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TRABALHO DE DISSERTAÇÃO

Ryan dos Santos Costa

Potencial antialérgico do *Ocimum gratissimum* Linn. e do seu constituinte químico, o ácido rosmarínico, em modelo de alergia respiratória ao ácaro *Blomia tropicalis*

Salvador, BA
2010

RYAN DOS SANTOS COSTA

DISSERTAÇÃO DE MESTRADO

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Orientadora: Profª Dra. Camila A. V. Figueiredo

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ATA DA SESSÃO PÚBLICA DO COLEGIADO DO PROGRAMA DE PÓS-GRADUAÇÃO EM IMUNOLOGIA PARA JULGAMENTO DO TRABALHO DE DISSERTAÇÃO DO MESTRANDO **RYAN DOS SANTOS COSTA**.

Aos dezessete dias do mês dezembro do ano de dois mil e dez, às quatorze horas, no auditório III no segundo andar do Instituto de Ciências da Saúde, a Banca Examinadora composta pelas Professoras: Dra. **Camila Alexandrina Viana de Figueiredo** Orientadora, Dra. **Silvia Lima Costa**, Dra. **Darizy Flávia Silva Amorim de Vasconcelos** se reúne em sessão pública para discutir, avaliar e julgar o trabalho de Dissertação intitulado, "**Potencial antialérgico do *Ocimum gratissimum* Linn. e do seu constituinte químico, o ácido rosmarínico, em modelo de alergia respiratória ao ácaro *Blomia tropicalis*** do Mestrando **RYAN DOS SANTOS COSTA**. Após a apresentação, foram feitos os comentários pelos examinadores. Havendo cumprido as exigências para a defesa, a Banca Examinadora conclui que o pós-graduando teve a sua defesa de Dissertação aprovado, emitindo pareceres individuais que serão anexados à ata. Nada mais havendo a tratar, encerra-se a sessão, da qual é lavrada a presente ata que após lida e aprovada vai assinada pelos componentes da Banca examinadora, pelo Mestrando e pela Coordenadora do Programa de Pós-Graduação. Salvador, 17 de dezembro de 2010.

Profa. Dra. Camila A.Viana de Figueiredo
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Mestrando

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

“Nada é veneno, tudo é veneno.

A diferença está na dose.”

Paracelsus

À minha avó Clarice, *in memoriam,*

fonte de serenidade e paz!

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CONSIDERAÇÕES

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

SUMÁRIO

LISTA DE ABREVIATURAS

BAL: Lavado Broncoalveolar

Bt: *Blomia tropicalis*

Célula Th2: Célula T “helper” 2

i.p.: Via intraperitoneal

IgE: Imunoglobulina da classe E

IL: Interleucina

Og: *Ocimum gratissimum Linn.*

RA: Ácido Rosmarínico

TCR: Receptor dde Célula T

v.o.: Via oral

Costa, Ryan dos Santos. Avaliação dos efeitos imunomoduladores do *Ocimum gratissimum* Linn. e do seu constituinte químico, o ácido rosmarínico, em modelo de alergia ao ácaro *Blomia tropicalis.* 60f. Il. 2010. Dissertação (Mestrado) da Universidade Federal da Bahia, Instituto de Ciências da Saúde, Programa de Pós-graduação em Imunologia, Salvador, 2010

RESUMO

A asma tem emergido como um importante problema de saúde pública da população urbana tanto de países desenvolvidos quanto dos países latino americanos. Para o tratamento desta doença há uma alta prevalência do uso de plantas medicinais devido a perda de eficácia e importantes efeitos colaterais relacionados às drogas classicamente utilizadas. A aplicação de plantas como medicamentos é tão velha quanto a humanidade e tem se tornado mais constante na ultima década. Neste contexto, *Ocimum gratissimum* Linn. (Og) é uma planta comumente utilizada na medicina popular brasileira para o tratamento de desordens inflamatórias como a asma. Baseado nisto, o presente estudo teve por objetivo avaliar os efeitos imunomodulatórios do *Ocimum gratissimum* e do seu fitoquímico polifenólico ácido rosmarínico (AR) em modelo murino de alergia respiratória induzida pelo ácaro *Blomia tropicalis* (Bt). A alergia respiratória foi induzida em camundongos A/J pela administração de antígeno de Bt e o tratamento foi realizado utilizando 100mg/Kg (v.o) do extrato metanólico do Og ou 200mg/Kg (i.p) do AR. Então foi analisada a alteração induzida por essas drogas nos parâmetros imunológicos relacionados com o processo alérgico, que são aumentados neste modelo, tais como a quantidade de leucócitos/eosinófilos no lavado broncoalveolar (BAL); a atividade da peroxidase eosinofílica no BAL e no pulmão; produção de muco; níveis de IgE no soro; alteração histopatológica no pulmão; e níveis de IL4 no BAL. O tratamento dos animais com Og e com o AR levou a redução estatisticamente significante na maioria dos parâmetros avaliados. Estes resultados sugerem que o extrato metanólico do *O. gratissimum* e o polifenol ácido rosmarínico possuem potencial antialérgico neste modelo murino de alergia respiratória caracterizada por inflamação eosinofílica.

PALAVRAS-CHAVE: ácido rosmarínico, asthma, *Blomia tropicalis,* inflamação eosinofílica, *Ocimum gratissimum* Lin*n.,* polifenóis

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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. To treat this widespread disease there is a high prevalence of usage of herbal medicine due to the lack of efficacy and the important side effects related to the classical drugs in use. The application of plants as medicines is as old as humankind and it has been steadily increasing over the past 10 years. In this context, *Ocimum gratissimum* (Og) is a plant widely used in Brazilian folk medicine to treat inflammatory disorders such as asthma. Based on that, the present study aims to study the immunomodulatory effects of *Ocimum gratissimum* and its polyphenolic phytochemical Rosmarinic acid (RA) in a murine model of respiratory allergy induced by *Blomia tropicalis* (Bt) mite. The respiratory allergy was induced in A/J mice by administration of Bt antigen and the treatment was done using 100mg/kg (v.o.) of the *O. gratissimum* methanolic extract or using 200 mg/kg (i.p.) of RA. Then we analyzed the changes induced by these drugs on immunological parameters related to the allergic process which are up-regulated in this allergic model, such as leukocytes/eosinophils in bronchoalveolar lavage (BAL); eosinophil peroxidase activity in BAL and lungs; mucus formation, IgE levels in serum, histopathological changes in the lung and IL-4 in BAL. The treatment of animals with Og and the RA led to a statistically significant reduction in the majority of parameters evaluated. These results suggest that the methanolic extract of *O. gratissimum* and the polyphenolRosmarinic acid have anti-allergic potential in this murine model of respiratory allergy charactherized by eosinophilic inflammation.

Keywords: asthma, *Blomia tropicalis,* eosinophilic inflammation, *Ocimum gratissimum* Lin*n.,* polyphenols, rosmarinic acid

1 INTRODUÇÃO

A asma é uma doença inflamatória crônica caracterizada por inflamação eosinofílica e hiperresponsividade das vias aéreas inferiores e por limitação variável ao fluxo aéreo, manifestando-se clinicamente por episódios recorrentes de sibilância, dispnéia e tosse (O'BYRNE, 2009). A asma constitui um importante problema de saúde pública, uma vez que tem alta prevalência, expõe o paciente a recorrentes hospitalizações e representa uma elevada carga econômica para o indivíduo, a sociedade e o governo (LEE, 2010).

A patogenia da asma é caracterizada pelo envolvimento dos linfócitos $CD4^+$ do tipo T *helper* 2 (Th2), bem como os mastócitos, através da liberação de citocinas que estão relacionadas com a inflamação alérgica, incluindo a interleucina-4 (IL-4), IL-5, e IL-13 (BARNES, 2008; KAY, 1997; KRUG et al., 1996; WILLS-KARP, 2000).

