Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz

INSTITUTO OSWALDO CRUZ

Pós-Graduação em Biologia Celular e Molecular

JAIME RIBEIRO FILHO

Propriedades antialérgicas e antiinflamatórias da curina, um alcaloide bisbenzilisoquinolínico isolado de *Chondrodendron platyphyllum* (Menispermaceae)

Tese apresentada ao Instituto Oswaldo Cruz como parte dos requisitos para obtenção do título de Doutor em Biologia Celular e Molecular na área de concentração em Imunologia e Farmacologia

Orientadora: Profa. Dra. Patrícia Torres Bozza

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Aos meus pais: Jaime e Helena;

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HipóTESE

Eu vim de uma terra distante e sofrida
Onde a despedida é sempre constante
Pessoas que lutam pra vencer na vida
Sorrindo e chorando ao mesmo instante

Eu vim de uma terra em que as pessoas
São simples, mas boas,e vivem em paz
Onde os amigos não brigam a toa
Se amam e não se esquecem jamais

Eu vim de uma terra em que o saber
É mais do que ler teses ou artigos
Aprende-se ouvindo pessoas mais velhas
Que contam novelas e contos antigos

Eu vim de uma terra em que as riquezas
Nem mesmo a pobreza consegue ofuscar
Bem mais que doutor, sou cabra da peste
Eu vim do nordeste, sou do Ceará

Jaime Ribeiro-Filho

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LISTA DE ABREVIATURAS

AA	Acido araquidônico
AHR	Hiperreatividade das vias aéreas
AINE	Antiinflamatório não esteroide
AMPc	Monofosfato de adenosina cíclico
AP-1	Proteína ativadora 1
APC	Célula apresentadora de antígeno
ASM	Músculo liso das vias aéreas
ATP	Trifosfato de adenosina
BAL	Lavado broncoalveolar
BBA	Alcalóide bisbenzilisoquinolínico
BLT1	Receptor 1 do leucotrieno B_4
CCR	Receptor de quimiocina
CD	Classe de diferenciação; marcador de superfície celular
CisLT	Cistenil leucotrieno
CisLTR1	Receptor 1 de Cisteinil leucotrieno
CL	Corpo lipídico
Con-A	Concanavalina-A
COX	Enzima ciclooxigenase
DC	Célula dendrítica
DE_{50}	Dose efetiva mediana
Dexa	Dexametasona
ERK	Proteina da família das MAP quinases
FcεRI	Receptor de alta afinidade para IgE
GATA-3	Proteina 3 de ligação ao GATA; fator de transcrição
GPCR	Receptor acoplado à proteina G
5-HT	5- Hidroxi-triptamina; Serotonina
ICAM	Molécula de adesão intercelular
IFN	Interferon
Ig	Imunoglobulina
IL	Interleucina
iNOS	Óxido nítrico sintase induzível
IP_3	Trifosfato de inositol
JNK	Proteina da família das MAP quinases
LABA	Agonista β_2-adrenérgico de longa ação
LB	Linfócito B

LBP	Proteina de ligação ao LPS
LFA-1	Antígeno 1 associado à função leucocitária
LPS	Lipopolissacarídeo
LO	Enzima lipoxigenase
LT	Leucotrieno
MAPK	Proteína quinase ativada por mitógeno
MCP	Proteína quimiotática para monócitos/macrófagos
MHC	Complexo de histocompatibilidade principal
MLCK	Quinase da cadeia leve de miosina
MYD88	Proteina adaptadora da transdução de sinal de receptores imunológicos
NET	Rede extracelular de neutrófilos
NFAT	Fator nuclear de células T ativadas
NF-κB	Fator nuclear kappa B
NO	Óxido nítrico
NOS	Òxido nítrico sintase
OVA	Ovalbumina
PAF	Fator ativador de plaquetas
PAMP	Padrão molecular associado ao patógeno
PG	Prostaglandina
PI3K	Fosfatidil inositol-3-quinase
PL	Fosfolipase
PK	Proteína quinase
PIP_2	Fosfatidil Inositol Bifosfato
PRR	Receptor de reconhecimento de padrão
PSGL-1	Ligante 1 da glicoproteina P-selectina
ROS	Espécies reativas de oxigênio
SNARE	Família de proteinas associadas a exocitose
TCR	Receptor de célula T
Th	Linfócitos T *helper* ou auxiliares
TLR	Receptor semelhante ao Toll
TLSP	Linfopoietina do estroma tímico
TNF	Fator de Necrose tumoral
Trif	Proteina adaptadora da transdução de sinal de receptores imunológicos
TX	Tromboxano
VCAM	Molécula de adesão Vascular

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RESUMO

TESE DE DOUTORADO

Jaime Ribeiro Filho

As doenças inflamatórias representam importantes problemas de saúde pública em termos de prevalência morbidade e mortalidade em todo o mundo. A asma alérgica é uma doença inflamatória crônica das vias aéreas, que resulta de uma resposta imune exacerbada contra antígenos comuns. A terapia antiinflamatória e antialérgica inclui o uso de fármacos que causam efeitos colaterais significantes ou não são efetivos em situações específicas, justificando a pesquisa de fármacos inovadores, seguros e eficazes. A curina é um alcaloide que atua como inibidor do influxo de cálcio, contudo as suas propriedades antialérgica e antiinflamatórias, bem como os efeitos tóxicos decorrentes do seu uso *in vivo* permanecem por serem caracterizadas. Este trabalho tem como objetivo caracterizar as atividades antialérgicas e antiinflamatórias da curina, bem como investigar a toxicidade induzida pelo tratamento oral com este alcaloide. Utilizando um modelo experimental de asma, nós demonstramos que a administração oral da curina inibiu significativamente o recrutamento e a ativação de eosinófilos e a AHR em animais desafiados com OVA, além de reduzir a produção de eotaxina e IL-13. O verapamil, um antagonista de canais de cálcio, apresentou propriedades antialérgicas semelhantes, e o pré- tratamento com a curina inibiu a resposta contráctil induzida por cálcio em anéis de traqueia *ex vivo*, sugerindo que o mecanismo pelo qual a curina exerce seus efeitos é através da inibição de uma resposta dependente de cálcio. A avaliação toxicológica mostrou que a curina administrada oralmente por 7 dias não induziu toxicidade evidente. A avaliação da atividade antiinflamatória e analgésica demonstrou que a curina inibiu a formação de edema da pata e a permeabilidade vascular, inibiu respostas nociceptivas na fase inflamatória, mas não na fase neurogênica, além de inibir a hiperalgesia induzida por carragenina. Em adição, a curina inibiu a produção de PGE_2, *in vitro*, sem afetar a expressão de COX-2 em macrófagos estimulados com LPS. Os efeitos do tratamento com a curina foram semelhantes aos efeitos da indometacina, mas diferentes dos efeitos do tratamento com morfina, sugerindo que o efeito analgésico da curina não resulta da inibição direta da ativação neuronal, mas depende de mecanismos antiinflamatórias que, pelo menos em parte, resultam da inibição da produção de PGE_2. Utilizando um modelo de pleurisia induzida por LPS, nós demonstramos que o tratamento oral com a curina inibiu significativamente o recrutamento de neutrófilos para o lavado pleural, associado com a inibição da produção de citocinas inflamatórias e de leucotrieno B_4 no lavado pleural. A curina também inibiu a produção de TNF-α, IL-1β, IL-6 e NO por macrófagos estimulados com LPS *in vitro*, e o verapamil, apresentou efeitos inibitórios semelhantes sugerindo que os efeitos inibitórios que a curina exerce sobre a ativação dos macrófagos, podem estar associados com a inibição do influxo de cálcio. Em conclusão, a curina apresentou efeitos antialérgicos, antiinflamatórios e analgésicos, na ausência de toxicidade evidente, que estão associados com a inibição da ativação de leucócitos e da produção de mediadores inflamatórios, cujos mecanismos envolvem uma inibição de uma resposta dependente de cálcio, e portanto tem potencial para o desenvolvimento de fármacos.

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THE ANTI-ALLERGIC AND ANTI-INFLAMMATORY PROPERTIES OF CURINE, A BISBENZILISOQUINOLINE ALKALOID ISOLATED FROM *Chondrodendron platyphyllum* (Menispermaceae)

ABSTRACT

TESE DE DOUTORADO

Jaime Ribeiro Filho

Inflammatory diseases are major public health problems in terms of prevalence of morbidity and mortality worldwide. Allergic asthma is a chronic inflammatory airway disease, which results from an exacerbated immune response against common antigens. The anti-inflammatory and anti-allergic therapy includes the use of drugs that cause significant side effects or are not effective under specific conditions, justifying the search for novel, safe and effective drugs. Curine is an alkaloid that acts as an inhibitor of calcium influx, but its anti-inflammatory and anti-allergic properties, as well as the toxic effects resulting from its administration *in vivo* remain to be elucidated. This study aims to characterize the anti-inflammatory and anti-allergic properties of curine as well as investigate the toxicity induced by the oral treatment with this alkaloid. Using an experimental model of asthma, we have demonstrated that orally administered curine significantly inhibited the recruitment and activation of eosinophils and AHR in mice challenged with OVA, besides reducing the production of eotaxin and IL-13. Verapamil, a calcium channel antagonist, showed similar anti-allergic properties and the pre-treatment with curine inhibited the calcium-induced tracheal contractile response *ex-vivo*, suggesting that the mechanism by which curine exerts its effects is through the inhibition of a calcium-dependent response. The toxicological evaluation showed that curine administered orally for 7 days did not induce significant toxicity. The evaluation of the analgesic and anti-inflammatory activities of curine demonstrated that it inhibited paw edema formation and vascular permeability, inhibited nociceptive responses in the inflammatory phase, but not in neurogenic phase, and inhibited carrageenan induced hyperalgesia. In addition, curina inhibited PGE_2 production *in vitro*, without affecting the expression of COX-2 in macrophages stimulated with LPS. The effects of the treatment with curine were similar to the effects of indomethacin, but different from the effects of morphine treatment, suggesting that the analgesic effect of curine does not result from direct inhibition of neuronal activation, but instead, relies on anti-inflammatory mechanisms which at least in part, result from inhibition of PGE_2 production. Using a model of LPS-induced pleurisy, we demonstrated that the oral treatment with curine significantly inhibited the recruitment of neutrophils into the pleural lavage, associated with inhibition of cytokine and leukotriene B_4 production in the pleural lavage. Also, curine inhibited the production of TNF-α, IL- 1β, IL -6 and NO by macrophages stimulated with LPS *in vitro*, and verapamil had similar inhibitory effects ,suggesting that the inhibitory effects that curine exerts on macrophage activation may be associated with the inhibition of calcium influx. In conclusion, curine the presented anti-allergic, anti-inflammatory and analgesic effects in the absence of significant toxicity, which are associated with the inhibition of leukocyte activation and production of inflammatory mediators, whose mechanisms involve inhibition of a calcium-dependent response and as such, has the potential for drug development.

1. INTRODUÇÃO

1.1. Inflamação

A inflamação é uma resposta do organismo a um dano tecidual caracterizada por alterações bioquímicas, vasculares e celulares, que tem como objetivo restaurar a integridade deste tecido (Lawrence *et al*., 2002). Os sintomas clássicos observados na inflamação incluem o calor (aumento da temperatura), a dor, a vermelhidão e o inchaço (edema). Quando o estímulo é persistente e o processo não é resolvido, outro sintoma e ainda mais grave pode ser acrescentado, a perda de função (Medzhitov, 2010).

A inflamação é conhecida pela humanidade há muito tempo. Existem textos médicos que fazem referência a esta condição desde o período histórico da antiguidade, muitos deles descrevendo sintomas de pacientes em eventos relacionados a epidemias e a ferimentos de guerra. Contudo, os sintomas clínicos clássicos da inflamação foram descritos pela primeira vez pelo médico romano Cornelius Celsus, no século 1 d. C. Em seu tratado de medicina, Celsus mencionou os sintomas que são comumente descritos como os quatro sinais cardinais da inflamação: *rubor*, *tumor*, *calore* e *dolore*, referindo-se a respectivamente: vermelhidão, e inchaço, calor e dor. O quinto sinal cardinal, perda de função, foi acrescentado por Rudolph Virchow (1858) em seu livro *Patologia celular* (Majno, 1975), após o estabelecimento das bases fisiológicas da inflamação, especialmente por meio dos trabalhos de Augusto Waller (1846) e Júlio Cohnheim (1867), que descreveram as alterações vasculares e a migração de leucócitos durante a inflamação (Majno e Joris, 2004).

Muitos outros eventos contribuíram de forma significante para o estabelecimento das bases imunológicas da inflamação, entre elas: a descoberta da fagocitose e teoria da imunidade celular desenvolvida por Elie Metchnikoff (1892), que destacou as funções benéficas da inflamação, tanto na defesa do hospedeiro, como manutenção da homeostase (Tauber, 2003); o desenvolvimento da teoria da imunidade humoral por Paul Ehrlich, baseado principalmente nas pesquisas sobre soroterapia de Emil von Behring e Shibasaburo Kitasato (1890); e a teoria da doença no final do século XIX por Robert Koch e Louis Pasteur, que destacam os microorganismos como importantes indutores da inflamação (Medzhitov, 2010).

A inflamação pode ocorrer em diferentes formas e modalidades, bem como ser regulada por diferentes mecanismos de indução, regulação e resolução. Deste modo, se por um lado a inflamação é um processo essencial para a defesa do hospedeiro contra patógenos (Lybbi *et al*., 2007), por outro lado ela pode ocorrer na ausência de infecção, um processo conhecido como inflamação estéril, que pode ser desencadeada por agentes químicos, como

agentes oxidantes, ácidos e bases ou agentes físicos como traumas, radiação, e temperaturas extremas (Chen & Nunes, 2010). Entretanto, independentemente da causa, a inflamação pode ser entendida como um processo essencial para a restauração da homeostase e portanto desempenha um importante papel fisiológico (Medzhitov *et al*., 2008) (Fig. 1.1).

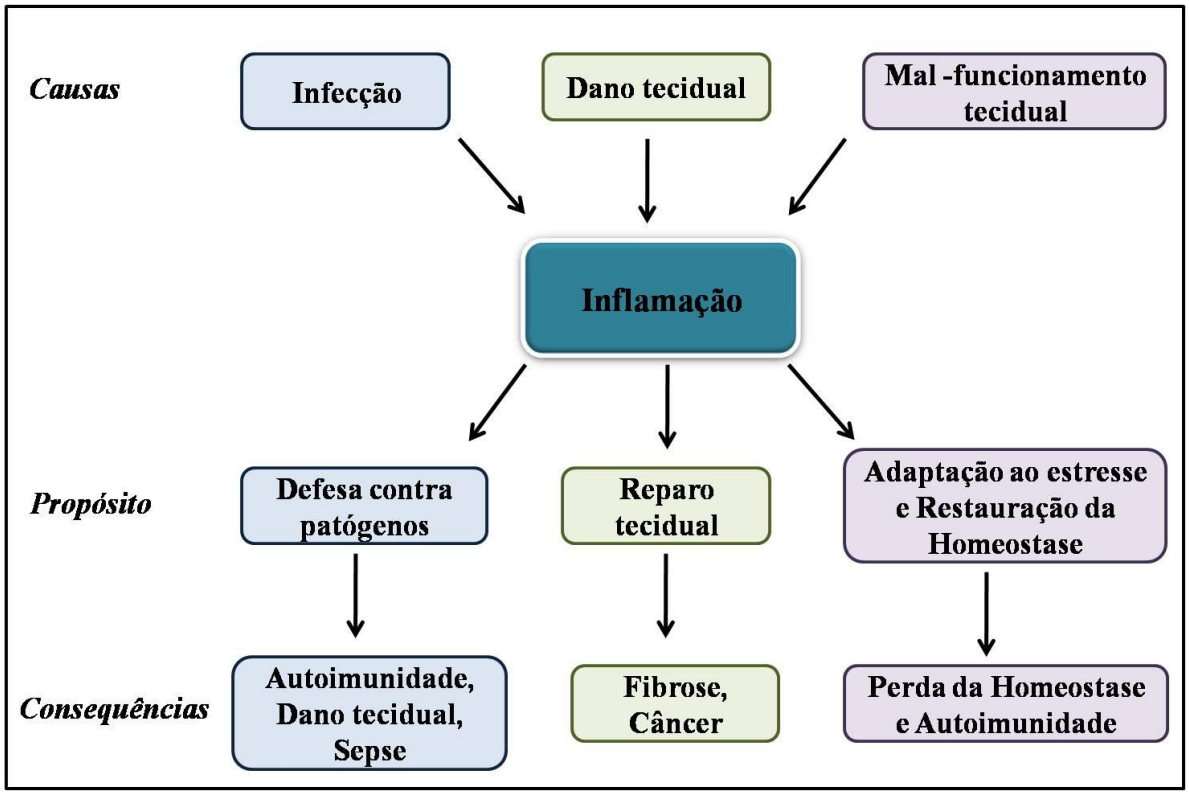

Figura 1.1. Causas, propósitos e consequências da inflamação. A inflamação é um fenômeno dinâmico no qual a finalidade fisiológica e as conseqüências patológicas são determinadas pelo tipo de estímulo indutor (adaptado de Medzitov, 2008. Nature).

Apesar de ser um processo ativamente controlado, frequentemente ocorrem falhas na resolução da inflamação, o que contribui significativamente para a patogênese de várias doenças crônicas, incluindo a asma e outras doenças alérgicas, a aterosclerose, a obesidade, o cancro, a doença pulmonar obstrutiva crônica, a doença inflamatória do intestino, a esclerose múltipla e a artrite reumatóide (Nathan & Ding, 2010). Nestes casos, a inflamação em si, passa a ser a causa da patologia, além de contribuir para a gravidade de outras co-morbidades (Nathan & Ding, 2010; Medzhitov, 2010).

1.1.1. Características Gerais das Respostas Inflamatórias

Os eventos iniciais das respostas inflamatórias são coletivamente denominados inflamação aguda, um termo classicamente utilizado para descrever uma resposta imediata e não inespecífica (Ryan & Majno, 1977). Neste sentido, os mecanismos envolvidos na ativação da inflamação aguda estão significativamente associados com a ativação de componentes da resposta imune inata, a qual é caracterizada por rapidez, pouca especificidade, ausência de memória e o predomínio de fagócitos (Janeway-Jr & Medzhitov, 2002). Atualmente o papel destes componentes está melhor estabelecido na resposta inflamatória contra infecções, embora ultimamente tenham havido avanços significativos na descrição do papel destes componentes na inflamação resultante de dano, estresse ou mal funcionamento tecidual na ausência de infecções (Medzhitov, 2008).

A resposta inflamatória a patógenos é dependente do reconhecimento de estruturas microbianas conhecidas como padrões moleculares associados a patógenos (PAMPs) por receptores da imunidade inata conhecidos como receptores de reconhecimento de padrão (PRRs) (Janeway, 1989). Os PAMPs incluem uma grande variedade de estruturas comuns a classes de microrganismos e incluem: o lipopolissacarídeo e o peptidoglicano de bactérias, o zimosan de fungos e as sequências CpG do DNA bacteriano, entre outras. Cada uma destas estruturas é reconhecida por PRRs específicos, tais como o receptores semelhantes ao Toll (TLR, do inglês: *"Toll like receptors"*), os receptores de varredura (ou scavengers), os receptores semelhantes ao Nod e os receptores de manose (Fraser *et al*., 1998; Jensen & Thomsen, 2012). Este tipo de interação permite ao sistema imune reconhecer estruturas estranhas e portanto, potencialmente perigosas ao organismos e também contribui para a ativação dos leucócitos, resultando na liberação de mediadores que iniciam respostas agudas bem como ativam respostas tardias (Fraser *et al*., 1998).

