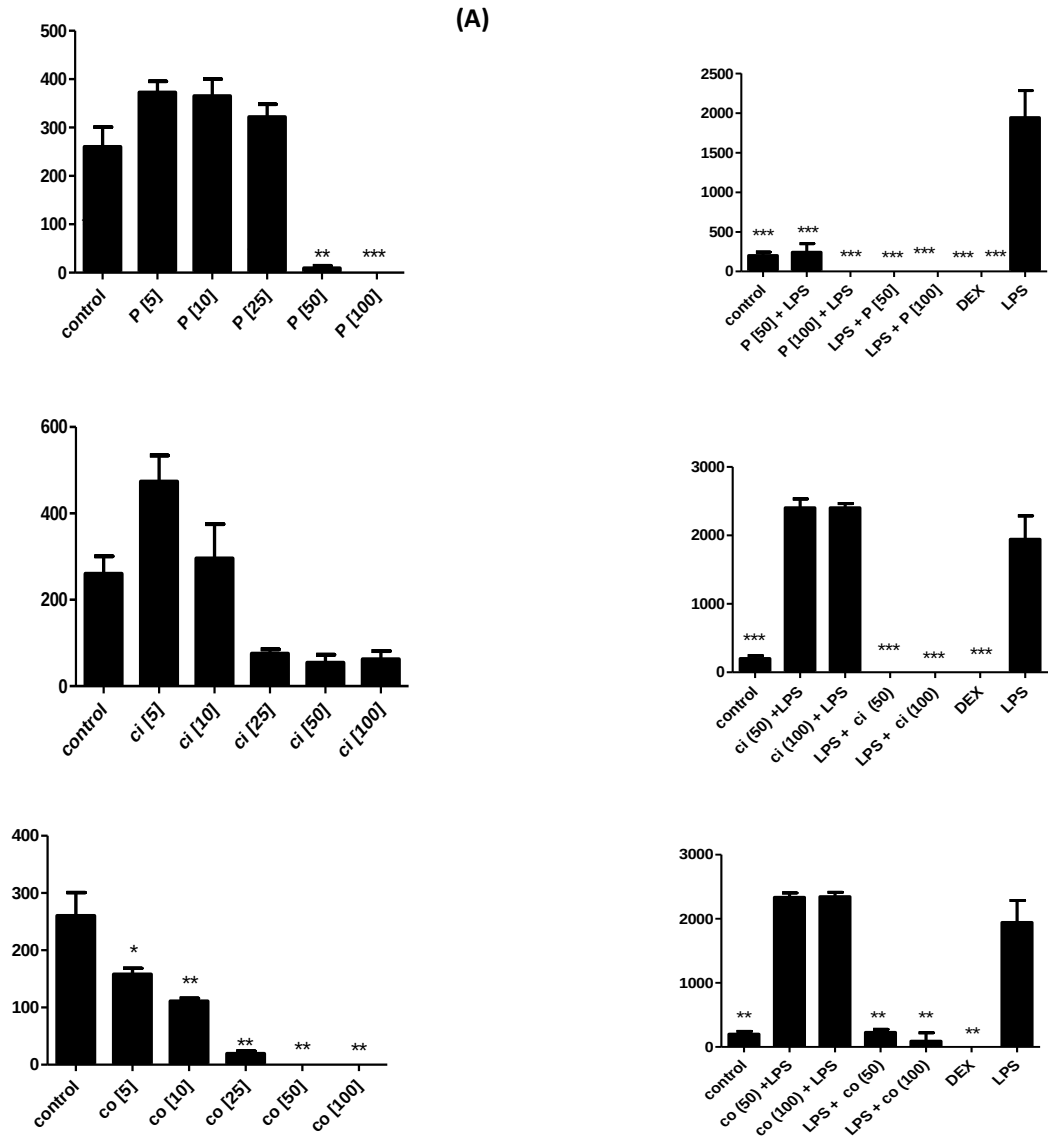

Fig. 3. (A) IL-10 production by peritoneal macrophages incubated with propolis (P), cinnamic (ci) and coumaric (co) acids at different concentrations (5, 10, 25, 50 and 100 μg/well) for 24 h at 37°C. **(B)** IL-10 production by peritoneal macrophages stimulated with LPS 2 h before or after incubation with P, ci and co acids for 22 h. DEX (10^{-4} mol/L) and LPS (5 μg/well) were used as negative and positive control, respectively. Data are expressed as means ± standard-deviation of 5-7 similar results. * significantly different from control ($P < 0.001$); ** significantly different from control or LPS ($P < 0.0001$).

4. Discussion

The chemical composition of our propolis sample was previously investigated, showing flavonoids (kaempferid, 5,6,7-trihydroxy-3,4-dimethoxyflavone, aromadendrine-4-methyl ether); a prenylated *p*-coumaric acid and two benzopyranes: *E* and *Z* 2,2-dimethyl-6-carboxyethenyl-8-prenyl-2H-benzopyranes; essential oils (spathulenol, (2*Z*,6*E*)-farnesol, benzyl benzoate and prenylated acetophenones); aromatic acids (dihydrocinnamic acid, *p*-coumaric acid, ferulic acid, caffeic acid, 3,5-diprenyl *p*-coumaric acid, 2,2-dimethyl-6-carboxy-ethenyl-8-prenyl-2H-1-benzo-pyran); di- and triterpenes, among others (Bankova et al., 1998).

Propolis, coumaric and cinnamic acids increased IL-1β production *in vitro*, suggesting their immunostimulatory action. Previous works of our laboratory have investigated the effects of propolis on the immune system, in attempt to understand its mechanisms of action on the initial events of the immune response. Orsatti et al. (2010b) verified that propolis upregulated Toll-like receptors (TLR-2 and TLR-4) expression and the production of pro-inflammatory cytokines (IL-1β and IL-6) by macrophages and spleen cells. In stressed mice, propolis exerted an immunorestorative effect, counteracting the inhibition of TLR-2 and TLR-4 expression (Pagliarone et al., 2009a). Dinarello et al. (2010) verified a reduced inflammation by blocking IL-1β with consequent risk of infections, suggesting the importance of this cytokine in the inflammatory/immune response. Herein, the effects of propolis may be the result of the synergistic action of its constituents. Cinnamic and coumaric acids induced a higher IL-1β production than propolis alone, and 5 µg/well of such acids was more efficient than the other concentrations. However, one should take into account that most of propolis compounds are found at quantities lower than 3% (Ivanovska et al., 1995).

On the other hand, propolis and its constituents inhibited IL-6 and IL-10 production by peritoneal macrophages depending on concentration, even in the presence of LPS. Since elevated levels of IL-6 are related to chronic inflammation, cardiac diseases and depression (Howren, 2009), our data suggest that propolis administration may represent a new option of treatment for such diseases. IL-10 is important for maintaining homeostasis of the host due to

its immunoregulatory action (Mosser et al., 2003). High levels of this anti-inflammatory cytokine are found in
immunosuppressed patients (Alter et al., 2010) and propolis inhibitory action on IL-10 production may be useful to prevent infections.

LPS-challenge protocols may represent two different conditions. First, when macrophages were incubated with propolis or its acids before LPS addition to the cell culture, one could expect a preventive role of the natural products, inhibiting LPS action. Second, the addition of propolis or its acids to macrophages that were previously incubated with LPS may represent a possible treatment of inflammatory diseases. Propolis seemed to be efficient either before or after macrophages incubation with LPS, suggesting its preventive and protective effects, respectively. Cinnamic and coumaric acids prevented LPS action only after its addition. Moreover, such acids showed a similar effect in all assays, probably because coumaric acid is derived from cinnamic acid (Galato et al., 2001).

We would like to emphasize that different experimental models, types of extracts, concentration and period of incubation (*in vitro)* or administration (*in vivo*) may influence propolis action on immunological assays (Pagliarone et al., 2009b; Orsatti et al., 2010b; Missima et al., 2009). Our data showed that propolis may exert both pro- or anti-inflammatory action, modulating the immune/inflammatory response, depending on the concentration. Propolis efficiency may occur due to the synergistic effect of its several compounds, and based on our results one may speculate that cinnamic and coumaric acids may be involved in propolis action on cytokines production.

Acknowledgements

The authors wish to thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP – 2008/06120-0) for the grant.

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Capítulo II

The effects of *Baccharis dracunculifolia* and caffeic acid on cytokines production by murine macrophages

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ABSTRACT

Aim of the study: *Baccharis dracunculifolia* has been described as the most important plant source of propolis in the southeastern Brazil. Herein, we investigated the immunomodulatory effect of *B. dracunculifolia* and its isolated compound – caffeic acid – on cytokines production (IL-1β, IL-6 and IL-10) by peritoneal macrophages *in vitro* in different protocols with LPS challenge.

Material and methods: Peritoneal macrophages from BALB/c mice were incubated with *B. dracunculifolia* and caffeic acid in different concentrations for 24h. The concentrations that inhibited cytokines production were tested before or after macrophages challenge with LPS, in order to evaluate a possible immunomodulatory action. Supernatants of cell cultures were used for cytokines determination by ELISA.

Results: *B. dracunculifolia* and caffeic acid stimulated IL-1β production. However, IL-6 production was significantly inhibited after *B. dracunculifolia* and caffeic acid (50 and 100 µg/well) incubation for 24 h. In LPS-challenge protocols, these natural products diminished LPS action when added either before or after LPS incubation. *B. dracunculifolia* (at all concentrations) and caffeic acid (50 and 100 µg/well) inhibited IL-10 production. *B. dracunculifolia* prevented LPS action either before or after LPS challenge, whereas caffeic acid prevented LPS effects only after LPS addition.

Conclusion: Our data showed that *B. dracunculifolia* exerted immunomodulatory action on cytokines production, which can be at least in part mediated by caffeic acid, since it may inhibit the transcription factor NF-κB. Further studies are still needed to evaluate *B. dracunculifolia* efficacy in inflammatory diseases, in order to explore its anti-inflammatory activity.

Keywords:
Baccharis dracunculifolia
Immunomodulation
Cytokines
Caffeic acid

1. Introduction

Baccharis dracunculifolia D.C. (Bd), popularly known as “alecrim do campo”, is the most important botanical origin of propolis from southeast Brazil. It has been reported to display anti-ulcer (Lemos et al., 2007), antimicrobial (Leitão et al., 2004) and immunomodulatory activities (Missima et al., 2007). Crude extracts and isolated compounds from *B. dracunculifolia* possess trypanocidal (Da Silva Filho et al., 2004), antileishmanial (Da Silva Filho et al., 2009), antimicrobial (Da Silva Filho et al., 2008) and antiviral (Búfalo et al., 2009) activities as well. One of the isolated compounds from this vegetal specie is caffeic acid (Ca), which can be associated to anti-inflammatory and antioxidant activities (Muriel et al., 2008; Song et al., 2008).