A citocina IL-4 possui um papel central no desenvolvimento da asma alérgica promovendo a diferenciação de células Th *naive* à linfócitos Th2, o que estimula a produção de citocinas Th2 responsáveis pelo processo inflamatório (BORISH et al., 2002). Esta citocina também está relacionada à indução da produção de IgE pelos linfócitos B (de VRIES; CARBALLIDO; AVERSA, 1999), além de potencializar a resposta mediada por IgE através da *up-regulation* dos receptores nos linfócitos B, mastócitos e basófilos (GASCAN, et al., 1991).

Outro importante papel do IL-4 é a inibição da apoptose dos eosinófilos, assim como da quimiotaxia destas células, o que leva a uma inflamação

caracteristicamente eosinofílica. (BORISH et al., 2001). A Interleucina-4 contribui também para a obstrução das vias aéreas por indução da secreção de muco (ANDREWS et al., 2006; POULIOT et al., 2005).

Tem sido demonstrado que a Interleucina-5 (IL-5) está relacionada com a diferenciação, ativação, expansão e recrutamento eosinofílico, excercendo efeito sobre o calibre das vias aéreas e a hiperresponsividade brônquica (MENZIES-GOW et al., 2007). Além disso, a IL-5 pode está relacionada com a remodelagem das vias aéreas, por contribuir para o aumento da deposição de colágeno nesta região. (BARNES, 2001).

IL-13 é uma citocina Th2 que desempenha importante papel no desenvolvimento da asma, pois contribui para a hiperresponsividade brônquica, aumento da produção de muco, inflamação e para o desenvolvimento da fibrose sub-epitelial. (MILLER et al., 2008).

Atualmente, a terapia disponível para a asma é baseada na broncodilatação, utilizando agonistas adrenérgicos ou antagonistas colinérgicos; ou baseada na supressão da inflamação, sendo os glicocorticóides as principais drogas disponíveis. Essas estratégias auxiliam no controle dos sintomas da asma, no entanto apresentam diversos efeitos colaterais e, principalmente, não levam à cura. (HOANG et al., 2010; WALSH, 2005).

A necessidade de desenvolver medidas eficazes de controle para a asma resultou na exploração de terapias alternativas baseadas em produtos naturais (BIELORY, 2004; KURUP, 2008). Desta forma, o território brasileiro, devido à sua

biodiversidade especialmente observada na região nordestina, apresenta-se como um importante sítio para a descoberta de novos produtos terapêuticos e a prospecção de novos protótipos a partir das espécies vegetais e suas diversidades químicas, possuindo várias espécies inéditas ou que não possuem, até o momento, a sua aplicação popular elucidada cientificamente. (ALBUQUERQUE & HANAZAKI, 2006, BARBOSA-FILHO, 1988; BARBOSA-FILHO, 1997), inclusive para o tratamento de alergias (SERRA et al. 1997).

Nesse sentido, levantamento etnofarmacológico realizado na cidade de Salvador-Bahia investigando os principais produtos administrado para o tratamento de asma em crianças apontou o *Ocimum gratissimum Linn.* (*Og*), popularmente conhecido como quioio, alfavacão ou manjericão-cheiroso, como um dos produtos vegetais mais utilizado. (COSTA et al., 2010).

O Ocimim gratissimum L. é uma espécie constituinte da família Lamiaceae, pertencente ao gênero *Ocimum* (PEREIRA & MAIA, 2007), Este gênero contém cerca de trinta espécies, as quais são encontradas nos trópicos e subtrópicos do Velho e do Novo Mundo, sendo que a espécie *Ocimum gratissimum* tem origem central na África (PATON, 1992; VIEIRA et al, 2002).

Na culinária de diversos países, a alfavaca – nome popular do *O. gratissimum* – é utilizada em saladas, sopas, pastas e como condimento (NWEZE & EZE, 2009). Na medicina popular, as folhas da alfavaca têm sido utilizada como anti-diarréica, para o tratamento da conjutivite, infecções da pele e para bronquite, dentre outras aplicações (IWU, 1993; ONAJOBI, 1986).

Algumas das propriedades biológicas da espécie *Ocimum gratissimum* L. têm sido comprovadas cientificamente, dentre elas destacam-se: propriedade antinociceptiva (RABELO et al, 2003); atividade antibacteriana (NAKAMURA et al, 1999); antagonista sobre a motilidade intestinal (MONTALVO; DOMÍNGUEZ, 1997); atividade antifúngica (LEMOS et al, 2005); dentre outras.

As atividades biológicas exercidas pelo *O. gratissimum* são atribuídas aos seus principais constituintes químicos dentre os quais os óleos essenciais, como o timol (GUENTER, 1948; VIEIRA et al, 2002), o geraniol (CHARLES; SIMON, 1992; VIEIRA et al, 2002), e o eugenol – principal óleo encontrado (BENITEZ, 2009). Estão presentes também flavonóides, como o xantomicrol e o cirsimaritin (VIEIRA et al, 2002) e compostos polifenólicos (OLA et al., 2009).

Estudo avaliando a composição de constituintes polifenólicos no *Ocimum gratissimum* da Nigéria apontou a presença do ácido rosmarínico como um dos compostos predominantes no extrato hidroalcoólico desta espécie (OLA et al., 2009) com propriedade imunomoduladora recentemente descrita (TAKANO et al, 2004, SANBONGI et al., 2004; KANG; YUN; WON, 2003).

O Ácido rosmarínico é um éster do ácido caféico e do ácido 3,4-dihidroxifenilatico que foi isolado pela primeira vez a partir da espécie *Rosmarinus officinalis* (Lamiaceae) (SCARPATI & ORIENTE, 1958; PETERSEN et al., 2009).

Sanbongi e cols. (2004) demonstrou que o ácido rosmarínico presente no extrato de *Perilla* sp. previne a inflamação eosinofílica de vias aéreas induzida por *Dermatophagoides farinae* em camundongos. Estes efeitos foram associados com

inibição do aumento local da expressão de citocinas Th2 e quimiocinaa e da produção aumentada de imunoglobulina alérgeno-específica. (SANBONGI et al., 2004).

Estudo realizado por Takano e cols. (2004) em humanos sugere que o ácido rosmarínico presente no extrato de *Perilla frutescens* pode ser uma intervenção efetiva para pacientes com rinoconjuntivite alérgica sazonal leve, através da inibição da inflamação dependente de leucócitos polimorfonucleares.

O ácido rosmarínico apresenta potencial anti-inflamatório e imunomodulador através da inibição das vias da lipoxigenase e ciclooxigenases, inibição da cascata do sistema complemento (Petersena, 2003), inibição dos eventos sinalizados pela indução do complexo TCR (receptor para antígenos em células T) (KANG; YUN; WON, 2003) e por indução de apoptose (HUR, YUN, WON, 2004).

Portanto, o presente trabalho apóia-se no uso popular do *Ocimum gratissimum* Linn., para o tratamento de alergias, associado ao forte indício do seu potencial imunomodulatório atribuído, em parte, ao acido rosmarínico, no intuito de desenvolver novas alternativas terapêuticas para alergias respiratórias.

O trabalho de dissertação será apresentado sob forma de artigo que se encontra formatado de acordo com a revista **Current Drug Targets** à qual o mesmo foi submetido para publicação.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar o potencial imunomodulador do *O. gratissimum* Linn. (Og), verificar se o ácido rosmarínico (RA) é um polifenol presente no extrato de Og, e explorar o mecanismo pelo qual o Og e o RA apresentam eficácia no modelo experimental de alergia respiratória ao ácaro *Blomia tropicalis* no intuito do desenvolver novas alternativas terapêuticas para o tratamento de desordens alérgicas.

