UNIVERSIDADE FEDERAL DA BAHIA
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KEINA MACIELE CAMPOS DOURADO

POTENCIAL TERAPÊUTICO DO EUGENOL EM MODELO EXPERIMENTAL DE ALERGIA RESPIRATÓRIA

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DISSERTAÇÃO DE MESTRADO

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Dissertação de mestrado apresentada ao curso de Pós-graduação em Imunologia do Instituto de Ciências da Saúde da Universidade Federal da Bahia, como requisito para obtenção do título de Mestre em Imunologia.

Orientadora: Profª. Drª. Camila A. Viana de Figueiredo
Co-orientadora: Profª. Drª. Darizy Flávia Silva Amorim de Vasconcelos

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"ATA DA SESSÃO PÚBLICA DO COLEGIADO DO PROGRAMA DE PÓS-GRADUAÇÃO EM IMUNOLOGIA PARA JULGAMENTO DA DEFESA PÚBLICA DA DISSERTAÇÃO DA MESTRANDA KEINA MACIELE CAMPOS DOURADO."

Ao primeiro dia do mês de fevereiro ano de dois mil e doze, às 14 horas no auditório III no 2º andar do Instituto de Ciências da Saúde, se reúne a Banca Examinadora composta das Professoras: Dra. **Darizy Flávia Silva Amorim de Vasconcelos** Co-orientadora, Dra **Maria de Fátima Dias Costa**, Dra **Vera Lúcia Costa Vale**, com a finalidade de discutir, avaliar e julgar o trabalho de Dissertação intitulado: **"Potencial Terapêutico do Eugenol em Modelo Experimental de Alergia Respiratória"** da Mestranda **KEINA MACIELE CAMPOS DOURADO.** Após a apresentação, foram feitos os comentários pelos examinadores. Havendo cumprido as exigências para a defesa, a Banca Examinadora conclui que a pós-graduanda teve a sua defesa de Dissertação APROVADA, emitindo pareceres individuais que serão anexados à ata. Nada mais havendo a tratar, é encerrada a sessão, da qual é lavrada a presente ata que após lida e aprovada vai assinada pelas componentes da Banca examinadora, pela Mestranda e pela Vice-Coordenadora do Programa de Pós Graduação. Salvador, primeiro de fevereiro do ano de dois mil e doze.

Dra. Darizy Flávia Silva Amorim de Vasconcelos
Co-orientadora

Dra. Maria de Fátima Dias Costa
Banca Examinadora

Dra. Vera Lúcia Costa Vale
Banca Examinadora

Keina Maciele Campos Dourado
Mestranda

Profa. Dra. Silvia Lima Costa
Vice-Coordenadora do PPGIm
ICS - UFBA

Dedico este trabalho à minha família, sempre muito presente e essencial na minha vida.

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A realização deste trabalho foi possível pela colaboração entre o Laboratório de Imunofarmacologia, o Laboratório de Alergia e Acarologia da Universidade Federal da Bahia e o Laboratório de Patologia e Biointervenção do Centro de Pesquisa Gonçalo Moniz – Fiocruz.

Basta ser sincero e desejar profundo, você será capaz de sacudir o mundo.
Tente outra vez.

(Raul Seixas)

LISTA DE ABREVIATURAS

BAL: Lavado bronco alveolar

Bk: bradicinina

CD4: Grupamento de diferenciação de linfócito T auxiliar

Cch: carbacol

COX2: cicloxigenase 2

ELISA: Ensaio imunoenzimático

Eug: Eugenol

EPO: Peroxidase eosinofílica

$E_{máx:}$ relaxamento máximo

GATA-3: Fator de transcrição

HRB: Hiper reatividade brônquica

HBSS: Hanks' balanced salt solution

IFN-ɣ: Interferon gama

IL: Interleucina

i.n.: Via intranasal

IgE: Imunoglobulina tipo E

LPS: Lipopolissacarídeo

NF-kB: Fator nuclear potencializador de células B ativadas

OVA: Ovalbumina

PAS: Ácido periódico Schiff

PBS: Solução fosfato tamponada bisódica

PWM: Pokweed (mitógeno)

RPM: Rotações por minuto

s.c.: via subcutânea

Th2: Linfócito T auxiliar do tipo 2

Th1: Linfócito T auxiliar do tipo 1

TRP: Transient Receptor Potential channels

TGF-β: Fator β de transformação do crescimento tumoral

v.o.: via oral

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RESUMO

A asma tem emergido como um importante problema de saúde pública da população urbana tanto de países desenvolvidos quanto dos países latino americanos. Embora os tratamentos atuais sejam capazes de controlar os sintomas e melhorar a função pulmonar na maioria dos pacientes, exacerbações agudas graves ainda ocorrem e contribuem significativamente para a morbidade e mortalidade da asma em todas as faixas etárias. Além disso, as drogas mais utilizadas, os corticosteroides, apresentam elevadas taxas de efeitos colaterais. Portanto, novas armas para o arsenal terapêutico necessitam serem desenvolvidas, sendo os produtos naturais uma importante fonte para tal. O eugenol (Eug) despertou interesse do nosso grupo por ser o principal componente químico do óleo essencial do *Ocimum gratissimum L.,* espécie da qual demonstramos previamente considerável potencial imunomodulador. Nesse contexto, o objetivo deste trabalho foi avaliar o potencial terapêutico (antiinflamatório e broncodilatador) do eugenol em um modelo experimental de asma. Para tal, camundongos AJ foram sensibilizados (100µg por animal - s.c.) e desafiados (10µg por animal - i.n), com extrato de ácaro de *Blomia tropicalis* (BtE). Os animais sensibilizados foram tratados ou não com Eug (40 ou 80mg/Kg) e foram analisados segundo os seguintes parâmetros: número de células totais no lavado bronco-alveolar (BAL); atividade de peroxidase eosinofílica (EPO) no pulmão; nível sérico de IgE anti-Bt; níveis de IL-4, IL-5, IL-13 no BAL e em cultura de esplenócitos, alterações histopatológicas no pulmão e produção de oxido nítrico (ON) por macrófagos peritoneais. Além disto, também foi avaliada a capacidade de relaxamento do músculo liso das vias aéreas pelo Eug. O tratamento dos animais com Eug levou a redução estatisticamente significante da inflamação das vias aéreas, com diminuição do infiltrado celular, EPO e muco no pulmão, assim como das citocinas Th2 e da produção de ON. Adicionalmente foi demonstrado um efeito dilatador do eugenol em aneis da traqueia isolada de camundongos. Estes resultados sugerem que o eugenol possui potencial como droga anti-asmática com ambas propriedades imunomodulatórias e broncodilatadoras, podendo este ser um candidato a compor o arsenal terapêutico desta patologia.

Palavras-chave: asma; *Blomia tropicalis*; produtos naturais; eugenol

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ABSTRACT

Asthma has emerged as an important public health problem of urban populations in developed countries as well as Latin America. Although current treatments are able to control symptoms and improve lung function in most patients, severe acute exacerbations still occur and contribute significantly to morbidity and mortality of asthma in all age groups. Moreover, the most common drugs, the corticosteroids, have high rates of side effects. Therefore, new weapons to the therapeutic arsenal need to be developed being natural products an important source to obtain that. We decided to explore the anti-allergic effect of eugenol (Eug) in the present study because this is the main molecule of the essential oil from *Ocimum gratissimum L*. which we and other authors have already described its immunomodulatory potential. In this context, the objective of this study was to evaluate the therapeutic potential (anti-inflammatory and bronchodilator) of eugenol on an asthma experimental model. To this end, AJ mice were sensitized (100 µg per animal -s.c.) and challenged (10µg per animal -i.n) with *Blomia tropicalis* (*BtE*) mite extract. Sensitized animals were treated or not with Eug (40 or 80mg/Kg) and the following parameters were analyzed: number of total cells in bronchoalveolar lavage (BAL); activity of eosinophil peroxidase (EPO) on the lung; serum level of specific IgE; levels of IL-4, IL-5, IL-13on BAL and spleen; histopathological changes in the lung and production of nitric oxide (NO) by peritoneal macrophages. In addition, the capability Eug in relaxing traqueal smooth muscle was also evaluated. Treatment of animals with Eug led to statistically significant reduction of airway inflammation, reducing the cellular infiltrate, EPO and mucus in the lungs, as well as production of Th2 cytokines and NO. Also was demonstrated a dilator effect of eugenol observed by the relaxation of isolated trachea up on carbachol stimulation. These results suggest that eugenol has potential as an anti-asthmatic drug with both bronchodilator and immunomodulatory properties, which can be a candidate to compose the therapeutic weapons to treat this pathology.

Keywords: asthma, *Blomia tropicalis*, natural products, eugenol

SUMÁRIO

1-INTRODUÇÃO

1- INTRODUÇÃO

A asma é uma doença caracterizada por uma inflamação crônica associada à hiper-responsividade das vias aéreas inferiores e limitação variável ao fluxo aéreo (O'BYRNE, 2009). É uma das doenças respiratórias mais comuns no mundo, com uma prevalência que vem aumentando nas últimas décadas. Segundo a OMS, afeta cerca de 300 milhões de pessoas, constituindo um importante problema de saúde pública (GINA, 2008; LEE, 2010).

Nos países em desenvolvimento, a asma é responsável por 5 a 10 % das mortes por causas respiratórias e no mundo o número de mortos devido a asma chega a 250.000 por ano (BATEMAN, et al. 2008). Segundo dados do *International Study for Asthma and Allergies in Childhood* (ISAAC, 1998), a estimativa de prevalência de asma no Brasil está entre as dez mais altas do mundo, situa-se em torno de 20%, gerando aproximadamente 350.000 internações hospitalares no SUS por ano (ISAAC, 1998; GINA, 2008). As capitais do Nordeste, incluindo Salvador, estão entre as cidades que apresentam maior prevalência dessa patologia (BAQUEIRO et al., 2007).

Muitos fatores influenciam na fisiopatogenia da asma, dando à doença um caráter multifatorial que contribui para a identificação de diferentes fenótipos da patologia, cujas manifestações clínicas podem refletir uma complexa interação entre um número muito variado de genes predisponentes, associada à relevantes influências ambientais. (BATEMAN, et al. 2008; COHN, 2004). Os fatores, que vão desde herança genética, atopia, idade e gênero à características socioeconômicas e ambientais, agem concomitantemente.

Observou-se que independente dos fatores de risco envolvidos, a inflamação crônica da mucosa brônquica está presente em todos os tipos da doença, inclusive nos quadros mais leves e estáveis com função pulmonar normal e sem sintomas atuais da doença (HALEY et al. 1998; SIMPSON, 2006).