Macrophages are crucial cells for host defense, since they effectively regulate innate and adaptive immune responses. Pathogen-associated molecular patterns can also initiate immune responses by activating pattern recognition receptors on antigen-presenting cells, such as macrophages. A typical example is the activation of TLR-4 by bacterial lipopolysaccharide (LPS), which promotes the production and release of inflammatory cytokines (Medzhitov et al., 2007).

Interleukin-1β (IL-1β) is a cytokine that plays a major role in inflammatory responses to infections and immune-mediated diseases, stimulating the production of mediators such as prostaglandin E_2, nitric oxide, cytokines, chemokines and adhesion molecules that are involved in inflammation (Jacques et al., 2006). Contrarily, IL-10 has a protective effect due to its ability to prevent an exaggerated inflammatory response, but overexpression of this cytokine is related to the predisposition to infectious complications (Kobayashi et al., 2001). With ambiguous functions, IL-6 is a pro-inflammatory cytokine; on the other hand, it has a role of self-limitation of inflammatory responses, because it can modulate the secretion of pro-inflammatory cytokines (Kaplanski et al., 2003).

Immunomodulation using medicinal plants provides an alternative to the conventional chemotherapy for several diseases. Thus, the aim of this study was to evaluate the *in vitro* effects of *B. dracunculifolia* on cytokines production, investigating the potential of this plant as an immunomodulatory agent. Caffeic

acid was also evaluated, in order to investigate a possible component responsible for *B. dracunculifolia* action. The concentrations of *B. dracunculifolia* or caffeic acid that inhibited cytokines production were also tested before or after macrophages challenge with LPS.

2. Material and methods

2.1. Baccharis dracunculifolia

Baccharis dracunculifolia leaves were collected in São Paulo State, Brazil. Plants were identified and voucher specimens were deposited in the Herbarium of the Chemical, Biological and Agricultural Pluridisciplinary Research Center (CPQBA), UNICAMP, São Paulo, Brazil. Extracts were obtained after maceration with ethanol-water (9:1) at room temperature. Hydroalcoholic solutions were filtered and concentrated in rotaevaporator. Specific dilutions were prepared in RPMI media for each assay.

2.2. Caffeic acid

Caffeic acid were purchased from Acros Organics (Morris Plains, NJ, USA), and diluited in RPMI media.

2.3. Animals and peritoneal macrophages

Male BALB/c mice weighing 25–30 g and aged between 8 and 12 weeks were used. Mice were kept in rooms at 21–25 ºC and 50% relative humidity, with a 12 h light/dark cycle. Food and water were provided *ad libitum*.

After inoculation of 3–5 mL of cold PBS in abdominal cavity and a soft abdominal massage for 30 s, the peritoneal liquid was collected and put in sterile plastic tubes (Falcon). This procedure was repeated 3 or 4 times for each animal and the tubes were centrifuged at 200 g for 10 min. Peritoneal macrophages were stained with neutral red (0.02%), incubated for 10 min at 37 °C and counted in a haematocytometer to obtain a final concentration of 2×10^6 cells/mL. Cells were resuspended in cell culture medium (RPMI 1640,

supplemented with 10% fetal calf serum, 2 mM L-glutamine, 20 mM HEPES – Sigma, USA) and cultured in a 96-well flat-bottomed plate (Corning, USA) at a final concentration of 2 x 10^5 cells per well. Cells were incubated at 37 °C and, after 2 h, non-adherent cells were removed (Sforcin et al., 2007).

This work agrees with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation, and was approved in August 8, 2008 (protocol n°46/08-CEEA).

2.4. Cytotoxicity assay

Prior to *in vitro* assays, cells were incubated with *Baccharis dracunculifolia* extract or caffeic acid at the concentrations 5, 10, 25, 50 and 100 µg/well, in order to carry out the assays only with noncytotoxic concentrations.

The evaluation of cytotoxicity was carried out by the crystal violet method (Ait-Mbarek *et al*. 2007). The macrophage culture received the stimuli as previously described and, after 24 h, supernatants were removed and 100 µL 0.5% crystal violet solution was added to the cells. After 10 min incubation at room temperature, the plates were washed and viable crystal violet-stained cells were lysed with 1% sodium dodecyl sulphate. Optical densities (OD) were read at 492 nm in an ELISA reader, and the percentage of cell viability was calculated using the formula: [OD test ⁄ OD control] x 100. Assays were carried out in triplicate.

2.5. In vitro assays

Peritoneal macrophages (2x10^5/well) were treated with *B. dracunculifolia* and caffeic acid at different concentrations (5, 10, 25, 50 and 100 µg/well) for 24 h at 37°C and 5% CO_2. Afterwards, the supernatants were harvested for cytokines (IL-1β, IL-6 and IL-10) measurement.

Concentrations that inhibited the production of IL-1β and IL-6 were used in other protocols, challenging the cells with LPS, as follows.

2.5.1. Baccharis dracunculifolia and cafeic acid incubation before LPS challenge

Macrophages were pre-treated with *B. dracunculifolia* (50 and 100 µg/well) or caffeic acid (25, 50 and 100 µg/well) at the concentrations that inhibited IL-6 and IL-10 production for 2 h and then incubated with LPS (5 µg/mL) for 22 h. After this period, the culture supernatants were harvested and stored at -70ºC for cytokines measurement (Shin et al., 2006).

2.5.2. Baccharis dracunculifolia and cafeic acid incubation after LPS challenge

Macrophages were stimulated with LPS (5 µg/mL) for 2 h and then incubated with *B. dracunculifolia* (50 and 100 µg/well) or caffeic acid (25, 50 and 100 µg/well) for 22 h. Afterwards, supernatants were collected and stored at -70ºC for cytokines determination (Shin et al. 2006).

Dexamethasone (DEX, 10^{-4} mol/L) (Zhuo et al.,2010) and LPS (5 µg/mL) were used as a negative and positive control, in order to inhibit and stimulate cytokine production, respectively.

2.6. Determination of cytokine production

IL-1β, IL-6 and IL-10 production was measured by enzyme-linked immunosorbent assay (ELISA), according to manufacturer's instructions (BD Biosciences, USA). Briefly, a 96-well flat bottom Nunc Maxisorp (Nunc/Apogent, USA) was coated with a 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 captured cytokine was detected using the specific conjugated detection antibody. The substrate reagent was added into each well and, after color development, the plate was read at 450 nm, using an ELISA plate reader (Tan et al., 2006).

2.7. Statistical analysis

Data were expressed as means ± standard-deviation of 5-7 similar assays. Analysis of variance (ANOVA) was employed, followed by Dunnet's multiple comparison method, with 0.05 as the significant level (Zar, 1999).

3. Results

3.1. Citotoxicity assay

B. dracunculifolia and caffeic acid did not affect cell viability as determined by crystal-violet test (data not shown).

3.2. Cytokines production

3.2.1. IL-1β production

IL-1β production was significantly increased ($P < 0.0001$) after *B. dracunculifolia* incubation for 24h (5, 10, 25, 50 and 100 µg/well) as well as after caffeic acid incubation (5 and 10 µg/well, $P < 0.0001$; 25 µg/well, $P < 0.001$) (Fig. 1).

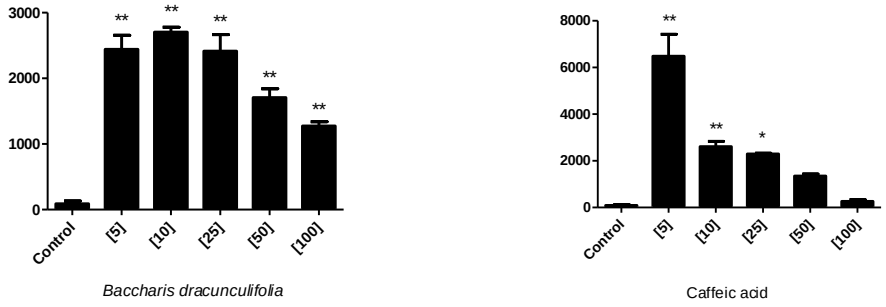

Fig. 1. IL-1β production by peritoneal macrophages incubated with *B. dracunculifolia* or caffeic acid at different concentrations (5, 10, 25, 50 and 100 µg/well) for 24 h at 37 ºC. * significantly different from control ($P < 0.001$); ** significantly different from control ($P < 0.0001$). Data are expressed as means ± standard-deviation of 5-7 smilar assays.

3.2.2. IL-6 production

As to IL-6, *B. dracunculifolia* (5, 10 and 25 µg/well) and caffeic acid (5 and 10 µg/well) stimulated significantly its production ($P < 0.0001$), whereas *B. dracunculifolia* (50 and 100 µg/well) and caffeic acid (25, 50 and 100 µg/well) exerted an inhibitory action on this cytokine production ($P < 0.0001$) (Fig. 2A). As seen in Fig. 2B, *B. dracunculifolia* (50 and 100 µg/well) and caffeic acid (25, 50 and 100 µg/well) diminished significantly LPS action when added either before or after LPS incubation ($P < 0.0001$)