2.2 OBJETIVOS ESPECÍFICOS

Os objetivos específicos do presente trabalho foram:

2.2.1 Padronizar o extrato do *Ocimum gratissimum* Linn. (Og) quanto a presença do polifenol denominado ácido rosmarínico (RA), através de HPLC;

2.2.2 Verificar o potencial anti-alérgico do Og e seu constituinte químico isolado, o ácido rosmarínico no modelo de alergia respiratória ao ácaro *Blomia tropicalis*;

2.2.3 Descrever os possíveis mecanismos pelos quais o Og e RA possuem atividade imunomoduladora.

3 RESULTADOS E DISCUSSÃO

Manuscrito: *Ocimum gratissimum* Linn. methanolic extract and rosmarinic acid attenuate eosinophilic airway inflammation in an experimental model of respiratory allergy to *Blomia tropicalis*

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Ocimum gratissimum Linn. methanolic extract and rosmarinic acid attenuate eosinophilic airway inflammation in an experimental model of respiratory allergy to *Blomia tropicalis*

Running title: *Ocimum gratissimum* Linn. and rosmarinic acid attenuate eosinophilic inflammation

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ABSTRACT

Allergic asthma has emerged as an important public health problem of urban populations in developed countries, including those of Latin America. Very often herbal medicines are used to treat this widespread disease, due to the poor efficacy and/or the important side effects related to the classical drugs in use. The application of plants as medicines is possibly as old as humankind, and it has been steadily increasing over the past 10 years. Along this line, *Ocimum gratissimum* is a plant widely used in Brazilian folk medicine to treat inflammatory disorders, such as asthma. The present study aimed at studying the immunomodulatory effects of an *O. gratissimum* methanolic extract (OgME) and its polyphenolic phytochemical rosmarinic acid (RA) in a murine model of respiratory allergy induced by the *Blomia tropicalis* mite. The respiratory allergy was induced in A/J mice by administration of *Blomia tropicalis* extract and the treatment was done using 100 mg/kg orally of OgME or using 200 mg/kg intraperitoneally of RA. Changes induced by these drugs on immunological parameters related to the allergic process, such as the numbers of leukocytes/eosinophils in bronchoalveolar lavage (BAL); eosinophil peroxidase activity in BAL and lungs; presence of mucus in respiratory tract, IgE levels in serum, histopathological changes in the lung, and interleukin 4 levels the in BAL, were evaluated. The treatment of animals with OgME and RA led to a statistically significant reduction in the majority of the evaluated phenomena. These results suggest that the OgME and the polyphenol RA have a therapeutic potential in this murine model of respiratory allergy characterized by eosinophilic inflammation induced by *B. tropicalis* antigens.

Keywords: asthma, *Blomia tropicalis*, eosinophilic inflammation, *Ocimum gratissimum*, polyphenols, rosmarinic acid

1. INTRODUCTION

Asthma is now one of the commonest chronic diseases in the world, affecting over 300 million people, and its prevalence is rising, particularly in developing countries [1]. The prevalence of allergic asthma in Brazil, where antigens from the *Blomia tropicalis* house dust mite are important sensitizing agents [2], is the 8th highest in the world [3]. Approximately 5–10% of patients have uncontrolled disease, despite taking inhaled therapy. These patients use a disproportionate amount of healthcare resources, as they are admitted to hospital, consume costly medication, and miss working days [4].

Historically, herbal medicine has a great importance in the treatment of asthma. Various derivatives from medicinal plants were identified as antiasthmatic medicines, and some of their mechanisms of action were very well studied, such as those of α2 agonists, anti-cholinergics, methylxanthines and chromones [6]. The understanding of the chronic inflammatory scenario found in the airways of asthmatic patients led to glycocorticoids being the gold standard drugs in the treatment of allergic asthma [7]. The main disadvantage of these drugs is their undesirable side effects.

Based on the lack of an effective drug for treating asthma without significant side effects, an ethnopharmacological survey was conducted by our research group in the city of Salvador, Bahia, in order to find out the main natural products administered for the treatment of asthma in children, aiming at identifying a plant species that could be the object of future studies as a source of anti-asthmatic drugs [8].

One of this species was the *Ocimum gratissimum* Linn (Labiatae), which is widely distributed in the tropics, is commonly used in folk medicine and has scientifically confirmed biological properties, such as antinociceptive [9], espamolytic [10, 11] and antibacterial [12] activities. Phytochemical studies revealed that the *O. gratissimum* is rich in polyphenols, such as rosmarinic acid (RA) [13], which has recently been shown to have immunomodulatory activity, by suppressing T-cell receptor signaling [14]. The rosmarinic acid obtained from another plant species, the *Perilla frutescens*, was able to prevent an eosinophilic airway inflammation in mice. These effects were associated with inhibition of the local expression of Th2 cytokines and of chemokines [15].

The objective of the present study, therefore, was to evaluate the effect of an *O. gratissimum* methanolic extract (OgME) and of RA in a murine model of respiratory allergy to *B. tropicalis* mite extract (BtE), and to investigate some of the immunological phenomena modulated by OgME and RA, in order to elucidate the mechanism by which it may be exerting its effect on experimental allergy.

2. MATERIAL AND METHODS

2.1. Animals

Male AJ mice (25-30g) 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. *Blomia tropicalis* extract

The *B. tropicalis* mites were cultivated in a fish food-containing standardized environment, purified with saturated NaCl and lysed in 0.15 M phosphate-buffered saline, pH 7.4 (PBS), in a blender (51BL30; Waring Commercial, Torrington, CO, USA). After centrifugation with ether (9,000g for 10 min), for removal of lipids, the protein content was determined by Lowry's method [16] and the extract was subsequently stored at -20° C until use. The BtE was standardized by determining the Blo t 5 allergen concentration, in a commercial capture ELISA (INDOOR Biotechnologies, Charlottesville, VI, USA). All used BtE batches contained 30-40 ng of this allergen per µg of protein

2.3. *Ocimum gratissimum* Linn. extracts and rosmarinic acid.

The methanolic and hexanic extracts of *O. gratissimum* were prepared according to a technique previously described by Soto and colleagues (2010) [17]. *O. gratissimum* leaves were obtained at the Laboratório de Tecnologia Farmacêutica (LTF), Federal University of Paraíba, Brazil, and kept in a cool and airy

environment for fifteen days for drying. The dry plant material was pulverized and crude extract were prepared by successive maceration process using methanol and hexane (3 times for 72 hours at room temperature). After filtration, extracts were concentrated in vacuum at 40º C. The RA [(R)-O-(3,4-dihydroxycinnamoyl) -3 - (3,4 - dihydroxyphenyl) lactic acid 3,4-dihydroxycinnamic acid (R)-1-carboxy-2-(3,4 - dihydroxyphenyl) ethyl ester - C18H16O8] was purchased from Sigma-Aldrich (catalog # 536954).

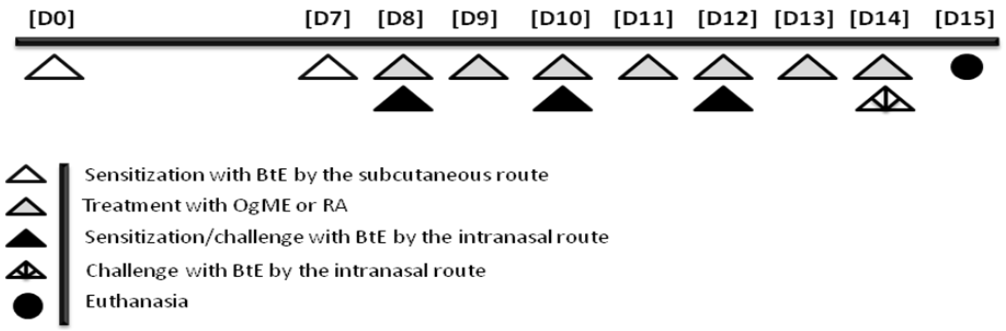
2.4. Standardization of *Ocimum gratissimum* Linn. extract

In order to better characterize the OgME preparation that was used in the present work, so as that it could be better compared with other preparations to be eventually used in future studies, the OgME was standardized in terms of RA concentration by high pressure liquid chromatography, with ultraviolet light detection, using a C18 column (250 x 4.6 mm ID, 5 μm particle size) and a C-18 pre-column (Phenomenex, Torrance, USA).