Um exemplo clássico deste fenômeno é o reconhecimento do lipopolissacarídeo bacteriano (LPS), um componente da parede celular de bactérias gram-negativas, pelo receptor semelhante ao toll (TLR, do inglês: *Toll like receptor*) - 4, expresso por macrófagos residentes no tecido (Lu *et al*., 2008). A sinalização do LPS via TLR-4 associado ao co-receptor CD-14 e a proteina ligante de LPS (LBP) ativa os macrófagos, induzindo a produção de citoquinas inflamatórias, tais como o TNF-a, a interleucina (IL)-1 e a IL-6; quimiocinas, como o CCL2 e CXCL8 (KC) e mediadores lipídicos, como a prostaglandina E_2 (PGE_2). Estes mediadores iniciam as alterações locais, inclusive a ativação de células epiteliais, células dendríticas e os mastócitos, além da ativação de componentes plasmáticos, como a bradicinina e os componentes da cascata de coagulação e as proteínas do sistema

complemento (Pechi *et al*., 2009), que amplificam o estímulo inicial provocando diversas alterações que favorecem o recrutamento, a migração e ativação de células inflamatórias no sítio de injúria, fornecendo um mecanismo de amplificação da resposta inflamatória. Estas alterações incluem a contração da musculatura lisa, o aumento da permeabilidade vascular e o aumento da expressão de moléculas de adesão, incluindo integrinas e selectinas (Libby, 2007). Em adição, a PGE_2, produzida principalmente por macrófagos e células endoteliais ativadas, pode ativar populações de neurônios no sistema nervoso central induzindo alterações comportamentais e febre, além de estar significativamente envolvida na dor inflamatória (Pechi *et al*., 2009).

No que diz respeito ao recrutamento de leucócitos, os neutrófilos são umas das primeiras células a migrar para o sítio inflamatório, onde exercem funções essenciais na eliminação dos patógenos, bem como na modulação da resposta inflamatória (Amulic *et al*., 2012). Algumas citocinas inflamatórias, incluindo o TFN- α, a IL-β e a IL-17 estimulam as células endoteliais a produzir moléculas de adesão, tais como: as P-selectinas, as E-selectinas e moléculas de adesão intercelular (ICAMs) da família das integrinas (Borregaard, 2010). Os neutrófilos expressam constitutivamente em sua superfície o ligante da glicoproteina P-selectina (PSGL-1) e a L-selectina (Kansas, 1996; McEver & Cummings, 1997). As mudanças ocorridas no fluxo sanguineo contribuem para a ligação destas moléculas com as P e E-selectinas das células endoteliais, resultando numa primeira adesão mediada dos neutrófilos ao endotelio vascular. Esta fase é seguida por um "rolamento" dos neutrófilos longo do endotélio, uma vez que esta primeira adesão é relativamente fraca. Além disto, esta interação mediada por selectinas é importante para a ativação dos neutrófilos (Mueller *et al*., 2010; Yago *et al*., 2010). Deste modo, a interação com selectinas, quimiocinas, citocinas e produtos bacterianos induzem diversas alterações nos neutrófilos, que incluem: a expressão de moléculas que promovem uma adesão firme dos neutrófilos, tais como as β2 integrinas (ex. LFA-1) que se ligam a ICAMs na superfície endotelial; alterações no citoesqueleto que permitem a que os neutrófilos se movam entre as células endoteliais e migrem em direção ao gradiente quimiotático (Amulic *et al*., 2012) (Fig. 1.2).

Na maioria dos casos, após a eliminação do agente agressor, a resposta inflamatória aguda é regulada negativamente para o estabelecimento da homeostase. Esta regulação é um processo ativo chamado de resolução da inflamação e envolve diversas mudanças, incluindo a mudança no perfil de mediadores produzidos (Nathan & Ding, 2010). Por exemplo, ocorre a interrupção da produção de prostaglandinas pró-inflamatórias e indução da produção de mediadores anti-inflamatórios, como as lipoxinas e resolvinas a medida em que os neutrófilos são substituídos por macrófagos que realizam fagocitose e liberam tais mediadores (Serhan &

Savill, 2005). Entretanto, quando o estímulo agressor persiste, a resolução não é eficiente e resposta inflamatória torna-se crônica. Neste caso, a resposta inflamatória causa danos mais graves ao organismo, originando um quadro patológico que necessita de intervenção terapêutica a longo prazo (Shacter & Weitzman, 2002). É o caso de várias patologias de origem inflamatória como, por exemplo, a asma, a artrite reumatoide e várias doenças autoimunes. Uma característica importante das inflamações crônicas é que elas frequentemente estão associadas levam ao remodelamento tecidual, que pode levar a perda de função do órgão afetado (Tabas & Glass, 2013).

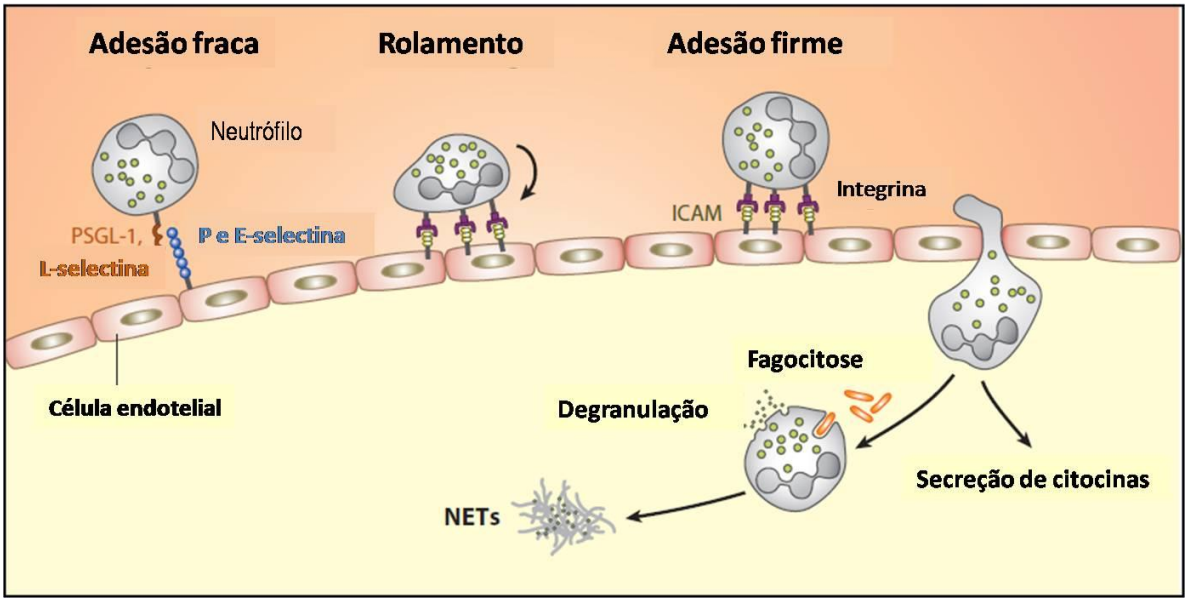

Figura 1.2. O recrutamento de neutrófilos para o sítio inflamatório. Os mediadores inflamatórios induzem diversas alterações que favorecem a migração celular. Isto envolve sequencialmente: adesão fraca e rolamento ao longo do endotélio mediados por selectinas, adesão firme mediada por integrinas, extravasamento entre as células endoteliais e migração seguindo gradientes de quimiocinas até o sítio inflamatório, onde exercem funções efetoras incluindo: fagocitose, secreção de enzimas e citocinas e formação de redes extracelulares de neutrófilos (NETs) (adaptado de Amulic *et al*., (2012). Annual Review of Immunology).

Na fase tardia da reposta inflamatória, muitos componentes da imunidade inata são conservados e os linfócitos diferenciados começam a orquestrar uma resposta específica que, como conhecido para respostas adaptativas, conserva memória imunológica (Iwasaki & Medzhitov, 2010). Dentro deste contexto, os eventos iniciais da inflamação aguda são determinantes para a montagem de uma resposta crônica. Uma evidência disso é que o tipo e a persistência do estímulo reconhecido pelas células apresentadoras de antígenos (APCs) influenciam a diferenciação dos linfócitos T CD4+ (Th, *do inglês*: *T helper*, ou auxiliares) virgens (Th0) em diferentes subtipos e deste modo, também afetam as características da resposta adaptativas (Murdoch & Lloyd, 2010; Soroosh & Doherty, 2009).

Os linfócitos Th desempenham funções importantes na perpetuação de doenças inflamatórias crônicas patologias por modular diversos processos imunológicos através da produção de citocinas características (Zhu *et al*., 2010). Os diferentes subtipos de linfócitos Th são diferenciados a partir de células $CD4^+$ naive por meio de um processo que envolve a ativação ou a repressão de determinados genes, resultando na expressão diferencial de fatores de transcrição, receptores de quimiocinas e citocinas que definem a linhagem e possibilitam a criação de uma alça de auto-sustentação (Murdoch & Lloyd, 2010). Até recentemente, as células Th eram subdivididas em duas populações funcionalmente distintas e mutuamente exclusivas: Th1, caracterizada pela produção de Interferon gama (IFN-γ) e pela proteção contra bactérias intracelulares, e Th2, caracterizadas pela produção de IL-4 e pela proteção contra patógenos extracelulares, incluindo helmintos. Contudo, atualmente, outras populações de células Th têm sido identificadas, entre elas as Th9, Th17 e as células T regulatórias (Treg) (O'Connor-Jr *et al*., 2010).

1.1.2. Mecanismos gerais de ativação celular e produção de mediadores inflamatórios

Uma grande variedade de células no nosso organismo é capaz de produzir e liberar mediadores inflamatórios em resposta a estímulos específicos. Os mediadores inflamatórios são substâncias endógenas que medeiam um ou mais eventos na resposta inflamatória através da estimulação de receptores específicos (Galley & Webster, 1996). Estes, incluem substâncias que são armazenadas em vesículas na célula e liberadas durante a ativação celular, como a histamina e a serotonina (5-hidroxitriptamina, ou 5-HT); substâncias que são produzidas imediatamente após o estímulo, como os mediadores lipídicos derivados do ácido araquidônico, incluindo as prostaglandinas, os leucotrienos e o fator ativador de plaquetas (PAF); e citocinas, substâncias proteicas que são produzidas como resultado da transcrição de genes em resposta a ativação celular (Bloemen *et al*., 2007). Estas são principalmente representadas por um grande número de interleucinas (ILs), como a IL-1β, IL-6, IL-10, IL-4, IL-5, IL-13, IL-8 entre outras, além do fator de necrose tumoral (TNF)-α e o Interferon (IFN)-γ; e as quimiocinas, que são citocinas com propriedades quimioatraentes, importantes no recrutamento de leucócitos (Fanning & Boyce, 2013; Feghali & Wright, 1997).

A ativação celular consiste na estimulação de processos celulares em resposta a estímulos externos. É um fenômeno que depende do tipo de estímulo, do tipo celular e dos receptores envolvidos (Cheng & Jiang, 2013). Contudo, de um modo geral a ativação celular é resultante da transdução de sinal induzida pela interação entre moléculas e receptores imunes específicos, incluindo a interação entre PAMPs e PRRs (Janeway-Jr & Medzhitov, 2002), a

ligação cruzada entre os antígenos e a Imunoglobulina E (IgE) ligada a receptores FcεRI (Turner & Kinet, 1999), a ligação entre moléculas de MHC e os receptores de células B e T (Jackson *et al*., 2013; Smith-Garvin *et al*., 2009), a ligação de citocinas aos respectivos receptores (Leonard & Lin, 2000) ou ainda pela estimulação de receptores por agonistas farmacológicos ativadores do influxo de Ca^{2+} (Rönnberg *et al*., 2013). Em concordância com a diversidade de estímulos e receptores envolvidos, diversas vias de sinalização podem ser ativadas numa resposta inflamatória. Entretanto a maioria destas vias compartilha características comuns. Em síntese, os receptores inflamatórios estão acoplados a adaptadores, tais como o MyDD88 (do inglês: *myeloid differentiation primary response gene 88*) e o TRIF (do inglês: *Toll/interleukin-1 receptor (TIR) domain-containing adaptor inducing IFN-β*) em macrófagos ativados por LPS (Brierley & Fish, 2005), ou Syk, Fyn e Lyn quinases no caso de mastócitos ativados por IgE (Gilfillan &Tkaczyk, 2006). Estes adaptadores medeiam a ativação de proteinas quinases intracelulares, que desempenham importantes funções como a abertura de canais de Ca^{2+} e a ativação de enzimas intracelulares capazes de modular a liberação de mediadores inflamatórios, bem como a atividade de fatores de transcrição, tais como o fator nuclear kappa B (NFκB), o fator nuclear de células T ativadas (NFAT) e a proteína ativadora 1 (AP-1), que regulam a produção de citocinas, receptores inflamatórios e moléculas de adesão (Lee & Yang, 2013). Dentre estas enzimas intracelulares, as proteínas quinases ativadas por mitógenos (MAPKs) desempenham funções centrais na inflamação. estas proteinas constituem uma família de serina / treonina quinases que medeiam respostas celulares para sinais de estresse externos. O aumento da atividade das MAPKs, em particular a MAPK p38 e a JNK (do inglês: *c-Jun N-terminal kinases*) em resposta a estímulos inflamatórios, bem como o seu envolvimento na regulação da síntese de mediadores inflamatórios em nível de transcrição e tradução, tornam-nas alvos potenciais para terapias anti-inflamatórias (Kaminska, 2005).

Os leucócitos ativados, especialmente os macrófagos e os neutrófilos também são caracterizados por um alto consumo de oxigênio e em consequencia passam a produzir inúmeras espécies reativas de oxigênio (ROS) e nitrogênio. Dentre estas espécies, o óxido nítrico (NO) é um mediador que desempenha de funções imunomoduladoras significantes em diversas doenças inflamatórias, inclusive na asma (Prado *et al*., 2011). Vários estímulos inflamatórios, incluindo o LPS e citocinas como o TNF- α, a IL-1 e IFN-γ estimulam a produção de NO por induzir a expressão da isoforma induzível da enzima óxido nítrico sintase (iNOS). O óxido nítrico é produzido a partir do metabolismo do aminoácido L-arginina pela NOS ou pela arginase (Moncada, 1991; Moncada &Higgs, 2002)

A ativação celular também leva a produção de uma classe especial de mediadores, cuja síntese independe de transcrição gênica, conhecidos como mediadores lipídicos. Estas substâncias constituem uma classe de lipídios bioativos que são produzidos localmente através de vias biossintéticas específicas em resposta a estímulos extracelulares (Murakami *et al*., 2011). Atualmente é sabido que estas moléculas desempenham importantes funções como sinalizadoras e reguladores de diversos processos celulares, de tal forma que o desequilíbrio em vias de sinalização reguladas por mediadores lipídicos contribui significativamente para a progressão de diversas doenças inflamatórias (Bozza *et al.*, 2009).

Dentre estes mediadores, destacam-se os eicosanoides derivados do ácido araquidônico, incluindo as prostaglandinas, os leucotrienos, as lipoxinas e as epoxinas (Kendall & Nicolaou, 2013). Estes mediadores não são estocados na célula, mas são produzidos quando estímulos promovem a ativação de acil-hidrolases, especialmente a fosfolipase A_2 (PLA_2) que liberam o ácido araquidônico da membrana, a partir de fosfolipídeos como a fosfatidilcolina e a fosfatidiletanolamina (Alhouayek *et al*., 2013). Uma vez metabolizado, o ácido araquidônico é rapidamente metabolizado por diferentes vias enzimáticas, incluindo a via das prostaglandinas sintases e das lipoxigenases. As prostaglandinas sintases, conhecidas como ciclooxigenases (COX), originam as prostaglandinas (PGG_2, PGH_2, PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2 e PGJ_2) e o tromboxano A_2 (TXA_2) (Narumiyia & FitzGerald, 2001). As prostaglandinas estão envolvidas numa série de funções inflamatórias. A PGE_2, particularmente, está implicada na formação de edema, na dor (causando sensibilização de nociceptores) e na febre (interferindo no controle da temperatura no hipotálamo) (Thun *et al*., 1991), enquanto a PGD_2 desempenha funções chaves nas respostas alérgicas (Oguma *et al*., 2008). A via da 5-lipoxigenase (5-LO) leva a síntese dos leucotrienos que exercem funções essenciais no desenvolvimento e persistência da resposta inflamatória, incluindo o LTB_4, uma molécula com propriedades quimiotáticas que desempenha funções essenciais no recrutamento de neutrófilos (Afonso *et al*., 2012). Os leucotrienos C_4, D_4 e E_4, que são coletivamente conhecidos como cisteinil leucotrienos. Estes mediadores desempenham diversas funções inflamatórias e contráteis, mediadas por receptores acoplados a proteina G (GPCRs) e exercem um papel central na patogênese da asma (Capra *et al*., 2007, Mukarami *et al*., 2011).

Diversos estudos tem demonstrado que durante a ativação celular, especialmente em leucócitos, ocorre o aumento do número e tamanho de organelas citoplasmáticas conhecidas como corpúsculos lipídicos (CL). Os CL são classicamente definidos como organelas citoplasmáticas ricas em lipídeos, rodeadas por uma monocamada de fosfolipídios (Jhonson *et al*., 1999; Murphy, 2001). Estudos na estrutura e função dos CL demonstraram que estas

estruturas são mais do que simples reservatórios de lipídios. Estas organelas possuem um amplo conteúdo protéico, incluindo proteínas estruturais, proteínas envolvidas no metabolismo de lipídios e no transporte de membranas. Além disto, os CL armazenam várias enzimas formadoras de eicosanóides como: PLA_2, 5-LO, 15-LO, COX, LTC_4 e PGE sintase e ainda citocinas, quimiocinas e proteínas quinases envolvidas em diversas vias de sinalização (BOZZA *et al*., 2009).

1.1.3. A relação entre inflamação, nocicepção e dor

A inflamação e a nocicepção estão conectadas por muitas vias, mecanismos e funções comuns. Em termos evolutivos, isto pode entendido pelo fato estes dois componentes serem determinantes na capacidade do organismos de garantir auto-integridade (Riedel & Neeck, 2001). A reação inflamatória promove diversas alterações que aumentam a sensibilidade de nociceptores e em consequência a dor é dos principais sintomas da inflamação (Tracey, 2002).

A nocicepção é o processo pelo qual os neurônios sensoriais primários detectam estímulos dolorosos (Julius & Basbaum, 2011). Por outro lado, a dor é uma experiência sensorial e emocional associada a ou dano tecidual potencial ou real (Merskey, 1991). Deste modo, a dor é uma experiência complexa e subjetiva que envolve não apenas a transdução de estímulos ambientais nocivos, mas o processamento cognitivo e emocional pelo cérebro e portanto, depende das experiências individuais relacionadas com lesões ao longo da vida (Julius & Basbaum, 2001; Merskey, 1991).