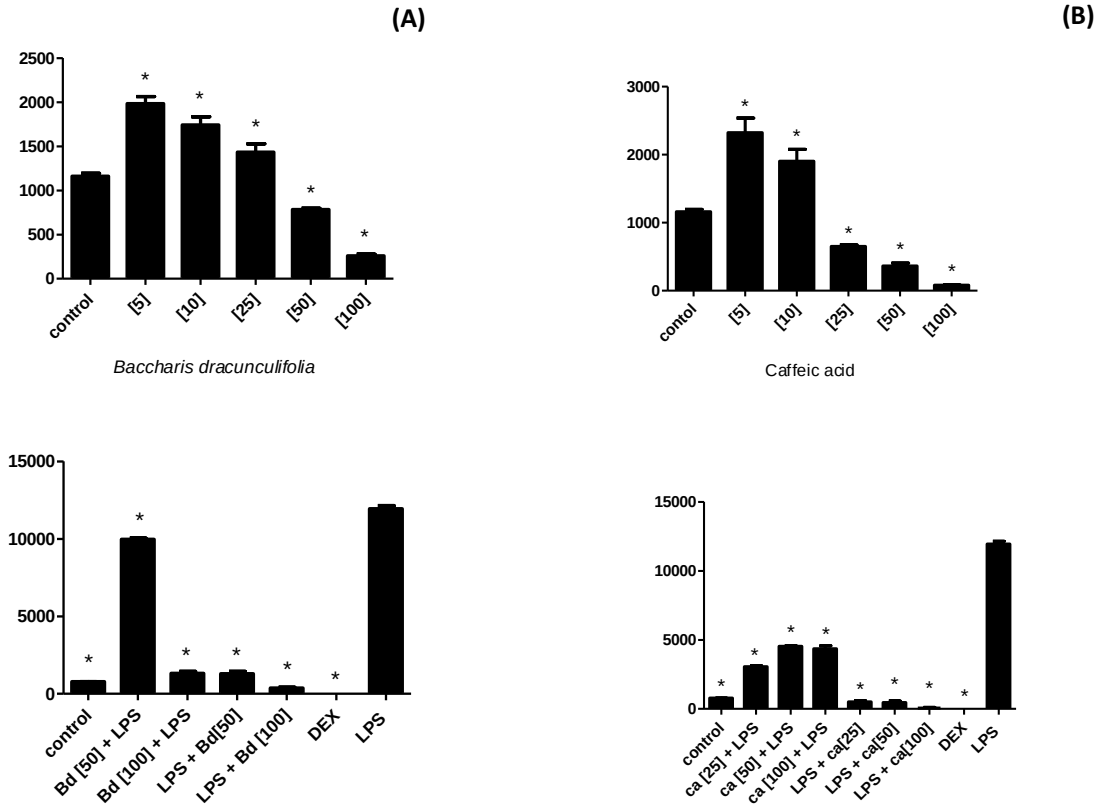

FIG. 2. (A) IL-6 production by peritoneal macrophages incubated with *Baccharis dracunculifolia* (Bd) or caffeic acid (ca) at different concentrations (5, 10, 25, 50 and 100µg/well) for 24 h at 37°C. **(B)** IL-6 production by peritoneal macrophages stimulated with LPS 2 h before or after incubation with *Baccharis dracunculifolia* (50 and100 µg/well) and caffeic acid (25, 50 and 100 µg/well) for 22 h. DEX (10^{-4} mol/L) and LPS (5 µg/well) were used as negative and positive control. * significantly different from control or LPS ($P < 0.0001$). Data are expressed as means ± standard-deviation of 5-7 similar assays.

3.2.3. IL-10 production

Fig. 3A shows that *B. dracunculifolia* (5, 10 µg/well, $P < 0.001$; 25, 50 and 100 µg/well, $P < 0.0001$) inhibited IL-10 production, while caffeic acid stimulated IL-10 production (5 µg/well, $P < 0.001$) and inhibited its production (50 and 100 µg/well, $P < 0.01$). In the next protocol, *B. dracunculifolia* prevented LPS action both before (100 µg/well) and after (50 and 100 µg/well) its challenge ($P < 0.0001$) (Fig. 3B). Caffeic acid (25, 50 and 100 µg/well) prevented efficiently LPS effects only after LPS addition ($P < 0.0001$) (Fig. 3B).

In all assays, DEX and LPS exerted their inhibitory and stimulatory activities, as negative and positive controls of cytokine production, respectively.

4. Discussion

B. dracunculifolia has been described as the most important plant source of propolis in the southeastern Brazil. Propolis possesses various biological activities, but the potential of *B. dracunculifolia* still need to be explored.

Due to its important role in the chemical constitution of propolis, our group has investigated the action of this plant on the immune system. In rats, *B. dracunculifolia* extract did not increase antibody production after immunization with bovine serum albumin when compared to control, but efficiently when compared to propolis-treated rats (Sforcin et al., 2005). On the other hand, *B. dracunculifolia* extract activated murine macrophages *in vitro* with a consequent H_2O_2 liberation, depending on concentration (Missima et al., 2007). This plant also exerted a cytotoxic action on human laryngeal epidermoid carcinoma (HEp-2) cells in vitro (Búfalo et al., 2010), and showed an efficient antiviral activity against poliovirus type 1 replication (Búfalo et al., 2009).

Our results demonstrated herein that *B. dracunculifolia* and caffeic acid stimulated IL-1β production, what may be related to macrophage activation. As to IL-6, Bd and Ca exerted an immunomodulatory action, since depending on concentration it stimulated or inhibited cytokines production. In LPS protocols, Bd and Ca diminished IL-6 production either before or after LPS addition to the

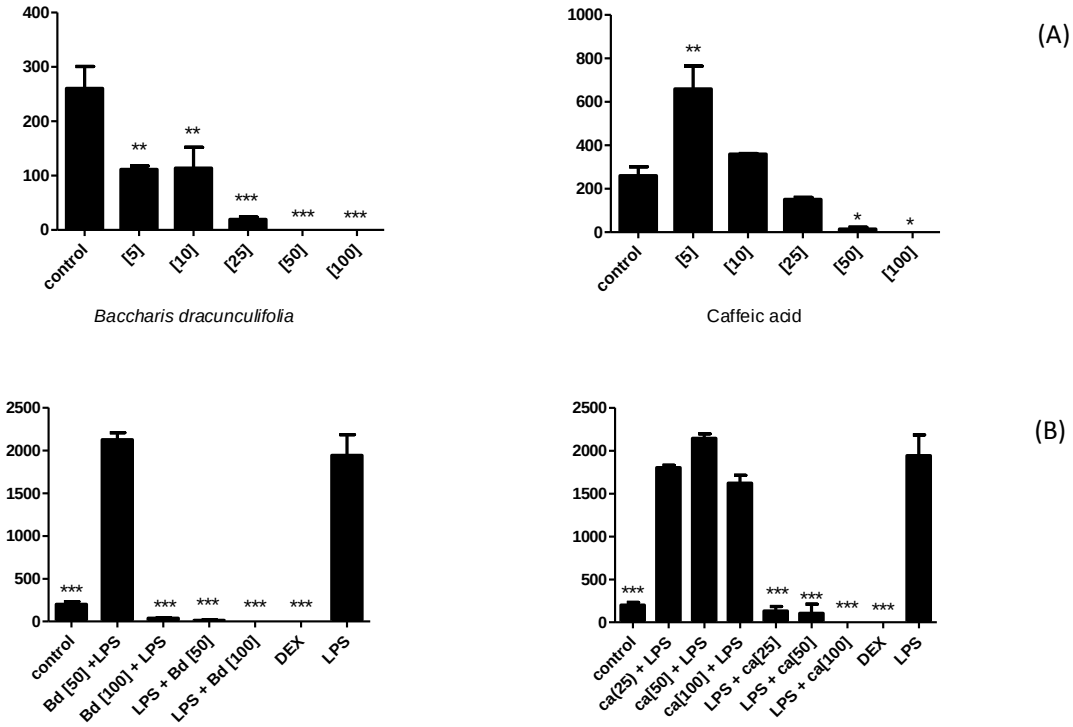

Fig. 3. (A) IL-10 production by peritoneal macrophages incubated with *Baccharis dracunculifolia* (Bd) or caffeic acid (ca) at different concentrations (5, 10, 25, 50 and 100 μg/well) for 24 h at 37°C. **(B)** IL-10 production by peritoneal macrophages stimulated with LPS 2 h before or after incubation with *Baccharis dracunculifolia* (50 and 100 μg/well) and caffeic acid (25, 50 and 100 μg/well) for 22 h. DEX (10^{-4} mol/L) and LPS (5 μg/well) were used as negative and positive control, respectively * significantly different from control ($P < 0.01$); ** significantly different from control ($P < 0.001$); *** significantly different from control or LPS ($P < 0.0001$). Data are expressed as means ± standard-deviation of 5-7 similar assays.

cell culture. Since the incubation of macrophages with Bd or Ca before LPS addition could lead a preventive role, whereas the addition of natural products after to represents a possible treatment, one may suppose that Bd and Ca could exert both preventive and therapeutic effects. Bd inhibited IL-10 production, while Ca stimulated and inhibited its production depending on

concentration. In LPS protocols, Bd and Ca were more efficient when added to the cultures after LPS incubation.

IL-10 is one of the elements of the immune system that inhibits the activation of macrophages (McCoy et al., 2010). Since *B. dracunculifolia* inhibited IL-10 production, one may speculate that it could exert a protective effect against an exacerbated inflammation, what could be investigated in further studies.

The mechanisms of action of Ca are not clear, but one may speculate that it could interfere on the activation of the transcription nuclear factor-kB (NF-κB) and Sp-1, which regulate the expression of various genes, including cytokines expression (Fitzpatrick et al., 2001; Marquez et al., 2003;Chanteux et al., 2007; Bose et al., 2008; Moon et al., 2009).

Several authors have reported the anti-inflammatory effects of caffeic acid and *B. dracunculifolia*, evaluating other mechanisms involved in inflammation, such as cyclooxygenase-2 suppression (Santos et al., 2009) and regression of footpad edema (Cifuente et al., 2001). We wish to report the immunomodulatory effects of *B. dracunculifolia* on cytokines production, which can be at least in part mediated by caffeic acid, since it may inhibit the transcription factor NF-κB. Further studies are still needed to evaluate *B. dracunculifolia* efficacy in inflammatory diseases, in order to explore its anti-inflammatory activity.

Acknowledgements

The authors wish to thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP – 2008/06120-0) for the grant.

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Capítulo III

Lemongrass and citral effect on cytokines production by murine macrophages

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ABSTRACT

Aim of the study: *Cymbopogon citratus*, an herb commonly known as lemongrass (LG), is an important source of ethnomedicines as well as citral, the major constituent of *C. citratus*, used in perfumery, cosmetic and pharmaceutical industries for controlling pathogens. Thus, the goal of this work was to analyze the effect of LG and citral on cytokines production (IL-1β, IL-6 and IL-10) in vitro, as well as before or after LPS incubation.

Material and methods: Peritoneal macrophages from BALB/c mice were treated with LG or citral in different concentrations for 24h. The concentrations that inhibited cytokines production were tested before or after macrophages challenge with LPS, in order to evaluate a possible anti-inflammatory action. Supernatants of cell cultures were used for cytokines determination by ELISA.

Results: As to IL-1β, only citral inhibited its release, exerting an efficient action before LPS challenge. LG and citral inhibited IL-6 release. *C. citratus* showed inhibitory effects only after LPS challenge, whereas citral prevented efficiently LPS effects before and after LPS addition. Citral inhibited IL-10 production and although LG did not inhibit its production, the concentration of 100 µg/well was tested in the LPS-challenge protocol, because it inhibited IL-6 production. LG inhibited LPS action after macrophages incubation with LPS, while citral counteracted LPS action when added before or after LPS incubation.

Conclusion: LG exerted an anti-inflammatory action and citral may be involved in its inhibitory effects on cytokines production. We suggest that a possible mechanism involved in such results could be the inhibition of the transcription factor NF-κB.