The mobile phase consisted of water acidified to pH 3.2 with formic acid (A) and acetonitrile (B) at a flow rate of 0.8 mL/min. The following elution method was performed: 85% to 75% of A in 18 min, 75% to 45% of A in 7 min, 45% to 15% of A in 5 min, 15% to 85 % of A in 5 min. A 20 μL sample was injected and the detection of RA was performed using light with a wavelength of 330 nm [13]. A control RA solution was injected at a concentration of 10.4 mg/mL.

2.5. Sensitization and challenge with antigen *Blomia tropicalis*

A/J mice (n=5) were initially sensitized with two subcutaneous injections (day 0 and day 7) of BtE (10 μg of protein), adsorbed to 4 mg/mL of $Al(OH)_3$ in saline (Fig. 1). Twenty-four hours after the last subcutaneous injection, the animals received three intranasal immunization boosters/challenges with BtE (10 μg/instilation) every other day, and, two days after the last immunization booster/challenge, they received a final intranasal challenge with 10 μg of BtE (Fig. 1). A negative control group received saline in both sensitization and challenge procedures. Twenty-four hours after the last challenge, the animals were euthanized with intraperitoneal injections of xilazine and ketamine (40 mg/kg/body weight).



2.6. Treatment with *Ocimum gratissimum* Linn. and rosmarinic acid

The different groups were treated daily from the 8th to the 14th days of the experimental protocol, one hour after the intranasal instilations in the 8th, 10th, 12th, and 14th days (Fig. 1). The animals were treated orally with 100 mg/kg of OgME [18], or intraperitoneally with 200 mg/kg of RA [19]. The groups of animals were named as: **Control**, non-sensitized and saline-treated mice; **BtE**, BtE-sensitized mice; **BtE/OgME**, BtE-sensitized and OgME-treated mice; **BtE/RA**, BtE-sensitized and RA-treated mice.

2.7. 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. Differential cell counts were obtained by using May–Grunwald–Giemsa - stained cytospin preparations. A differential count of at least 100 cells was made in a blind fashion in accordance with standard morphologic criteria.

2.8. Eosinophil peroxidase (EPO) activity

The EPO activity in the cells obtained from the BAL was measured according to a previously described method [21]. 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 150 µL of the chromogen and substrate solution (1.5 mmol/L of o-phenylenediamine and 6.6 mM of H_2O_2 in 0.05 M Tris-HCl, pH 8.0). After 30 min at room temperature, the reaction was stopped with the addition of 75 µL of 0.2 M citric acid, and the absorbance of the sample determined at 492 nm in an ELISA reader.

2.9. Levels of interleukin (IL-) 4, interferon gamma (IFN-γ) and tumor necrosis factor alfa (TNF-α) in the bronchoalveolar lavage

The concentrations of IL-4, IFN-γ, and TNF-α in the BAL were quantified by a standard ELISA, as recommended by the manufacturer (BD Pharmingen, USA).

2.10. Histopathological analysis

The degree of peribronchiolar and perivascular inflammation was evaluated as described previously [15]. Briefly, lung tissues were fixed by inflation with freshly prepared 10% (v/v) paraformaldehyde. The specimens were dehydrated and embedded in paraffin. Tissue sections (5 μm) were stained with haematoxylin and eosin, for the assessment of cellular infiltration, and with periodic acid Schiff to assess mucus presence, under optical microscopy with 200 x magnification.

2.11. Measurement of anti-BtE IgE antibody levels in the BAL

Antibody levels were determined by ELISA using samples collected 24 hours after the last BtE-challenge. In brief, wells of a 96-well microtitre high-binding plate (Costar) were coated with BtE (100 μg/well) overnight, at 4° C. The wells were washed three times with PBS containing 0.05% Tween 20 (PBS-T) and blocked during 1 hour with PBS-T containing 10% fetal bovine serum at room temperature (RT). After several washes with PBS-T, the mouse sera were added and incubated overnight at 4° C. After this incubation period and washes, a biotin-conjugated rat anti-mouse IgE (BD Pharmingen, San Diego, CA, USA) was added in each well 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.

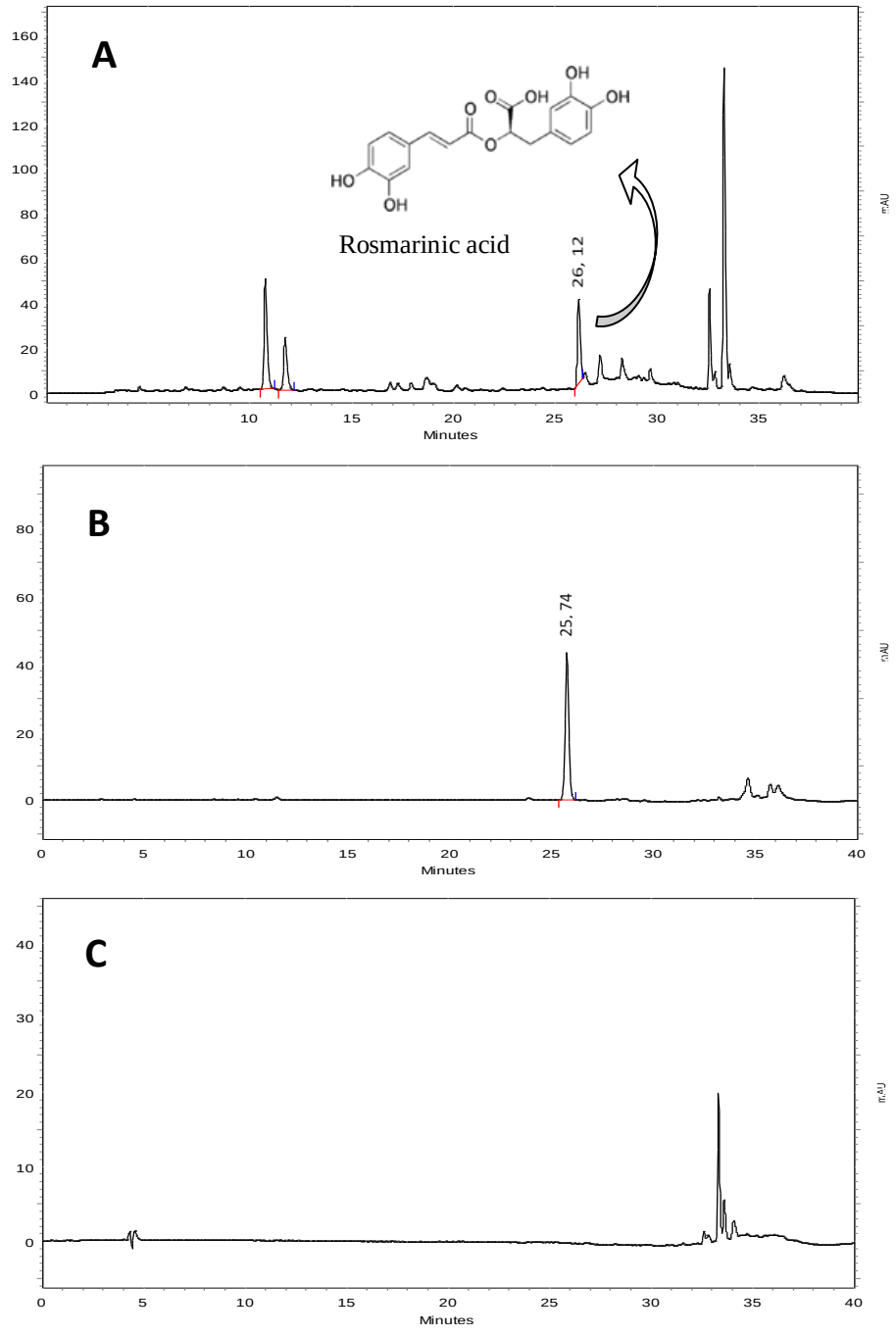
2.12. Statistical analysis

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 $\leq$ 0.05 were considered statistically significant. Each experiment was repeated at least two times.