Em consequência da inflamação, as vias aéreas tornam-se hiperresponsivas e se estreitam facilmente em resposta a inúmeros estímulos. Isso desencadeia sintomas clínicos como dispneia, tosse, espirro intermitente e opressão torácica, sendo esses sintomas mais comuns a noite. Entretanto o aspecto clínico pode ser variável e, histopalogicamente, pode apresentar diferentes padrões celulares (ABBAS; LICHTMAN, 2005; HOWARTH, 1997).

Apesar dos diferentes fenótipos identificados, a asma alérgica está muitas vezes associada à atopia (definida como a produção anormal de imunoglobulina E (IgE) em resposta à estímulos ambientais). Nesse caso, é uma sensibilização a alérgenos que resulta em uma diferenciação dos linfócitos Th0 em células Th2, gerando o ambiente inflamatório (BARNES, 2008; KAY, 2005; SIMPSON, 2006; WILLS-KARP, 2000).Os linfócitos Th2 induzem as células B a secretarem IgE e frente a uma segunda exposição, os alérgenos inalados iniciam a resposta inflamatória por meio da sua interação com IgE ligada aos mastócitos e basófilos.(ABBAS; LICHTMAN, 2005)

O perfil de resposta Th2 é iniciado, entre outros fatores, pela estimulação de células CD4+ a produzir IL-4 (interleucina 4) e essa, por sua vez, orquestra as células e moléculas necessárias para o desenvolvimento da inflamação brônquica característica da asma (BORISH et al., 2002). Uma vez que a IL-4 esteja acoplada ao seu receptor nas células B, resulta em uma

translocação para o núcleo do *signal transduction-activated transcription* (STAT-6), o qual estimula a transcrição de sequências codificadoras que resultam na produção de IgE. Esta citocina também está relacionada à inibição da apoptose dos eosinófilos, assim como da quimiotaxia destas células (BORISH et al., 2001).

A polarização Th2 não só estimula as respostas mediadas por anticorpos IgE, mas também ativa mastócitos, promove a eosinofilia tecidual e a hiperreatividade brônquica. Todos esses eventos associados às citocinas (IL-4, IL-5, IL-9 e IL-13) possuem papel importante na doença (FINKELMAN, 2010). A IL-5 está relacionada com a diferenciação, ativação, expansão e recrutamento de eosinófilos, exercendo também efeito sobre o calibre das vias aéreas e a hiperresponsividade brônquica (MENZIES-GOW et al., 2007). A IL-13 contribui para a hiperresponsividade brônquica, para o aumento da produção de muco, inflamação e para o desenvolvimento da fibrose sub-epitelial (MILLER et al., 2008).

Outros mediadores inflamatórios são liberados pelos mastócitos (histamina, leucotrienos, triptase e prostaglandinas), pelos linfócitos T (IL-2,GM- CSF), pelos eosinófilos (proteína básica principal, peroxidase eosinofílica (EPO), mediadores lipídicos e citocinas), pelos macrófagos (fator de necrose tumoral – TNF-alfa, IL-6, óxido nítrico), pelos neutrófilos (elastase) e pelas células epiteliais (endotelina-1, mediadores lipídicos, óxido nítrico) (IV diretrizes para manejo da asma, 2006).O aumento destas substâncias geram uma lesão tecidual com o aparecimento de alterações estruturais caracterizadas por fibrose subepitelial (espessamento da membrana basal),

hiperplasia da musculatura lisa da via aérea com produção exacerbada de muco e neo -formação vascular. Essas alterações podem estar relacionadas à obstrução do fluxo aéreo e são determinantes da gravidade da asma e da irreversibilidade da redução da luz brônquica. (JOSKOVA, 2011; BATEMAN, 2008; COHN, 2004; HOWARTH, 1997).

Essa resposta inflamatória crônica associada ao remodelamento faz com que os pacientes asmáticos geralmente tenham hiperreatividade brônquica. Nesse caso, a concentração de um estímulo necessária para aumentar a resistência das vias inflamatórias é de apenas 1 a 2% da concentração que causaria o mesmo efeito em pessoas saudáveis. Essa hiperreatividade é inespecífica, podendo ocorrer frente a fatores endógenos (p. ex. histamina) ou exógenos (p. ex. odores, ar frio e poluentes). (GOODMAN, 2006).

Apesar dos avanços no estudo na fisiopatogênia da asma, até o momento não existem drogas que levem a cura. A terapia disponível atualmente está centrada na supressão da inflamação, tendo os glicocorticoides (GC) como as principais drogas disponíveis. Outros fármacos que relaxam a musculatura lisa dos brônquios são utilizados para obter alivio mais rápido e direto dos sintomas (BUSSE, 2011; GOODMAN, 2006). Apesar dos benefícios inegáveis de GC para o tratamento de doenças inflamatórias, a exemplo da asma, exacerbações agudas graves ainda ocorrem e contribuem significativamente para a morbidade e mortalidade da asma em todas as faixas etárias. Além disso, os efeitos colaterais são bastante intensos, principalmente, quando o uso desses medicamentos é por via oral ou seu é uso crônico, como acontece em muitos pacientes com asma e outras alergias (HOANG et al.,

2010; WALSH, 2005). Sendo assim, a busca de terapias alternativas tem sido de crucial importância, na tentativa de se buscar novos fármacos que tenham os mesmos benefícios com relação à capacidade antiinflamatória e broncodilatadora da terapia atual, e que apresentem menos efeitos colaterais.

Essa necessidade de desenvolver medidas eficazes de controle para a asma resultou no estudo de terapias alternativas, sendo os produtos naturais uma importante fonte para tal. A alergia é considerada como a segunda patologia em que a população mundial dá preferência ao uso de medicamentos complementares, apenas perdendo para lombalgias (BIELORY, 2004). Segundo Shafer (2004), na Alemanha ocidental, 30% dos portadores de alergias utilizam medicamentos alternativos, sendo os fitoterápicos os mais importantes deles.

O uso dos produtos naturais iniciou-se há milhares de anos por populações de vários países com o intuito de tratar diversas patologias. Eram utilizados pela população como forma alternativa ou complementar aos medicamentos sintéticos (VEIGA-JUNIOR, 2008). Segundo a OMS (Organização Mundial de Saúde) 65 a 80% da população mundial, especialmente em países em desenvolvimento, utilizam produtos à base de plantas medicinais no tratamento de doenças, na atenção primaria à saúde (RAHMAN; SINGHAL, 2002).

No Brasil o uso de plantas medicinais pela população, principalmente aquelas de baixa renda é alto devido ao difícil acesso da população à assistência médica e farmacêutica e ao custo dos medicamentos industrializados (SIMÕES et al, 1998). Assim, muitos trabalhos científicos vêm

sendo realizados, principalmente nas Universidades do Nordeste, para caracterizar a flora brasileira, riquíssima, apresentando várias espécies inéditas ou que não possuem a sua aplicação popular elucidada cientificamente, quanto às suas propriedades farmacológicas para muitas enfermidades (YOUNES et al., 2007; BARBOSA-FILHO, 1988; BARBOSA-FILHO, 1997), inclusive as doenças alérgicas.

A OMS incentiva estes tipos de estudos, almejando um intercâmbio entre o conhecimento científico e o conhecimento popular para o uso racional das plantas medicinais (VENDRUSCOLO, 2005). Muitas áreas estão envolvidas na pesquisa e seleção de novas plantas medicinais e substâncias oriundas de plantas, dentre elas a etnobotânica e a etnofarmacologia, que buscam informações a partir do conhecimento de diferentes povos e etnias; a fitoquímica, que isola, caracteriza e purifica princípios ativos e a farmacologia, que estuda os efeitos biológicos de extratos e/ou seus constituintes químicos isolados. A integração destas áreas na pesquisa de plantas medicinais conduz a um caminho promissor e eficaz para descobertas de novos medicamentos (MACIEL et al.; VENDRUSCOLO et al., 2005).

Nesse sentido, um levantamento etnofarmacológico foi realizado na cidade de Salvador-Bahia para investigar os principais produtos consumidos pela população para o tratamento de asma em crianças. *Ocimum gratissimum Linn.* (*Og*), popularmente conhecido como quioio, alfavacão ou manjericão-cheiroso, destacou-se como um dos produtos vegetais mais utilizados (COSTA et al., 2010).

O gênero *Ocimum L*. é encontrado nos trópicos e subtrópicos do Velho e do Novo Mundo e contém cerca de trinta espécies, sendo que a espécie *Ocimum gratissimum* (Og) tem origem central na África (PATON, 1992; VIEIRA et al, 2002). O potencial imunomodulador deste gênero já foi demonstrado (MEDIRATTA; SHARMA; SINGH, 2002), e outras atividades biológicas da espécie *Ocimum gratissimum* L. têm sido descritas na literatura, dentre elas destaca-se: propriedade antinociceptiva (RABELO et al, 2003); atividade antibacteriana (NAKAMURA et al, 1999); antagonista sobre a motilidade intestinal (Montalvo; DomÍnguez, 1997) e atividade antifúngica (LEMOS et al, 2005). O potencial antialérgico do extrato do Og foi recentemente descrito em um modelo murino de asma (COSTA, 2010). O Og reduziu a inflamação eosinofílica no pulmão de camundongos alérgicos assim como os demais padrões utilizados para avaliar a alergia, como por exemplo, a peroxidase eosinofilica no BAL e produção de muco.(COSTA, 2010)

As atividades biológicas exercidas pelo *O. gratissimum* podem ser atribuídas aos seus principais constituintes químicos. Por exemplo, Trevisan (2006) avaliou à capacidade antioxidante dos óleos essenciais de cinco espécies do gênero *Ocimum*. O rendimento dos óleos das folhas das cinco espécies foi variável sendo que o melhor rendimento obtido foi o do *Ocimum gratissimum* (3,5%). A capacidade antioxidante foi positivamente associada com uma alta proporção de compostos que possuem um anel fenólico, como o eugenol (TREVISAN et al, 2006).

Dentre os componentes do *O. gratissimum* destacam-se os óleos essenciais (VIEIRA et al, 2002). Estão presentes também flavonóides, como o

xantomicrol e o cirsimaritin (VIEIRA et al, 2001; VIEIRA et al, 2002) e compostos polifenólicos (OLA et al., 2009).