Keywords:
Cymbopogon citratus
Lemongrass
Citral
Immunomodulation
Cytokines

1. Introduction

Cymbopogon citratus, an herb commonly known as lemongrass (LG), is an important source of ethnomedicines. The tea from its leaves has been widely used as an antiseptic, antifever, antidyspeptic, carminative, tranquilizer and stomachic agent (Barbosa et al., 2008). Several studies have also demonstrated the anti-inflammatory, antimicrobial, antiseptic, diuretic and fungistatic activities of *C. citratus* (Carbajal et al., 1989). Citral (3,7-dimethyl-2,6-octadienal) is the major constituent of *C. citratus* and has been used in perfumary, cosmetic and pharmaceutical industries for controlling pathogens (Guynot et al., 2003).

Macrophages are one of the first lines of host defense. These cells undergo a series of physiological changes in response to infections or exposure to pathogen-derived products such as lipopolysaccharide (LPS). Upon activation, macrophages are more adept to kill pathogens. These cells are an important source of inflammatory cytokines, which represent an important strategy of host defense (Cao et al., 2006). As an example, interleukin (IL)-1β, a pro-inflammatory cytokine, is a key mediator of inflammation, inducing fever and the acute-phase response. IL-1β has important functions in the innate immune defense against microbes, trauma and stress, and is also an effector molecule involved in tissue destruction and fibrosis (Mandrup-Poulsen et al., 2010).

IL-6 is a potent and pleiotropic regulatory cytokine that mediates a plethora of physiological functions. IL-6 is known to influence cell growth, differentiaton and migration during immune responses, hematopoiesis and inflammation (Frick et al., 2010).

Overproduction of proinflammatory cytokines may cause immunopathologies while defective production of these cytokines results in uncontrolled infection. Macrophages may control the overproduction of pro-inflammatory cytokines by producing anti-inflammatory cytokines such as IL-10, since it can inhibit the transcription and translation of a variety of inflammatory cytokines, reduce antigen presentation and inhibit or bias T cell activation (Anderson and Mosser, 2002).

Several studies have reported the potential of *C. citratus* and citral in several areas, such as neurobehavorial, but little is known about their influence on the immune system. In a previous work we reported that the treatment of mice with LG water extract inhibited macrophages to produce IL-1β and IL-6 production, suggesting the anti-inflammatory action of this spice *in vivo* (Sforcin et al., 2009). Thus, this study aimed to investigate the immunomodulatory effect of *C. citratus* on cytokines production (IL-6, IL-1β and IL-10) by peritoneal macrophages in vitro. After, the concentrations of *C. citratus* that inhibited cytokines production were tested before or after macrophages challenge with LPS. Citral effects were also evaluated, in order to investigate a possible compound responsible for *C. citratus* action.

2. Material and methods

2.1. Lemongrass extract

The aerial parts of *C. citratus* were collected in the Lageado Experimental Farm, UNESP, Campus of Botucatu. The plant material was identified and a voucher specimen (BOTU 25663) was stored in the Herbarium Botu of the Department of Botany, UNESP, Campus of Botucatu.

Fresh plant material was air-dried at 40 ºC for 48 h. The dried leaves (400.0g) were powdered in a blender and submitted to maceration for 72 h in 4L of aqueous methanol 70% (v/v) at room temperature. The macerated material was filtered and concentrated in rotaevaporator. This dried extract was lyophilized and specific dilutions were prepared in RPMI media for each assay.

2.2. Citral

Citral was kindly provided by Dr. Jairo K. Bastos, School of Pharmaceutical Sciences of Ribeirão Preto, USP, Brazil. Specific dilutions of citral were prepared in RPMI media for the experiments.

2.3. Animals and peritoneal macrophages

Male BALB/c mice weighing 25–30 g and aged between 8 and 12 weeks were used. Mice were kept in rooms at 21–25 ºC and 50% relative humidity, with a 12 h light/dark cycle. Food and water were provided *ad libitum*.

Peritoneal macrophages were obtained by inoculation of 3–5 mL of cold PBS in abdominal cavity. After a soft abdominal massage for 30 s, the peritoneal liquid was collected and put in sterile plastic tubes (Falcon). This procedure was repeated 3 or 4 times for each animal and the tubes were centrifuged at 200 g for 10 min. Cells were stained with neutral red (0.02%), incubated for 10 min at 37 °C and counted in a haematocytometer to obtain a final concentration of 2 x 10^6 cells/mL. Cells were resuspended in cell culture medium (RPMI 1640, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 20 mM HEPES – Sigma, USA) and cultured in a 96-well flat-bottomed plate (Corning, USA) at a final concentration of 2x10^5 cells per well. Cells were incubated at 37 °C and, after 2 h, non-adherent cells were removed (Sforcin et al., 2007).

This work agrees with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation, and was approved in August 8, 2008 (protocol n° 46/08-CEEA).

2.4. Cytotoxicity assay

Prior to *in vitro* assays, cells were incubated with LG extract or citral at the concentrations 5, 10, 25, 50 and 100 μg/well, in order to carry out the assays only with noncytotoxic concentrations. The evaluation of cytotoxicity was carried out by crystal violet method (Ait-Mbarek et al. 2007).

The macrophage culture received the stimuli as previously described, and after 24 h, supernatants were removed and 100 μL 0.5% crystal violet solution was added to the cells. After 10 min incubation at room temperature, the plates were washed and viable crystal violet-stained cells were lysed with 1% sodium dodecyl sulphate. Optical densities (OD) were read at 492 nm in an

ELISA reader, and the percentage of cell viability was calculated using the formula: [OD test ⁄ OD control] x 100. Assays were carried out in triplicate.

2.5. In vitro assays

Macrophages were incubated with LG or citral at different concentrations (5, 10, 25, 50 and 100 µg/well) for 24 h, supernatants were harvested and stored at -70 ºC for cytokines (IL-1β, IL-6 and IL-10) measurement. Concentrations that inhibited IL-1β and IL-6 production followed two other protocols, challenging the cells with LPS.

2.5.1. Lemongrass and citral incubation before LPS challenge

Macrophages were pre-treated with LG (100 µg/well) or citral (25, 50 and 100 µg/well for IL-1β; 5, 10, 25, 50 and 100 µg/well for IL-6 and IL-10) at the concentrations that inhibited IL-1β, IL-6 and IL-10 production for 2 h and then incubated with LPS (5 µg/mL) for 22 h. After this period, the culture supernatants were harvested and stored at -70 ºC for cytokines measurement (Shin et al., 2006).

2.5.2. Lemongrass and citral incubation after LPS challenge

Macrophages were stimulated with LPS (5 µg/mL) for 2 h and then incubated with LG (100 µg/well) or citral (25, 50 and 100 µg/well for IL-1β; 5, 10, 25, 50 and 100µg/well for IL-6 and IL-10) for 22 h. Afterwards, supernatants were collected and stored at -70 ºC for cytokines determination (Shin et al., 2006).

Dexamethasone (DEX, 10^{-4} mol/L) (Zhou et al., 2010) and LPS (5 µg/mL) were used as a negative and positive control, in order to inhibit and stimulate cytokine production, respectively.

2.6. Determination of cytokine production

IL-1β, IL-6 and IL-10 production was measured by enzyme-linked immunosorbent assay (ELISA), according to manufacturer's instructions (BD Biosciences, USA). Briefly, a 96-well flat bottom Nunc Maxisorp (Nunc/Apogent, USA) was coated with a 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 captured cytokine was detected using the specific conjugated detection antibody. The substrate reagent was added into each well and, after color development, the plate was read at 450 nm, using an ELISA plate reader (Tan et al., 2006).

2.7. Statistical analysis

Data were expressed as means ± standard-deviation of 5-7 siilar assays. Analysis of variance (ANOVA) was used, followed by Dunnet's multiple comparison method, with 0.05 was chosen as the significant level (Zar, 1999).

3. Results

3.1. Citotoxicity assay

Cymbopogon citratus extract and citral did not affect cell viability as determined by crystal-violet test (data not shown).

3.2. Cytokines production

3.2.1. IL-1β production

As to IL-1β, *C. citratus* (5 and 10 µg/well) stimulated significantly its production ($P < 0.01$), whereas the concentrations 25, 50 and 100 µg/well did not affect its production ($P > 0.05$) (Fig. 1A). Citral (5 µg/well) stimulated this cytokine production ($P < 0.001$), whereas 10 and 25 µg/well did not affect its production ($P > 0.05$) and 50 and 100 µg/well inhibited significantly its release ($P < 0.01$) (Fig. 1B).

Thus, in the next protocol, since citral exerted an inhibitory action in IL-1β production, macrophages were challenged with LPS before or after incubation with citral. As seen in Fig. 1C, citral prevented LPS action, exerting an efficient action before LPS challenge ($P < 0.0001$). *C. citratus* extract was not evaluated in LPS-challenge assays since it did not inhibit IL-1β production.

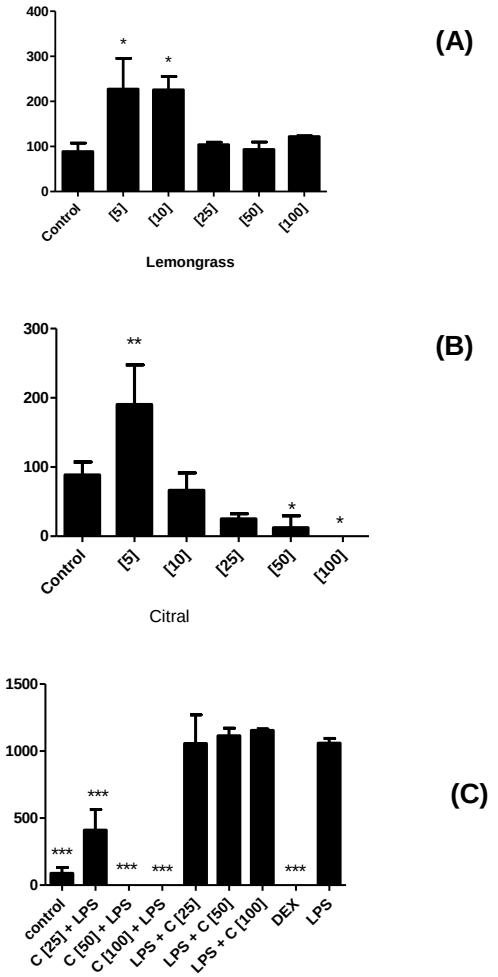

Fig. 1. IL-1β production by peritoneal macrophages incubated with **(A)** Lemongrass or **(B)** citral (C) at different concentrations (5, 10, 25, 50 and 100 μg/well) for 24 h at 37ºC. **(C)** IL-1β production by peritoneal macrophages stimulated with LPS 2 h before or after incubation with C (25, 50 and 100 μg/well) for 22 h. DEX (10^{-4} mol/L) and LPS (5 μg/well) were used as negative and positive control, respectively. * significantly different from control ($P < 0.01$); ** significantly different from control ($P < 0.001$); *** significantly different from LPS ($P < 0.0001$). Data are expressed as means ± standard-deviation of 5-7 similar assays.