A dor pode ser classificada em pelo menos três categorias: fisiológica, inflamatória e neuropática (Markenson, 1996). A dor fisiológica é originada pela geração de potenciais de ação de neurônios sensoriais especializados nos tecidos periféricos, que detectam uma grande variedade de estímulos nocivos, incluindo estímulos químicos (*ex.* ácidos) e físicos (*ex.* calor excessivo) (Julius & Basbaum, 2001), e é transferida ao sistema nervoso central através de uma cadeia de nocicepção transferência formada por três neurônios, que são chamados sequencialmente de neurônios de primeira, segunda e terceira ordem (Handwerker & Kobal, 1993). A dor inflamatória é causada pela estimulação inespecífica de neurônios sensoriais nos tecidos periféricos por mediadores inflamatórios e por fim, a dor neuropática é causada por lesão do sistema nervoso central ou periférico ou central. Contudo, ambas as formas de dor são caracterizadas por hipersensibilidade no local da lesão e nos tecidos adjacentes. Como consequência, estímulos que normalmente nunca causar dor podem passar a fazê-lo, uma condição conhecida como alodínia, ou estímulos nocivos podem causar dor maior e mais prolongada do que o esperado, caracterizando a hiperalgesia (Riedel & Neeck, 2001).

As lesões resultantes da reação inflamatória aumentam a experiência dolorosa a estímulos térmicos ou mecânicos, principalmente devido a liberação de mediadores a partir de terminações sensoriais primárias e por células não-neuronais, incluindo fibroblastos, mastócitos, neutrófilos e plaquetas (Bevan, 1999). Alguns destes mediadores, incluem os prótons, o trifosfato de adenosina (ATP), a serotonina, o fator de crescimento do nervo (NGF, do inglês: *nerve growth factor*) e especialmente a bradicinina e a PGE_2 (Fig 1.3). Estes mediadores agem através de vias de sinalização dependentes de canais iônicos ou de receptores acoplados a proteina G, presentes na superfície de neurônios nociceptores, provocando o influxo de cátions como Na^+ e Ca^{2+}, causando despolarização e diminuindo o limiar excitatório (Woolf & Salter, 2000) (Fig 1.3).

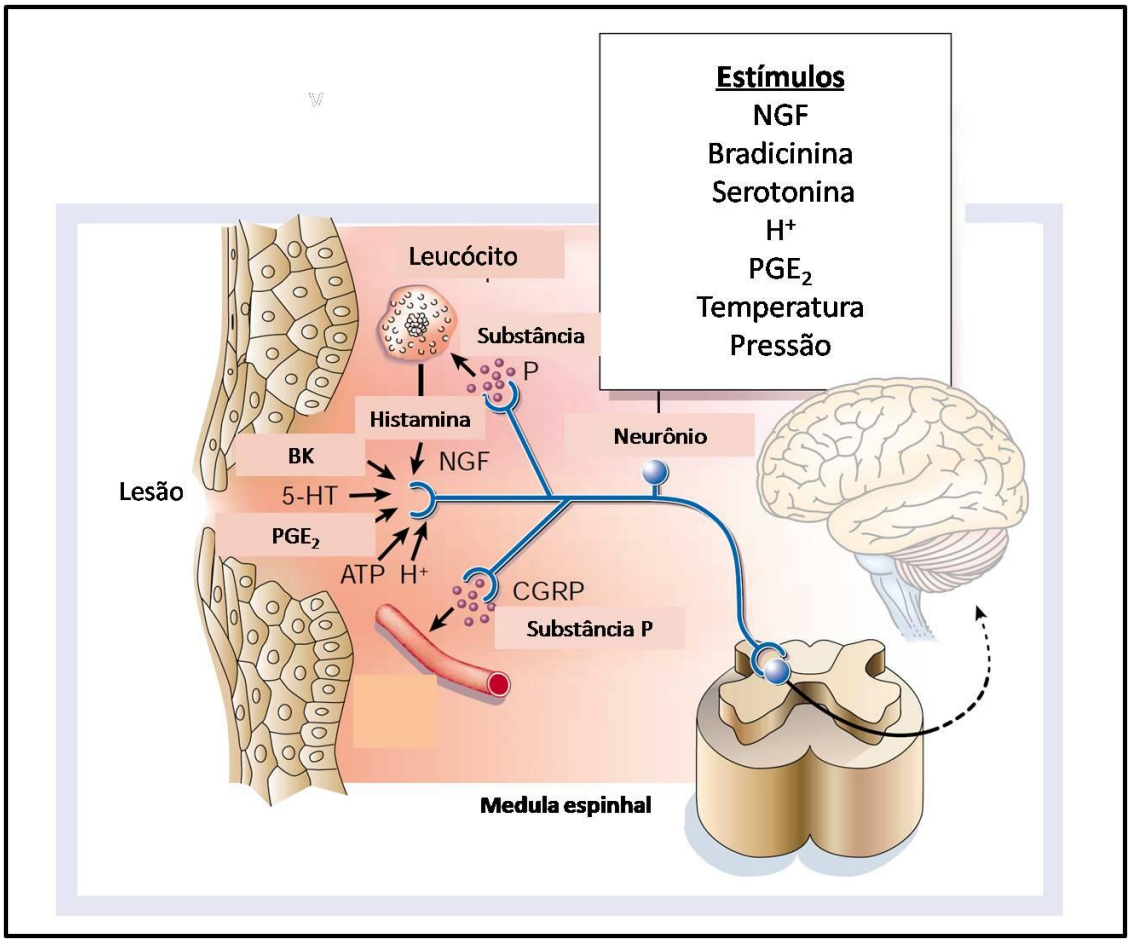

Figura 1.3. O papel dos mediadores inflamatórios na ativação de nociceptores. As lesões resultantes da reação inflamatória aumentam a experiência dolorosa a estímulos térmicos ou mecânicos, principalmente devido a liberação de mediadores que sensibilizam os nociceptores, diminuindo o limiar excitatório (adaptado de Julius & Basbaum, 2001. Nature).

A maioria dos tipos de dor pode ser significativamente inibidas por agonistas de receptores opióides, como a morfina. Estes fármacos são potentes analgésicos que agem inibindo as vias da dor no sistema nervoso central. Contudo, os opiáceos possuem o uso limitado por seu alto potencial para causar dependência e induzir mecanismos adaptativos que levam a tolerância (Kreek *et al*., 2012; Ueda & Ueda, 2009). No entanto, que diz respeito ao tratamento da dor inflamatória, os antiinflamatórios não esteroidais (AINEs) estão entre os fármacos mais indicados (Whittle, 2003)

Os AINEs estão entre as drogas mais amplamente utilizadas para o tratamento da febre, dor e inflamação moderada em todo o mundo. Estes fármacos constituem um grupo heterogêneo de substâncias que inibem a COX (Howe, 2007). Embora os AINEs apresentem um mecanismo de ação semelhante, estas drogas diferem em muitos aspectos, incluindo a potência farmacológica, a segurança e a seletividade (Patrono & Rocca, 2009). Os AINEs clássicos, tais como a aspirina, a indometacina e o diclofenaco inibem tanto a enzima constitutiva COX-1, como a isoforma COX-2, que é induzida em tecidos periféricos por diversos estímulos inflamatórios, como por exemplo, pela ação da IL-1β, cujo mecanismo envolve ativação de MAPKs como a ERK (do inglês: *extracellular-signal-regulated kinases*) e a p38 (Molina-Holgado *et al*., 2000).

A eficácia dos AINEs como antiinflamatórios e analgésicos é atribuida principalmente a inibição da produção da PGE_2, uma vez que um grande número de estudos indicam que dentre os mediadores produzidos pela ação da COX, a PGE_2 é aquele contribui mais significativamente na para o desenvolvimento dos sintomas inflamatórios. A PGE_2 promove sensibilização dos nociceptores periféricos por aumentar os níveis de AMP cíclico através da ativação de receptores acoplados à proteina G (Gs). Contudo alguns estudos indicam que este mediador também pode estar presente na medula espinhal, o que permitiria alguma ação em nível central (Julius & Basbaum, 2001).

Apesar da eficácia em combater os sintomas da maioria das inflamações moderadas, os AINEs clássicos apresentam efeitos colaterais gastro-intestinais graves, que limitam o seu uso a longo prazo. Os inibidores seletivos da COX-2 (coxibes) foram desenvolvidos com o objetivo de apresentar baixos efeitos GI, no entanto, esses medicamentos apresentaram eventos cardiovasculares graves (Inotai *et al.*, 2010). Recentemente, os AINEs doadores de NO, tais como nitro-aspirina, foram desenvolvidos com base na evidência de que o óxido nítrico desempenha um papel importante na regulação do fluxo sanguíneo, evitando danos GI e cardíaca (Whittle, 2003).

1.1.4. A inflamação alérgica

A inflamação alérgica é resultante de uma resposta imune exacerbada contra substâncias que, para a maioria das pessoas são inócuas. As doenças alérgicas mais comuns incluem a asma, a rinoconjuntivite, a sinusite, a alergia alimentar, a dermatite atópica, o angioedema, a urticária, a anafilaxia e alergia a drogas e insetos (Holgate & Polosa, 2008). A etiologia destas doenças é complexa e está associada a susceptibilidade genética de indivíduos a montar respostas mediadas por IgE a estímulos ambientais específicos, uma condição chamada de atopia (Alen, 2006; Sicherer & Sampson, 2007; Murdoch & Lloyd, 2010).

Os dados epidemiológicos mais recentes indicam que as doenças alérgicas possuem prevalência, morbidade e mortalidade crescente em todo o mundo. Estima-se que estas doenças afetem cerca de 20% da população mundial (Edwards *et al*., 2009). No Brasil, a asma afeta cerca de 10% dos indivíduos, demandando altos custos com a terapia , o que destaca esta doença como um dos principais problemas de saúde pública da atualidade (SBPT, 2012).

Uma característica especial das reações alérgicas a antígenos específicos é a necessidade de uma etapa prévia conhecida como sensibilização. A sensibilização alérgica consiste numa série de eventos que resultam na produção de IgE e sua ligação a receptores Fcε de alta afinidade (FcεRI) em mastócitos ou basófilos teciduais (Willart & Hammad, 2010). Neste processo, a exposição ao alérgeno induz o seu reconhecido e captura principalmente por células dendríticas (DCs) residentes no epitélio. Alguns estudos indicam que este reconhecimento é dependente de TLRs (Lambrecht *et al*., 1998) e leva a transdução de sinais essenciais para a maturação das células dendríticas (Chomarat *et al*., 2003; Yadav *et al*., 2006). A maturação das DCs, induzida pelo reconhecimento do alérgeno, envolve mudanças na expressão de diversas proteinas, incluindo as moléculas de MHC (complexo de histocompatibilidade principal) de classe II e moléculas co-estimulatórias como CD80 (B7-1) e CD86 (B7-2) que são fundamentais para a apresentação de antígenos aos linfócitos Th0 (Murdoch & Lloyd, 2010). A sinalização induzida pela interação entre MHC e TCR e entre as moléculas co-estimulatórias e CD 28 expresso por linfócitos, estimula a translocação do fator de transcrição NFAT1, o qual induz a expressão da proteína 3 ligante do GATA (GATA 3), o principal regulador da diferenciação de linfócitos Th0 em Th2. Este processo é essencial para o desenvolvimento das respostas alérgicas, pois os linfócitos Th2 secretam citocinas que orquestram a resposta inflamatória a longo prazo, tais como a IL-13, a IL-4 e a IL-5 (Rodriguez-Palmero *et al*., 1999). A diferenciação das células Th2 é também favorecida pelo próprio epitélio que secreta citocinas como a (IL)-25, a IL-33 e a Linfopoietina do estroma tímico (TLSP) que estimulam as DCs a induzirem a diferenciação de linfócitos Th2, além de

estimularem IL-4 especialmente por basófilos. A IL-4, por sua vez, é fundamental para a ativação de genes de citocinas do perfil Th2 (Paul &Zhu, 2010). No contexto da sensibilização, a IL-13 e a IL-4, secretadas por linfócitos Th2, induzem a mudança de classe de imunoglobulina para a produção de IgE por Linfócitos B ativados. Uma vez produzida e secretada, a IgE liga a receptores FcεRI em mastócitos e basófilos (Holgate & Polosa, 2008).

A reação alérgica propriamente dita é iniciada por eventos que levam a ativação de mastócitos provocando a desgranulação destas células e a liberação de mediadores inflamatórios pré-formados, tais como a histamina, além de várias citocinas e quimiocinas. Os produtos liberados na fase imediata iniciam a fase tardia, que envolve o recrutamento de células inflamatórias tais como: macrófagos, eosinófilos, neutrófilos e linfócitos T CD4+, especialmente os linfócitos Th2 (Cockcroft *et al.,* 2007; Gould & Sutton, 2008). Em modelos experimentais, o ato de fornecer estímulo(s) que desencadeie(m) uma reação alérgica é comumente chamado de desafio.

Em indivíduos sensibilizados, a ligação cruzada do alérgeno ao complexo IgE-FcεRI desencadeia uma cascata de eventos bioquímicos levando à desgranulação (Galli *et al.*, 1999; Gould & Sutton, 2008). Esse processo envolve a elevação da concentração citosólica do Ca^{2+}, seguida pela ativação de quinases tais como: a proteína quinase C (PKC), proteína quinase ativada por mitógeno (MAPK) e fosfatidilinositol-3-quinase (PI3K) (Huber *et al.,* 2000), com posterior translocação das vesículas contendo os grânulos e, em última instância, fusão com a membrana plasmática em um processo mediado, em parte, por proteínas de ancoramento da família das SNAREs (Puri *et al.*, 2003; Gilfillan &Tkaczyk, 2006)

Experimentalmente, eventos semelhantes podem ser produzidos pela estimulação direta dos mastócitos com compostos como a substância P, o peptídeo degranulador de mastócitos, o neuropeptídeo Y, agonistas de canais de cálcio e o composto sintético 48/80 (Tatemoto *et al.,* 2006). As reações alérgicas que envolvem a liberação sistêmica e súbita de mediadores, podem desencadear um quadro clínico conhecido como choque anafilático (Kemp & Lockey, 2002). O choque anafilático é uma reação grave na qual o indivíduo apresenta sintomas como falta de ar, hipotensão, arritmia cardíaca, vômitos, urticária, dor de cabeça e inconsciência, podendo levar o indivíduo ao óbito (Wade *et al.*, 1989).

As características fisiopatológicas das doenças alérgicas são diretamente influenciadas pelo ambiente no qual elas ocorrem. Deste modo, uma característica fundamental da maioria das alergias cutâneas é a coceira (Buddenkotte & Steinhoff, 2010). Neste contexto, a coceira é principalmente resultante da estimulação de receptores específicos em neurônios por uma grande variedade de mediadores, tais como: histamina, a serotonina, a substância P, o LTB_4, a IL-31, prostaglandinas e proteases (Garibyan *et al*., 2013; Greaves *et al*., 2010). A coceira

severa constitui um importante problema porque induz o "*scratching behavior*" (em português: *comportamento de coceira*) resultando na formação de lesões na pele, que pioram a situação dos indivíduos alérgicos (Wahlgren, 1991).

A asma alérgica é uma doença caracterizada por uma inflamação crônica das vias aéreas que está associada a um intenso recrutamento e ativação de leucócitos, remodelamento tecidual e hiperreatividade das vias aéreas (AHR) (Fig 1.4) (Barnes, 2008; Paul & Zhu, 2010). A AHR é uma característica chave em episódios de asma. Esta condição é definida como um estreitamento das vias aéreas em resposta a estímulos não específicos, que geralmente não afetam as vias aéreas de indivíduos normais (Barnes, 2008; Lauzon *et al*., 2012). A AHR é resultante de interações complexas entre os leucócitos ativados e a musculatura lisa das vias aéreas e estudos prévios demonstraram que e os mediadores inflamatórios, incluindo a IL-13 e os cisteinil leucotrienos (CisLTs) desempenham funções importantes neste processo (Yang *et al*., 2005).

No que diz respeito ao recrutamento e ativação de leucócitos na asma alérgica, diversos estudos tem demonstrado que os eosinófilos possuem um papel crítico na patogênese desta doença (Gleich *et al*., 1993; Weller, 1997; Rothenberg, 1998). Estas células são recrutadas para o pulmão e ativadas em resposta a estímulos de mediadores como a IL-5 e a eotaxina. Uma vez ativados, os eosinófilos produzem inúmeras citocinas (incluindo a IL-13), além de proteases (como a proteina básica principal e a peroxidase eosinofílica) e mediadores lipídicos, que amplificam o processo inflamatório e causam injuria tecidual, remodelamento e AHR. No que diz respeito ao papel da eotaxina neste processo, Vieira-de-Abreu e colaboradores (2005) demonstraram que esta quimiocina efetivamente induz a tanto o recrutamento como a ativação de eosinófilos, aumentando a geração de corpúsculos lipídicos nestas células. Embora estas organelas possam ser encontradas em eosinófilos não-estimulados, um aumento na formação de cospúsculos lipídicos durante a inflamação alérgica é importante para a produção de eicosanoides, especialmente de CisLTs, que causam vários efeitos inflamatórios, incluindo: broncoconstrição, hipersecreção de muco, aumento da permeabilidade microvascular, hiperresponsividade brônquica e infiltrado eosinofílico (Lewis *et al*., 1990; Laitinen *et al*., 1993). Desta forma, o recrutamento e subseqüente ativação de eosinófilos nos sítios de inflamação alérgica são eventos-chaves na patogênese da asma e outras doenças alérgicas (Weller, 1997).

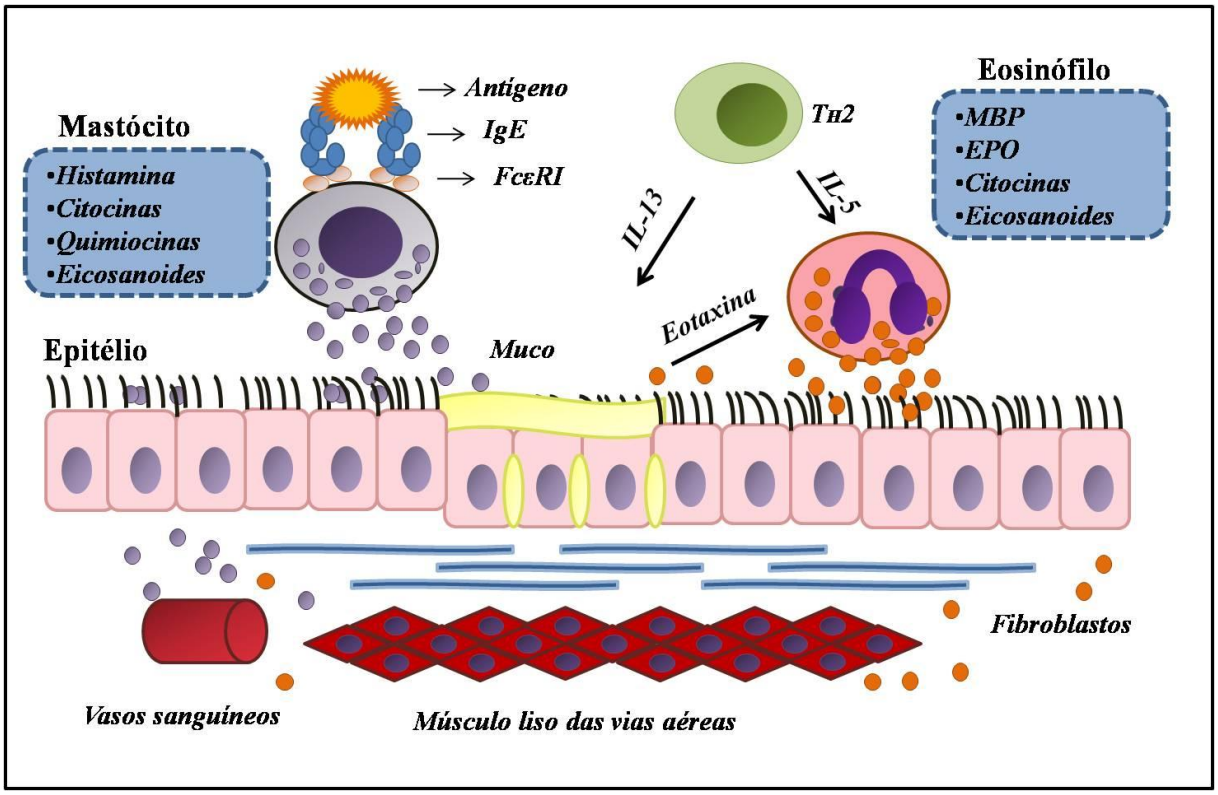

Figura 1.4. A inflamação alérgica pulmonar na asma. O ambiente inflamatório no pulmão asmático é caracterizada pela presença de leucócitos ativados, incluindo mastócitos e linfócitos Th2 e eosinófilos, que são recrutados para o sítio inflamatório, onde desempenham várias funções efetoras através da liberação de citocinas, eicosanoides e proteases que perpetuam a inflamação e causam mudanças estruturais no epitélio e na musculatura lisa, levando a AHR e ao remodelamento das vias aéreas (adaptado de Paul & Zhu, 2010. Nature Reviews Immunology).