Os óleos essenciais de *O. gratissimum* podem ser divididos em dois grupos, o primeiro com alto teor de eugenol, e o segundo com alto teor de Timol (GUENTHER, 1948). A composição química dos óleos essenciais de *O. gratissimum* relatada na literatura confirma essa divisão. Segundo Benitez, 2009, eugenol, timol e geraniol são os principais constituintes do óleo volátil encontrado em *Ocimum gratissimum*, destacando o eugenol como componente principal do óleo, correspondendo a cerca de 80% de todo o óleo essencial da planta (VIEIRA et al, 2002; BENITEZ, 2009)

Oeugenol (4-Alil-2-Metoxifenol) é um composto aromático, membro da classe dos fenilpropanóides. É encontrado em diversas espécies, principalmente no cravo, noz-moscada, canela, manjericão e folha de louro (BHUIYAN, 2010). Recentes trabalhos vêm demonstrando suas propriedades imunomoduladoras (MAGALHÃES et al, 2010; LEE et al, 2007 ; LI et al, 2006; TREVISAN et al, 2006; LEE et al, 2008; KIM et al, 2007).

Recentemente, Magalhães (2010) avaliou o potencial antiinflamatório deste composto em ratos e demonstrou que 160mg/kg de eugenol foi capaz de reduzir a inflamação induzida por LPS, diminuindo a lesão pulmonar, a deposição de fibras de colágenos e o influxo de neutrófilos.

Um estudo realizado por Lee e colaboradores (2007), teve como objetivo investigar os efeitos do eugenol em linhagem de macrófagos humanos (U937), sob o estímulo do lipopolissacarídeo (LPS). O eugenol bloqueou a liberação de

interleucina-1beta (IL-1-beta), fator de necrose tumoral alfa (TNF-alfa), e prostaglandina E2 de macrófagos estimulados com LPS, além de suprimir a expressão do RNA mensageiro da IL-1beta induzida viaLPS, TNF-alfa, e ciclooxigenase 2 (COX2) em cultura demacrófagos. Os resultados sugerem um potencial antiinflamatório do eugenol (LEE et al, 2007).

Em outro estudo avaliando a atividade antiinflamatória do eugenol e isoeugenol foi demonstrado que estes compostos inibem, de forma dose-dependente, a produção de óxido nítrico (ON) induzida por LPS em cultura de macrófagos RAW264.7 por um mecanismo relacionado à inibição da síntese da proteína iNOS (NO sintase induzível). O estudo demostrou que a ação antinflamatória de compostos contendo Eugenol pode ser explicada pela inibição da produção de ON e expressão de COX-2 e, consequentemente de mediadores pró-inflamatórios (LI et al, 2006).

Cerca de 100 extratos naturais de plantas coletadas na Coréia tiveram sua atividade antialérgica testada através da avaliação dos efeitos sobre a degranulação dos mastócitos em cultura e em ensaio de anafilaxia cutânea passiva (PCA) *in vivo*. O extrato de folhas de *Camellia japonica* (LECJ) apresentou potente efeito sobre a degranulação de mastócitos de roedores e macrófagos humanos estimulada por antígeno e suprimiu a expressão e secreção de TNF-alfa e IL-4 em ratos. Quercetina-3-beta-D-glicosídeo e eugenol foram identificados, em análise de cromatografia líquida, como os principais componentes ativos da LECJ (LEE et al, 2008).

Os efeitos anti-anafiláticos do eugenol foram avaliados em ratos. O eugenol inibiu a anafilaxia sistêmica induzida pelo composto 48/80 com uma

dose de 10 microgramas g-1 de peso corporal (PC). Embora os níveis séricos de histamina aumentarem significativamente após injeção do composto 48/80 em todos os grupos de ratos, ratos injetados com eugenol apresentaram uma redução significativa nos níveis de histamina do soro, inibindo a anafilaxia cutânea passiva ativada por IgE. Estes resultados sugerem que o eugenol tem propriedades antianafiláticas impedindo a degranulação dos mastócitos (KIM et al, 2007).

Tem sido mostrado que compostos antioxidantes fenólicos, como o eugenol, inibem o fator transcripcional NF kappa B (NFκB), responsável pela transcrição de citocinas inflamatórias e indução da iNOS e COX2 (RAHMAN, 2006; CHAINY, 2000). Estudo *in vitro* utilizando cultura de macrófagos, mostrou que dímeros do eugenol inibiram a atividade transcripcional induzida por LPS, embora o eugenol não (MURAKAMI, 2005). Em contraste, KAUR et al (2010) mostrou que *in vivo* o eugenol modulou a ativação induzida do NFκB na pele de camundongos .

Além das propriedades imunomodulatórias, trabalhos recentes tem sugerido uma ação potencial direta do eugenol no relaxamento da musculatura lisa das vias aéreas (MAGALHÃES, 2011), uma vez que propriedades relaxantes de composto relacionados ao eugenol têm sido descritas na traquéia e no músculo liso vascular. LIN (1999) mostrou que o isoeugenol apresentou um efeito relaxante, dose depende, sobre a traquéia de cobaia, contraída com carbacol (10μM). O eugenol também é descrito como modulador de canais Transient Receptor Potential channels (TRP) (MOSHE PARNAS, 2009). Uma vez que alguns TRPs estão altamente relacionados a contractibilidade do

músculo liso, agindo diretamente sobre o influxo de cálcio (SOUSA et al, 2011), o efeito do eugenol sobre esses, pode resultar em um efeito relaxante sobre a musculatura lisa.

Mediante o potencial antialérgico do *Ocimum gratissimum L*. demonstrado pelo nosso grupo, associado aos importantes indícios na literatura que apontam para uma possível propriedade imunomoduladora e broncodilatadora do eugenol, principal óleo essencial encontrado nesta espécie,o objetivo deste trabalho é avaliar o potencial terapêutico do eugenol, investigando para tanto sua capacidade imunomoduladora e broncodilatadora, em modelo de alergia respiratória ao ácaro *Blomia tropicalis*(Bt).

Embora o modelo experimental de alergia a ovalbumina (Ova) seja o mais abundantemente utilizado na literatura para o estudo da asma, os ácaros são os agentes biológicosalergizantes mais importantes sendo o Bt o ácaro mais prevalente nos países tropicais (BAQUEIRO et al., 2006; CARVALHO et al., 2004 e CHUA et al., 2007). Acredita-se que esse ácaro podem ser responsável pelo aumento das reações IgE mediadas em pacientes sensibilizados, possivelmente devido à sua alta prevalência e associação filogenética (SATO et al., 2002).

O trabalho de dissertação será apresentado sob forma de artigo cientifico.

2-OBJETIVOS

2. OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar o potencial terapêutico do eugenol em modelo experimental deasma alérgica induzida pelo ácaro *Blomia tropicalis.*

2.2 OBJETIVOS ESPECÍFICOS

Os objetivos específicos do presente trabalho foram:

-Avaliar o potencial imunomodulador do eugenol em modelo de alergia ao ácaro *Blomia tropicalis*;

- Investigar a influência do eugenol na inflamação eosinofílica no pulmão induzida por Blomia tropicalis;

- Verificar o potencial broncodilatador do Eugenol em modelo experimental de traquéia isolada;

- Verificar o potencial inibitório do eugenol sob citocinas Th2 *in vitro* e sob a produção de NO em cultura de macrófagos;

3-RESULTADOS E DISCUSSÃO

MANUSCRITO: Effects of Eugenol (4-Alil-2-Metoxifenol) in murine model of allergic respiratory disease induced by Blomia tropicalis mite

AUTORES: DOURADO, Keina Maciele Campos[a], OLIVEIRA, Tatiane[a], CERQUEIRA-LIMA, Ana Tereza[a], COSTA, Ryan Santos[a], CARNEIRO, Tamires Cana Brasil[a], SILVA, Darizy Flávia[a], PONTES-DE-CARVALHO, Lain Carlos[b], ALCÂNTARA-NEVES, Neuza Maria[a] and FIGUEIREDO, Camila Alexandrina[a]

Effects of Eugenol (4-Alil-2-Metoxifenol) in murine model of allergic respiratory disease induced by *Blomia tropicalis* mite

DOURADO, Keina Maciele Campos[a], OLIVEIRA, Tatiane[a], CERQUEIRA-LIMA, Ana Tereza[a], COSTA, Ryan Santos[a], CARNEIRO, Tamires Cana Brasil[a], SILVA, Darizy Flávia[a], PONTES-DE-CARVALHO, Lain Carlos[b], ALCÂNTARA-NEVES, Neuza Maria[a] and FIGUEIREDO, Camila Alexandrina[a*]

[a]Instituto de Ciências da Saúde, Universidade Federal da Bahia, Bahia, Brazil; [b]Centro de Pesquisas Gonçalo Moniz,Fundação Oswaldo Cruz, Bahia, Brazil.

***Corresponding author:**

Camila Alexandrina Figueiredo

Departamento de Biorregulação

Instituto de Ciências da Saúde

Universidade Federal da Bahia,

Campus do Canela, CEP 41110-100, Salvador, BA, Brazil

Tel/Fax: +557132838948

e-mail address: cavfigueiredo@gmail.com (CA Figueirêdo)

ABSTRACT

Allergic asthma has emerged as an important public health problem of urban populations in developed countries, including those of Latin America**.** Very often her natural products are used to treat this disease, due to the lack of efficacy and the important side effects related to the classical drugs in use. Eugenol is an aromatic compound, a member of the phenyl propanoids. Since eugenol and related compounds exhibit anti-inflammatory properties and a possible relaxing effect on smooth muscle we examined the pharmacological potential of eugenol in a murine model of respiratory allergy to *B. tropicalis* mite extract (Bt). To this end, AJ mice were sensitized (100 µg per animal -s.c.) and challenged (10µg per animal -i.n) with *Blomia tropicalis* (*BtE*) mite extract. Sensitized animals were treated or not with Eug (40 or 80mg/Kg) and the following parameters were analyzed: number of total cells in broncho alveolar lavage (BAL); activity of eosinophil peroxidase (EPO) on the lung; serum level of specific IgE; levels of IL-4, IL-5, IL-13 on BAL and spleen; histopathological changes in the lung and production of nitric oxide (NO) by peritoneal macrophages. In addition, the capability Eug in relaxing traqueal smooth muscle was also evaluated. Treatment of animals with Eug led to statistically significant reduction of airway inflammation, reducing the cellular infiltrate, EPO and mucus in the lungs, as well as production of Th2 cytokines and NO. Also was demonstrated a dilator effect of eugenol observed by the relaxation of isolated trachea up on carbachol stimulation. These results suggest that eugenol has potential as an anti-asthmatic drug with both bronchodilator and immunomodulatory properties, which can be a candidate to compose the therapeutic weapons to treat this pathology.