3. RESULTS

3.1. Rosmarinic acid is present in the methanolic extract of *O. gratissimum* leaves

Figure 2 shows the chromatogram of the OgME (Fig. 2A), a RA solution (Fig. 2B) and a leaf hexane extract of *O. gratissimum* (Fig. 2C), demonstrating that the separation of a compound in the OgME in the same retention time of the RA in the sample standard (Fig. 2A and B). The estimated percentage of RA in the OgME was 0.21%. On the other hand, the chromatogram of the hexane extract of *O. gratissimum* (Fig. 2C) showed no RA characteristic peak, indicating the absence of the compound in that extract.

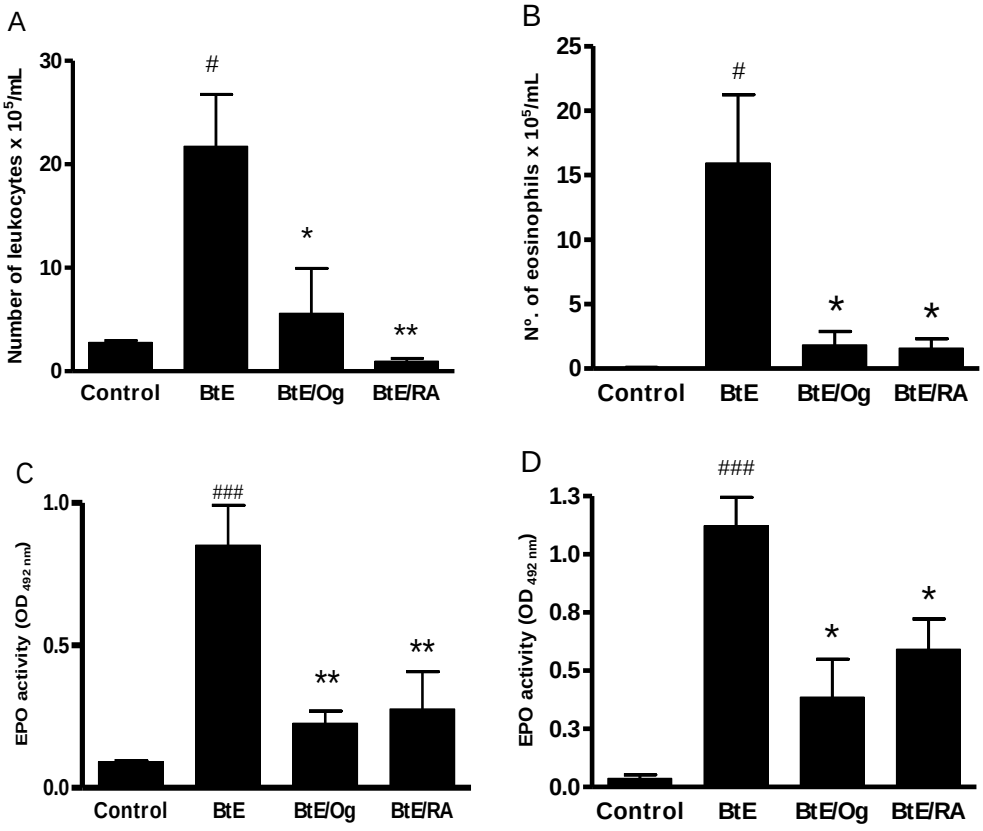


3.2. Treatment with *O. gratissimum* methanolic extract and rosmarinic acid reduce the BtE-induced BAL eosinophilia

To assess the effects of OgME and RA on the eosinophilic exudate in BAL of the BtE-sensitized and challenged mice, the presence of cells in the BAL was assessed 24 hours after the last challenge. BtE-challenged mice displayed a significant increase of both total cells and eosinophils in relation to the control group ($P < 0.05$) (Fig. 3). Oral administration of 100 mg/kg of OgME, daily and 1 hour after the BtE challenges, significantly suppressed the number of eosinophils and total inflammatory cells, in relation to the untreated BtE-immunized and challenged mice ($P < 0.05$; Fig. 3A and B). The intraperitoneal administration of 200 mg/kg of RA was also able to significantly suppress the number of eosinophils ($P < 0.05$) and total inflammatory cells ($P < 0.01$; Fig. 3A and B).

3.3. Treatment with *O. gratissimum* methanolic extract and rosmarinic acid reduce eosinophil peroxidase levels in BAL and lungs

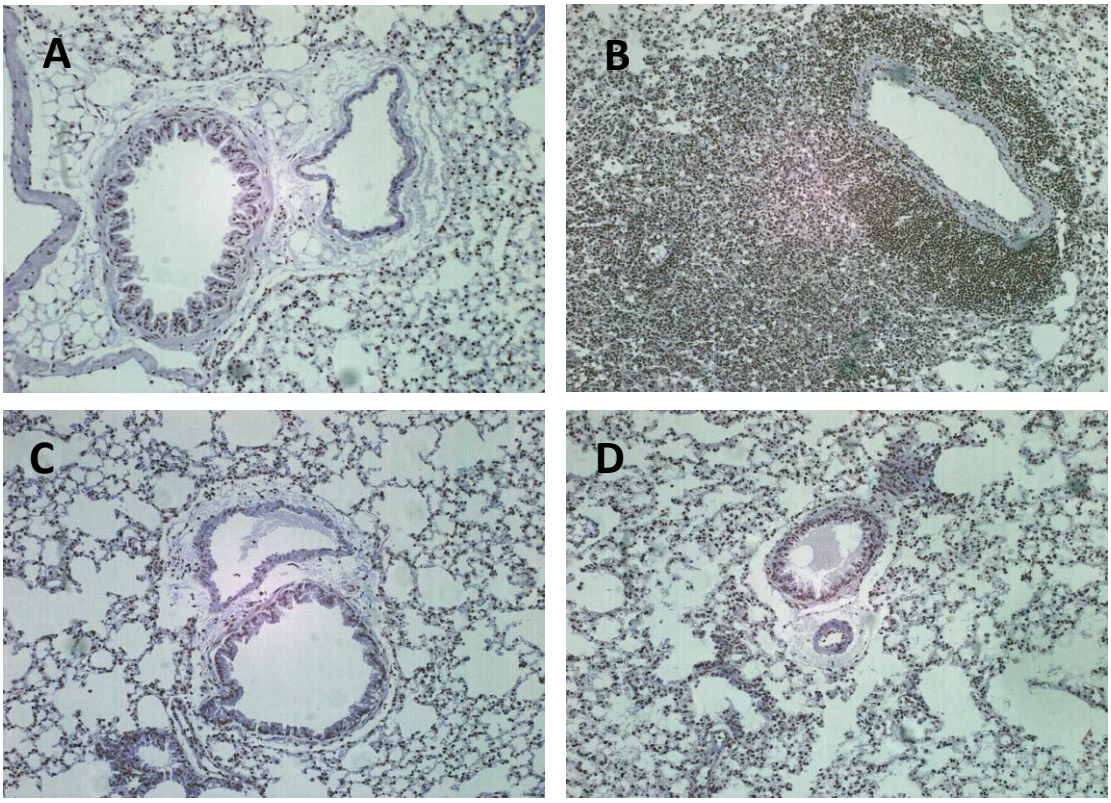
The sensitization of animals with BtE produced a significant increase of EPO activity in the BAL ($P < 0.001$) and in the lungs ($P < 0.001$) when compared to the control group (Fig. 3C and D).