1.1.4.1. A Terapia farmacológica da Asma

A terapia farmacológica da asma envolve a utilização de fármacos que aliviam e controlam os sintomas da doença (Holgate e Polosa , 2008). Os medicamentos que são amplamente utilizados no tratamento da asma hoje incluem potentes corticosteróides inalatórios, tais como a budesonida e a fluticasona; agonistas β2 - adrenérgicos de longa ação (LABA), tais como o salmeterol e o formoterol , e modificadores de leucotrienos, incluindo o zafirlucaste, o montelucaste e o zileuton; também existem terapias combinadas, que incluem corticosteróides inalatórioss e LABAs em um dispositivo de liberação única (Szefler *et al*., 2011).

Os corticosteróides são fármacos anti- inflamatórios potentes que regulam a expressão de citocinas, quimiocinas e moléculas de adesão , modulando a atividade de fatores de transcrição tais como NF-kB e a AP-1. Os Corticosteróides inalatórios são muito eficazes em

inibir a inflamação das vias aéreas, e eles representam uma importante ferramenta no manejo da asma (Ivancsó *et al*., 2013). No entanto, esses medicamentos não são eficazes , em condições específicas, incluindo exacerbações induzidas por vírus ou fumaça (Harrison *et al*., 2004).

Os agonistas β_2- adrenérgicos promovem broncodilatação por meio da produção do 3'5'- monofosfato de adenosina cíclico (AMPc) e a ativação da proteína quinase A (PKA). Os agonistas β_2 -adrenérgicos de curta ação são mais comumente usados em associação com corticosteróides inalatórios como uma terapia complementar para asma (Holgate e Polosa , 2008). Os inibidores da fosfodiesterase também aumentam o AMPc, mas esses tratamentos tem sido menos comumente utilizados, devido ao baixo índice terapêutico de algumas fármacos (Boswell - Smith *et al*., 2006).

Os Antagonistas do receptor 1 de cisteinil leucotrienos (CisLTR1) estão disponíveis atualmente e são importantes ferramentas terapêuticas na asma. Estes fármacos agem bloqueando muitas atividades dos CisLTs, incluindo a broncoconstrição, e são usados principalmente como terapia complementar aos corticosteróides inalatórios (Polosa, 2007). Por último, os inibidores de mastócitos, os anticorpos monoclonais bloqueadores de citocinas e as imunoterapias específicas para o alérgeno são também importantes no tratamento da asma e outras doenças alérgicas (Edwards e Howell , 2000; Holgate e Polosa, 2008).

Apesar da variedade de terapias actualmente disponíveis anti- asmáticos, a procura de novos produtos químicos estruturalmente e para o desenvolvimento de medicamentos seguros e eficazes para o tratamento da asma e outras doenças alérgicas permanece um importante campo de investigação.

1.2. O desenvolvimento de fármacos a partir de produtos naturais

Os produtos naturais possuem um papel importante na História da medicina e em especial, na História da farmacologia. Ainda na antiguidade o ser humano descobriu o potencial curativo das plantas e passou a utilizá-las no tratamento de suas enfermidades (Teske & Trentini, 1994). O isolamento da morfina (um constituinte do ópio) a partir da planta *Papaver somniferum* (Papoula) por Friedrich Sertürner, há cerca 200 representou um marco no desenvolvimento de fármacos, uma vez que constituintes purificados poderiam ter suas propriedades farmacológicas e/ou tóxicas estudadas e administradas em doses precisas (Hamilton & Baskett, 2000).

O desenvolvimento da pesquisa na área farmacêutica, especialmente depois da Segunda Guerra mundial em que havia um grande interesse na descoberta de antibióticos

ampliou bastante o isolamento e identificação de novas estruturas biologicamente ativas, de modo que em 1990, cerca de 80% dos medicamentos eram produtos naturais ou análogos desenvolvidos com base na estrutura deles, incluindo: Antibióticos, antiparasitários, antimaláricos, agentes de controle de lipídeos, imunossupressores (essenciais em transplantes) e antineoplásicos. Estas descobertas tiveram um enorme impacto na qualidade e expectatuva de vida da população, que passou de 40 anos no início do XX para mais de 77 anos, atualmente (Harvey, 2008).

Apesar do impacto positivo no desenvolvimento da indústria farmacêutica, os dados mais recentes indicam que a proporção de medicamentos desenvolvidos a partir de produtos caiu para aproximadamente 50% em 2008 (Li &Vederas, 2009). Uma das razões para esta queda foi a mudança, nos últimos anos, no cenário farmacêutico, que passou a ter mais exigências com relação a testes de eficácia e segurança dos produtos por parte das agências reguladoras, o que aumentou o tempo e custo no desenvolvimento de novos fármacos, além de questões específicas relacionadas a patentes e relações entre empresas farmacêuticas (Koehn &Carter, 2005; Malik, 2008). Outra dificuldade na pesquisa com produtos naturais é a falta de métodos eficientes em encontrar candidatos a fármacos com atividade desejável, além da falta de tecnologia apropriada para sintetizar e modificar estruturalmente algumas moléculas de origem natural, devido a abundância de substituintes e centros estereoquímicos (Butler, 2004). Contudo, a perspectiva é de que num futuro, novas tecnologias sejam desenvolvidas para superar a maioria destas dificuldades e portanto o desenvolvimento de fármacos a partir de produtos naturais permanece um importante campo de investigação científica (Li &Vederas, 2009).

Os alcalóides compreendem a maior classe de produtos naturais derivados de plantas. Estas substâncias são produzidos pelo metabolismo secundário das plantas e vários estudos indicam que estes metabólitos são dotados de uma grande diversidade de atividades farmacológicas. Os alcaloides substâncias de caráter alcalino que contém um ou mais átomos de nitrogênio, geralmente em combinação, como parte de um sistema cíclico (Harborne, 1991). Segundo Barbosa-Filho e colaboradores (2006), somente no século XX, mais de 170 alcalóides foram testados em todo o mundo quanto à atividade antiinflamatória. Destes, cerca de 140 compostos mostraram-se farmacologicamente ativos em diferentes modelos experimentais, sendo a maioria destes compostos pertencente à classe de alcaloides isoquinolínicos.

Vários estudos têm mostrado que os alcaloides bisbenzilisoquinolínicos (BBAs) possuem atividade imunomoduladora contra doenças inflamatórias e alérgicas (Seow *et al*., 1986; Teh *et al*., 1990). Nos últimos anos, o nosso grupo de pesquisa tem investigado o

potencial terapêutico de produtos naturais e resultados promissores teme sido obtido, em especial no que diz respeito ao uso da planta *Cissampelos sympodialis* EICHL (Menispermaceae) e o alcaloide bisbenzilisoquinolínico warifteina em modelos animais de inflamação e alergia. Os efeitos imunomoduladores produzidos pelo extrato da planta bem como pela warifteina incluem a inibição da proliferação de esplenócitos e aumento produção de IL-10 (Piuvezam *et al*., 1999), inibição da produção de IgE específica para ovalbumina (OVA) (Bezerra-Santos *et al*., 2004) e inibição do choque anafilático induzido por OVA (Bezerra-Santos *et al*., 2005); inibição do recrutamento e ativação de eosinófilos *in vivo*, associado com redução da produção de eotaxina e cisLTs (Bezerra-Santos *et al*., 2006); inibição da ativação de mastócitos in vitro (Costa *et al*., 2008), além da modulação da AHR e do remodelamento das vias aéreas em modelo de asma experimental (Bezerra-santos *et al*., 2012).

1.2.1. A planta *Chondrodendron platyphyllum* e o alcaloide curina

Chondrodendron platyphyllum A. St. Hil (Miers) (Menispermaceae) é uma planta medicinal encontrada no nordeste do Brazil. Esta planta é conhecida popularmente como "abútua", "abútua grande" e "uva do mato" e tem sido usada na medicina popular para tratar uma grande variedade de condições, incluindo a malária, febre, dor, edema, uretrite, cistite, úlceras e distúrbio menstrual (Correa, 1984; Gotfredsen, 2013). De acordo com Silva (2006), *C. platyphyllum* tem sido utilizado em conjunto com *Zanthoxylum articulatum* A. St. Hil. para compor o medicamento fitoterápico Uva-mato ®, que é utilizado para tratar cãibras abdominais, renal e e dor muscular.

A análise fitoquímica da raiz de *C. platyphyllum* revelou que esta planta é rica em alcaloides bisbenzilisoquinolínicos, um grupo de compostos naturais com propriedades farmacológicas interessantes, como propriedades anti-inflamatórias, anti-alérgicas e analgésicas (Souto, 2011). Pelo menos três alcalóides, incluindo a curina, a isocurina e a 12-O-metilcurina foram identificados a partir desta planta e curina (Fig. 1.5) é o constituinte principal (Dias *et al*., 2002).

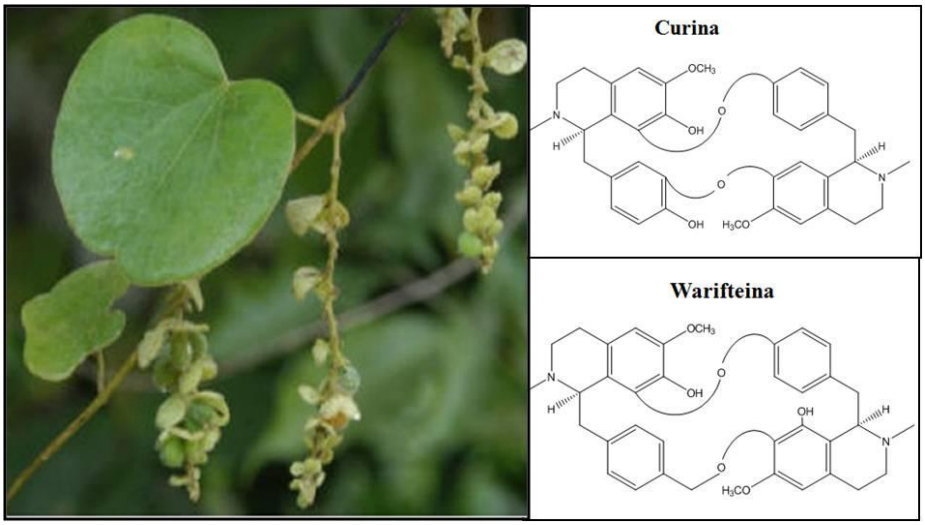

Figura 1.5. A planta *Chondrodendron platyphyllum* e os alcaloides curina e warifteina

Estudos anteriores demonstraram que os alcalóides extraídos de *Chondrodendron platyphyllum* são farmacologicamente ativos (Guedes *et al*., 2002). Dias e colaboradores (2002) demonstraram que a curina e a isocurina possuem efeitos vasodilatadores e sugeriram que os efeitos da curina estão associados com a inibição de canais de cálcio. Medeiros e colaboradores (2011) demonstraram que a curina diminuiu as correntes transientes de Ca^{2+} intracelular em células A7r5, possivelmente através de um bloqueio direto de canais de Ca^{2+} do tipo L. No entanto, apesar da interessante atividade farmacológica da curina e da sua semelhança estrutural com a warifteína, as propriedades antiinflamatórias e antialérgicas da curina e os seus potenciais efeitos tóxicos permanecem por ser caracterizados. Utilizando modelos murinos de inflamação e asma alérgica e não encontrando nenhuma toxicidade detectável, o presente estudo relata os efeitos benéficos da curina como um composto antiinflamatório e anti-alérgico ativo por via oral.

2. OBJETIVOS

2.1 Analisar os efeitos tóxicos induzidos pelo tratamento oral com a curina.

2.2. Investigar o efeito da curina na ativação de eosinófilos e na hiperreatividade das vias aéreas em um modelo de asma alérgica experimental.

2.3. Caracterizar o efeito anti-inflamatório e analgésico da curina em camundongos.

2.4. Investigar o efeito da curina na ativação de macrófagos e no recrutamento de neutrófilos em um modelo experimental de inflamação induzida por LPS.

3. ARTIGOS ANEXADOS

3.1. Artigo I

CURINE INHIBITS EOSINOPHIL ACTIVATION AND AIRWAY HYPER-RESPONSIVENESS IN A MOUSE MODEL OF ALLERGIC ASTHMA. Ribeiro-Filho, J.; Calheiros, A. S.; Vieira-de-Abreu, A.; Carvalho, K. I. M.; Mendes, D. S.; Bandeira-Melo, C.; Martins, M. A.; Dias, C. S.; Piuvezam, M. R.; Bozza, P. T. Toxicology and Applied Pharmacology, v. 273, p. 19-26, 2013.

Neste primeiro artigo nós caracterizamos o efeito antialérgico da curina utilizando um modelo de asma alérgica experimental e avaliamos diversos parâmetros toxicológicos em animais tratados por via oral com curina. Nós demonstramos que a administração oral da curina inibiu significativamente a inflamação eosinofílica , formação de corpos de lípidicos em eosinófilos e AHR em animais desafiados com OVA em comparação com os animais do grupo não tratado. O tratamento com a curina também reduziu a produção de eotaxina e IL-13. O verapamil , um antagonista de canais de cálcio, apresentou propriedades anti -alérgicas semelhantes, e o pré- tratamento com a curina inibiu a resposta contráctil induzida por cálcio em anéis de traqueia *ex vivo,* o que sugere que o mecanismo pelo qual a curina exerce os seus efeitos é através da inibição de uma resposta dependente de cálcio. A avaliação toxicológica mostrou que a curina administrada oralmente por 7 dias em doses até 10 vezes superiores a sua dose efetiva mediana (DE_{50}) , não alterou significativamente os parâmetros bioquímicos, hematológicos , comportamentais e físicos em animais experimentais em comparação com os animais tratados com solução salina. Estes resultados indicam que a curina possui atividade anti- alérgica que se dá por meio de mecanismos que envolvem a inibição de IL -13 e eotaxina e inibição do influxo de Ca^{2+}, sem induzir toxicidade evidente.

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Curine inhibits eosinophil activation and airway hyper-responsiveness in a mouse model of allergic asthma

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ABSTRACT

Allergic asthma is a chronic inflammatory airway disease with increasing prevalence around the world. Current asthma therapy includes drugs that usually cause significant side effects, justifying the search for new anti-asthmatic drugs. Curine is a bisbenzylisoquinoline alkaloid that modulates calcium influx in many cell types; however, its anti-allergic and putative toxic effects remain to be elucidated. Our aim was to investigate the effects of curine on eosinophil activation and airway hyper-responsiveness (AHR) and to characterize its potential toxic effects. We used a mouse model of allergic asthma induced by sensitization and challenge with ovalbumin (OVA) to evaluate the anti-allergic effects of oral treatment with curine. The oral administration of curine significantly inhibited eosinophilic inflammation, eosinophil lipid body formation and AHR in animals challenged with OVA compared with animals in the untreated group. The curine treatment also reduced eotaxin and IL-13 production triggered by OVA. Verapamil, a calcium channel antagonist, had similar anti-allergic properties, and curine pre-treatment inhibited the calcium-induced tracheal contractile response *ex-vivo*, suggesting that the mechanism by which curine exerts its effects is through the inhibition of a calcium-dependent response. A toxicological evaluation showed that orally administered curine did not significantly alter the biochemical, hematological, behavioral and physical parameters measured in the experimental animals compared with saline-treated animals. In conclusion, curine showed anti-allergic activity through mechanisms that involve inhibition of IL-13 and eotaxin and of Ca^{++} influx, without inducing evident toxicity and as such, has the potential for the development of anti-asthmatic drugs.

Introduction

Allergic asthma is an important public health problem in terms of prevalence, morbidity and mortality, affecting approximately 20% of the world's population and requiring costly therapy (Chatila, 2004; Edwards et al., 2009). The pathophysiology of asthma is complex and results from an inappropriate immune response to common allergens; this response is characterized by chronic airway inflammation that is associated with intense leukocyte recruitment and activation at the site of injury and airway hyper-responsiveness (AHR) (Barnes, 2008; Paul and Zhu, 2010).

The current standard for the treatment of asthma involves the use of drugs that relieve and control the symptoms of the disease (Holgate and Polosa, 2008). The medications that are broadly used in asthma management today include potent inhaled corticosteroids, such as budesonide and fluticasone; long-acting β_2-adrenergic agonists (LABAs), such as salmeterol and formoterol; and leukotriene modifiers, including zafirlukast, montelukast and zileuton; there are also combination therapies that include inhaled corticosteroids and LABAs in a single delivery device (Szefler, 2011). Corticosteroids are potent anti-inflammatory drugs that regulate the expression of cytokines, chemokines and adhesion molecules by modulating the activity of transcription factors such as nuclear factor-κB (NF-κB) and activator protein 1 (AP1). Inhaled corticosteroids are very effective at inhibiting airway inflammation, and they represent an important tool in asthma management (Ivancsó et al., 2013). However, these drugs are not effective under specific conditions, including virus- or smoke-induced exacerbations (Harrison et al., 2004). β_2-Adrenergic agonists effectively promote bronchodilation through the production of cyclic adenosine 3′5′-monophosphate (cAMP) and the activation of protein kinase A (PKA). Inhaled short- and long-acting β_2-adrenergic agonists are

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currently most commonly used in association with inhaled corticosteroids as a supplementary therapy for asthma (Holgate and Polosa, 2008). Phosphodiesterase inhibitors also increase cAMP, but these treatments have been less commonly used of late because of the low therapeutic index of some drugs (Boswell-Smith et al., 2006). Antagonists of cysteinyl leukotriene receptor 1 (CysLTR1) are currently available and are important therapeutic tools in asthma. These drugs act by blocking many activities of CysLTs, including bronchoconstriction, and are mainly used as a supplementary therapy to inhaled corticosteroids (Polosa, 2007). Finally, mast cell inhibitors, cytokine-blocking monoclonal antibodies and allergen-specific immunotherapies are also important in the treatment of asthma and other allergic diseases (Edwards and Howell, 2000; Holgate and Polosa, 2008).

Despite the currently available array of anti-asthmatic therapies, the search for structurally novel chemicals and for the development of safe and effective drugs for the treatment of asthma and other allergic conditions remains an important field of investigation. Several studies have shown that bisbenzylisoquinoline alkaloids (BBAs) have immunomodulatory activity against allergy and inflammation (Seow et al., 1986; Teh et al., 1990). Our group has demonstrated that the alkaloid warifteine has anti-allergic properties, including the inhibition of anaphylaxis, the inhibition of eosinophil recruitment and activation, the ability to modulate airway hyper-responsiveness and remodeling (Bezerra-Santos et al., 2005, 2006, 2012) and the ability to inhibit histamine release (Costa et al., 2008).