Keywords: asthma, *Blomia tropicalis*, natural products, eugenol

1.INTRODUCTION

Asthma is a chronic inflammatory disorder associated with hyperresponsiveness of the lower airways and variable airflow limitation (HASHIMOTO, 2011; O'BYRNE, 2009). It is characterized by a Th2-dominant response where interleukin-4 (IL-4), IL-5, and IL-13 are involved in coordinating, amplification and perpetuation of the inflammatory response and by attracting additional inflammatory cells, mainly eosinophils, increased mucus production and increased serum immunoglobulin E (IgE) antibody (Ab) levels (BARNES, 2006; KAY, 2005; KAY, 2006).

Although current treatments are able to control symptoms and improve lung function in most patients, severe acute exacerbations still occur and contribute significantly to morbidity and mortality of asthma in all age groups (BUSSE, 2011; HASHIMOTO, 2011). Historically, herbal medicine has a great importance in the treatment of asthma. Four of the five classes of drugs currently used to treat asthma - namely, α2 agonists, anticholinergics, methylxanthines and cromones - have origins in herbal treatments going back at least 5 years (BIELORY, 1999; BEZZERA-SANTOS et al., 2006).

Eugenol (4-ALLYL-2-METHOXYPHENOL) is an aromatic compound, a member of the phenyl propanoids. It is found in several species, especially in cloves, nutmeg and *Ocimum gratissimun* (BHUIYAN, 2010). This oil is commonly used as a flavoring agent in cosmetics and food products and, in particular, in dentistry in zinc oxide-eugenol chelating cement. Previous studies demonstrated that eugenol and other phenolic compounds show antioxidative and anti-inflammatory activities by inhibiting prostaglandin synthesis and neutrophil chemotaxis (MA Q, 2002; MURAKAMI, 2003; MURAKAMI, 2005). It has been shown that this compound has anti-inflammatory activities such as blocking the release of IL-1-beta, tumor necrosis factor (TNF-alpha)

and prostaglandin E2 in macrophages stimulated with LPS (LEE et al, 2007), inhibiting systemic anaphylaxis (KIM et al, 1997) and decreasing lung injury induced by LPS *in vivo* (MAGALHÃES et al, 2010). Also, it has been shown that phenolic compounds such as eugenol have a relaxing effect on smooth muscle of guinea pig tracheal and may have bronchodilator effect (LIN et al., 1999)

Since eugenol and related compounds exhibit anti-inflammatory properties and a possible relaxing effect on smooth muscle we examined, therefore, the pharmacological potential of eugenol in a murine model of respiratory allergy to *B. tropicalis* mite extract (Bt).

2. MATERIAL AND METHODS

2.1. Animals

Females AJ mice (20-25g) were used throughout the study. Animals were maintained with free access to food and water. They were obtained from the animal facilities of the Fundação Oswaldo Cruz, Bahia, Brazil. Groups of 5 animals were used in each experiment. All the experimental procedures were approved by the Ethical Committee for Use of Experimental Animals of the Faculdade de Odontologia, Universidade Federal da Bahia, Brazil (protocol number: 02/09).

2.2. Experimental design

To investigate the anti-allergic effect of eugenol we used the experimental model of allergy to *Blomia tropicalis* (Bt) dust mite as previously described (BAQUEIRO et al, 2010). Briefly, A/J mice (n=5) were initially sensitized with two subcutaneous injections (day 0 and day 7) of Bt (100 μg of protein), adsorbed to 4 mg/mL of Al(OH)3

in saline. Twenty-four hours after the second subcutaneous injection, the animals received four intranasal immunization boosters/challenges with Bt (10 µg/instilation) every other day. One day after the last challenge, the animals were euthanized with intraperitoneal injections of xilazine and ketamine (40 mg/kg/body weight). Mice which were sensitized and challenged with $Al(OH)_3$ in saline alone (vehicle) were considered normal controls. The figure 1 presents the experimental protocol for sensitization, challenge and treatment used in this work.

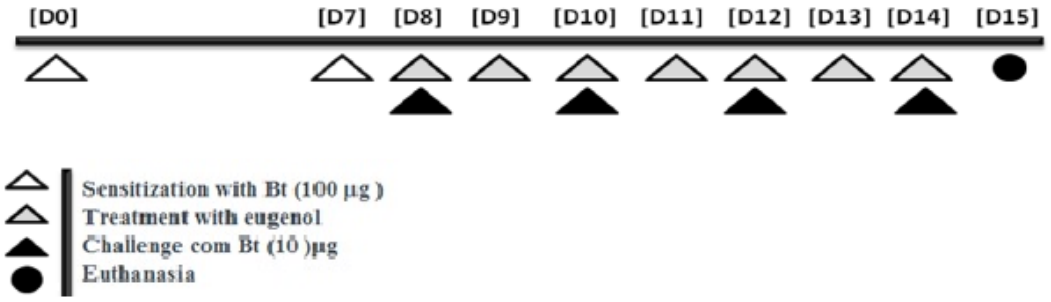


Fig (1): Experimental protocol for sensitization, challenge and treatment.

2.3 Experimental groups and eugenol oral treatment

The animals were daily treated orally with 40 mg/kg or 80mg/kg of eugenol, obtained commercially from Sigma-Aldrich®, dissolved in 1% of tween in saline (vehicle) from the 8th to the 14th days of the experimental protocol and one hour after the intranasal challenges with Bt (Fig. 1). The groups of animals were defined as: **Control**, non-sensitized and vehicle-treated mice; **Bt**, Bt-sensitized mice and vehicle-treated mice; **Bt/Eug 40**, Bt-sensitized and eugenol 40mg/kg treated mice; **Bt/Eug 80**, Bt-sensitized and eugenol 80mg/kg treated mice. To determine the *in vivo* doses, pilot studies were carried out using 160mg/kg **(**based in previous studies evaluating anti-inflammatory effect of this compound (MAGALHÃES et al, 2010)), 80mg/Kg. As 160

mg/kg and 80 mg/kg had the same effect (data not shown), we evaluated lower doses (80 and 40 mg/kg) in an attempt to obtain a dose-response curve.

2.4. Bronchoalveolar Lavage (BAL)

The trachea was canulated and the lungs were carefully washed three times with 0.5 mL of PBS containing 1% of bovine serum albumin. The total numbers of leukocytes in the BAL were immediately determined in a hemocytometer, using Trypan blue.

2.5. Eosinophil peroxidase (EPO) activity

The EPO activity in lung cells was evaluated according to a previously described method (CHOI et al., 2009). Briefly, cell suspensions were frozen and thawed three times in liquid nitrogen. After centrifugation at 4^{o} C for 10 min at 1000 g, the cell lysates were placed into wells of 96-well plates (75 μL/well), followed by the addition of the chromogen and substrate solution (1.5 mmol/L of o-phenylenediamine and 6.6 mmol/L of H_2O_2 in 0.05 mol/L Tris-HCl, pH 8.0). After 30 min at room temperature, the reaction was stopped with the addition of 0.2 mol/L citric acid, and the absorbance of the sample determined at 492 nm in an ELISA reader.

2.6. Histopathological analysis

The histopathological changes and the degree of inflammation in peribronchiolar and perivascular region were assessed as described previously (TAKANO, 2004). Briefly, lung tissues were fixed at the time of withdrawal with 10% (v/ v) para formaldehyde. The tissue was dehydrated and embedded in paraffin for achieving the cuts (5 mm). The slides with fixed sections were stained with hematoxylin and eosin

for evaluation of cellular infiltration and with periodic acid Schiff to assess mucus, under light microscopy with x 40 magnification.

2.7. Measurement of anti-Bt IgE antibody levels in serum

Anti-Bt IgE antibody levels in the serum of mice from the different experimental groups were determined by ELISA. 96-well micro titre high-binding plate (Costar) were coated with Bt (100 µg/well) overnight, at 4° C. The wells were washed 3 times with PBS containing 0.05% Tween 20 (PBS-T) and blocked during 1 hour with PBS-T containing 10% fetal calf serum (FCS) at room temperature (RT). After this incubation period and washes with PBS-T, the samples were added and incubated overnight at 4° C. After several washes, a biotin-conjugated rat anti-mouse IgE (BD Pharmingen, San Diego, CA, USA) was added in and incubated during 1 hour at RT. A solution of avidin-horseradish peroxidase was then added to each well for 30 min. After washes, a solution containing 3,3′,5,5′-tetramethylbenzidine and hydrogen peroxide was added and incubated during 30 min at RT and the reaction was stopped with 4M sulfuric acid. The absorbance of the sample was determined at 492 nm in an ELISA reader.

2.8. *In vitro* cytokine production in spleen and BAL cells

In vitro estimation of cytokines by spleen cells was performed according to the method described by Bezerra-Santos (2004). In brief, spleen cell suspensions from non-sensitized and Bt-sensitized groups were prepared. Cells were then washed twice in RPMI medium by centrifugation at 200×g for 10 min. The obtained pellet was resuspended in RPMI medium supplemented with 200mM l-glutamine, 100 units/ml penicillin, 100µg/ml streptomycin, 5 -Mercaptoethanol and 10% fetal calf serum (Gibco, Pisley, UK). Viable cell number was determined in a hemocytometer by

exclusion of trypan blue. Five wells for each concentration were plated in 96-well flat-bottomed tissue culture plates (Costar, Cambridge, MA, USA) in a final volume of 200 µl per well containing 5×10^5 cells. pokeweed (PWM) (Sigma, EUA) was added at 5µg/ml either in the absence or in the presence of different concentrations of eugenol (12,5-200µM). Cultures were then incubated for 2 days at 37 ◦C in an atmosphere with 5% CO2. Supernatant of the cell culture was removed for cytokine measurement. Regarding to the production of cytokines on BAL cells, after the collection of BAL as described in the item 2.4, the supernatants were also collected and kept in -20°C until use. Cytokines (IL-4, IL-5 and IL-13) in both BAL and spleen were quantified by enzymatic immunoassay with their respective antibody pairs following manufacturer's instructions (BD Pharmingen).

2.9. *In vitro* production of nitric oxide (NO)

Estimation of NO levels in the supernatant from treated/untreated cells was performed as previously described by Chandrasekaran (2010). In brief, peritoneal macrophages were pre-incubated with 50, 100 or 200 µM of eugenol for 1 h and LPS (5 µg/ml) for 24h. After the incubation period, the supernatants were collected and analyzed for nitrite by Griess reaction. Briefly, equal volumes of culture supernatant and Griess reagent were mixed and incubated at room temperature for 5 min, and then the absorbance was measured at 540nm in a microplate reader. The amount of nitrite in the sample was determined using sodium nitrite standard curve.