This last datum directly correlated with the number of eosinophils at the inflammation site (data not shown). Treatment with 100mg/kg of OgME or with 200 mg/kg of RA decreased EPO activity in both BAL ($P < 0.01$) and lung tissue ($P < 0.05$) of BtE-immunized and challenged mice (Fig. 3).

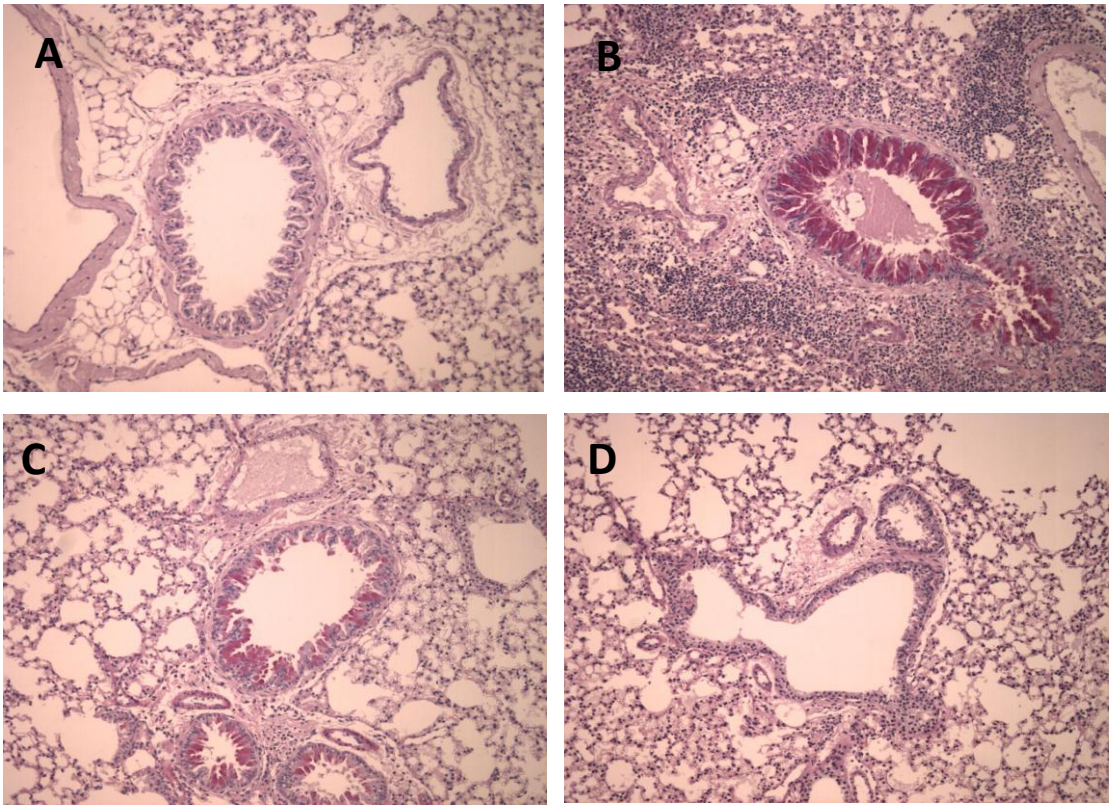
3.4. Treatments with *O. gratissimum* methanolic extract and rosmarinic acid ameliorate the pathological changes of BtE-immunized animals

Histological evaluation of lung tissue revealed typical pathologic features of allergic asthma in the BtE-immunized mice, characterized by numerous inflammatory cells, including eosinophils, infiltrated around the bronchioles (Fig. 4B). Treatment with OgME and RA markedly reduced the inflammatory cell infiltration within the peribronchiolar and perivascular regions (Fig. 4C and D).



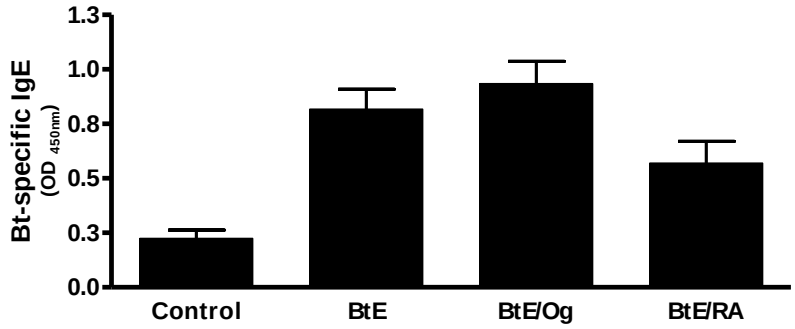
3.5 Treatment with *O. gratissimum* methanolic extract and rosmarinic acid reduce the amount of mucus in the airways

To evaluate airway hypersecretion of mucus and goblet-cell hyperplasia, lung sections were stained with PAS. Mucus production was significantly induced in the airway of BtE-immunized and challenged mice (Fig. 5B). Treatment with OgME (Fig 5C) and RA (Fig. 5D) markedly suppressed mucus secretion in the lung tissue.



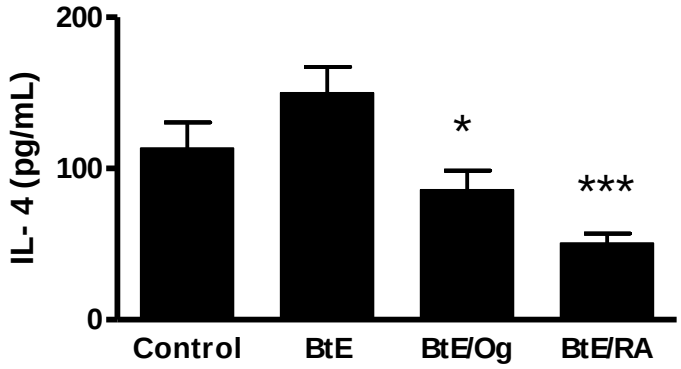
3.6. Treatment with rosmarinic acid, but not with *O. gratissimum* methanolic extract, tended to decrease the levels of BtE-specific IgE antibodies in the sera of BtE-immunized mice

Figure 6 shows the levels of anti-BtE IgE antibodies in the sera of OgME- and RA-treated, BtE-immunized mice. BtE-immunized mice produced higher levels of specific IgE antibodies than control, non-immunized animals. Treatment with OgME did not reduce the IgE antibody levels. Treatment with rosmarinic acid led to a decrease in the levels of anti-BtE IgE antibodies. However, these results were not statistically significant.



3.7. Treatments with *O. gratissimum* methanolic extract and rosmarinic acid reduce levels of IL-4, and do not affect levels of IFN-γ and TNF-α, in the BAL of BtE-immunized mice

To determine the possible mechanisms associated with the OgME and RA effects in airway inflammation, levels of the T-helper (Th) type 2 cytokine IL-4, and of the Th1 cytokines IFN-γ and TNF-α, were evaluated. Levels of IL-4 in the BAL were higher in BtE-immunized and challenged mice than in the control group, although the difference was not statistically significant. The oral treatment with OgME and RA led to significant reductions in levels of this Th2 cytokine in the BAL of BtE-immunized animals in relation to those of untreated, BtE-immunized animals ($p < 0.05$ and < 0.001, respectively) (Fig. 7). Only very low levels of IFN-γ and TNF-α were detected in the BAL, and these levels were not affected by treatment with OgME or RA (data not shown).



4. DISCUSSION

The inflammatory response to allergens in the asthmatic lung is a consequence of infiltration of the airway wall 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 [22]. The resolution of inflammation is an essential process for the establishment of appropriate host responses and the return to homeostasis [23].

The present study was conducted using a murine model of allergic airway disease induced by the sensitization to *Blomia tropicalis* mite extract, which was previously characterized by our research group as leading to an increased number of eosinophils in the BAL fluid, to a marked influx of inflammatory cells into the lung around blood vessels and airways, and to airway luminal narrowing [24]. This allowed us to investigate the potential antiallergic effect of an *Ocimum gratissimum* extract, and of its polyphenolic constituent, the rosmarinic acid, in an experimental model of airway and lung inflammation induced by a clinically relevant aeroallergen.