Curine (Fig. 1A) is a BBA that is the major constituent of the root bark of *Chondrodendron platyphyllum* (Menispermaceae). This compound has a molecular structure that is similar to that of warifteine. Recently, Medeiros et al. (2011) demonstrated that curine may have direct effects on L-type Ca^{2+} channels in vascular smooth muscle cells. However, despite curine's interesting pharmacological activity and its structural similarity to warifteine, this molecule's anti-allergic properties and potential for toxic effects remain to be investigated. Using a mouse model of allergic asthma and finding no detectable toxicity, our study is the first to report that curine is beneficial as an orally active anti-allergic compound.

Methods

Curine purification. *C. platyphyllum* Hil St. (Miers) was collected in the municipality of Santa Rita, Paraíba, Brazil. The voucher specimen of this plant is deposited in the Herbarium Prof. Lauro Pires Xavier, number 3631-P, and was identified by Prof. Dr Maria de Fatima Agra. Spectroscopically pure curine was isolated from the root bark of *C. platyphyllum* as described by Mambu et al. (2000). The curine solution was prepared using 1 mg of the crystal in 50 μl of 1 N HCl and 500 μl of distilled water.

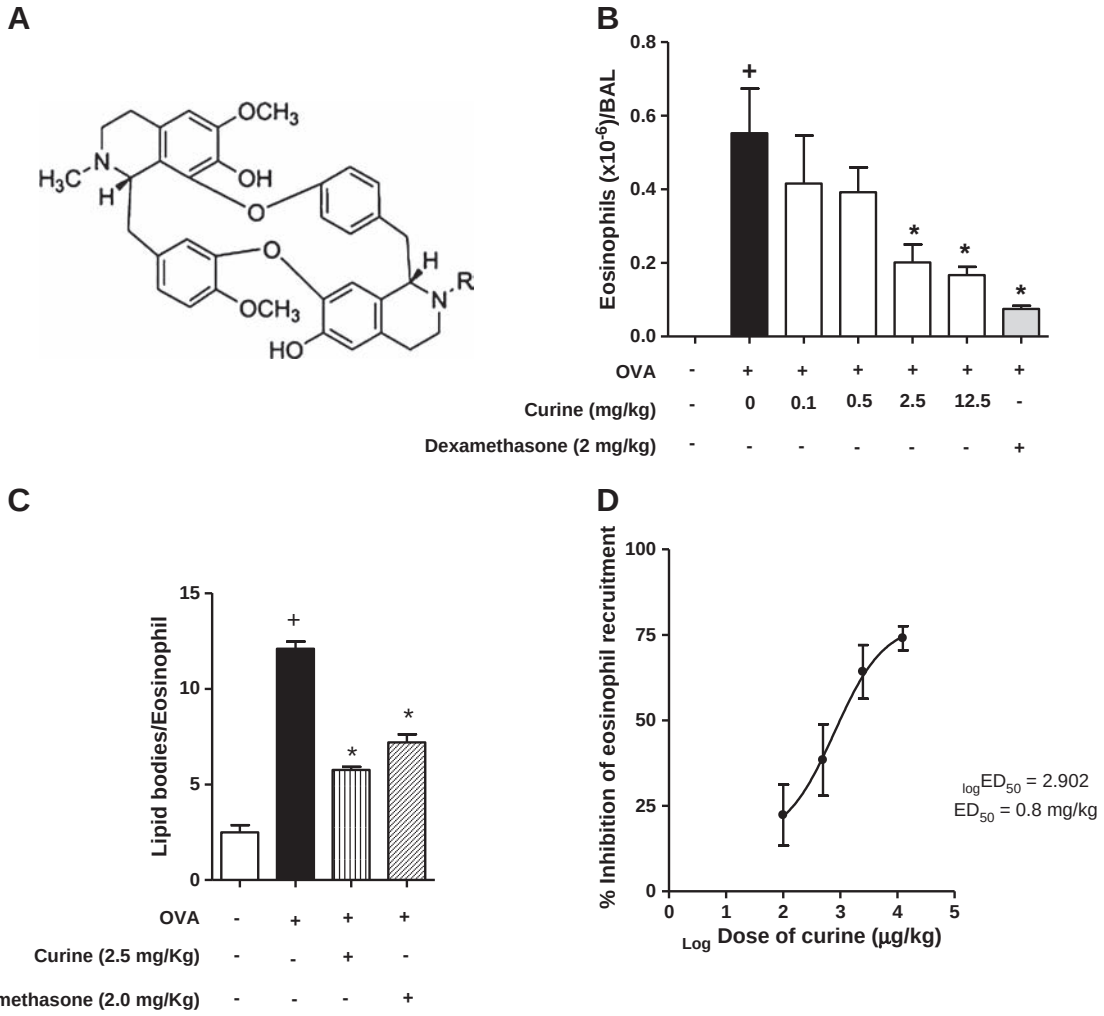

Fig. 1. The effect of curine on eosinophil recruitment and activation. BALB/c mice (n = 5–7) were treated orally with increasing doses of curine (0.1, 0.5, 2.5 or 12.5 mg/Kg), or dexamethasone (2 mg/Kg) 1 h before each challenge. Twenty-four hours after the last challenge, the BAL was collected and the eosinophils and lipid bodies were counted using a light microscope. A) the chemical structure of curine; B) the number of BAL eosinophils; C) the number of lipid bodies per eosinophil; D) a dose–response curve of curine on the inhibition of eosinophil recruitment. These results are expressed as the mean ± SEM of at least 5 animals. + Significantly different ($p < 0.05$) from the unchallenged group; * significantly different from the untreated, OVA-challenged group.

The pH was adjusted to 7–8 with 1 N NaOH. The volume was adjusted to 1000 μl, and the dilutions were made in phosphate-buffered saline (PBS).

Animals. Male BALB/c or Swiss mice weighing 20–30 g were obtained from the Oswaldo Cruz Foundation breeding unit. The animals were maintained with food and water *ad libitum* in a room with the temperature ranging from 22 to 24 °C and a 12 h light/dark cycle. This study was carried out in accordance with the recommendations of the Brazilian National Council for the Control of Animal Experimentation (CONCEA). The protocols were approved by the Animal Welfare Committee of the Oswaldo Cruz Foundation (CEUA/FIOCRUZ protocol # L-002/08).

Treatments. To perform the dose–response curve with eosinophil recruitment as a measured outcome, animals were treated orally with different doses of curine (0.1, 0.5, 2.5 or 12.5 mg/Kg) or dexamethasone (2 mg/Kg) orally (p.o.) 1 h before each challenge (pre-treatment protocol). Alternatively, the mice were treated with curine (2.5 mg/Kg) 1 h after the last challenge (post-treatment protocol). The dose of curine (2.5 mg/Kg) was chosen based on the results obtained from the pre-treatment dose–response curve. Verapamil (2.5 mg/Kg) was given as a control calcium channel antagonist. For the *ex-vivo* experiments, 9 μM curine was used. This concentration was found to be the approximate IC_{50} for the inhibition of calcium influx in the experiments performed by Medeiros et al. (2011). To evaluate the toxicological parameters, animals were treated orally once a day for 7 consecutive days with curine (2.5 or 8 mg/Kg) or dexamethasone (2 mg/Kg). The control group was treated orally with PBS. Importantly, the 8 mg/Kg dose of curine is 10-fold higher than its median effective dose (ED_{50}).

Toxicological evaluation. The male Swiss mice were treated as described above; their weight, behavior, and primary physical aspects (*e.g.*, activity, breath, piloerection, convulsions and diarrhea) were monitored daily. Twenty-four hours after the last treatment, the animals were euthanized by exposure to an atmosphere of CO_2, and the blood was collected by cardiac puncture. EDTA was added to the blood samples for the hematological analyses of leukocytes, red blood cells (RBC), platelets, hematocrit, hemoglobin, the mean corpuscular hemoglobin concentration (MCHC), the mean corpuscular volume (MCV) and the mean corpuscular hemoglobin (MCH). Blood samples were centrifuged at 6000 *g* for 8 min at 4 °C to separate out the serum. The serum concentrations of alkaline phosphatase, alanine transaminase (ALT), aspartate transaminase (AST), bilirubin, creatinine, creatinine kinase, cholesterol, glucose, total proteins and uric acid were determined. The relative weight and the macroscopic aspects of the stomach, liver, spleen, thymus, intestines, kidneys and lungs were analyzed, and the presence of gastric ulcer was assessed through macroscopic and histological analyses.

Airway inflammation in actively sensitized mice. Allergic airway inflammation was induced as described by Lloyd et al. (2001). The animals were sensitized intraperitoneally (i.p.) with ovalbumin (OVA — 10 μg/mouse) and $Al(OH)_3$ (10 mg/mL) in a 0.9% NaCl solution (saline 0.2 mL) on days 1 and 10. From day 19 to day 24 after sensitization, the mice were challenged daily for 20 min with OVA (5%) in PBS by aerosol. Aerosolized PBS was administered to some of the sensitized mice as a negative control. These procedures were performed in a 30 × 20 × 10 cm acrylic chamber, and the aerosol was generated by an ultrasonic nebulizer. Twenty-four hours after the last challenge, the animals were euthanized by exposure to an atmosphere of CO_2, and the trachea was surgically exposed and cannulated. The bronchoalveolar lavage (BAL) was collected from mice by washing the lungs with 1 mL of PBS.

Leukocyte counts and lipid body enumeration. Total leukocyte counts were performed using a Neubauer chamber under a light microscope after diluting the BAL samples in Turk fluid (2% acetic acid). Differential counts were performed. To enumerate lipid bodies, the cells, while still moist, were fixed in 3.7% formaldehyde (diluted in Ca^{2+}/Mg^{2+}-free HBSS; pH 7.4), rinsed in 0.1 M cacodylate buffer (pH 7.4), stained with 1.5% OsO_4 for 30 min, rinsed in distilled H_2O, immersed in 1.0% thiocarbohydrazide for 5 min, rinsed in 0.1 M cacodylate buffer, restained with 1.5% OsO_4 for 3 min, rinsed in distilled water and then dried and mounted (Bozza et al., 1997). The cell morphology was observed and the lipid bodies were enumerated by microscopy with an objective lens at 100× magnification. Twenty-five consecutively scanned eosinophils were evaluated, and the results were expressed as the mean numbers of lipid bodies per eosinophil.

Cytokine quantification. Twenty-four hours after the last challenge, the lungs of the mice were surgically removed. Each lung was treated with 800 μl of a complete cocktail solution of protease inhibitors (Roche Diagnostics GmbH / Mannheim', Germany) containing both reversible and irreversible protease inhibitors and 20 μM EDTA. The cocktail solution was prepared by dissolving 1 tablet in 50 ml of 100 mM phosphate buffer, pH 7.0. The lungs were then macerated, the samples were centrifuged at 6000 *g* for 20 min at 4 °C, and the supernatants were collected. The total protein concentration of these samples was determined using a kit for the determination of proteins (BCA, from Pierce, Rockford, IL). The concentrations of IL-13 and eotaxin were measured using DuoSet kits according to the manufacturer's instructions (R&D Systems).

Analysis of airway hyper-responsiveness. Airway hyper-responsiveness (AHR) in the mice was analyzed using non-invasive whole body plethysmography (Buxco, Sharon, CT) 24 h after the last OVA challenge. AHR was measured after the aerosolization of PBS followed by increasing concentrations of methacholine (0, 6, 12.5 and 25 mg/mL; Sigma-Aldrich), each of which was injected into the chamber for 2 min. The AHR data were expressed as an average enhanced pause (Penh). There was an interval of 10 min between each aerosol exposure, and within this period of time, the Penh values had returned to baseline.

Analysis of the trachea contractile response ex vivo. Tissue preparation. Male Wistar rats (8 weeks) were euthanized in a CO_2 chamber; the tracheas were removed and were immediately immersed in Krebs nutritive solution (118 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 24 mM $NaHCO_3$ and 11 mM glucose). The tracheas were dissected and cut into segments of 3–4 rings. The segments were mounted in isolated organ baths containing 10 mL of Krebs solution maintained at 37 °C and aerated with a carbogenic gas mixture (95% O_2 and 5% CO_2). To achieve a constant level of spontaneous tone, an initial tension of 1 g was applied. Contractions were measured isometrically with a force-displacement transducer (Ugo Basile, Comerio, Italy) and were recorded by an isolated organ data acquisition program (Proto5; Letica Scientific Instruments, Barcelona, Spain).

Calcium-concentration response curve. Tissues were stabilized over a period of 60 min; the bath solution was changed every 10 min. At the end of the equilibration period, the response to carbachol (2.5 mM) was recorded. After the removal of the carbachol and the restoration of the tone to a stable baseline, the tissues were exposed to repeated cycles of stimulation with 60 mM KCl and washing in a calcium-free Krebs solution until there was complete desensitization of the contractile response evoked by 60 mM KCl. Then, the tracheal segments were immersed in Ca^{2+}-free Krebs solution containing 60 mM KCl, and the concentration of extracellular Ca^{2+} was gradually increased by the cumulative addition of $CaCl_2$ (0.01 to 100 mM) in the presence or absence of 9 mM curine or vehicle. All of the responses were expressed as a percentage of the response to 2.5 mM carbachol (Foster et al., 1984).

Statistical analysis. Data were analyzed by one-way ANOVA followed by Tukey's test using GraphPad Prism software (GraphPad, San Diego, CA). The values were expressed as the means ± S.E.M. Differences with $p < 0.05$ were considered significant.

Results

Curine inhibits eosinophil recruitment and activation in a mouse model of allergic asthma

An allergic challenge with OVA (5%) in actively sensitized mice induced an intense influx of eosinophils into the BAL that was associated with the development of airway inflammation (Fig. 1B). To demonstrate the anti-allergic effects of pre-treatment with curine, the animals were pre-treated orally with curine (2.5 mg/Kg) or dexamethasone (2 mg/Kg) 1 h before each OVA challenge. This treatment significantly reduced both the total number of eosinophils in the BAL (Fig. 1B) and the numbers of cytoplasmic lipid bodies within those cells (Fig. 1C) compared with the untreated OVA-challenged group. In this model, curine had a dose-dependent effect, with a median effective dose (ED_{50}) of 0.8 mg/Kg (Fig. 1D).

Curine decreases AHR in a mouse model of allergic asthma

Airway hyper-responsiveness is a hallmark of asthma. Fig. 2 shows that an allergic challenge in actively sensitized BALB/c mice induced a significant increase in Penh values compared with the unchallenged group. Daily pre-treatment with curine or dexamethasone (2 mg/Kg) reduced the allergic reaction-triggered Penh values, indicating that curine, similarly to dexamethasone, has an inhibitory effect on the development of allergic AHR. Of note, when expressing data as % baseline the results were the same (not shown). Importantly, curine failed to inhibit the methacholine response in the non-allergic vehicle group (not show), demonstrating that curine does not alter the bronchoconstriction induced by methacholine in the absence of inflammation.

Curine inhibits the production of eotaxin and IL-13 in vivo

Eotaxin and IL-13 play key roles in allergic responses, including the regulation of eosinophil migration and activation and the development of AHR (Lloyd et al., 2001). As shown in Fig. 3, the BAL fluid of actively sensitized OVA-challenged mice contained increased concentrations of these cytokines when compared with unstimulated mice. As shown in Figs. 3A and B, curine and dexamethasone pre-treatments both decreased the eotaxin (Fig. 3A) and IL-13 (Fig. 3B) BAL concentrations observed in the untreated OVA-challenged group, revealing a part of the mechanism by which curine inhibits eosinophil recruitment and activation and AHR.

Effects of curine on the calcium-induced tracheal contractile response

Previous studies have shown that curine has an inhibitory effect on calcium influx in different cell types (Dias et al., 2002; Medeiros et al., 2011). Therefore, we investigated the involvement of calcium-dependent mechanisms on the anti-allergic effects of curine. In the experiments with trachea ring preparations maintained in Ca^{2+}-free medium and depolarized with 60 mM KCl, the cumulative addition of Ca^{2+} (0.01 to 100 mM) produced a concentration-dependent contraction that was inhibited by up to 50% by pre-treatment with 9 μM curine (Fig. 4). This observation suggests that curine inhibits the influx of calcium by blocking the voltage-dependent Ca^{2+} channels in rat tracheal smooth muscle and is consistent with previously published data (Medeiros et al., 2011).

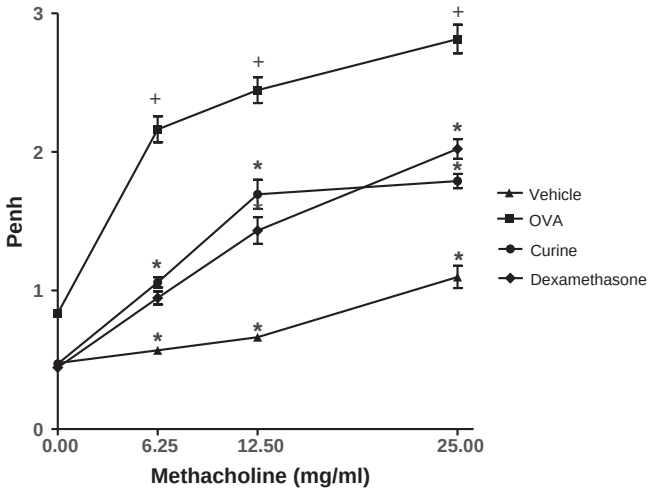

Fig. 2. The effect of curine on airway hyper-responsiveness (AHR). OVA-sensitized BALB/c mice (n = 7–8) were treated orally with curine (2.5 mg/Kg), or dexamethasone (2 mg/Kg) 1 h before each challenge. AHR was analyzed by aerosolization with PBS followed by increasing concentrations of methacholine, 24 h after the last allergic challenge. The AHR is expressed as the rate of enhanced pause (Penh). These results are expressed as the mean ± SEM. + Significantly different ($p < 0.05$) from the unchallenged group; * significantly different from the OVA-challenged and untreated group.

Comparison between the anti-allergic effects of curine and verapamil

In recent work, Khakzad et al. (2012) demonstrated the anti-inflammatory activity of verapamil, a calcium channel antagonist, using a model of allergic asthma. To confirm the role of calcium influx inhibition as an anti-allergic mechanism, we compared the effect of verapamil to that of curine on AHR and eosinophil recruitment, two important features of asthma. When administered under the same conditions (dose, time and pathway), verapamil and curine showed similar anti-allergic effects (Fig. 5), suggesting that the anti-allergic effects of curine may be due, at least in part, to the inhibition of calcium influx.

Curine post-treatment inhibits eosinophil recruitment and activation in OVA-challenged mice

To investigate the therapeutic effects of curine on ongoing airway inflammation, we administered a single dose of curine 1 h after the last allergic challenge (post-treatment). Balb/c mice post-treated with curine had a reduced number of eosinophils (Fig. 6A) and similarly reduced numbers of eosinophil lipid bodies (Fig. 6B) in the BAL compared with mice in the untreated group. Even when the treatment was given after the development of inflammation, curine inhibited eosinophil recruitment and activation; such findings demonstrate the therapeutic effectiveness and the clinical relevance of curine.