2.10. Assessment of the effects of eugenol on the airway smooth muscle

Initially, normal AJ mice were euthanized by overdose of sodium thiopental and then the trachea was rapidly removed, cleaned of connective tissue and washed three times with Krebs-bicarbonate solution (composition in mM: NaCl 119, NaHCO3 25, CaCl2 ×H2O 1.6, KCl 4.7, KH2PO4 1.2, MgSO4×7H2O 1.2 and glucose 11.1). After, the trachea was sectioned into rings measuring about 2 mm, containing on average three to four cartilage bands. The rings were suspended on metal rods, attached to a force transducer (FORT10 WPI, Sarasota, USA) and placed in tanks for isolated organ, maintained at 37 ° C and aerated with a mixture carbogen 95%O_2 and 5% CO_2. The rings were subjected to stabilization for a period of 1h to 0.5g. After the stabilization period, the rings were contracted with 10μM of carbachol (Cch) to assess the contractile state of the tissue. To evaluate the presence of functional epithelium, the rings were contracted with carbachol and after reaching a plateau of contractile state, were stimulated with bradykinin (Bk) (10^{-6}M). After stabilization and assessment of the presence of functional epithelium, the rings were again contracted with carbachol (10μM) and were added cumulatively, increasing concentrations of eugenol. Concentration response curve was constructed and the data will be analyzed

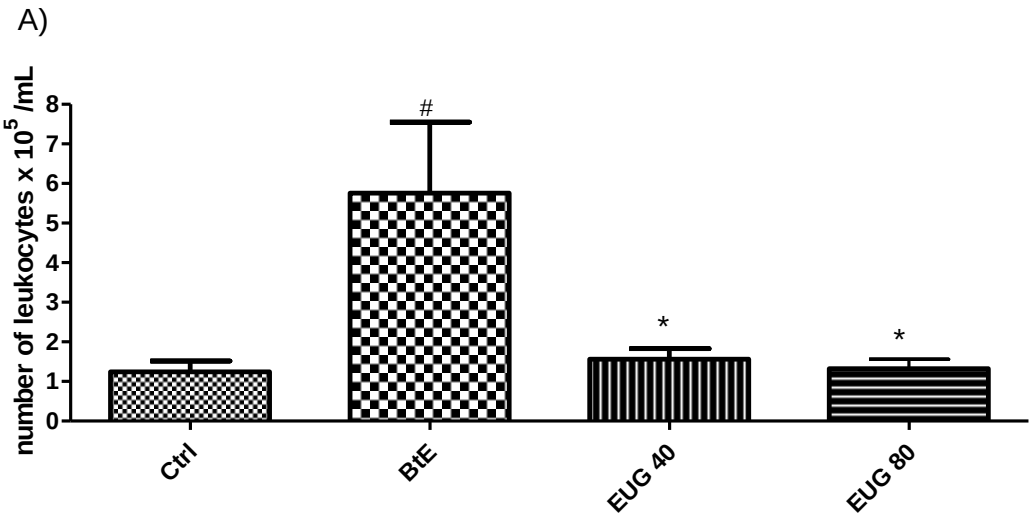
2.12. Statistical analysis

The one-way analysis of variance (ANOVA) and Tukey´s post-test (for data with normal distribution) were used to determine the statistical significance between the experimental groups. Differences in P values ≤ 0.05 were considered statistically significant. Each experiment was repeated at least two times.

3. RESULTS

3.1.Treatment with eugenol reduces the cellular infiltration induced by Bt in BAL

To assess the effects of eugenol on the inflammatory cell infiltration in BAL of the Bt-sensitized and challenged mice, the presence of cells in the BAL was assessed 24 hours after the last challenge. Bt-challenged mice displayed a significant increase of total cells in relation to the control group ($P < 0.05$) (Fig. 2A). Oral administration of 40 mg/kg and 80 mg/kg of eugenol daily significantly suppressed the number of total inflammatory cells, in relation to the untreated Bt-immunized and challenged mice ($P < 0.05$; Fig. 2A). No significant difference between doses was observed.



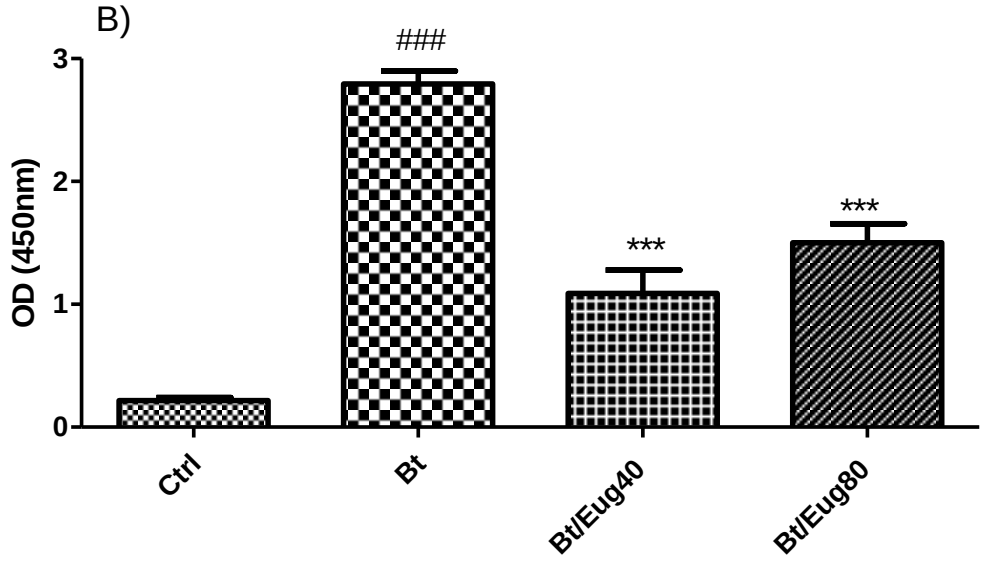


Fig (2): Effect of Eug on cellularity in BAL and eosinophil peroxidase. **A)** Numbers of leukocytes in the BAL; and **B)** Kinetics of eosinophil peroxidase (EPO) activity in lung tissue from vehicle-treated and sensitized mice (**Control**) or Bt-sensitized and challenged animals (**Bt**) and Bt-sensitized and challenged, and eug (40mg/kg or 80mg/kg) treated mice (**Bt/Eug40 or Bt/Eug80).** Columns represent the mean values of the results obtained from five animals, and error bars represent the standard error from the means. #P < 0.05 vs control; ### p <0.001 vs control; * p <0.05; *** p <0.001 vsBt group. ANOVA-Tukey.

3.2 Treatment with eugenol reduces eosinophil peroxidase levels in lungs

The sensitization of animals with Bt produced a significant increase of EPO activity in the lungs (P < 0.001) when compared to the control group (Fig. 2B). Treatment with both doses of eugenol decreased EPO activity in lung tissue (P < 0.001) of Bt-immunized and challenged mice (Fig. 2B).

3.3. Treatment with eugenol decreases the inflammatory cell infiltration and amount of mucus in lungs of Bt-immunized animals

The figure 3 and 4 show the typical pathologic features of allergic asthma in lung tissue from Bt-immunized mice, characterized by the infiltration of numerous inflammatory

cells in the peribronchiolar and perivascular regions (Fig. 3B) and airway hypersecretion of mucus (Fig. 4B). Treatment with both doses of eugenol (40 and 80mg/Kg) markedly reduced the inflammatory cell infiltration around the bronchioles (Fig. 3C and D) as well as suppressed mucus secretion in the lung tissue (Fig. 4C and D).

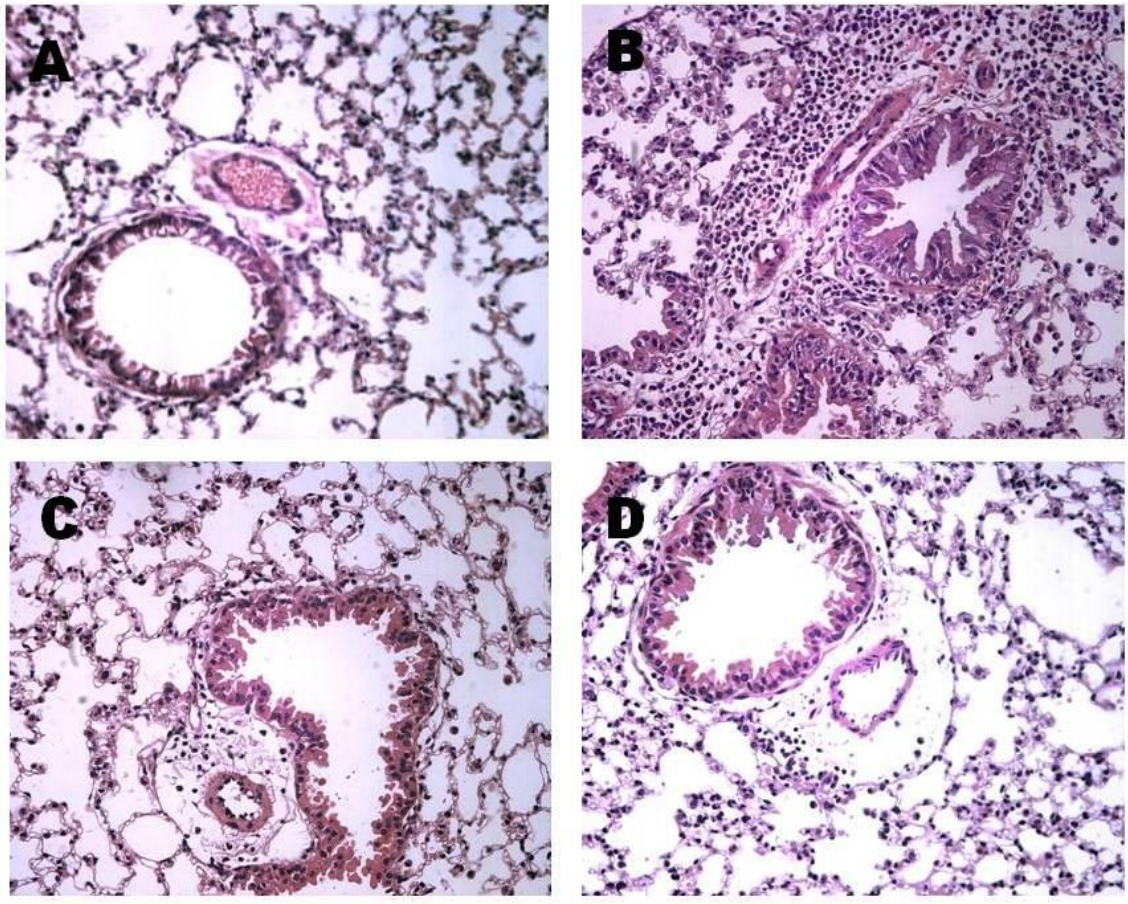


Fig (3): Effect of the treatment with Eug on leukocyte infiltration in lung tissues of mice sensitized and challenged with *Blomia tropicalis* extract (Bt). Sections were stained with hematoxylin-eosin (magnification ×400). (**A**) Lung section from a control, saline-treated mice; (**B**) Lung section from a Bt- immunized and challenged, saline-treated mice; (**C**) Lung section from a Bt-immunized and challenged, Eug 40mg/kg - treated mice; (**D**) Lung section from a Bt-immunized and challenged, Eug 80mg/kg treated mice.