Ocimum gratissimum extracts have been shown to contain large amounts of polyphenolic compounds (flavonoids, stilbenes, phenolic acids and others), including RA [25, 26, 13]. Polyphenols have been shown to exert antiallergic, antiinflammatory, and bronchodilatory effects, by reducing the levels of inflammatory cytokines, chemokines, eosinophils and anti-allergen antibodies [27, 28, 29, 30]. To assess the presence and the amount of RA in the OgME used in this study, we studied the extract by high pressure liquid chromatography, which allowed us to confirm the presence of RA in the OgME, but not in the hexane extract. The estimated amount of RA in the methanol extract was 0.2 %, corroborating a previously published study [13].

An ethnopharmacology survey conducted by our research group, describing plant species used in folk medicine to treat allergies [8], identified *O. gratissimum* as one of these plants. To date, however, no scientific study has confirmed this. Some biological activities exerted by *O. gratissimum* are attributed to its polyphenols, such as the rosmarinic acid that is present in the plant [13]. In the present study, the treatment with OgME (orally) and RA (intraperitoneally) in BtE-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 and eosinophils in BAL and lung; (ii) inflammatory cell infiltration in the peribronchiolar and perivascular pulmonary region; (iii) presence of mucus inside airways; (iv) levels of IL-4 in the BAL.

The anti-inflammatory and immunomodulatory activities of RA has been ascribed to its inhibition of the lipoxygenase and cyclooxygenase pathways, interference with the complement cascade [31] and, mainly, the suppression of T-cell antigen receptor signaling [32]. These activities may explain, at least in part, the airway antiallergic activity of RA observed in this study.

According to BEL-RHLID and collaborators [20], RA is readily hydrolysed (up to 99%) by the gut microflora. Consistent with this finding, the oral administration of 200 mg/kg of RA, using the same protocol used in the present paper, had no effect on the development of respiratory allergy (Costa R.S. and collaborators, unpublished results). Since RA constituted only about 0.2 % (w/w) of the dry OgME, the amount of RA that was orally administered in the present paper with the 100 mg/kg dose of OgME was approximately 0.2 mg/kg, i.e. 1000 fold less than the amount of RA that had been previously found to be ineffective. It is therefore highly unlikely that the anti-allergic activity of the OgME by the oral route was due the presence of RA.

Eosinophilia is a relevant pathological feature of allergic diseases, contributing to airway damage through release of several cytotoxic mediators, including EPO, eosinophil-derived major basic protein, eosinophil cationic protein and bronchoconstrictor mediators, such as leukotriene C4 [33]. Accordingly, the increased presence of eosinophils and their secreted products in the asthmatic lung often correlates with severity and exacerbation of disease [34]. Additionally, the eosinophils have been shown to be a source of cytokines that are directly involved in the development of type I hypersensitivity, including IL-4, IL-5, and IL-13, suggesting that they have important roles in the immunopathology of allergic asthma [35, 36, 37].

Several studies attribute the anti-allergic property of natural products to their ability to reduce the eosinophilic inflammatory process [38, 39, 40, 33]. For example, an extract from *Perilla frutescens*, which is a species belonging to the taxonomical family of *O. gratissimum*, attenuates allergic airway inflammation by inhibiting Th2 cytokines and eosinophil infiltration into the airways. This activity was also attributed to the RA that was present in the extract [29], despite the fact that the extract was

administrated by the oral route. Different mechanisms have been proposed to explain the reduction in lung eosinophilia induced by plant-derived products, such as the suppression of the synthesis and inhibition of the effects of eosinophil survival factors, and the direct induction of eosinophil apoptosis [41].

In order to study the mechanism by which OgME and RA modulated eosinophil infiltration, we investigate the effect of these drugs on IL-4, IFN-γ and TNF-α production. The production of IL-4 was indeed reduced by the treatments. IL-4 is a crucial cytokine for the development of Th2 immune responses, which, through the activity of IL-5, promotes the recruitment of eosinophils [42].

Increased mucus production by goblet cells in the airway epithelium is associated with airway inflammation and asthma. The data presented here demonstrated that OgME and RA reduced the amount of mucus present in the airways in the BtE-induced experimental model of airway inflammation. IL-13 and IL-4 play an important role in the production of mucus [43]. Thus, the decrease in mucus in the airways of mice treated with OgME and RA may be due to the inhibition of Th2 cytokines by these drugs, which is supported by the reduced levels of IL-4 levels that were found in the BAL of the treated mice. In addition, IL-4 directly drives B lymphocytes to synthesize IgE [44], and thus, the reduction of this cytokine may exert an important anti-asthmatic effect. High anti-allergen IgE antibody levels in the serum or BAL have been associated with airway hyperresponsiveness both in adults and in children with asthma, and is associated with the severity of the disease [45]. These antibodies activate events related to eosinophil and mast cell degranulation [46]. A statistically significant increase in circulating IgE antibodies was not observed in the experimental model of airway inflammation used in the present study, despite the detection of increased levels of IL-4 in the BAL. This discrepancy could be explained by a local release of IL-4, which would not stimulate the systemic production of IgE. Although the treatment with RA had a tendency to reduce the levels of anti-BtE IgE antibodies, this reduction was not statistically significant. A possible effect of OgME and of RA in the production of *B. tropicalis*-specific IgE antibodies would have to be assessed in an experimental model of *B. tropicalis*-induced respiratory allergy in which these antibodies were significantly produced. In order to verify if the OgME and RA effect was due to the down-modulation of the Th2 immune response by a Th1 immune response, we also measured two Th1-type cytokines, IFN-γ and TNF-α, but none of them was affected by either the treatment with OgME or the treatment with RA.

The results of the present study, obtained in an experimental model in which clinically relevant and common allergens are used, namely an extract containing allergens from the *B. tropicalis* house dust mite, strongly support the potential usefulness of OgME and RA as antiinflammatory agents for the treatment of allergic asthma. In addition, they justify the carrying out of experiments to further elucidate the molecular mechanisms underlying the OgME and RA immunomodulatory effects, and to identify the component of the OgMe that is biologically active by the oral route.

6. LIST OF ABBREVIATIONS

BAL: Bronchoalveolar lavage

BtE: *Blomia tropicalis* extract

ELISA - Enzyme-linked immunosorbent assay

EPO: Eosinophil peroxidase

IFN-γ: Interferon gamma

IL: Interleukin

OgME: *Ocimum gratissimum* Linn. methanolic extract

RA: Rosmarinic acid

TNF-α: Tumor necrosis factor-alpha

7. CONFLICT OF INTEREST

All authors declare they have no competing financial interests.

8. ACKNOWLEDGMENTS

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FIGURES LEGENDS

Fig. (1). Experimental protocol for induction of respiratory allergy using aluminium hydroxide-adsorbed *Blomia tropicalis* extract (BtE) and assessment of the treatment with *O. gratissimum* methanolic extract (OgME, 100 mg/kg, orally) and rosmarinic acid (RA, 200 mg/kg, intraperitoneally). [D0] to [D15], days 0 to 15 after the start of the experiments.

Fig. (2). Chromatogram of samples subjected to high performance liquid chromatography. Optical densities for UV light are shown at the Y axis. (**A**) Chromatogram of *O. gratissimum* methanol extract. (**B**) Chromatogram of rosmarinic acid. (**C**) Chromatogram of *O. gratissimum* hexane extract. Retention times are shown above peaks in (A) and (B).