Curine fails to induce toxic effects in orally treated mice

Outbred Swiss mice treated orally with curine daily for seven days were evaluated for toxicological parameters. Outbred mouse stocks were used for the toxicological evaluation because of their genetic heterogeneity and their common use in toxicological studies (Faqi, 2012). We found that curine treatment did not induce significant alterations in biochemical (Table 1), hematological (Supplement Table S1) or physical and behavioral parameters and in gastric ulcer formation (Supplement Table S2) compared with saline treatment, even at a dose of 8 mg/Kg (a dose 10 times higher than the ED_{50}). These data indicate that curine is able to inhibit allergic inflammation at doses at which it does not induce significant toxicity. On the other hand, dexamethasone treatment induced an increase in the concentrations of total proteins, cholesterol and ALT compared with saline treatment, reflecting the side effects broadly described in the literature. Of note, no alterations in physical

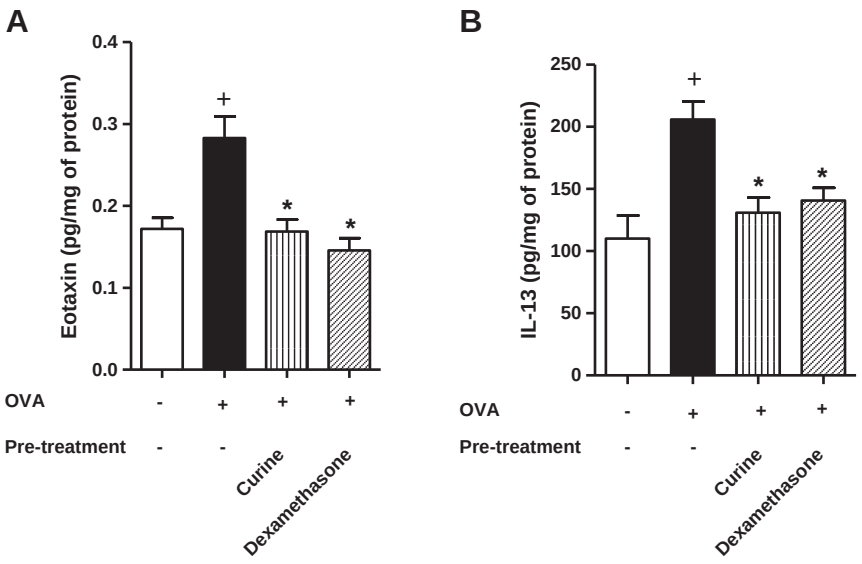

Fig. 3. The effect of curine on eotaxin and IL-13 production. BALB/c mice (n = 5–6) primed with OVA were treated orally with curine (2.5 mg/Kg), or dexamethasone (2 mg/Kg) 1 h before each challenge. Twenty-four hours after the last challenge, the lungs were collected and homogenized. The production of eotaxin (A) was analyzed using an EIA protocol, and the production of IL-13 (B) was analyzed by ELISA. These results are expressed as the mean ± SEM from at least 6 animals. + Significantly different ($p < 0.05$) from the unchallenged group; * significantly different from the OVA-challenged and untreated group.

and behavioral parameters or leukocyte counts were observed in the Balb/c mice treated with curine at 2.5 mg/kg (not shown).

Discussion

Allergic diseases, including asthma, have multifactorial etiologies, and their prevalence, morbidity and mortality have been increasing around the world (Edwards et al., 2009). Asthma is usually treated with a combination of anti-inflammatory and bronchodilator drugs. Inhaled corticosteroids are currently the most important drugs used to control airway inflammation. Because of their potent inhibitory effects on cell activation, cytokine and lipid mediator production and adhesion molecule and inflammatory receptor expression, they are simultaneously able to block several essential steps of the inflammatory cascade (Barnes, 2006, 2011; Barnes and Adcock, 2009; Rhen and Cidlowski, 2005). However, corticosteroid therapy induces important side effects (Schacke et al., 2004), and some patients are completely corticoid resistant or fail to show clinical improvement after treatment with high doses of oral glucocorticoids (Barnes et al., 1995). This situation can limit the use of corticosteroids and impairs the patients' life quality. Some reports suggest that the ineffectiveness of corticosteroids in some severe forms of asthma is due to their failure to decrease the level of TH1 cytokines, such as tumor-necrosis factor (TNF) (Truyen et al., 2006). Classic bronchodilator drugs including β_2-adrenergic agonists and phosphodiesterase inhibitors have long been used to control the shortness of breath that is characteristic of asthmatic attacks; however, these drugs are also not free from side effects (Boswell-Smith et al., 2006). Currently, leukotriene antagonists have been employed successfully in asthma therapy. Nevertheless, they are not effective as a monotherapy and are therefore mainly used as a supplementary therapy to inhaled corticosteroids (Polosa, 2007). Mast cell inhibitors, monoclonal antibodies, 5-lipoxygenase inhibitors and allergen-specific immunotherapy have also found a place in asthma therapy (Edwards and Howell, 2000; Holgate and Polosa, 2008). However, novel, safe and effective drugs are needed for the treatment of asthma, and the assessment of new compounds with potentially unique mechanisms of action remains an important area of research.

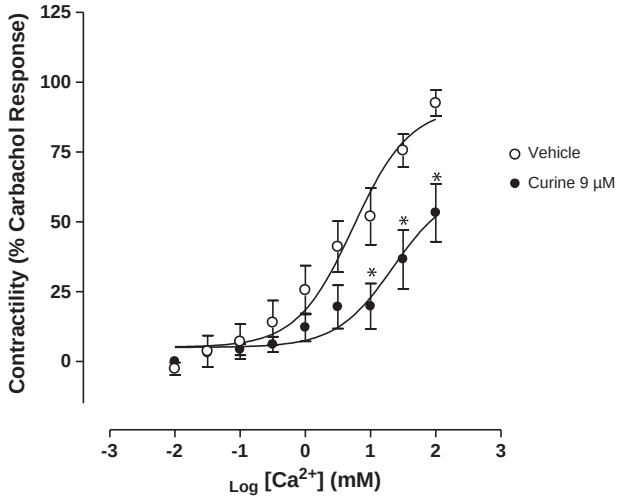

Fig. 4. The effects of curine on the calcium-induced tracheal contractile response. A contractile response was triggered after the cumulative application of calcium in tracheal segments maintained in Krebs solution rich in K^+ (60 mM) and free of calcium. Pre-treatment with curine (9 µM) led to a decrease in tension after the addition of calcium. Each point represents the mean ± S.E.M. from at least six segments. The magnitude of the voltage induced contraction of trachea calcium preparations was expressed as a percentage of the contractile response evoked by 2.5 mM carbachol. * Significantly different from the vehicle.

In this work, we have for the first time demonstrated the anti-allergic properties and toxicological characterization of curine, an orally active bisbenzylisoquinoline alkaloid. To investigate the anti-allergic properties of curine, we used a well-established mouse model of asthma induced by allergic sensitization and challenge with OVA. Eosinophilic infiltration into the airways is a hallmark of this model; allergic challenge induces eosinophil maturation and differentiation from precursors in the bone marrow and their migration to sites of inflammation in response to mediators such as eotaxin and IL-5 (Gleich, 2000). Therefore, we performed a dose–response experiment with oral treatment with curine, and we observed that such treatment dose-dependently inhibited the number of eosinophils in the BAL, supporting the inhibitory role that curine plays in eosinophil recruitment.

To further understand the additional effects of curine, we analyzed the formation of lipid bodies in eosinophils. Increased lipid body assembly within eosinophils is closely associated with cellular activation and LTC_4 production in an allergic response and therefore plays important roles in inflammatory conditions and may be used as a marker of

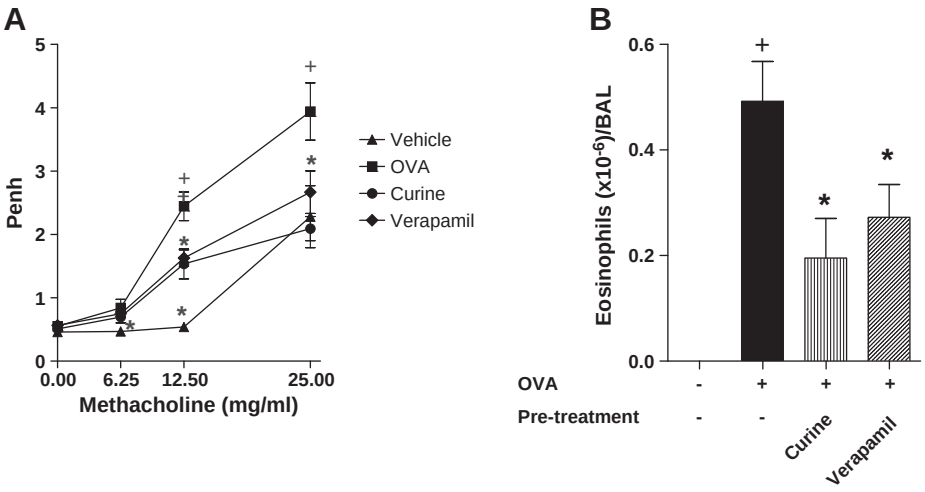

Fig. 5. A comparison between the anti-allergic effects of curine and verapamil. BALB/c mice (n = 6–8) primed with OVA were treated orally with curine (2.5 mg/kg) or verapamil (2.5 mg/kg) 1 h before each OVA challenge. A) AHR was analyzed by aerosolization with PBS followed by increasing concentrations of methacholine 24 h after the last allergic challenge. The AHR was expressed as the rate of enhanced pause (Penh). B) The eosinophils in the BAL were counted using a light microscope. These results are expressed as the mean ± SEM. + Significantly different ($p < 0.05$) from the unchallenged group; * significantly different from the OVA-challenged and untreated group.

eosinophil activation (Mesquita-Santos et al., 2006; Vieira-de-Abreu et al., 2005). We demonstrated not only that the animals treated with curine showed a reduced number of eosinophils in the BA but also that these eosinophils had fewer cytoplasmic lipid bodies, indicating that curine plays an inhibitory role on both migration and activation of these cells. Our group has demonstrated that the inhibition of lipid bodies is an important parameter that can be used to evaluate the anti-inflammatory effects of drugs; furthermore, evaluating the inhibition of lipid bodies may be useful in understanding the pharmacology and therapeutic efficacy of new interventions in numerous conditions such as asthma, allergies, cancer, atherosclerosis, diabetes and other diseases in which the formation of lipid bodies is a negative clinical parameter (Bozza et al., 2009). We observed that treatment with curine as well as dexamethasone significantly reduced the production of eotaxin in the model we used. It has been shown that eotaxin plays a key role in eosinophil migration and activation (Vieira-de-Abreu et al., 2005), which supports the effects of curine described herein. AHR is a particular feature of asthma, resulting from complex interactions between inflammatory and airway smooth muscle (ASM) cells, leading to recurrent episodes of shortness of breath, wheezing and coughing (Lauzon et al., 2012). Here, we showed that curine has an important inhibitory activity on AHR, decreasing the observed Penh values to levels similar to those observed in the animals treated with dexamethasone. To elucidate the mechanisms involved in the anti-allergic effects of curine, we analyzed the production of key mediators of allergic responses, such as IL-13. Previous studies have demonstrated that blocking IL-13 in mice prevents the development of AHR (Wills-Karp et al., 1998). Furthermore, Kuperman et al. (2005, 2002) demonstrated that the overexpression of IL-13 in mice was sufficient to cause AHR, possibly through a direct effect on the airway epithelium. Importantly, IL-13 is also involved in the modulation of various pathophysiological aspects of asthma, such as class switching to IgE, mucus production, inflammation,

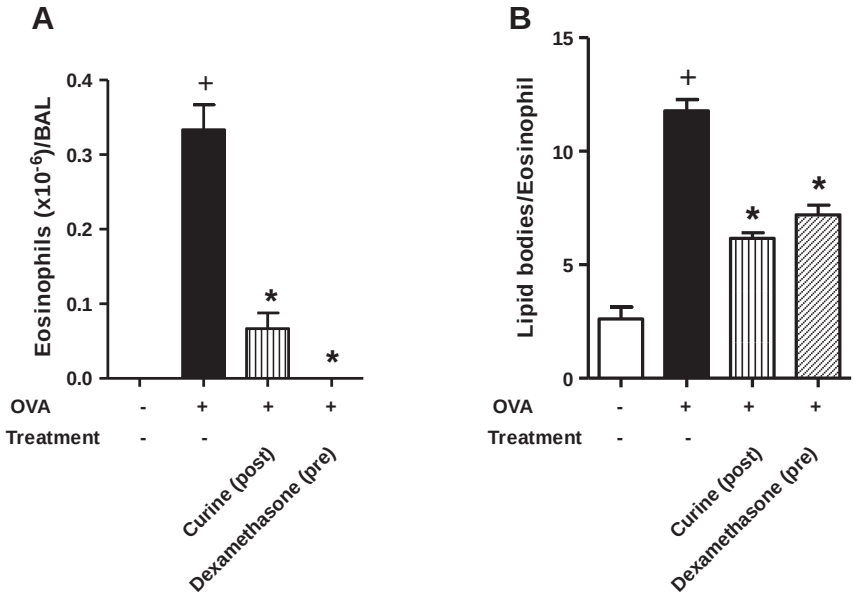

Fig. 6. The effect of post-treatment curine on eosinophil recruitment and activation. OVA-sensitized BALB/c mice (n = 5–6) received a single dose of curine (2.5 mg/Kg) 1 h after the last allergic challenge. Dexamethasone (2 mg/Kg) was administered 1 h before each challenge with OVA. The BAL was collected 24 h after the last challenge. The eosinophils (A) and lipid bodies (B) were counted using a light microscope. These results are expressed as the mean ± SEM. + Significantly different ($p < 0.05$) from the unchallenged group; * significantly different from the OVA-challenged and untreated group.

Table 1
The biochemical analyses of serum from male Swiss mice treated with curine (2.5 or 8 mg/kg) or dexamethasone (2 mg/kg) for 7 days.

Parameter (unit)	Vehicle	Curine (2.5 mg/kg)	Curine (8 mg/kg)	Dexamethasone (2 mg/kg)
Alkaline Phosphatase	222.40 ± 22.70	212.20 ± 32.80	218.90 ± 19.45	284.20 ± 38.13
ALT	79.50 ± 15.37	62.80 ± 4.25	82.67 ± 12.37	101.30 ± 13.37
AST	98.83 ± 15.91	94.10 ± 8.76	112.10 ± 11.17	175.10 ± 25.44*
Bilirubin	0.64 ± 0.03	0.67 ± 0.04	0.81 ± 0.04	1.15 ± 0.09*
Creatinine	0.20 ± 0.00	0.22 ± 0.01	0.22 ± 0.02	0.20 ± 0.00
Creatinine Kinase	289.00 ± 90.75	261.60 ± 60.55	267.40 ± 41.97	227.90 ± 26.16
Cholesterol	62. 29 ± 2.50	65.67 ± 3.90	63.60 ± 4.52	106.60 ± 10.30*
Glucose	264.00 ± 25.26	249.60 ± 18.47	297.50 ± 15.29	292.80 ± 72.41
Total Proteins	5.29 ± 0.09	5.42 ± 0.08	5.54 ± 0.10	7.27 ± 0.17*
Uric acid	3.73 ± 0.47	3.90 ± 0.54	5.03 ± 0.42	2.90 ± 0.79

These values are expressed as the mean ± S.E.M. AST = aspartate transaminase. ALT = alanine transaminase.
* Significantly different from the vehicle group ($p < 0.05$).

airway remodeling and the contraction of ASM through an interaction with specific receptors on the cell surface (Bloemen et al., 2007).

Allergic mechanisms involve signaling and the activation of many cell types, including leukocytes and epithelial and airway muscle cells (Paul and Zhu, 2010). Eosinophilic inflammation and AHR are important features of asthma, and both processes require calcium-dependent mechanisms. Accordingly, recent work has demonstrated that verapamil, a calcium channel antagonist, significantly inhibited goblet cell hyperplasia, mucus hypersecretion and inflammation in a model of allergic asthma (Khakzad et al., 2012). Medeiros et al. (2011) demonstrated that curine might have a direct effect on L-type Ca^{2+} channels in vascular smooth muscle cells. In this work, we investigated the involvement of calcium-dependent mechanisms on the anti-allergic effects of curine. We demonstrated that curine pre-treatment significantly inhibited a calcium-induced trachea contractile response *ex vivo*, suggesting that curine inhibits the influx of calcium by blocking voltage-dependent Ca^{2+} channels in rat tracheal smooth muscle. Interestingly, in non-allergic mice, curine did not alter the bronchoconstriction induced by methacholine (*in vivo*), which is mediated by calcium release from intracellular stores (Foster et al., 1984). These data support our hypothesis that curine inhibits calcium channels in the cell membrane. Additionally, *in vivo* treatments with curine or verapamil (an L-type calcium-channel antagonist) using the same dose, time and method of administration had similar effects on AHR and eosinophilic inflammation, suggesting that the anti-allergic effects of curine may be, at least in part, dependent on the inhibition of calcium influx. Indeed, calcium-dependent signaling plays key roles both in asthma-related cytokine production and in their function. Antigen-triggered cytokine and chemokine expression is largely dependent on the activation of NFAT, a transcription factor that requires Ca^{2+} for its activation (Macian, 2005). Rothenberg et al. (1996) demonstrated that eotaxin signaling through its receptor, CCR3, effectively induced a calcium influx into eosinophils *in vitro*. Additionally, Moynihan et al. (2008) demonstrated that IL-13 modulated AHR by inducing SOCS expression and a calcium influx into human airway smooth muscle cells.

Finally, we investigated the possible toxicity of curine by oral treatment because this parameter has not previously been addressed experimentally. To that end, Swiss mice were treated daily for seven days with curine at doses of 2.5 and 8 mg/Kg. The dose of 2.5 mg/Kg was chosen because it had a significant anti-allergic effect throughout the experiments and the dose of 8 mg/Kg is 10 fold higher than the ED_{50}. We also included a group treated with dexamethasone (2 mg/Kg, p.o.) here as we did for other experiments. All of the treatments were compared with the oral treatment with PBS with HCl and NaOH added (pH 7), which was used as a vehicle for the dilution of the curine. The toxicological evaluation showed that the treatment with curine induced no changes in the hematologic or biochemical parameters. Additionally, the treatment did not induce the formation of gastric ulcers, and there were no physical or behavioral changes, indicating that under these conditions, curine showed no evident toxicity.

Aiming to provide additional therapeutic information concerning the anti-allergic properties of curine, we have demonstrated that curine post-treatment was effective in the inhibition of eosinophil recruitment and activation; this observation highlights the potential of curine as an anti-allergic compound, as post-treatment more accurately replicates what happens in clinical situations.

In conclusion, orally administered curine showed potent anti-allergic activity without inducing evident toxicity and, as such, has the potential for the development of anti-asthmatic drugs.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.taap.2013.08.015.

Disclosure of conflict of interest

The authors state that they have no conflict of interest.