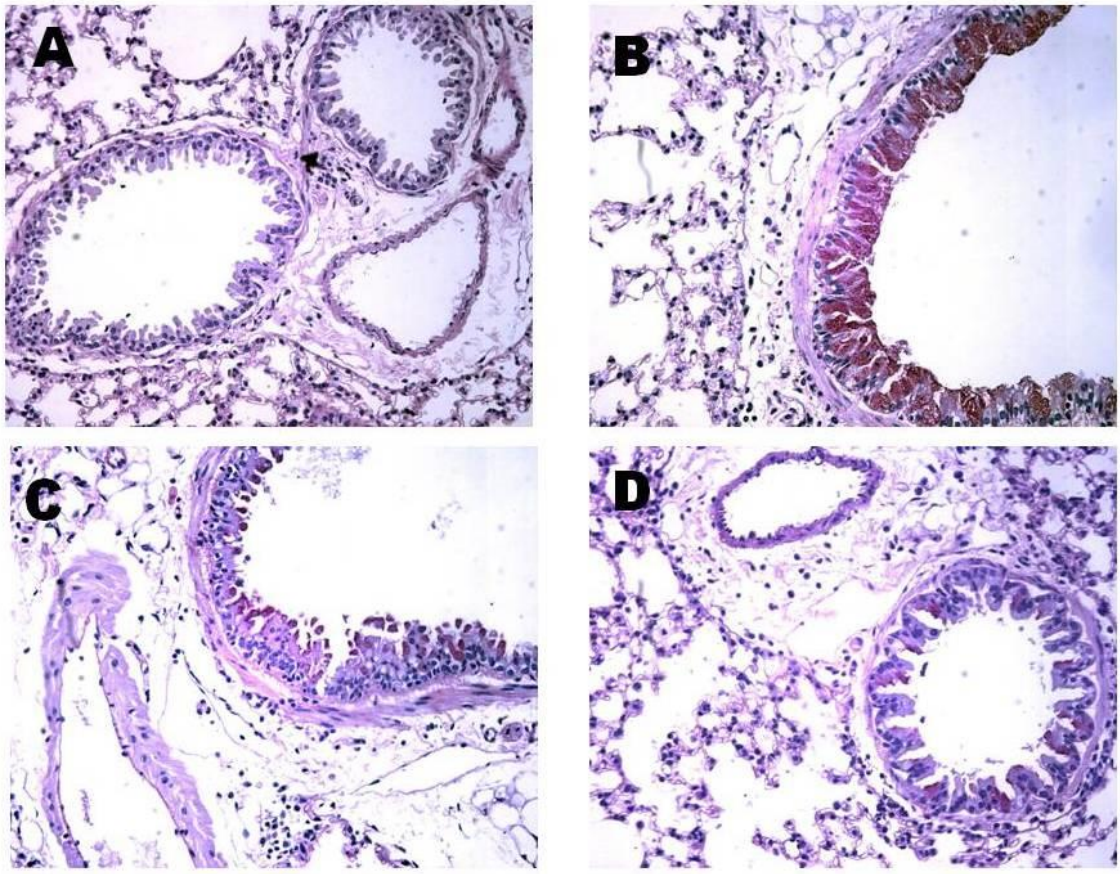


Fig (4): Effect of the treatment with Eug on the production of mucus in the lung tissue of mice sensitized with Bt antigen. Sections were stained with periodic acid-Schiff (magnification×400). (**A**) Lung section from a control, saline-treated mice; (**B**) Lung section from a Bt- immunized and challenged, saline-treated mice; (**C**) Lung section from a Bt-immunized and challenged, Eug 40mg/kg -treated mice; (**D**) Lung section from a Bt-immunized and challenged, Eug 80mg/kg treated mice.

3.4. Treatments with eugenol do not decrease the levels of Bt-specific IgE antibodies in the sera of Bt-immunized mice

To assess if eugenol affects specific antibody production, we evaluated the effect of eugenol on the production of specific IgE in Bt-sensitized animals. Bt-immunized mice produced higher levels of specific IgE antibodies than non-immunized animals ($p<0.001$). Treatment with eugenol did not reduce significantly serum IgE levels (Fig. 5).

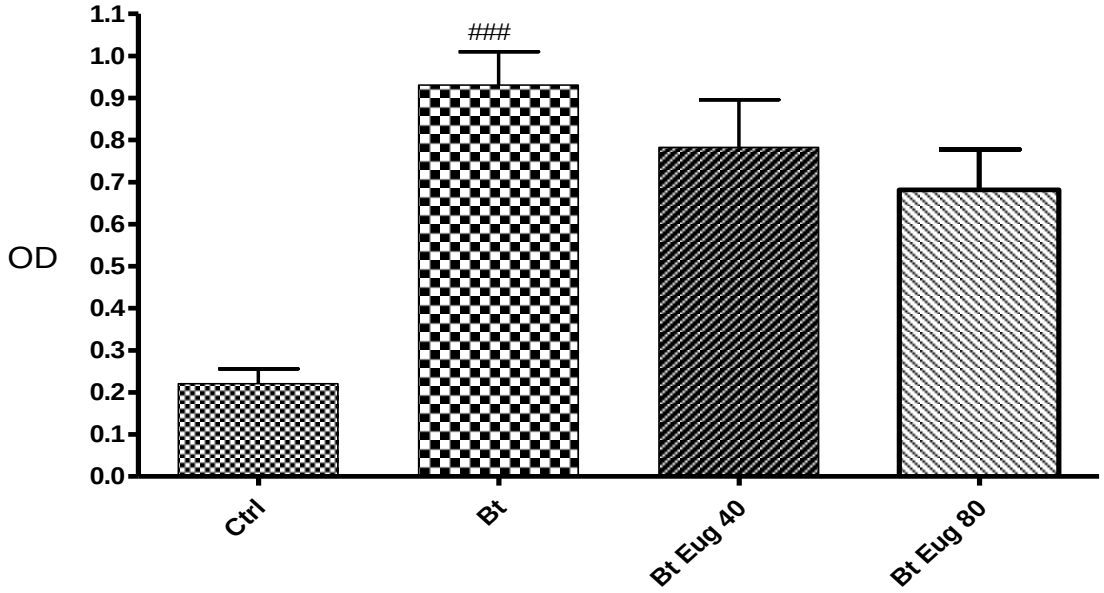
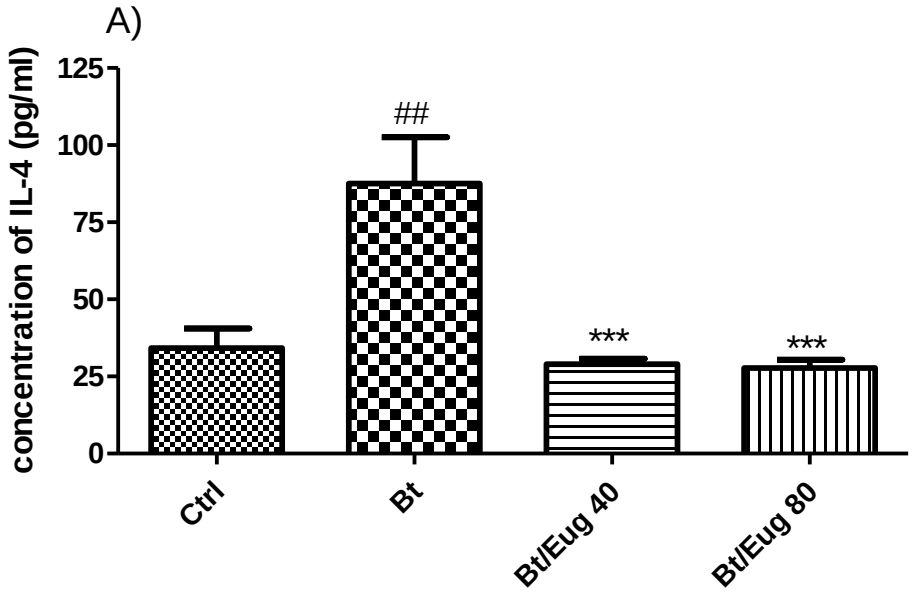


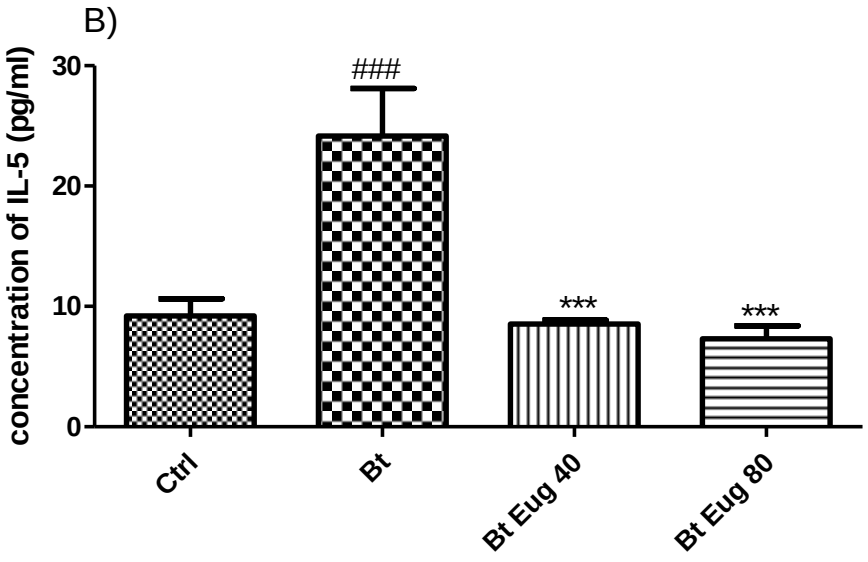
Fig (5): Effect of Eug on the levels of IgE anti-*Blomia tropicalis*. Antibody levels were measured by indirect ELISA. **Control** (sensitized and treated animals with vehicle; **Bt** (Bt-challenged mice and treated with vehicle), **Bt/Eug 40** (Bt-challenged mice and orally treated with 40mg/kg of eugenol), **Bt/Eug 80** (Bt-challenged mice and orally treated with 80mg/kg of eugenol). Columns represent the mean values of the results obtained from six animals, and error bars represent the error deviations from the means. ## $p<0.01$vs control.