Fig. (3): Effect of treatment with *O. gratissimum* methanolic extract (OgME) and with rosmarinic acid (RA) in the numbers of inflammatory cells, mainly eosinophils, in the airways, and in levels of eosinophil peroxidase (EPO) in airways and lung tissue, of *Blomia tropicalis* extract (BtE)-immunized and challenged mice. (**A**) number of leukocytes in the bronchoalveolar lavage (BAL); (**B**) number of eosinophils in the BAL; (**C**) levels of EPO in the BAL; (**D**) levels of EPO in lung homogenates. Groups: **Control**, vehicle-treated animals; **BtE**, BtE-sensitized and challenged, and vehicle-treated mice; **BtE/OgME**, BtE-sensitized and challenged, and OgME-treated mice; **BtE/RA**, BtE-sensitized and challenged, and RA-treated mice. Columns represent the mean values of the results obtained from six

animals, and error bars represent the standard error from the means. $^{\#}P < 0.05$ vs control; $^{\#\#\#}$ $p < 0.001$ vs control; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs BtE group. ANOVA-Tukey.

Fig. (4). Effect of the treatment with *O. gratissimum* methanolic extract (OgME) and rosmarinic acid (RA) on leukocyte infiltration in lung tissues of mice sensitized and challenged with *Blomia tropicalis* extract (BtE). Sections were stained with hematoxylin-eosin (magnification ×200). (**A**) Lung section from a control, saline-treated mice; (**B**) lung section from a BtE- immunized and challenged, saline-treated mice; (**C**) lung section from a BtE-immunized and challenged, OgME-treated mice; (**D**) Lung section from a BtE-immunized and challenged, RA-treated mice.

Fig. (5). Effect of treatment with *O. gratissimum* methanolic extract (OgME) and rosmarinic acid (RA) on the production of mucus in the lung tissue of mice sensitized with BtE antigen. Sections were stained with periodic acid-Schiff (magnification ×200). (**A**) Lung section from a control, saline-treated mice; (**B**) lung section from a BtE- immunized and challenged, saline-treated mice; (**C**) lung section from a BtE-immunized and challenged, OgME-treated mice; (**D**) lung section from a BtE-immunized and challenged, RA-treated mice. Arrows indicate the presence of mucus.

Fig. (6). Levels of anti-*Blomia tropicalis* extract (BtE) IgE antibodies in BtE-immunized mice treated with methanolic extract of *O. gratissimum* (OgME) and rosmarinic acid (RA). Antibody levels were measured by indirect ELISA. Control, animals that received only the vehicle during the sensitization and treatment procedures; BtE, BtE-challenged mice thar were treated with vehicle; BtE/OgME, BtE-challenged mice that were orally treated with 100mg/kg of OgME), BtE/RA (BtE-challenged mice that were intraperitoneally treated with 200mg/kg of RA). Columns represent the mean values of the results obtained from six animals, and error bars represent the standard error from the means.

Fig. (7). Effect of the treatment with *O. gratissimum* methanolic extract (OgME) and rosmarinic acid (RA) on the levels of IL-4 in the BAL of BtE-challenged A/J mice. IL-4 quantification was done by sandwich ELISA. **Control**, vehicle-treated animals; **BtE**, BtE-sensitized and challenged, and vehicle-treated mice; **BtE/OgME**, BtE-sensitized and challenged, and OgME-treated mice; **BtE/RA**, BtE-sensitized and challenged, and RA-treated mice. Columns represent the mean values of the results obtained from six

animals, and error bars represent the standard error of the means. (* $p < 0.05$, and *** $p < 0.001$ vs BtE group), ANOVA-Tukey.

4 CONCLUSÕES

4.1. Através da técnica de HPLC foi demonstrado que o ácido rosmarínico é um composto polifénólico presente no *Ocimum gratissimum* Linn. Portanto, essa substancia é uma potencial candidata a ser explorada em nível clínico como uma alternativa terapêutica para a asma e outras alergias

4.2 O *Ocimum gratissimum* Linn., bem como o polifenól ácido rosmarínico, possuem capacidade de atenuar a inflamação eosinófílica e a produção de muco nas vias aéreas e, portanto, apresentam potencial anti-alérgico em modelo de alergia respiratória.

4.3 A atividade antialérgica do *Ocimum gratissimum* Linn. e do ácido rosmarínico é exercida através da redução dos níveis da citocina Th2, IL-4, que está envolvida no processo alérgico.

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ANEXO 1

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Artigo

Produtos naturais utilizados para tratamento de asma em crianças residentes na cidade de Salvador-BA, Brasil

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RESUMO: O conhecimento popular é o passo inicial para a investigação científica de atividades terapêuticas de remédios caseiros. Diversas patologias podem ser tratadas ou amenizadas através de preparações de origem natural e muitos fármacos disponíveis são oriundos de fontes naturais. Este trabalho tem como objetivo avaliar o uso de remédios caseiros derivados de plantas para tratamento de asma em crianças residentes no município de Salvador. Os dados foram obtidos a partir de estudo realizado em Salvador sobre fatores de risco, uso de medicações e vias imunológicas relacionadas à asma em crianças. Foram calculadas as freqüências de uso de preparações caseiras para tratamento de asma por esta população e realizado uma revisão bibliográfica sobre os efeitos das plantas mais usadas. Dentre as espécies mais citadas, destacam-se o *Allium sativum* (alho) que teve a maior freqüência de utilização na preparação dos remédios caseiros (25%), seguido da *Allium cepa* (cebola, 19,74%). Após a revisão crítica de literatura, constatou-se que a maioria das espécies é utilizada com base em relatos fundamentados no saber popular, sendo assim carente de evidências científicas para as atividades farmacoterapêuticas esperadas. Neste sentido, há necessidade de mais estudos farmacológicos para comprovação das atividades terapêuticas peculiares a cada produto de origem natural bem como para avaliar possíveis efeitos tóxicos destes produtos.

Unitermos: medicina popular, asma, etnofarmacologia.

ABSTRACT: "Natural products used for asthma treatment in children living in Salvador-BA, Brazil". The popular knowledge is the initial step for the scientific inquiry of therapeutical activities of herb-based remedies. Several pathologies can be treated or brightened up through this kind of preparations and also many of the available drugs in the market have natural sources. The objective of this work was to evaluate the use of herb-based remedies for treatment of asthma in children in the city of Salvador. Data were collected by a standard questionnaire during a transversal study carried out in Salvador on risk factors, use of medications and immunological pathways involved in asthma. Among the most frequently mentioned species, the *Allium sativum* had the highest frequency of use in the preparation of home remedies (25%), followed by the *Allium cepa* (19.74%). The literature review showed that the majority of the species is empirically used based on popular knowledge and lacks on scientific evidences that prove their pharmacotherapeutic activities and safety for human use. In this way, this work not only new species unexplored in the context of anti-asthmatic drugs but it also highlights the need for new pharmacological studies in order to identify and prove the popular use of herb-based remedies.

Keywords: medicine knowledge, asthma; ethnopharmacology.

INTRODUÇÃO

A asma é uma doença inflamatória crônica caracterizada por hiperresponsividade das vias aéreas inferiores e por limitação variável ao fluxo aéreo, manifestando-se clinicamente por episódios recorrentes de sibilância, dispnéia e tosse (O'Byrne, 2009). A asma constitui um problema importante de saúde pública, uma vez que tem alta prevalência, expõe o paciente a recorrentes hospitalizações e representa uma elevada carga econômica para o indivíduo, a sociedade e o governo (Lee, 2010; Franco et al., 2009). Alta prevalência de asma tem sido

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ANEXO 2

Comprovante da Comissão de Ética na experimentação Animal

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CERTIFICADO

Certificamos que o Projeto de Pesquisa "EFEITOS IMUNOMODULADORES DE EXTRATOS DE PRODUTOS NATURAIS DE ORIGEM VEGETAL E PARASITÁRIA EM MODELO DE ALERGIA E AUTOIMUNIDADE- 02/09" de autoria de CAMILA FIGUEIRÈDO, foi analisado pela Comissão de Ética na Experimentação Animal e considerado APROVADO em reunião ordinária realizada nesta data.

Salvador, 12 de maio de 2009

Prof. Antonio Luiz B. Pinheiro
Presidente

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