3.2.2. IL-6 production

IL-6 production was significantly ($P < 0.0001$) increased after *C. citratus* incubation for 24 h (5, 10, 25 and 50 µg/well), whereas 100 µg/well inhibited its production ($P < 0.001$) (Fig. 2A). Citral (5, 10, 25, 50 and 100 µg/well) decreased ($P < 0.0001$) IL-6 production by macrophages (Fig. 2A).

The inhibitory concentrations of *C. citratus* (100 µg/well) and citral (5, 10, 25, 50 and 100 µg/well) were analyzed in the next protocol. Fig. 2B shows that *C. citratus* showed inhibitory effects only after LPS challenge, and IL-6 production was lower than that induced by LPS alone ($P < 0.0001$). Citral prevented efficiently LPS effects before and after LPS addition ($P < 0.0001$) at all concentrations (Fig. 2B).

3.2.3. IL-10 production

There were no significant differences in IL-10 production by macrophages treated with LG ($P > 0.05$), whereas citral (25, 50 and 100 µg/well) inhibited its release ($P < 0.01$) (Fig. 3A).

Although lemongras did not inhibit IL-10 production, the concentration of 100 µg/well was tested in the LPS-challenge protocol, because it inhibited IL-6 production. In fact, one may verify in Fig. 3B that LG (100 µg/well) inhibited significantly LPS action ($P < 0.001$), after macrophages incubation with LPS. Citral (5, 10, 25, 50 and 100 µg/well) counteracted significantly LPS action when added before or after LPS incubation ($P < 0.0001$) except when cells were incubated with citral (5 µg/well) and subsequently challenged with LPS ($P > 0.05$) when compared to LPS alone (Fig. 3B).

In all assays, DEX and LPS exerted their inhibitory and stimulatory activities, as negative and positive controls of cytokine production, respectively.

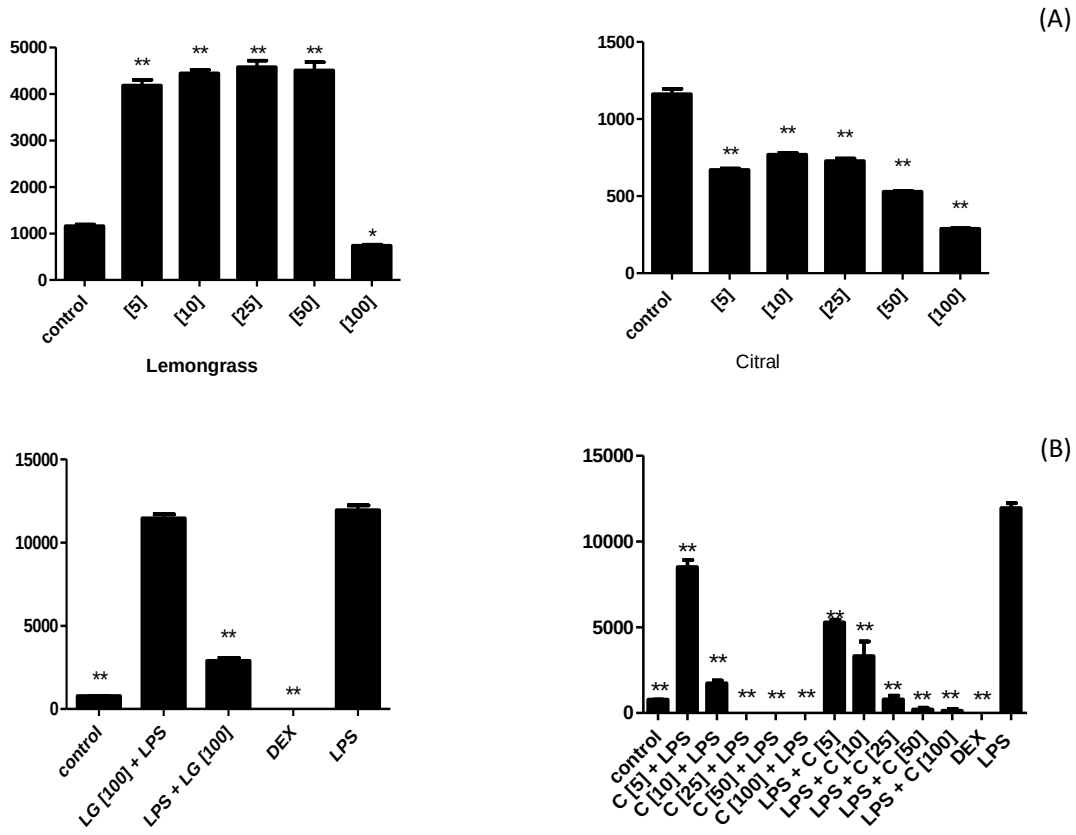

Fig. 2. (A) IL-6 production by peritoneal macrophages incubated with LG (LG) or citral (C) at different concentrations (5, 10, 25, 50 and 100μg/well) for 24 h at 37°C. **(B)** IL-6 production by peritoneal macrophages stimulated with LPS 2 h before or after incubation with LG (100 μg/well) and C (5, 10, 25, 50 and 100 μg/well) for 22 h. DEX (10^{-4} mol/L) and LPS (5 μg/well) were used as negative and positive control, respectively. * significantly different from control ($P < 0.001$); ** significantly different from control or LPS ($P < 0.0001$). Data are expressed as means ± standard-deviation of 5-7 similar assays.

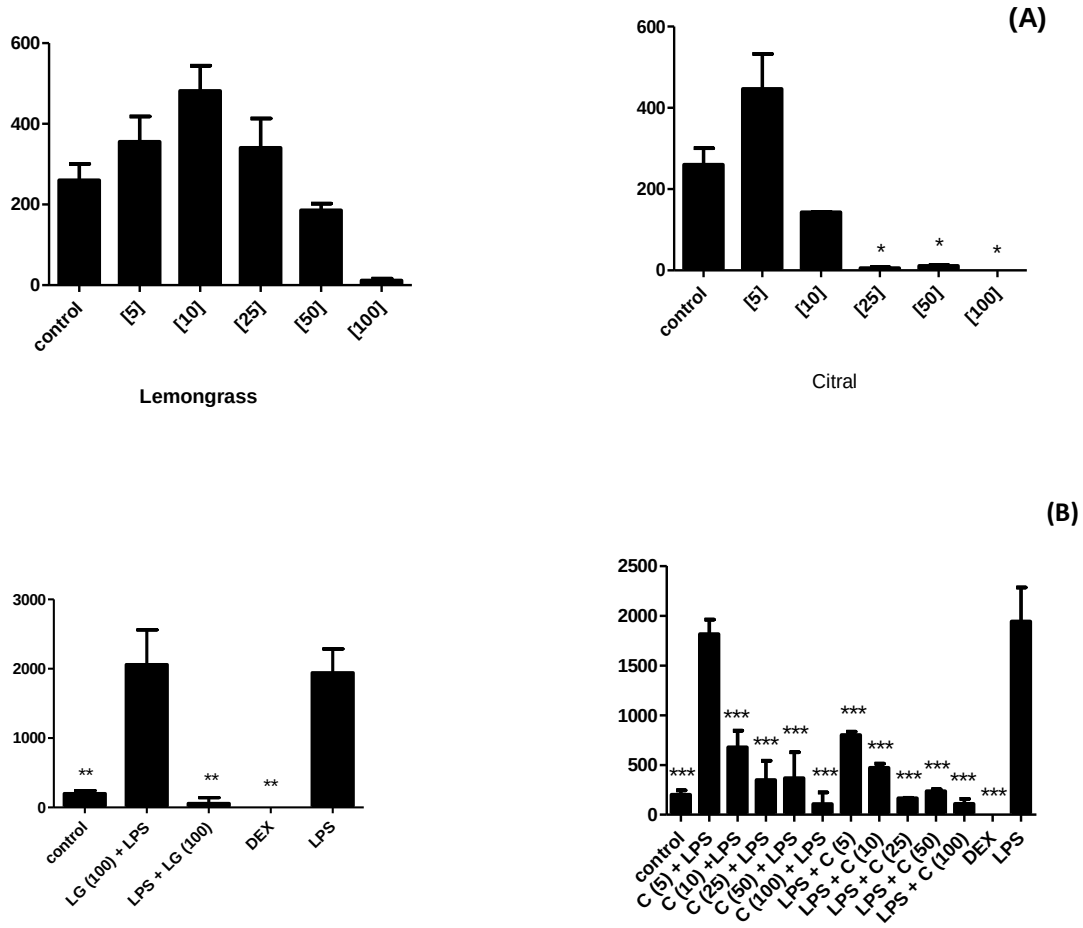

Fig. 3. (A) IL-10 production by peritoneal macrophages incubated with LG (LG) or citral (C) at different concentrations (5, 10, 25, 50 and 100 μg/well) for 24 h at 37°C. **(B)** IL-10 production by peritoneal macrophages stimulated with LPS 2 h before or after incubation with LG (100 μg/well) and C (5, 10, 25, 50 and 100 μg/well) for 22 h. DEX (10^{-4} mol/L) and LPS (5 μg/well) were used as negative and positive control, respectively * significantly different from control ($P < 0.01$); ** significantly different from LPS ($P < 0.001$); ***significantly different from LPS (B) ($P < 0.0001$). Data are expressed as means ± standard-deviation of 5-7 similar assays.

4. Discussion

Immunomodulators may be defined as agents that affect the immune system by regulating molecules, such as cytokines, hormones,

neurotransmitters and others peptides, stimulating or inhibiting the events of the immune response (Spelman et al., 2006). In this study, we investigated the immunomodulatory action of *C. citratus* and citral on cytokines production.

Previous works from our laboratory revealed that the treatment of mice with LG water extract inhibited macrophages to produce IL-1β and IL-6. LG essential oil inhibited these cytokines production by macrophages *in vitro* (Sforcin et al., 2009). These data encouraged us to investigate the effects of LG before and after macrophages challenge with LPS, in order to evaluate its efficacy as an anti-inflammatory agent.