3.5.Treatement with eugenol reduce levels IL-4, IL-5 and IL-13 in the BAL and splenocytes

To determine the possible mechanisms associated with the eugenol effects in airway inflammation, levels of the T-helper (Th) type 2 cytokine, typical of allergic asthma, were evaluated. Levels of IL-4, IL-5 and IL-13 in the BAL were higher in Bt-immunized and challenged mice than in the control group ($p<0.001$) (Fig. 6). The oral treatment with eugenol shows significant reductions in levels of this Th2 cytokine in the BAL of Bt-immunized animals in relation to those untreated, Bt-immunized animals ($p < 0.01$) (Fig. 6A, B and C). The same reduction was observed in splenocytes.

A significant increase in cytokine production by splenocytes stimulated with PWM was observed when using the spleen from animals sensitized and challenged with Bt compared with non-stimulated splenocytes. *In vitro* treatment with concentration of eugenol (12,5-200 μM) significantly reduced cytokine production (Fig. 7A, B, and C)





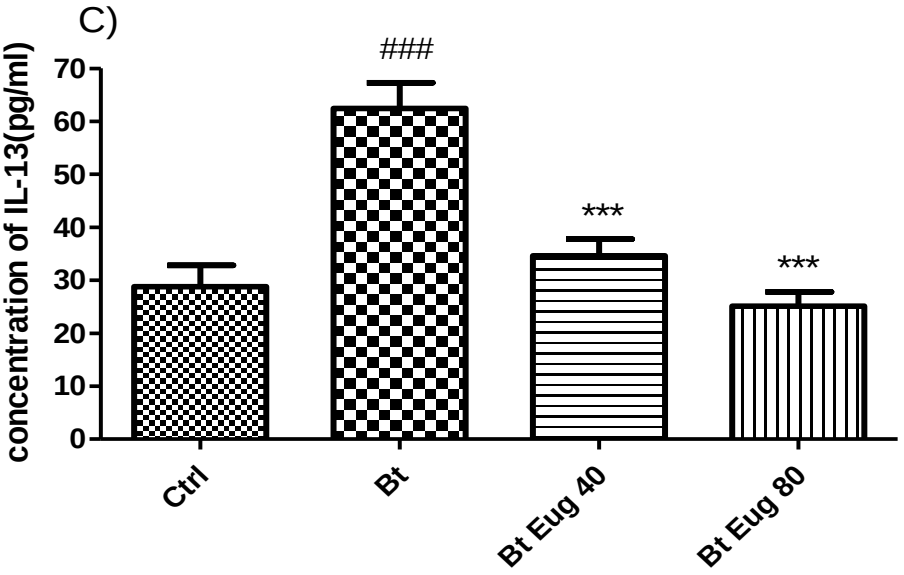


Fig (6): Effect of the treatment with Eug on the levels of **A)** IL-4, **B)** IL-5, and **C)** IL-13 in the BAL of Bt-challenged mice A/J. citokynes quantification was done by sandwich ELISA. **Control**, vehicle-treated animals; **Bt**, Bt-sensitized and challenged, and vehicle-treated mice; **Bt/eug 40**, Bt-sensitized and challenged, and eug 40mg/kg - treated mice; **Bt/80** Bt-sensitized and challenged, and eug 80mg/kg treated mice. Columns represent the mean values of the results obtained from six animals, and error bars represent the standard error from the means. (### P <0.001 vs control, and *** p <0.001 vs Bt group), ANOVA-Tukey.

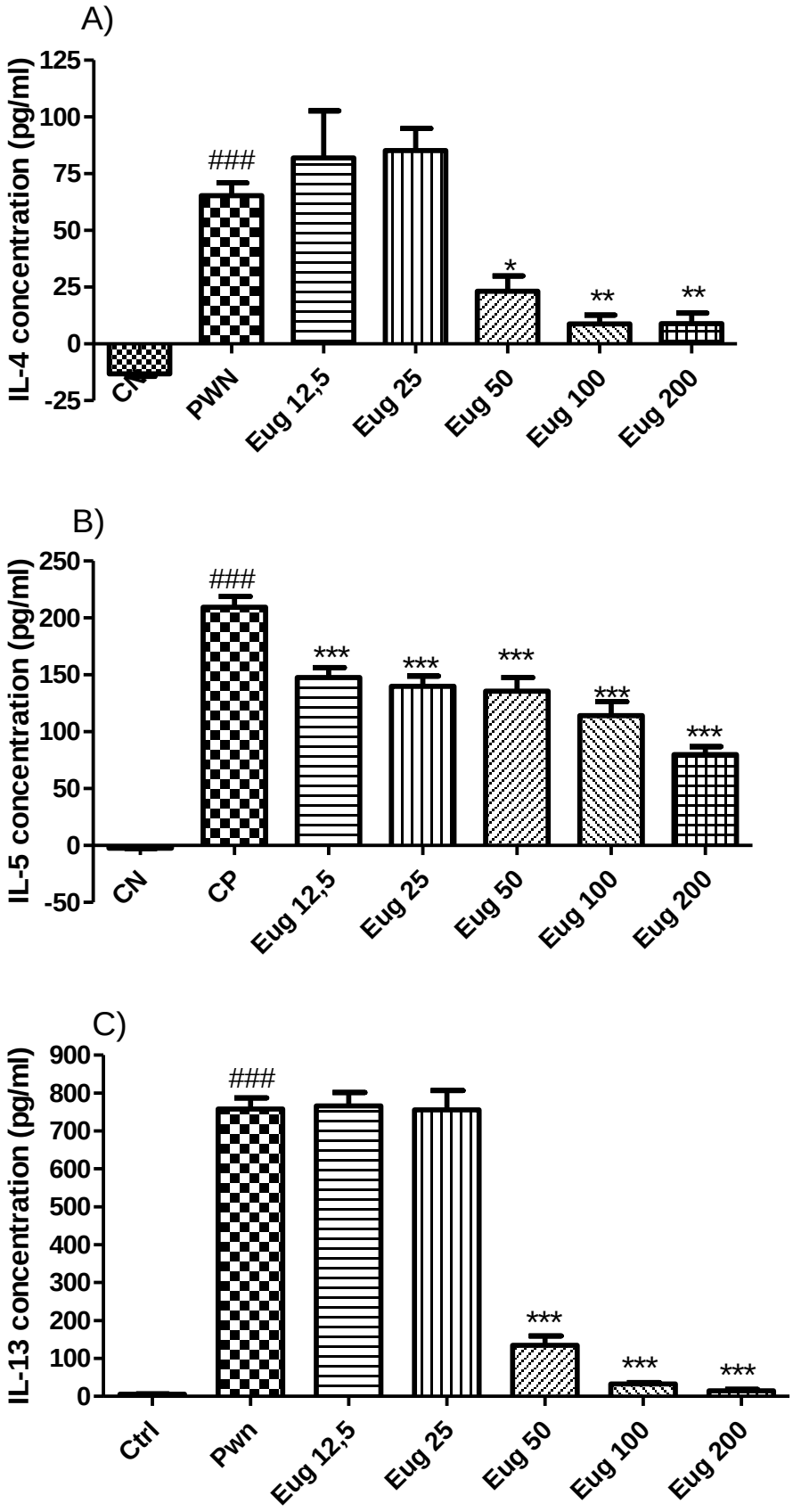

A)
IL-4 concentration (pg/ml)
125
100
75
50
25
0
-25
###
*
**
**
CN
PWN
Eug 12,5
Eug 25
Eug 50
Eug 100
Eug 200
B)
IL-5 concentration (pg/ml)
250
200
150
100
50
0
-50
###
***
***
***
***
***
CN
CP
Eug 12,5
Eug 25
Eug 50
Eug 100
Eug 200
C)
IL-13 concentration (pg/ml)
900
800
700
600
500
400
300
200
100
0
###
***
***
***
Ctrl
Pwn
Eug 12,5
Eug 25
Eug 50
Eug 100
Eug 200

Fig (7): Effect of the treatment with eugenol on the levels of **A)** IL-4, **B)** IL-5, and **C)** IL-13 in the splenocytes supernatant from animals Bt-sensitized without stimulation (Control), stimulated with PWM (PWM) and stimulated with PWM and treated with Eug (12.5-200micM). (### P <0.001 vs control, and *** p <0.001 vs Bt group), ANOVA-Tukey.

3.6. Eugenol reduces the production NO production in vitro

The production of NO by peritoneal macrophages was also evaluated *in vitro.* Considerable levels of NO were detected in the supernatant of macrophages that were treated with 5µg/mLof LPS. Treatment with all concentrations eugenol used in the experiment was able to suppress production of NO in LPS-treated macrophages. (Fig. 8)

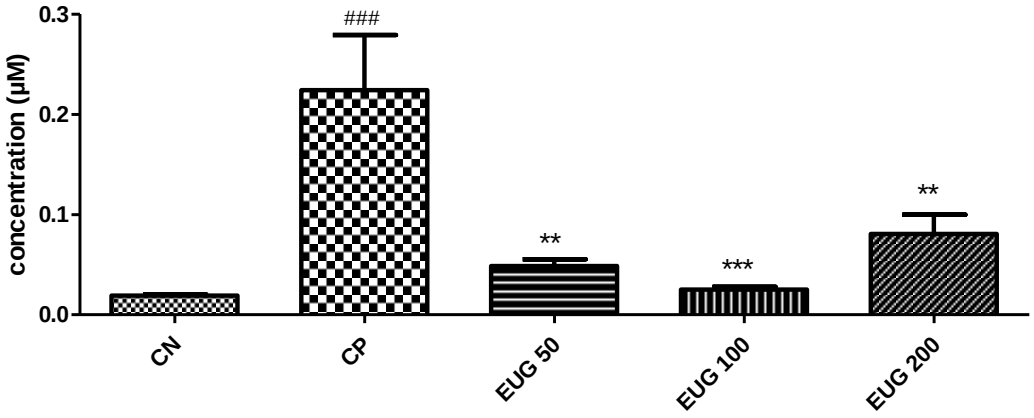


Fig (8): Eug decreases the production of NO by peritoneal macrophages. The following groups were presented: peritoneal macrophages not stimulated (CN), stimulated with LPS (CP) and stimulated with LPS and Eug (50, 100 or 200 µM).

3.7. Eugenol relaxing effect on airway smooth muscle.

Adding cumulative Eug (10-9 - 10-3M) we observed a relaxant effect on tracheal smooth muscle isolated from mice without functional epithelium, pre contracted with muscarinic agonist carbachol (10µM). The data set can be seen from the figure 9 that

shows the concentration-response curve of Eug where the percentage of maximum relaxation (Emax) induced by Eug was Emax =112 ± 3.9.