Acknowledgments

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References

Barnes, P.J., 2006. How corticosteroids control inflammation. Br. J. Pharmacol. 148, 245–254.
Barnes, P.J., 2008. Immunology of asthma and chronic obstructive pulmonary disease. Nat. Rev. Immunol. 8, 183–192.
Barnes, P.J., 2011. Glucocorticosteroids: current and future directions. Br. J. Pharmacol. 163, 29–43.
Barnes, P.J., Adcock, I.M., 2009. Glucocorticoid resistance in inflammatory diseases. Lancet 373, 1905–1917.
Barnes, P.J., Greening, A.P., Crompton, G.K., 1995. Glucocorticoid resistance in asthma. Am. J. Respir. Crit. Care Med. 152, 125–140.
Bezerra-Santos, C.R., Peçanha, L.M.T., Piuvezam, M.R., 2005. *Cissampelos sympodialis* Eichl. (Menispermaceae) inhibits anaphylactic shock reaction in murine allergic model. Braz. J. Pharmacogn. 15, 287–291.
Bezerra-Santos, C.R., Vieira-de-Abreu, A., Barbosa-Filho, J.M., Bandeira-Melo, C., Piuvezam, M.R., Bozza, P.T., 2006. Anti-allergic properties of *Cissampelos sympodialis* and its isolated alkaloid warifteine. Int. Immunopharmacol. 6, 1152–1160.
Bezerra-Santos, C.R., Vieira-de-Abreu, A., Vieira, G.C., Ribeiro-Filho, J., Barbosa-Filho, J.M., Pires, A.L., Martins, M.A., Souza, H.S., Bandeira-Melo, C., Bozza, P.T., Piuvezam, M.R., 2012. Effectiveness of *Cissampelos sympodialis* and its isolated alkaloid warifteine in airway hyperreactivity and lung remodeling in a mouse model of asthma. Int. Immunopharmacol. 13, 148–155.
Bloemen, K., Verstraelen, S., Van-Den-Heuvel, S., Witters, H., Nelissen, I., Schoeters, G., 2007. The allergic cascade: review of the most important molecules in the asthmatic lung. Immunol. Lett. 113, 6–18.
Boswell-Smith, V., Cazzola, M., Page, C.P., 2006. Are phosphodiesterase-4 inhibitors just more theophylline? J. Allergy Clin. Immunol. 117, 1237–1243.
Bozza, P.T., Yu, W., Penrose, J.F., Morgan, E.S., Dvorak, A.M., Weller, P.F., 1997. Eosinophil lipid bodies: specific, inducible intracellular sites for enhanced eicosanoid formation. J. Exp. Med. 186, 909–920.
Bozza, P.T., Magalhães, K.G., Weller, P.F., 2009. Leukocyte lipid bodies — biogenesis and functions in inflammation. Biochem. Biophys. Acta 1791 (6), 540–551.
Chatila, T.A., 2004. Interleukin-4 receptor signaling pathways in asthma pathogenesis. Trends Mol. Med. 10, 493–499.

Costa, H.F., Bezerra-Santos, C.R., Barbosa-Filho, J.M., Martins, M.A., Piuvezam, M.R., 2008. Warifteine, a bisbenzylisoquinoline alkaloid, decreases immediate allergic and thermal hyperalgesic reactions in sensitized animals. Int. Immunopharmacol. 8, 519–525.
Dias, C.S., Barbosa-Filho, J.M., Lemos, V.S., Cortes, S.F., 2002. Mechanisms involved in the vasodilator effect of curine in rat resistance arteries. Planta Med. 68, 1049–1051.
Edwards, A.M., Howell, J.B., 2000. The chromones: history, chemistry and clinical development. A tribute to the work of Dr R. E. C. Altounyan. Clin. Exp. Allergy 30, 756–774.
Edwards, M.R., Bartlett, N.W., Clarke, D., Birrell, M., Belvisi, M., Johnston, S.L., 2009. Targeting the NF-κB pathway in asthma and chronic obstructive pulmonary disease. Pharmacol. Ther. 121, 1–13.
Faqi, A., 2012. A Comprehensive Guide to Toxicology in Preclinical Drug Development, First ed. Academic Press, New York.
Foster, R.W., Okpalugo, B.I., Small, R.C., 1984. Antagonism of Ca^{2+} and other actions of verapamil in guinea-pig isolated trachealis. Br. J. Pharmacol. 81, 499–507.
Gleich, G.J., 2000. Mechanisms of eosinophil-associated inflammation. J. Allergy Clin. Immunol. 105, 651–663.
Harrison, T.W., Oborne, J., Newton, S., Tattersfield, A.E., 2004. Doubling the dose of inhaled corticosteroid to prevent asthma exacerbations: randomised controlled trial. Lancet 363, 271–275.
Holgate, S.T., Polosa, R., 2008. Treatment strategies for allergy and asthma. Nat. Rev. Immunol. 8, 116–124.
Ivancsó, I., Böcskei, R., Müller, V., Tamási, L., 2013. Extrafine inhaled corticosteroid therapy in the control of asthma. J. Asthma Allergy 6, 69–80.
Khakzad, M.R., Mirsadraee, M., Mohammadpour, A., Ghafarzadegan, K., Hadi, R., Saghari, M., Meshkat, M., 2012. Effect of verapamil on bronchial goblet cells of asthma: an experimental study on sensitized animals. Pulm. Pharmacol. Ther. 25, 163–168.
Kuperman, D.A., Lewis, C.C., Woodruff, P.G., Rodriguez, M.W., Yang, Y.H., Dolganov, G.M., Fahy, J.V., Erle, D.J., 2005. Dissecting asthma using focused transgenic modeling and functional genomics. J. Allergy Clin. Immunol. 116, 305–311.
Lauzon, A.M., Bates, H.T., Donovan, G., Tawhai, M., Sneid, J., Sanderson, M.J., 2012. A multi-scale approach to airway hyperresponsiveness: from molecule to organ. Front. Physiol. 3, 1–25.
Lloyd, C.M., Gonzalo, J.A., Nguyen, T., Delaney, T., Tian, J., Oettgen, H., Coyle, A.J., Gutierrez-Ramos, J.C., 2001. Resolution of bronchial hyperresponsiveness and pulmonary inflammation is associated with IL-3 and tissue leukocyte apoptosis. J. Immunol. 166, 2033–2204.
Macian, F., 2005. NFAT proteins: key regulators of T-cell development and function. Nat. Rev. Immunol. 5, 472–484.
Mambu, L., Martin, M.T., Razafimahefa, D., Ramanitrahasimbola, D., Rasoanaivo, P., Frappier, F., 2000. Spectral characterization and antiplasmodial activity of bisbenzylisoquinolines from *Isolona ghesquiereina*. Planta Med. 66, 537–540.
Medeiros, M.A.A., Pinho, J.F., De-Lira, D.P., Barbosa-Filho, J.M., Araújo, D.A.M., Cortes, S.F., Lemos, V.S., Cruz, J.S., 2011. Curine, a bisbenzylisoquinoline alkaloid, blocks L-type Ca^{2+} channels and decreases intracellular Ca^{2+} transients in A7r5 cells. Eur. J. Pharmacol. 669, 100–107.
Mesquita-Santos, F.P., Vieira-de-Abreu, A., Calheiros, A.S., Figueiredo, I.H., Castro-Faria-Neto, H.C., Weller, P.F., Bozza, P.T., Diaz, B.L., Bandeira-Melo, C., 2006. Cutting edge: prostaglandin D_2 enhances leukotriene C_4 synthesis by eosinophils during allergic inflammation: synergistic *in vivo* role of endogenous eotaxin. J. Immunol. 176, 1326–1330.
Moynihan, B.J., Tolloczko, B., Bassam, S.E., Ferraro, P., Michoud, M., Martin, J.G., Laberge, S., 2008. IFN-γ, IL-4 and IL-13 modulate responsiveness of human airway smooth muscle cells to IL-13. Respir. Res. 9 (1), 84.
Paul, W., Zhu, J., 2010. How are TH_2-type immune responses initiated and amplified. Nat. Rev. Immunol. 10, 225–235.
Polosa, R., 2007. Critical appraisal of antileukotriene use in asthma management. Curr. Opin. Pulm. Med. 13, 24–30.
Rhen, T., Cidlowski, J.A., 2005. Antiinflammatory action of glucocorticoids — new mechanisms for old drugs. N. Engl. J. Med. 353, 1711–1723.
Rothenberg, M.E., Robert-Ownbey, T., Mehlhop, P.D., Loiselle, P.M., Van de Rijn, M., Bonventre, J.V., Oettgen, H.C., Leder, P., Luster, A.D., 1996. Eotaxin triggers eosinophil-selective chemotaxis and calcium flux via a distinct receptor and induces pulmonary eosinophilia in the presence of interleukin 5 in mice. Mol. Med. 2 (3), 334–348.
Schacke, H., Schottelius, A., Docke, W.D., Strehlke, P., Jaroch, S., Schmees, N., Rehwinkel, H., Hennekes, H., Asadullah, K., 2004. Dissociation of transactivation from transrepression by a selective glucocorticoid receptor agonist leads to separation of therapeutic effects from side effects. Proc. Natl. Acad. Sci. U. S. A. 101, 227–232.
Seow, W.K., Li, S.T.Y., Thong, Y.H., 1986. Inhibitory effect of tetrandrine on human neutrophil and monocyte adherence. Immunol. Lett. 13, 83–88.
Szefler, S.J., 2011. Advancing asthma care: the glass is only half full. J. Allergy Clin. Immunol. 128 (3), 485–494.
Teh, B.S., Seow, W.K., Li, S.Y., Thong, Y.H., 1990. Inhibition of prostaglandin and leukotriene generation by the plant alkaloids tetrandrine and berbamine. Int. J. Immunopharmacol. 12, 321–326.
Truyen, E., Coteur, L., Dilissen, E., Overbergh, L., Dupont, L.J., Ceuppens, J.L., Bullens, D.M., 2006. Evaluation of airway inflammation by quantitative TH1/TH2 cytokine mRNA measurement in sputum of asthma patients. Thorax 61, 202–208.
Vieira-de-Abreu, A., Assis, E.F., Gomes, G.S., Castro-Faria-Neto, H.C., Weller, P.F., Bandeira-Melo, C., Bozza, P.T., 2005. Allergic challenge-elicited lipid bodies compartmentalize *in vivo* leukotriene C_4 synthesis within eosinophils. Am. J. Respir. Cell Mol. Biol. 33, 254–261.
Wills-Karp, M., Luyimbazi, J., Xu, X., Schofield, B., Neben, T.Y., Karp, C.L., Donaldson, D.D., 1998. Interleukin-13: central mediator of allergic asthma. Science 282, 2258–2261.

Table S1. Haematological analyses of serum of swiss male mice treated with curine (2.5 or 8 mg/kg) or dexamethasone (2 mg/kg) for 7 days

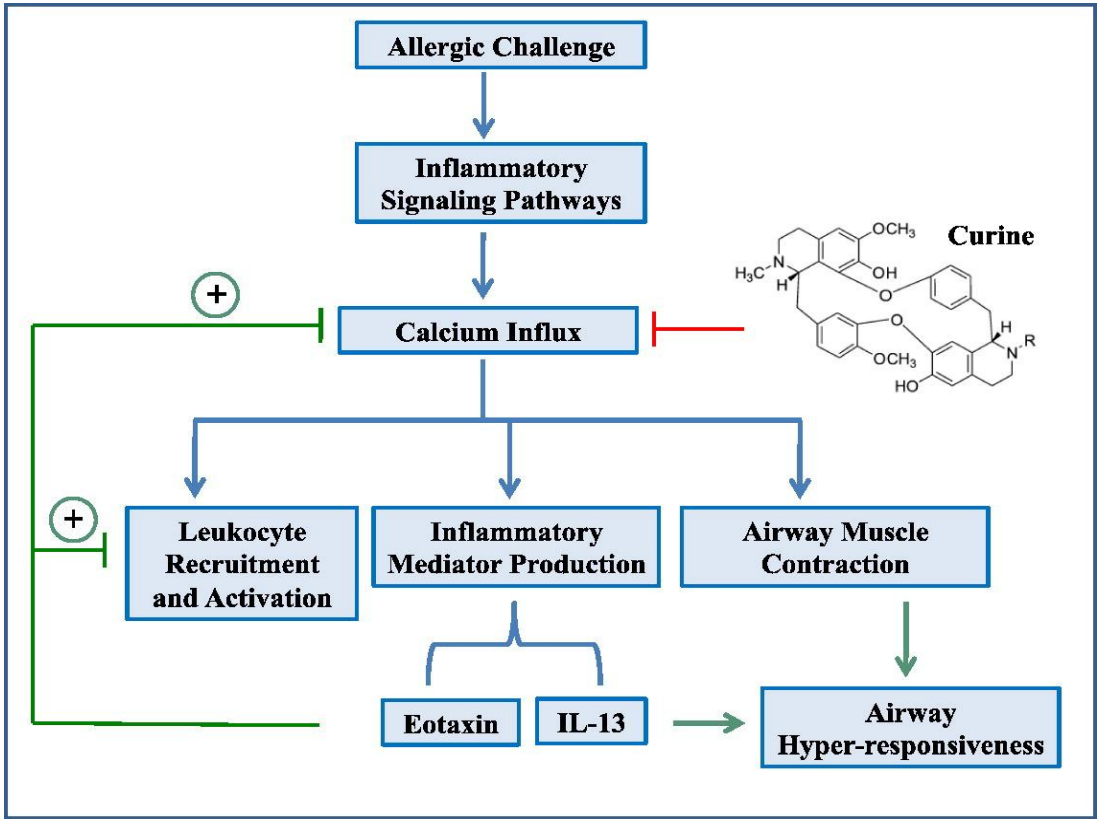
Parameter (unit)	Vehicle	Curine (2.5 mg/kg)	Curine (8 mg/kg)	Dexamethasone (2 mg/kg)
Hematocrit (%)	58.01 ± 0.94	55.13 ± 1.90	56.79 ± 0.95	62.91 ± 1.22
Hemoglobin (g/dL)	16.10 ± 0.30	15.89 ± 0.60	16.27 ± 0.31	17.60 ± 0.34
Leukocytes ($10^3/mm^3$)	9.42 ± 0.59	10.02 ± 1.28	9.83 ± 1.36	6.42 ± 0.82*
MCHC (g/dL)	27.74 ± 0.19	28.80 ± 0.23	28.64 ± 0.17	25,.99 ± 1.94
MCH (pg)	15.83 ± 0.13	15.72 ± 0.16	19.06 ± 3.81	14.63± 1.06
MCV (fm^3)	57.13 ± 0.66	54.62 ± 0.69	53.77 ± 0.76	55.86 ± 0.47
Platelets ($10^3/mm^3$)	1216.00 ± 98.34	1296.00 ± 87.36	1350 ± 168.50	1110.00 ± 128.60
RBC ($10^6/mm^3$)	10.17 ± 0.23	10.12 ± 0.39	10.67 ± 0.18	11.17 ± 0.27

Values are expressed as mean ± S.E.M.. * significantly different from vehicle group ($p < 0.05$). MCHC= Mean corpuscular hemoglobin concentration; RBC= red blood cells; MCV= mean corpuscular volume, MCH=mean corpuscular hemoglobin,

Table S2. Macroscopical analyses of swiss male mice treated with curine (2.5 or 8 mg/kg) or dexamethasone (2 mg/kg) for 7 days.

Parameter (unit)	Vehicle	Curine (2.5 mg/kg)	Curine (8 mg/kg)	Dexamethasone (2 mg/kg)
Stomach ulcers (nº)	0.00 ± 0.00	0.00 ± 0.00	0.40 ± 0.40	0.00 ± 0.00
Weight increase (g)	0. 50 ± 0.89	1.00 ± 0.75	1.90 ± 0.40	-0.33 ± 0.58
Behavior	Normal	Normal	Normal	Normal
Feces	Normal	Normal	Normal	Normal
Skin changes	None	None	None	None
Organs[1] aspects	Normal	Normal	Normal	Normal

Values are expressed as mean ± S.E.M. * significantly different from vehicle group ($p < 0.05$) . 1- Liver, spleen, thymus, lung, intestine and kidneys.



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3.2. Artigo II

THE ANTI-INFLAMMATORY AND ANALGESIC EFFECTS OF CURINE, A BISBENZYLISOQUINOLINE ALKALOID ISOLATED FROM *Chondrodendron platyphyllum* (MENISPERMACEAE). Leite, F. C.; Ribeiro-Filho, J.; Costa, H. F.; Salgado, P. R. R.; Calheiros, A. S.; Carneiro, A. B.; Almeida, R. N.; Dias, C. S.; Bozza, P. T.; Piuvezam, M. R., 2013. Journal of Natural Products. Submetido.

Como no trabalho anterior nós demonstramos que a curina possui uma atividade antialérgica significante, inclusive no que diz respeito a migração e ativação de leucócitos, e os dados etnofarmacológicos indicam que a planta *C. platyphyllum* é popularmente utilizada para tratar sintomas inflamatórios, neste segundo artigo nós investigamos os efeitos anti-inflamatórios e analgésicos da curina em camundongos. A curina inibiu significativamente a formação de edema da pata por diminuir a permeabilidade vascular, inibiu a resposta de contorção abdominal induzida por ácido acético, inibiu o comportamento de lambida de pata durante a fase inflamatória, mas não durante a fase neurogênica do teste de formalina, e inibiu a hiperalgesia induzida por carragenina. Em adição, a curina inibiu a produção de PGE_2, *in vitro*, sem afetar a expressão de COX-2 em macrófagos estimulados com LPS. Os efeitos do tratamento com a curina foram semelhantes aos efeitos da indometacina , mas diferentes dos efeitos do tratamento com morfina , sugerindo que o efeito analgésico da curina não resulta da inibição direta da ativação neuronal, mas em vez disso depende de mecanismos antiinflamatórias que, pelo menos em parte, resultam da inibição da produção de PGE_2 .

The anti-inflammatory and analgesic effects of curine, a bisbenzylisoquinoline alkaloid isolated from *Chondrodendron platyphyllum* (Menispermaceae)

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ABSTRACT

Curine (1) is a bisbenzylisoquinoline alkaloid that is isolated from *Chondrodendron platyphyllum* (Menispermaceae), a plant that is used to treat malaria, inflammation and pain. Recent reports have demonstrated the anti-allergic effects of curine at non-toxic doses. However, the anti-inflammatory and analgesic properties remain to be elucidated. This study investigated the anti-inflammatory and analgesic effects of curine in mice. We analyzed the effects of an oral treatment with curine in the formation of paw edema, vascular permeability, abdominal contortion, licking behavior and hyperalgesia using different inflammatory stimuli. Curine significantly inhibited the formation of paw edema by decreasing vascular permeability, inhibited the acetic acid-induced writhing response, inhibited the licking behavior during inflammation but not during the neurogenic phase of the formalin test, and inhibited carrageenan-induced hyperalgesia. Finally, curine inhibited PGE_2production *in vitro* without affecting COX-2 expression. The effects of curine treatment were similar to the effects of indomethacin but were different from the effects of morphine treatment, suggesting that the analgesic effects of curine do not result from the direct inhibition of neuronal activation but instead depend on anti-inflammatory mechanisms that, at least in part, result from the inhibition of PGE_2 production. In conclusion, curine presents anti-inflammatory and analgesic effects at non-toxic doses and has the potential for use in anti-inflammatory drug development.

INTRODUCTION

Chondrodendron platyphyllum A. St. Hil (Miers) (Menispermaceae) is a medicinal plant found in northeastBrazil. This plant is popularly known as "abútua", "abútua grande" and "uva do mato" and has been used in folk medicine to treat a great variety of conditions, including malaria, fever, pain, edema, urethritis, cystitis, ulcers and menstrual disorder (Correa, 1984; Gotfredsen, 2013). According to Silva (2006),*C. platyphyllum* has been used in conjunction with *Zanthoxylum articulatum A. St. Hil.* to composethe herbal medicine *Uva do mato®,* which is used to treat abdominal,kidney and muscle cramps, and muscle pain. The phytochemical analysis of the *C. platyphyllum* root revealed that *C. platyphyllum* is rich in bisbenzylisoquinoline alkaloids, a group of natural compounds with interesting pharmacological properties, such as anti-inflammatory, anti-allergic and analgesic properties (Souto, 2011). At least three alkaloids, including curine, isocurine and 12-O-metilcurine have been identified from this plant, and curine is the major constituent (Dias *et al*., 2002).