The chemical composition of *C. citratus* (extract and essential oil) was previously described (Sforcin et al., 2009), and citral and linalool were the major constituents of our sample. Thus, we also evaluated citral effects in our assays, in order to investigate a possible compound responsible for *C. citratus* action.

Lemongrass stimulated IL-1β production, whereas citral stimulated and inhibited its production depending on concentration. In our study, we included LPS-challenge protocols in order to observe a possible preventive or therapeutic action. Citral was more efficient to inhibit IL-1β production when incubated before LPS addition to the cells, suggesting its preventive activity.

LG stimulated and inhibited IL-6 production by macrophages, depending on concentration, while citral inhibited IL-6 release. In LPS protocols, LG was more efficient when added after LPS incubation, whereas citral seemed to be efficient both before or after LPS addition.

LG and citral inhibited IL-10 production. In LPS protocols LG exerted therapeutic action by counteracting LPS stimulatory action, while citral showed preventive and therapeutic effects.

Figueirinha et al. (2010) demonstrated that *C. citratus* leaf infusion significantly inhibited other inflammatory parameters: nitric oxide (NO) production and inducible NO synthase expression by LPS-stimulated mouse skin dendritic cells, suggesting its anti-inflammatory activity. The pharmacological properties of citral were also investigated (Lertsatitthanakorn et al., 2006). Regarding its anti-inflammatory effects, Lee et al. (2007) reported that citral blocked LPS-induced activation of NF-κB by inhibiting the phosphorylation of IκB, which in turn inhibited p65 and p50 translocation to the nucleus – an initial process for gene expression of several cytokines. Anti-

inflammatory effects of several natural products such as *Butea monosperma* and *Marasmius oreades* and their compounds gallatanins, butin, isobutin and butein have been reported to inhibit NF-κB activation (Lee et al., 2007; Rasheed et al., 2010), suggesting that the transcription factor NF-κB is an obvious target of anti-inflammatory therapeutics (Gadjeva et al., 2010).

Taken together, our data is in agreement with those found in literature, pointing out to the anti-inflammatory effects of citral, which may be involved in LG inhibitory effects on cytokines production, and a possible mechanism involved in LG and citral actions could be the inhibition of the transcription factor NF-κB. Since inflammation plays an important role in host homeostasis, immunomodulatory agents may be useful to maintain or restore this balance. The potential of both LG and citral as immunomodulatory and anti-inflammatory agents should be further explored, in order to understand under which conditions they might be useful tools for prevention or treatment of inflammatory diseases.

Acknowledgements

The authors wish to thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP – 2008/06120-0) for the grant.

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Capítulo IV

In vitro effects of clove and eugenol on cytokines production by murine macrophages

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ABSTRACT

Aim of the study: Clove (*Syzygium aromaticum*) is an aromatic dried bud, commonly used as a spice to add flavor to food preparations. The extract and the essential oil isolated from clove are widely used because of its medicinal properties. Eugenol (4-allyl-2-methoxyphenol) is the most important component of clove, showing several biological properties. Thus, the goal of this work was to analyze the effect of clove and eugenol on cytokines production (IL-1β, IL-6 and IL-10) *in vitro*, as well before or after LPS incubation.

Material and methods: Male BALB/c mice were used and peritoneal macrophages were incubated with clove or eugenol at different concentrations (5, 10, 25, 50 and 100 µg/well) for 24 h. Concentrations that inhibited the production of pro-inflammatory cytokines (IL-1β and IL-6) followed two other protocols, challenging the cells with LPS before or after incubation with clove or eugenol. Culture supernatants were harvested for cytokines (IL-1β, IL-6 and IL-10) measurement by ELISA.

Results: Clove (100 µg/well) inhibited IL-1β, IL-6 and IL-10 production and exerted an efficient action either before or after LPS challenge for all cytokines. Eugenol did not affect IL-1β production but inhibited IL-6 and IL-10 producion. The action of eugenol (50 and 100 µg/well) on IL-6 production prevented efficiently LPS effects either before or after its addition, whereas on IL-10 production it counteracted significantly LPS action when added after LPS incubation.

Conclusion: Clove exerted immunomodulatory effects by inhibiting LPS action, and a possible mechanism of action probably involves the suppression of NF-κB pathway by eugenol, since it was the major compound found in clove extract.

Keywords:
Syzygium aromaticum
Clove
Eugenol
Immunomodulation
Cytokines

1. Introduction

Clove (*Syzygium aromaticum*, syn. *Eugenia aromaticum* or *Eugenia caryophyllata*) is an aromatic dried bud of a tree from the family *Myrtaceae*, commonly used as a spice to add flavor to food preparations (Kim et al., 1998).

The extract and the essential oil isolated from clove are widely used because of its medicinal properties, since it is active against a large number of bacteria (Chaieb et al., 2007). Its antifungal (Chami et al., 2005), anticarcinogenic (Zheng et al., 1992), antiallergic (Kim et al., 1998) and antimutagenic activities have been reported as well (Miyazawa and Hisama, 2001). Clove essential oil increased the total white blood cell count in mice and restored cellular and humoral immune responses in cyclophosphamide-immunossuppressed mice in a dose-dependent manner (Carrasco et al., 2009).

Natural products containing bioactive phytochemicals are potentially important sources of antiinflammatory drugs (Raskin et al*.,* 2002). Eugenol (4-allyl-2-methoxyphenol) is a phenolic compound representing the most important component of clove. In Asian countries, eugenol has been traditionally used as an antiseptic, analgesic and antibacterial agent (Morsy et al., 2008). Its antiviral (Benecia et al., 2000), antioxidant (Sondak et al., 2001), anti-inflammatory (Lee et al., 2007) and antitumoral (Ghosh et al., 2005; Okada et al., 2005) activities have been also investigated.

Macrophages are cells of the innate immunity that respond to a variety of stimuli (Mosser, 2003). The innate immune response is typically triggered by pathogen-associated molecular patterns that are recognized by different receptors on the surface of macrophages, leading to intracellular pathways followed by stimulation of cytokines production, including proinflammatory (interleukin-1), regulatory (IL-6) and anti-inflammatory (IL-10) cytokines (Tzeng et al*.,* 2003).

The proinflammatory cytokines of the IL-1 family, most notably IL-1β and IL-1α, display a very important role for antimicrobial host defense, binding the same receptor and activating the adaptive response. IL-1 is largely responsible for the acute phase response, which includes fever, acute protein synthesis, anorexia, and somnolence (Netea et al., 2010). Interleukin-6 is a regulatory cytokine by regulating the expression of immune/inflammatory genes and

regulating cell proliferation, differentiation and survival (Ahmed et al., 2000). IL-10 is a potent anti-inflammatory cytokine that is crucial for dampening the inflammatory response after pathogen invasion, protecting the host from excessive inflammation. One mechanism whereby IL-10 mediates its anti-inflammatory effect is through the down-regulation of proinflammatory genes such as those encoding IL-1, IL-12 and TNF-α (McCoy et al., 2010).

Several studies have reported the potential of clove and eugenol against specific pathogens, but little is known concerning their influence on the immune system. In a previous work we reported that its administration to mice was shown to inhibit macrophages to produce IL-1β and IL-6, suggesting the anti-inflammatory action of this spice in vivo (Rodrigues et al., 2009). Thus, this study aimed to investigate the immunomodulatory effect of clove and eugenol on cytokines production (IL-6, IL-1β and IL-10) by peritoneal macrophages *in vitro*. After, the concentrations of clove or eugenol that inhibited cytokines production were tested before or after macrophages challenge with LPS.

2. Material and methods

2.1. Clove extract and chemical characterization

Authentic flower buds of *S. aromaticum* were purchased from the local market of Botucatu, São Paulo, Brazil. Clove buds (350 g) were ground in a knife mill, and extracted in methanol 70% (v/v) at room temperature. After 3 days, the extract was filtered and concentrated in rotoevaporator. The dried extract was lyophilized and specific dilutions were prepared in RPMI media for each assay.

Clove chemical composition was previously investigated and eugenol was the major component present in the extract (Rodrigues et al., 2009).

2.2. Eugenol

Eugenol was purchased from Sigma Chemicals (St. Louis, MO) and identified by comparing their retention index (RI relative to C9–C22 n-alkanes) to those reported in the literature, as well as by comparison of the obtained

mass spectra of the peaks to those reported in the literature or available in the Wiley 7.0 data system library (Adams, 2001).

2.3. Animals and peritoneal macrophages

Male BALB/c mice weighing 25–30 g and aged between 8 and 12 weeks were used. Mice were kept in rooms at 21–25 ºC and 50% relative humidity, with a 12 h light/dark cycle. Food and water were provided *ad libitum*.

Peritoneal macrophages were obtained by inoculation of 3–5 mL of cold PBS in abdominal cavity. After a soft abdominal massage for 30 s, the peritoneal liquid was collected and put in sterile plastic tubes (Falcon). This procedure was repeated 3 or 4 times for each animal and the tubes were centrifuged at 200 g for 10 min. Cells were stained with neutral red (0.02%), incubated for 10 min at 37 °C and counted in a haematocytometer to obtain a final concentration of 2 x 10^6 cells/mL. Cells were resuspended in cell culture medium (RPMI 1640, supplemented with 10% fetal calf serum, 2mM L-glutamine, 20mM HEPES – Sigma, USA) and cultured in a 96-well flat-bottomed plate (Corning, USA) at a final concentration of 2 x 10^5 cells per well. Cells were incubated at 37 °C and, after 2 h, non-adherent cells were removed (Sforcin et al., 2007).

This work agrees with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation, and was approved in August 8, 2008 (protocol n° 46/08-CEEA).

2.4. Cytotoxicity assay

Prior to *in vitro* assays, cells were incubated with clove extract or eugenol in the concentrations 5, 10, 25, 50 and 100 µg/well, in order to carry out the assays only with noncytotoxic concentrations. The evaluation of cytotoxicity was carried out by crystal violet method (Ait-Mbarek et al. 2007).

The macrophage culture received the stimuli as previously described, and after 24 h, supernatants were removed and 100 µL 0.5% crystal violet solution was added to the cells. After 10-min incubation at room temperature, the plates were washed and viable crystal violet-stained cells were lysed with

1% sodium dodecyl sulphate. Optical densities (OD) were read at 492 nm in an ELISA reader, and the percentage of cell viability was calculated using the formula: [OD test ⁄ OD control] x 100. Assays were carried out in triplicate.

2.5. In vitro assays

Macrophages were incubated with clove or eugenol at different concentrations (5, 10, 25, 50 and 100 µg/well) for 24 h and the supernatants were harvested and stored at -70 ºC for cytokines (IL-1β, IL-6 and IL-10) measurement. Concentrations that were able to inhibit the production of IL-1β and IL-6 followed two other protocols, challenging the cells with LPS.