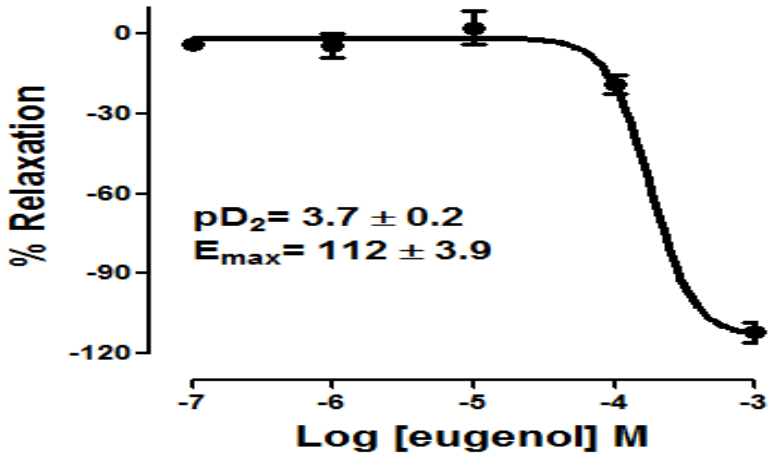


Fig (9): Concentration-response curve of the Eug relaxing effect in airway smooth muscle.

4. DISCUSSION

The inflammatory response to allergens in the atopic asthmatic lung is a consequence of infiltration on the airway by inflammatory cells, especially eosinophils and is associated with the increased expression of several inflammatory proteins in lung tissue, including cytokines, such as IL-4, IL-5 and IL-13 (LEE et al, 2010). The resolution of inflammation is an essential process for the establishment of appropriate host responses and the return to homeostasis (LEE et al 2009).

The use of biologically active natural products is gaining increasing popularity day by day over conventional medicine as a striking alternative approach for the treatment of various diseases. However, limited scientific evidence regarding the effectiveness of these natural derivatives, and lack of mechanistic understanding has prevented their incorporation into mainstream medicine and their application in human therapy. Eugenol, an o-methoxyphenol, is of interest for many recent research because of its

anti-inflammatory and chemopreventive effects based on the antioxidant capability of its phenolic group.

The present study was conducted using a murine model of allergic airway disease induced by the sensitization to a common allergen, the *Blomia tropicalis* mite, which was previously characterized by our research group as an inductor of increasing number of eosinophils in the BAL fluid, a marked influx of inflammatory cells into the lung around blood vessels and airways, and airway luminal narrowing (BAQUEIRO, 2010). This allowed us to investigate the potential antiallergic effect of eugenol in an experimental model of airway and lung inflammation induced by a clinically relevant aeroallergen.

As we previously described sensitization with 100μg of Bt produced inflammatory cells, high levels of EPO and Bt-specific IgE, as well as induced the production of cytokines such as IL-5, IL-13 and IL-4 (BAQUEIRO et al., 2010).

The treatment with eugenol in Bt-sensitized and challenged mice resulted in a significant inhibition of airway and lung stroma inflammation, characterized by reduction in: (i) numbers of total inflammatory cells in BAL and lung; (ii) inflammatory cell infiltration in the peribronchiolar and perivascular pulmonary region; (iii) presence of mucus inside airways; (iv) levels of EPO in the lung; but did not alter Bt-specific IgE in serum.

Several studies attribute the anti-allergic property of natural products to their ability to reduce the inflammatory cell infiltrate(CERQUEIRA-LIMA et al., 2010; JUNG et al., 2008; EL GAZZAR et al., 2006; BRADDING, 2008), and reduce eosinophils degranulation measured through eosinophil peroxidase (MEDEIROS et al., 2008).

The eosinophilic infiltrate is stimulating pro-inflammatory chemokines and cytolytic enzymes, including eosinophil cationic protein and major basic protein that break the integrity of airway epithelium (TODO-BOM, 2006). Accordingly, the increased presence of inflammatory cells and their secreted products in the asthmatic lung often correlates with severity and exacerbation of disease (FUJIMOTO, 1997). Reducing the release of EPO, when animals were treated with eugenol, may be of relevance to the improvement of inflammation and (or) tissue remodeling in allergic asthma. The result of suppressing the activity of EPO follows the reduction of inflammatory cell infiltration induced by treatment with eugenol in animals sensitized with Bt.

The anti-inflammatory and immunomodulatory activities of eugenol has been described to its inhibition of the lipoxygenase and cyclooxygenase1' pathways (KAUR, 2010; MAGALHÃES, 2010), inhibition of iNOS, and, mainly, inhibiting the production of inflammatory cytokines (MAHAPATRA, 2011; BASKARAN, 2010, MAGALHÃES et, 2010; LI et, 2006) These activities may explain, at least in part, the airway antiallergic activity of eugenol observed in this study.

In order to explore the mechanism where by eugenol modulated eosinophils infiltration and activation we investigate the effect of this drug on IL-4, IL-5 and IL-13 production. Eugenol treatment at the doses tested decreased the levels of these cytokines both *in vitro* (spleen) and *in vivo* (BAL). The reduction of IL-4 and IL-5 explains, at least in part, the reduction of inflammation in the lung as well as reducing the levels of EPO, since IL-4 is the main cytokine involved in inflammatory response Th2 and IL -5 is the principal involved in the maturation, activation and migration of eosinophils. Previous studies in humans show that the addition of anti-IL-5 therapy for asthma significantly accelerated apoptosis of eosinophils decreases pulmonary eosinophilia (SIMON, 1997).

IL-4 is also related to production of IgE, the main immunoglobulin class associated with allergic diseases (TURNER H, KINET JP, 1999), however, the reduction was not observed in the production of IgE. The lack of modulation of IgE despite the decrease in IL-4 and IL-13 could be related to the short-term treatment. IL-13 and IL-4 play too an important role in the production of mucus. Increased mucus production by goblet cells in the airway epithelium is associated with airway inflammation and asthma. Thus, the reduced production of IL-13 may reflect the decreased production of mucus and improve lung function, as was observedin animals treated with Eug..The mechanism by which eugenol inhibit the production of Th2 cytokines is not yet clear. Previous studies have shown that this compound reduces the production of inflammatory cytokines by inhibiting the activation of factor NFkB related transcription of inflammatory proteins (KAUR, 2010). This may be one of the probable mechanisms of the anti-allergic effect of eugenol. This hypothesis will be investigated in future studies of allergy in the model studied

In addition to adaptative mechanisms we have also investigated the *in vitro* potential of eugenol on the modulation of nitric oxide (ON) in cultured peritoneal macrophages. ON in the normal physiological concentrations, is related to relaxation of airway smooth muscle, however when produced in high concentrations by the dioxygenase, called NO inducible synthesis (iNOS), as occurs in patients with asthma, determines hyperemia, edema and exudation, contributing to the narrowing of the airway. Therefore, the decrease of NO via the iNOS prove useful in the treatment of asthma (PARK, 2008). The culture of peritoneal macrophages stimulated with LPS showed an increased production of nitric oxide when comparing non-stimulated macrophages. When cells stimulated with LPS were treated with different concentrations of eugenol (12.5-200μM) it reduced the NO production significantly

which validates the action of these drugs on the regulation of nitric oxide. This study corroborates previous studies that describe the inhibitory role of eugenol in the production of NO via iNOS. (LI, 2006)

In addition to anti-inflammatory effect, the experiments with the isolated trachea showed that the Eug induced a relaxing effect of tracheal smooth muscle pre-contracted with muscarinic agonist carbachol, thus demonstrating, at least, a potential bronchodilator. These results are consistent with studies showing that phenolic compounds are able to relax smooth muscle and improve lung function (LIN, 1999). At this point, we do not know exactly where eugenol may be acting to allow us to elaborate a final mechanism in airway smooth muscle. However, it is well reported that a number of monoterpenes have also been described as both agonists and antagonists of different members of the transient receptor potential (TRP) channel family, which are non-selective cation channels (PARNAS et al, 2009). Eugenol is a monoterpene, and it could also target TRP channels on airway smooth muscle, as that TRPC, altering local intracellular Ca2+ concentration, and consequently inducing relaxation (SOUSA et al, 2011). However, additional studies will be needed to completely clarify the actual mechanism.

The results of the present study, obtained in an experimental model strongly support the potential usefulness of eugenol as antiinflammatory and bronchodilatador agents for the treatment of allergic asthma. Additional studies are in progress in our laboratory in order to further elucidate the exact mechanism of action whereby eugenol exerts its activities.

5. LIST OF ABBREVIATIONS

BAL: broncho alveolar lavage
BK: bradykinin
CD4: Grouping of T helper lymphocyte differentiation
Cch, carbachol
COX2: Cyclooxygenase 2
ELISA: enzyme immunoassay test
Eug: Eugenol
EPO: eosinophil peroxidase
Emax: maximum relaxation
GATA-3: Transcription factor
BHR: bronchial hyper-reactivity
HBSS, Hanks' balanced salt solution
Ɣ-IFN: Interferon-gamma
IL: Interleukin
i.n.: Via intranasal
IgE: Immunoglobulin E-type
LPS: lipopolysaccharide
NF-kB, nuclear factor of activated B cells potentiating
OVA: Ovalbumin
PAS: Periodic Acid Schiff
PBS: phosphate buffered solution bisódica
PWM: Pokweed (mitogen)
RPM: Revolutions per minute
s.c.: subcutaneous
Th2: T helper lymphocyte type 2
Th1: T-helper type 1
TRP: Transient Receptor Potential Channels
TGF-β, β transformation factor of tumor growth
v.o.: oral

6. CONFLICT OF INTEREST

All authors declare they have no competing financial interests.

7. ACKNOWLEDGMENTS

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4-CONCLUSÕES

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O eugenol possui capacidade de atenuar a inflamação eosinófílica e a produção de muco nas vias aéreas e, portanto, apresentam potencial anti-alérgico exercido através da redução dos níveis das citocina Th2, IL-4, IL-5 e IL-13, que estão envolvidas no processo alérgico.Adicionalmente, esse composto causou um relaxamento da traqueia isoladade camundongo,epitélio independente, demostrando um potencial broncodilatador.

Os resultados obtidos neste trabalho evidenciam o potencial terapêutico do eugenol no tratamento da asma alérgica.Estudos adicionais são encorajados na tentativa de elucidar os mecanismos pelos quais o eugenol é efetivo neste modelo tendo em visto o estudo desta drogaem nível clínico.

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