2.5.1. Clove and eugenol incubation before LPS challenge

Macrophages were pre-treated with clove (100 µg/well) or eugenol (50 and 100 µg/well) at the concentrations that inhibited IL-1β, IL-6 and IL-10 production for 2 h and then incubated with LPS (5 µg/mL) for 22 h. After this period, the culture supernatants were harvested and stored at -70 ºC for cytokines measurement (Shin et al., 2006).

2.5.2. Clove and eugenol incubation after LPS challenge

Macrophages were stimulated with LPS (5 µg/mL) for 2 h and then incubated with clove (100 µg/well) or eugenol (50 and 100 µg/well) for 22 h. Afterwards, supernatants were collected and stored at -70 ºC for cytokines determination (Shin et al., 2006).

Dexamethasone (DEX, 10^{-4} mol/L) (Zhou et al., 2010) and LPS (5 µg/mL) were used as a negative and positive control, in order to inhibit and stimulate cytokine production, respectively.

2.6. Determination of cytokine production

Supernatants were collected and IL-1β, IL-6 and IL-10 production was measured by enzyme-linked immunosorbent assay (ELISA), according to manufacturer's instructions (BD Biosciences, USA). Briefly, a 96-well flat bottom

Nunc Maxisorp (Nunc/Apogent, USA) was coated with a 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 captured cytokine was detected using the specific conjugated detection antibody. The substrate reagent was added into each well and, after color development, the plate was read at 450 nm, using an ELISA plate reader (Tan et al., 2006).

2.7. Statistical analysis

Data were expressed as means ± standard-deviation of 5-7 similar assays. Analysis of variance (ANOVA) and Dunnet's multiple comparison method were used. A probability (*P*) of 0.05 was chosen as the significance level (Zar, 1999).

3. Results

3.1. Cytotoxicity assay

Clove extract and eugenol did not affect cell viability as determined by crystal-violet test (data not shown).

3.2. Cytokines production

3.2.1. IL-1β production

As to IL-1β, clove (5, 10, 25 µg/well) stimulated significantly its production, whereas the concentration of 100 µg/well inhibited significantly its release ($P < 0.001$) (Fig. 1A). Eugenol (5 µg/well) stimulated this cytokine production ($P < 0.0001$), whereas 10, 25, 50 and 100 µg/well did not affect its production ($P > 0.05$) (Fig. 1B).

Thus, in the next protocol, since clove (100 µg/well) exerted an inhibitory action in IL-1β production, macrophages were challenged with LPS before or after incubation with this natural product. As seen in figure 1C, clove prevented LPS action as well as reverted its effects, exerting an efficient action either

before or after LPS challenge ($P < 0.0001$). Eugenol was not evaluated in LPS-challenge assays since it did not inhibit IL-1β production.

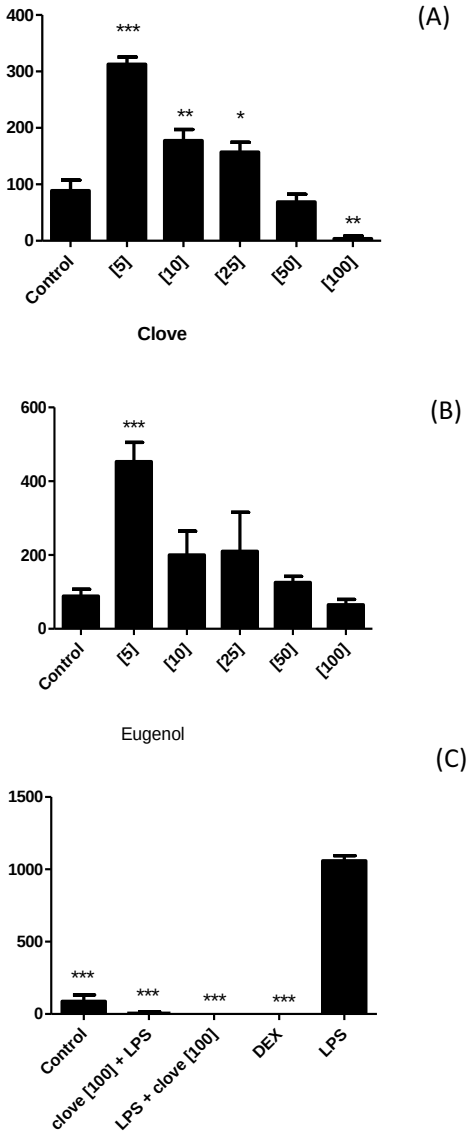

Fig. 1. IL-1β production by peritoneal macrophages incubated with **(A)** clove or **(B)** eugenol at different concentrations (5, 10, 25, 50 and 100 μg/well) for 24 h at 37ºC. **(C)** IL-1β production by peritoneal macrophages stimulated with LPS 2 h before or after incubation with clove (100 μg/well) for 22 h. DEX (10^{-4} mol/L) and LPS (5 μg/well) were used as negative and positive control, respectively. * significantly different from control and LPS ($P < 0.01$); ** significantly different from control ($P < 0.001$); *** significantly different from control (B) or LPS (C) ($P < 0.0001$). Data are expressed as means ± standard-deviation of 5-7 similar assays.

3.2.2. IL-6 production

IL-6 production was significantly ($P < 0.0001$) increased after clove incubation for 24 h (5, 10, 25 and 50 µg/well), whereas 100 µg/well inhibited its production ($P < 0.001$) (Fig. 2A). Eugenol (5, 10 and 25 µg/well) increased ($P < 0.0001$) IL-6 production by macrophages, while 50 and 100 µg/well exerted an inhibitory effect on its production (Fig. 2A).

The inhibitory concentrations of clove (100 µg/well) and eugenol (50 and 100 µg/well) were analyzed in the next protocol. Figure 2B shows that clove prevented LPS action both before and after its challenge, and IL-6 production was lower than that induced by LPS alone ($P < 0.0001$). Eugenol prevented efficiently LPS effects before (100 µg/well) and after (50 and 100 µg/well) LPS addition ($P < 0.0001$) (Fig. 2B).

3.2.3. IL-10 production

Clove stimulated significantly IL-10 production after incubation with 5 and 10 µg/well ($P < 0.01$), whereas 25, 50 and 100 µg/well did not affect its production (Fig. 3A). Eugenol (5 and 10 µg/well) stimulated IL-10 production ($P < 0.0001$), while 50 and 100 µg/well inhibited its release ($P < 0.01$) (Fig. 3A).

Although clove did not inhibit IL-10 production, the concentration of 100 µg/well was tested in the LPS-challenge protocol, because it inhibited IL-1β and IL-6 production. In fact, one may verify in Fig. 3B that clove (100 µg/well) inhibited significantly LPS action ($P < 0.0001$), either before or after macrophages incubation with LPS. Eugenol (50 and 100 µg/well) counteracted significantly LPS action when added after LPS incubation ($P < 0.0001$) (Fig. 3B).

In all assays, DEX and LPS exerted their inhibitory and stimulatory activities, as negative and positive controls of cytokine production, respectively.

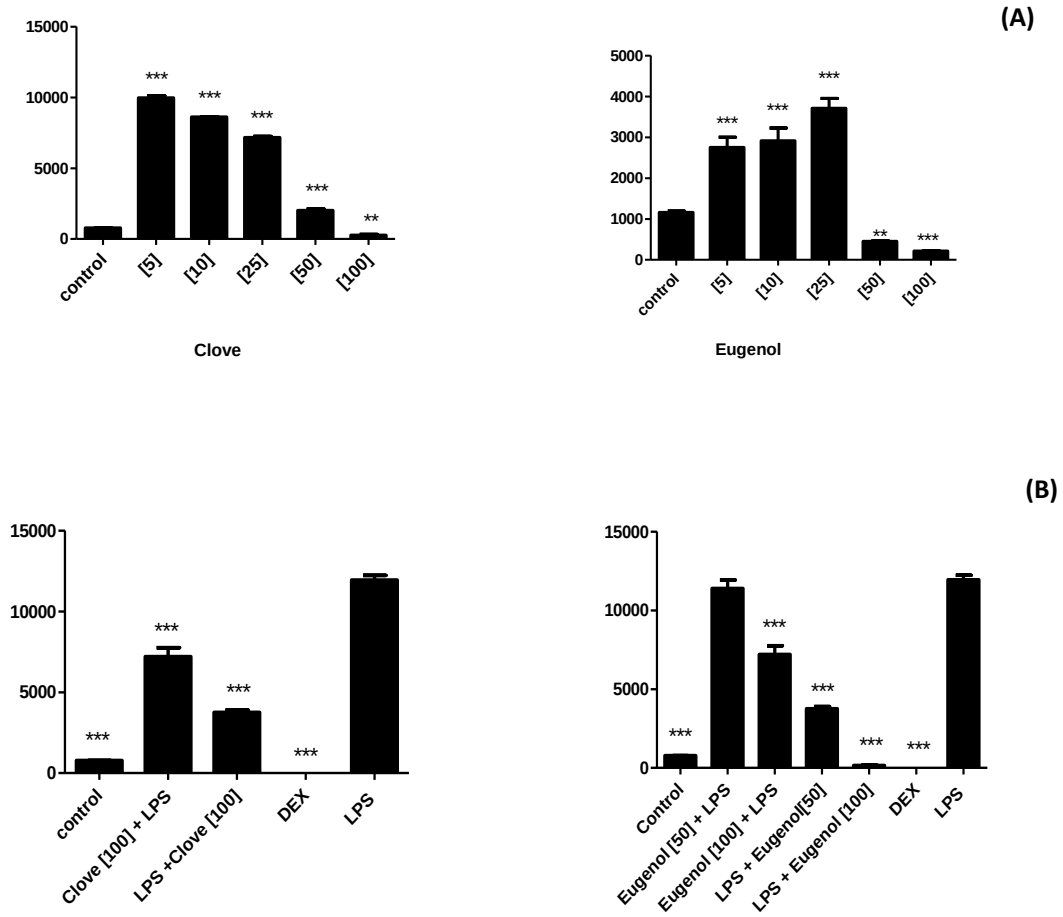

Fig. 2. (A) IL-6 production by peritoneal macrophages incubated with clove or eugenol at different concentrations (5, 10, 25, 50 and 100μg/well) for 24 h at 37°C. **(B)** IL-6 production by peritoneal macrophages stimulated with LPS 2 h before or after incubation with clove (100 μg/well) and eugenol (50 and 100 μg/well) for 22 h. DEX (10^{-4} mol/L) and LPS (5 μg/well) were used as negative and positive control, respectively. * significantly different from control and LPS ($P < 0.01$); ** significantly different from control ($P < 0.001$); *** significantly different from control (A) or LPS (B) ($P < 0.0001$). Data are expressed as means ± standard-deviation of 5-7 similar assays.

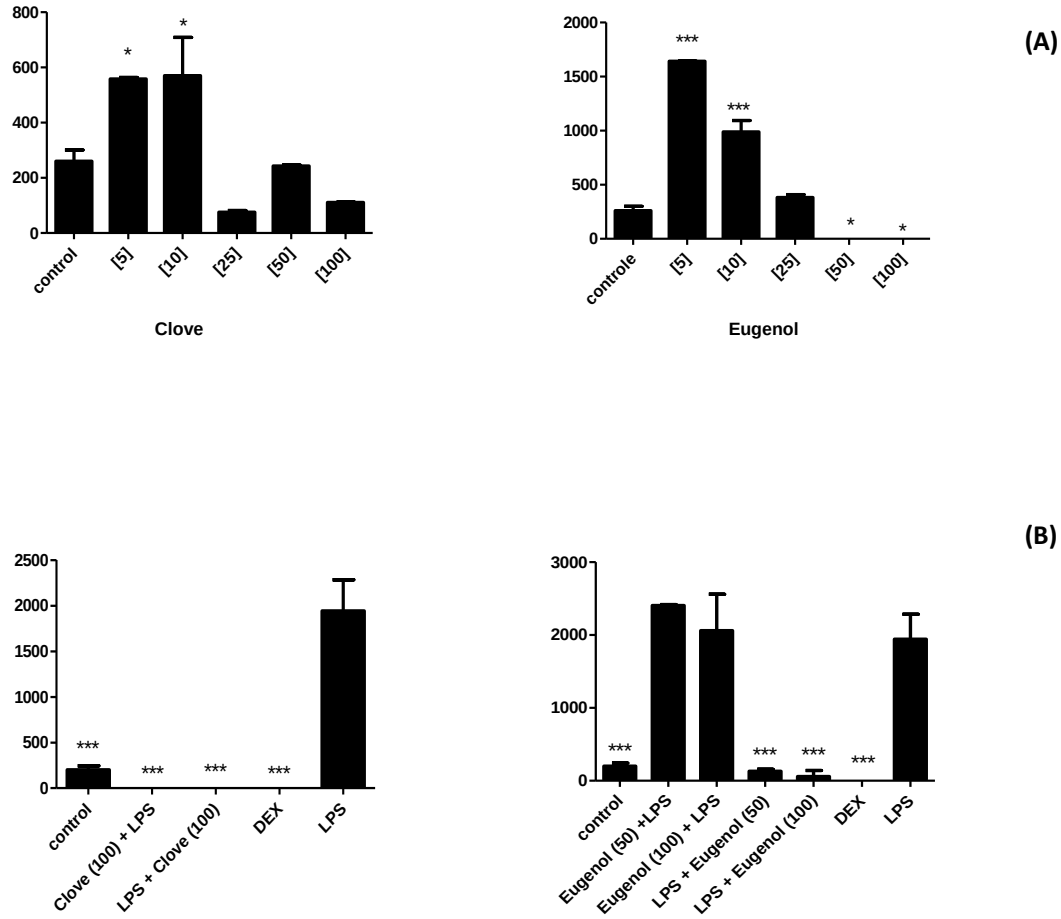

Fig. 3. (A) IL-10 production by peritoneal macrophages incubated with clove or eugenol at different concentrations (5, 10, 25, 50 and 100 μg/well) for 24 h at 37°C. **(B)** IL-10 production by peritoneal macrophages stimulated with LPS 2 h before or after incubation with clove (100 μg/well) and eugenol (50 and 100 μg/well) for 22 h. DEX (10^{-4} mol/L) and LPS (5 μg/well) were used as negative and positive control, respectively. * significantly different from control ($P < 0.01$); ** significantly different from control ($P < 0.001$); ***significantly different from control (A) or LPS (B) ($P < 0.0001$). Data are espressed as means ± standard-deviation of 5-7 similar assays.

4. Discussion

Plants are a rich source of bioactive components that are traditionally useful for treatment and prevention of various infectious and inflammatory diseases (Baskaran et al., 2009). Current researches have demonstrated the

effects of garlic (*Allium sativa*) (Clement et al., 2010), *Curcuma longa* (Yue et al., 2010), black pepper (*Piper nigrum*) (Majdalawieh et al., 2010), ginseng (*Panax ginseng*) (Ni et al., 2010), and cardomon (*Elletaria cardamomum*), but little is known concerning clove effects on the immune system. Clove has been widely used not only as a spice but also for its several biological properties, and eugenol is its majoritary phenolic compound. In the present study, we investigated the immunomodulatory effects of clove and eugenol on cytokines production.

Clove administration (200mg/kg) to mice over a short term (3 days) did not influence the Th1/Th2 cytokine balance (Bachiega et al., 2009). However, Park et al. (2007) demonstrated that eugenol and isoeugenol inhibited IL-2 expression and T cell proliferation in vitro, down-regulating the transcription factors NF-AT and NF-κB.

Works from our laboratory revealed that mice treatment with water extract of clove was found to inhibit macrophages to produce both IL-1β and IL-6. The essential oil of clove also inhibited the production of such cytokines in vitro (Rodrigues et al., 2009). These data encouraged us to investigate the effects of clove and eugenol before or after macrophages challenge with LPS, in order to evaluate their efficacy as anti-inflammatory agents.

First, both clove and eugenol showed an immunomodulatory action: whereas lower concentrations stimulated IL-1β, IL-6 and IL-10 production, higher ones inhibited their generation by macrophages, what was not associated to citotoxicity, since clove and eugenol did not affect cell viability.

Thus, the inhibitory concentrations of clove and eugenol were evaluated in LPS-challenge protocols, in order to explore their anti-inflammatory action: the incubation of macrophage with natural products before LPS addition may represent a possible preventive action, and the addition of natural products after LPS incubation could be associated to a therapeutic effect. Clove (100 µg/well) inhibited significantly IL-1β, IL-6 and IL-10 production. In LPS-challenged cells, it exerted an efficient inhibitory action on LPS stimulatory action either before or after LPS addition, suggesting a possible preventive and therapeutic action. Eugenol (50 and 100 µg/well) exerted inhibitory effects on IL-6 and IL-10 production by macrophages. The incubation of these cells with eugenol in such

concentrations before and after LPS challenge revealed its potential to inhibit LPS effects as well.

A variety of intracellular and extracellular stimuli such as LPS, cytokines, reactive oxygen species, hypoxia, infections, among others, may induce phosphorylation and proteasomal degradation of IκB, with release of the NF-κB heterodimer that translocates to the nucleus and binds to κB elements, regulating the expression of more than 200 genes. In the resting state, NF-κB is found sequestered in the cytoplasm as a heterodimer of p65 and p50 subunits complexed with the inhibitory κB alpha (IκBα) protein (Manikandan et al., 2010). Aggarwal and Shishodia (2004) mentioned that agents that can suppress NF-κB activation would have the potential to prevent or delay the onset or treatment of NF-κB-linked diseases. These authors verified that eugenol and isoeugenol suppressed NF-κB activation by suppressing IκBα degradation. Based on these observations, one may speculate that the mechanism by which clove and eugenol inhibited LPS effects involved NF-κB suppression, since the secretion of cytokines was induced by LPS alone.

Several studies also pointed out the anti-inflammatory effects of clove and eugenol, reporting that these products are able to modulate several inflammatory markers such as cycloxigenase-2 (Baskaran et al., 2010), nitric oxide, inducible nitric oxide synthase (iNOS) and prostaglandin E2 (Kaur et al., 2010); leukotrien C4 (Raghavenra et al., 2006); mast cell degranulation (Kim et al., 1997) and the transcription factors NF-AT (Park et al., 2007) and NF-κB (Lee et al., 2007).

Chemoprevention by eugenol may also occur due to down-regulation of IκBα phosphorylation, a critical step in NF-κB activation thereby blocking the NF-κB signaling cascade (Manikandan et al., 2010). In lung inflammation, eugenol effectively improved functional and structural pulmonary changes induced by LPS, modulating lung injury by inhibition of NF-κB activation and TNF-α release (Magalhães et al., 2010)

Nowadays, there is a growing interest in identifying plant components with immunomodulatory activity that may be employed in complementary and alternative medicine. In our work, clove exerted immunomodulatory effects by inhibiting LPS action, and a possible mechanism of action probably involves

NF-κB suppression by eugenol, since it was the majoritary compound found in clove extract. Further studies are still needed to check clove and eugenol efficacy in inflammatory diseases, in order to explore their potential as anti-inflammatory agents.

Acknowledgements

The authors wish to thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP – 2008/06120-0) for the grant.

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Conclusão

Como conclusões de todos os ensaios desta dissertação, temos:

1. A Própolis pode exercer efeito modulador sobre a resposta imune e inflamatória. Esta atividade pode ocorrer devido ao sinergismo de seus inúmeros componentes e, de acordo com nossos resultados, podemos especular que o ácido cumárico e o ácido cinâmico podem estar envolvidos na imunomodulação exercida por este produto apícola.
2. Observamos que *B. dracunculifolia* também exerceu efeito imunomodulador quanto à produção de citocinas, o que pode ser mediado em parte pela ação do ácido cafeico. Ademais, esta planta exerceu ação semelhante à da Própolis, visto que a *Baccharis dracunculifolia* é a sua principal fonte vegetal em nossa região, esta similaridade à atividade deste apiterápico sugere sua possível utilização para os mesmos propósitos que o da Própolis.
3. O capim-limão, na maior concentração testada (100 µg/well) exerceu efeito inibitório na produção de citocinas, sendo este efeito mais pronunciado em ensaios com o citral, um de seus compostos isolados.
4. O cravo-da-Índia exerceu efeito imunomodulador quanto à produção de citocinas. Resultado semelhante foi observado para o seu composto majoritário, o eugenol.
5. De todos os produtos naturais e compostos isolados avaliados, a maior ação inibitória ocorreu após a incubação de macrófagos com citral, pois dependendo da citocina, as menores concentrações já exerceram ação inibitória, tanto antes como após o desafio com LPS. Este fato tem implicações práticas reforçando relatos anteriores da ação anti-inflamatória deste composto e, embora não avaliado em nosso trabalho, o provável alvo para esta ação é a possível inibição da ativação do fator de transcrição NF-κB, o qual está envolvido na produção de citocinas.
6. Nossos resultados sugerem que o potencial dos produtos naturais merece ser explorado em futuras investigações avaliando sua eficiência em doenças inflamatórias.