Previous studies have shown that the alkaloids extracted from *Chondrodendron platyphyllum* are pharmacologically active (Guedes*et al.*, 2002). Dias and collaborators (2002) have demonstrated that curine and isocurine have a vasodilator effect and have suggested that the effects of curine were associated with the inhibition of calcium channels. Medeiros and collaborators (2011) demonstrated that curine decreased intracellular Ca^{2+} transients in A7r5 cells, possibly through a direct blockade of L-type Ca^{2+} channels. Recently, we demonstrated the anti-allergic effects of an oral treatment with curine using a mouse model of allergic asthma. The oral administration of curine significantly inhibited eosinophilic inflammation, eosinophil lipid body formation, cytokine production and airway hyper-responsiveness (AHR) *in vivo*. Verapamil, a calcium channel antagonist, had similar anti-allergic properties, and curine pre-treatment inhibited the calcium-induced tracheal contractile response *ex-vivo*, suggesting that the mechanism by which curine exerts effects is through the inhibition of a calcium-dependent response. Importantly, oral treatment with curine for 7 consecutive days in doses up to 10-fold higher than the median effective dose (ED_{50}) did not induce changes in the hematologic or biochemical parameters. Additionally, the treatment did not induce the formation of gastric ulcers, and no physical or behavioral changes were observed, indicating that curine is not toxic under these conditions (Ribeiro-Filho *et al*., 2013).

Despite the popular use of *C. platyphyllum* as an anti-inflammatory and analgesic plant and the interesting anti-allergic effect and low toxicity of curine, the scientific evidence of the analgesic effect of this compound remains to be provided. Such findings justify the characterization of the pharmacological properties of curine using other models of inflammation and nociception. In addition, the currently available inflammatory drugs frequently cause significant side effects that limit the long-term therapy (Bjarnason*et al.*, 1993; Allen, 2006) and are not effective under specific conditions (Barnes*et al*., 1995).Therefore, novel, safe and effective anti-inflammatory drugs are needed. Considering the popular use and the pharmacological activity of curine in the absence of toxicity, the present work aims to investigate the anti-inflammatory and analgesic effects of curine on mice.

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RESULTS

Curine inhibits edema formation by decreasing vascular permeability in mice

We used two different inflammatory agents to evaluate the role of curine on edema formation. As shown in Figure 1A, carrageenan injections significantly induced the formation of paw edema, which was significantly inhibited by the pre-treatments with curine or indomethacin. Similarly, zymosan injections induced the expressive formation of paw edema, which was significantly inhibited by both treatments (Figure 1B), demonstrating the inhibitory effect of curine on edema formation that is triggered by inflammatory agonists. To further examine the inhibitory effect of curine on edema formation, we analyzed the effects of curine in the vascular permeability induced by

acetic acid, because this has been demonstrated to be a standard protocol (Whittle, 1964). The intraperitoneal injection of acetic acid induced significantly moreplasma extravasations thanthe vehiclein the control group, attested by the optical density of the Evans blue dye (Figure 2). Curine and indomethacin significantly and similarly decreased the vascular permeability compared to the non-treated animals, suggesting that the role that curine plays in edema formation is associated with the inhibition of the vascular permeability.

Curine inhibits acetic acid-induced writhing response

Because we demonstrated the inhibitory effect that curine plays on edema formation, we attempted to characterize the effects of curine on inflammatory pain. We analyzed the effect of curine in the writhing response induced by acetic acid. We used indomethacin, an NSAID, and morphine, a central acting analgesic drug, as standard pharmacological controls. As shown in Figure 3, the writhing response was significantly inhibited by all of the treatments compared to non-treated group. Interestingly, curine and indomethacin presented similar but only partially inhibitory effects, whereas the writhing response was completely inhibited by morphine, suggesting that curine presents an analgesic effect that is associated with anti-inflammatory mechanisms.

Curine inhibits the inflammatory phase of the formalin test

We used the formalin test to characterize the analgesic effect of curine in nociception mediated by neurogenic or inflammatory mechanisms. Formalin injections induce a biphasic response. The first phase is triggered in the first 5 min after the stimulus, causing direct neural activation and pain. The second phase occurs between 15 - 30 min after the stimulus and is triggered by the action of mediators released in the inflammatory reaction. Inhibitory mechanisms suppress pain between these phases (Hunskaar & Hole, 1987). Mice treated with curine or indomethacin presented no difference in the licking time in the neurogenic phase compared to non-treated mice (Figure 4A), whereas the licking behavior was significant inhibited by morphine. However, the second phase was significantly inhibited by curine, indomethacin and morphine (Figure 4B), indicating that the analgesic effect of curine is dependent on anti-

inflammatory mechanisms and is not a result of a direct inhibition of neuronal activation.

The effect of curine on carrageenan-induced hyperalgesia

Hyperalgesia is characterized by an enhanced response to noxious stimuli and is a hallmark of inflammatory pain (Sammons *et al*., 2000). Because we demonstrated that curine inhibits the inflammatory phase of the formalin test, we used the hot plate test to confirm the inhibitory effect of curine in the inflammatory pain triggered by carrageenan. Figure 5 shows that the challenge with carrageenan significantly decreased the heat withdrawal latency compared to the challenge with PBS in non-treated animals. However, there were no significant differences between the carrageenan challenge and the PBS challenge in animals treated with curine or indomethacin, supporting the potent analgesic role of curine in inflammatory conditions.

Curine inhibits PGE_2 production *in vitro*

Based in the significant inhibitory effect of curine in hyperalgesia and the similarity between the curine and indomethacin effects in our experiments, we hypothesized that curine mechanisms might involve inhibition of the production of PGE_2, a key mediator of hyperalgesia in inflammatory conditions (Kidd and Urban, 2001). Therefore, we investigated the effect of curine on PGE_2 production *in vitro* using peritoneal macrophages stimulated with lipopolysaccharide (LPS). Figure 6A shows that the LPS stimulated cells presented a consistent increase in COX-2 expression that was not affected by curine pre-treatments. However, the supernatants from curine treated cells presented lower concentrations of PGE_2 compared to the supernatant of the non-treated cells (Figure 6B), indicating that in this model, curine inhibits PGE_2 production without affecting the expression of COX-2. These findings suggest that the anti-inflammatory and analgesic effects of curine are, at least in part, dependent on PGE_2 production inhibition.

DISCUSSION

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely used drugs for the treatment of fever, pain and moderate inflammation. These drugs constitute aheterogeneous group of substances that inhibit the enzyme cyclooxygenase (COX) (Howe, 2007). Although NSAIDs present a similar mechanism of action, these drugs differ in many aspects, including pharmacologic potency, safety and selectivity (Patrono & Rocca, 2009). Classical NSAIDs, such as aspirin, indomethacin and diclofenac, inhibit both the constitutive enzyme COX-1 and the isoform COX-2, which is induced in peripheral tissues by many inflammatory stimuli. The anti-inflammatory properties of NSAIDs are primarily attributed to the inhibition of the prostaglandins (PGs) synthesis, which are important mediators of inflammation and pain. PGE_2plays a key role in edema formation, by increasing vascular permeability, and hyperalgesia, by decreasing the threshold of activation of nociceptive neurons (Kidd and Urban, 2001). However, classical NSAIDs also have gastro-intestinal (GI) side effects. Selective COX-2 inhibitors (coxibs) have been developed to present low GI effects; however, these drugs presented serious cardiovascular events (Inotai *et al*., 2010). Recently, nitric oxide - donating NSAIDs, such as nitro-aspirin, have been developed based in evidence that nitric oxide plays an important role in regulating blood flow and vascular functions, preventing GI and cardiac damage (Whittle, 2003). Despite the currently available array of medications, the development of novel, safe and effective drugs for the treatment of inflammatory conditions remains an important area of research.

In this study, we have demonstrated for the first time the anti-inflammatory and analgesic properties of curine, an active bisbenzylisoquinoline alkaloid isolated from *Chondrodendron platyphylum* (Menispermaceae).We demonstrated that oral pre-treatment with curine significantly inhibits mouse paw edema induced by carrageenan and zymosan, and acetic acid induced vascular permeability, indicating that curine plays an inhibitory role in edema formation that is associated with a decrease in vascular permeability. The administration of carrageenan, zymosan and acetic acid is known to trigger a local inflammatory reaction with the release of inflammatory mediators that induce vascular changes that cause plasma extravasations and edema formation (Campos & Calixto, 2000). Here, we suggest that curine inhibitsedema formation by modulating the inflammatory reaction. Previous studies have reported that curine is able to reduce leukocyte activationby decreasing the production of inflammatory cytokines

in other models of inflammation (Ribeiro-Filho *et al*., 2013). Accordingly, the anti-inflammatory properties of other BBA has been previously described (Ivanovska *et al*.; 1996; Kondo *et al*., 1992; Luo *et al*., 1998; Kupeli*et al*.,2002; Wong *et al*., 1991), and our group has demonstrated the anti-allergic and anti-inflammatory properties of warifteine, a BBA isolated from *Cissampelos sympodialis* (Menispermaceae) (Bezerra-Santos *et al*., 2006, 2012; Costa *et al*., 2008).

Importantly, pre-treatment with curine inhibited both the writhing response induced by acetic acid and the licking behavior in the inflammatory phase of the formalin test, demonstrating the analgesic properties of curine. However, curine did not inhibit the neurogenic phase of the formalin test, indicating that the analgesic effect of curine is associated with anti-inflammatory mechanisms and is not a result of the direct inhibition of neuronal activation. Accordingly, our experiments have shown that curine presents phenotypic outcomes similar to those observed for indomethacin, a NSAID, but different from morphine, a central acting analgesic drug. In addition, curine significantly inhibited the hyperalgesic response triggered by carrageenan, which is highly dependent on anti-inflammatory mechanisms (Tjølsen*et al*., 1992). Furthermore, several reports have indicated that hyperalgesia is essentially dependent on the action of PGE_2 (Hunskaar & Hole, 1987). Thus, considering that PGE_2 is importantly involved in the development of the inflammatory and nociceptive responses that were evaluated in the present study and that curine and indomethacin (which the analgesic effect is highly dependent on the inhibition of PGE_2production) presented similar analgesic effects, we demonstrated that curine inhibited PGE_2production without affecting COX-2expression, suggesting that curine inhibits other enzymes in the arachidonic acid metabolism cascade upstream of PGE_2 synthesis. Our data demonstrate that curine has anti-inflammatory and analgesic effects that are at least partially dependent on the inhibition of PGE_2 production. In activated macrophages, phospholipase A_2 (PLA_2) promotes the release of the arachidonic acid from membrane phospholipids, which is associated with the induction of COX-2 expression and leads to increased PGE_2 production (Tabas & Glass, 2013; Williams & Galli, 2000). NSAIDs primarily inhibit PGE_2 synthesis by blocking the COX-2 activity without affecting the expression of this enzyme (Vane & Botting, 1996). The effects of natural products on PGE_2 production have been previously described; however, due to the great diversity of structures, different mechanisms were found, including the inhibition of COX-2 expression (Bae *et al*., 2012; Kim *et al*., 2012; Yun *et al*., 2009), the inhibition of COX-2 activity (Dannhardt

et al., 2000; Estevão *et al*., 2012; Yang *et al*., 2012) and PLA_2 inhibition (Folmer *et al*., 2010).

We have recently reported the anti-allergic effects of curine (Ribeiro-Filho *et al*., 2013). We demonstrated that curine oral treatment *in vivo* significantly inhibits eosinophilic inflammation, eosinophil lipid body formation, and AHR and cytokine production (IL-13 and eotaxin). Verapamil, a calcium channel antagonist, has similar anti-allergic properties, and curine pre-treatment inhibits the calcium-induced tracheal contractile response *ex-vivo*, indicating that the anti-allergic effect of curine is associated with the inhibition of the calcium influx. These results suggest that curine affects many inflammatory signaling pathways, including those involved in the production of PGE_2 (van Rossum & Petterson, 2009). Importantly, oral treatment with curine for 7 consecutive days did not change hematologic (such as leukocytes, red blood cells platelets, hematocrit and hemoglobin), or biochemical (such as alkaline phosphatase, alanine transaminase, aspartate transaminase, bilirubin, creatinine, creatinine kinase, cholesterol, glucose, total proteins and uric acid) parameters. Additionally, curine treatment did not induce the formation of gastric ulcers, and no physical or behavioral changes were observed, indicating that curine is not toxic when used in doses up to 10-fold higher than the ED_{50} under these conditions (Ribeiro-Filho *et al*., 2013). These findings have had an important therapeutic impact because curine has anti-inflammatory effects at non-toxic doses. Furthermore, the currently available anti-inflammatory drugs, including NSAIDs, frequently alter some of the parameters evaluated; therefore, curine could be an important alternative for the development of novel safe and effective drugs.

In conclusion, orally administered curine presents anti-inflammatory and analgesic effects, scientifically endorsing the popular use of *C. platyphyllum* and demonstrating that curine has potential as a new anti-inflammatory drug.

EXPERIMENTAL SECTION

Curine purification

Chondrodendron platyphyllum Hil St. (Miers) was collected in Santa Rita, Paraíba, Brazil. The voucher specimen was deposited in the Herbarium of Prof. Lauro Pires Xavier (UFPB - João Pessoa, Brazil), number 3631-P, and was identified by Prof.

Dr. Maria de Fatima Agra. *C. platyphyllum* bark was dried and pulverized in a HARLEY type grinder and was extracted under exhaustive percolation with ethanol 95º GL for 3-4 days. The extract was concentrated under a vacuum at temperature ranging from 50ºC to 60ºC to obtain the crude ethanol extract. This extract was then dissolved in 3% HCl, filtered through Celite (545 FISCHER SCIENTIFIC) and submitted to $CHCl_3$ extraction alternated with NH_4OH (pH 8) basification. After washing with water and $MgSO_4$,the solvent was evaporated, andthis $CHCl_3$ extract became the total tertiary alkaloid fraction (TTA). The TTA column chromatography followed by TLC purification yielded the bisbenzylisoquinoline alkaloid curine (0.031%). The structure was established by spectroscopic data analysis and comparison with the literature data. NMR 13C and NMR 1H data from these crystals demonstrated that the product was predominantly curine when compared to the literature data (Manbu *et al*., 2000). EIMS: m/z (rel. int.,%): [M] + 594 (28), 442 (10), 398 (7), 369 (4), 341 (2), 299 (22), 298 (100), 297 (23), 296 (17), 266 (7), 253 (15), 208 (23), 180 (23), 180 (43), 160 (23), 136 (33), 134 (18), 91 (14), 83 (10), 81 (12), 57 (50). HREIMS:m/z594.2733 [M]+; calculated for C36H38N2O6.1HNMR(500 MHz, CDCl3):δH6.57 (1H, s, H-5′), 5.87 (1H, H-8′), 6.74 (1H, s, H-5), 6.27 (1H, d,J=2.0 Hz, H-10), 6.83(1H, ddJ=8.4, 2.2 Hz, H-14),7.06 (1H, dd,J=8.4, 2.2 Hz,H-14′), 6.79 (1H, dd,J=8.0, 2.0 Hz, H-13′), 6.70 (1H, dd,J=8.0, 2.4 Hz, H-13), 6.54 (1H, dd,J=8.0, 2.0 Hz, H-10),3.92 (1H, d,J=8.5 Hz, H-1′), 3.83 (3H, s, OMe), 3.78 (3 H,s,OMe), 2.42 (3H, s, N-Me), 2.18 (3H, s, N-Me), 3.80 (1H,m, H-1), 3.10 and 3.30 (4H, m, H-3 y H-3′), 2.66 and 2.79(4H, m, H-4, H-4′).13C NMR (400 MHz, CDCl3):δC155.4 (s, C-12′), 148.0 (s, C-6′), 146.7 (s, C-6), 145.9 (s, C-12), 143.1 (s, C-11), 143.1 (s, C-7′), 137.9 (s, C-8), 137.2 (s, C-7), 132.6 (s, C-9), 132.2 (d, C-10′), 131.7 (s, C-9′), 129.9 (d, C-14′), 128.6 (s, C-4a′), 128.1 (s, C-8a′), 125.7 (d, C-14), 125.4 (s, C-8a), 124.6 (s, C-4a), 121.0 (d, C-10), 117.9 (d, C-8′), 115.4 (d, C-13), 114.9 (d, C-13′), 111.9 (d, C-5′), 107.7 (d, C-5), 64.4 (d, C-1′), 59.3 (d, C-1), 56.0 and 55.8 (s, 2×OMe), 46.5 (t, C-3′), 44.2 (t, C-3), 42.5 and 42.4 (q, 2×N-Me), 25.2 (t, C-4′), 23.2 (t, C-4). The curine solution was prepared using 1 mg of the crystal in 50 µLof 1 N HCl and 500 µL of distilled water. The pH was adjusted to 7-8 with 1 N NaOH. The volume was brought to 1000 µL with phosphate buffered saline (PBS).

Animals

Male or female Swiss mice and male C57Bl/6 mice weighing 25- 30 g were obtained from the Federal University of Paraiba and the Oswaldo Cruz Foundation breeding units, respectively. The animals were maintained with food and water *ad libitum* in a room with the temperature ranging from 22ºC to 24ºC and a 12 h light/dark cycle. This study was carried out in accordance with the recommendations of the Brazilian National Council of Control of Animal Experimentation (CONCEA). The protocols were approved by the Animal Welfare Committee of the Oswaldo Cruz Foundation (CEUA/FIOCRUZ protocol # L-033/09) and the Ethical Committee for Experimental Animals (CEPA Nº 0504/08, UFPB). Groups of 5 to 10 animals were used in each experiment.

Treatments

For the *in vivo* experiments, the animals were orally (p.o.) pre-treated with curine (2.5 mg/ kg). Indomethacin (10 mg/kg, po), an NSAID, and morphine (10 mg/kg, po), a central acting analgesic drug, were used as standard pharmacological controls. PBS (po) was used as negative control. All treatments were performed 1 h before each challenge. For the *in vitro* experiments, cells were treated with curine (1 or 10 μM), 1 h before the stimulus. Non-treated cells received supplemented RPMI medium as detailed below. The dose of curine (2.5 mg/Kg) was chosen based on the results obtained from the pre-treatment dose-response curve performed by Ribeiro-Filho and colleagues (2013) in a mouse model of allergic asthma.

Mouse paw edema induction

Acute inflammation of the mouse hind paw was induced as described (Posadas *et al*., 2004; Leite *et al*., 2007). Briefly, 1 h after the pre-treatments, Swiss mice were subcutaneously (s.c.) injected with 20 μL of carrageenan (500 μg/paw) or zymosan (200 μg/paw) into the plantar region of the left hind paw. As control, PBS (20μL) was injected into the right hind paw. The paw thickness of each animal was measured before and after the challenge using a plethysmometer (UgoBasile), and the results were expressed as the difference in volume between the left and the right paw.

Acetic acid-induced peritoneal vascular permeability in mice

The peritoneal vascular permeability in mice was adapted from the procedure described by Whittle (1964). Briefly, 30 min after the pre-treatments, Swiss mice received an intraperitoneal (i.p.) injection of 1% Evans blue solution (0.1 ml/10 g) followed by 0.6% acetic (ip) 30 min later. The animals were sacrificed 50 min late. The peritoneal cavity was washed with PBS (10 mL), and the peritoneal fluid was collected. The vascular permeability was expressed as the optical density (O.D.) of Evans blue dye in an UV/VIS spectrophotometer at 610 nm.

The Acetic Acid-Induced Writhing Response

Abdominal contortions were induced as previously described (Nardi *et al*., 2006). Intraperitoneal injections of acetic acid are known to induce abdominal writhing in mice followed by hind limb twitching, which are indicative actions of nociception (Koster *et al*., 1959). One hour after the treatments, the animals received an injection (i.p.) of 1.0% acetic acid solution (0.1 mL/10 g). After being challenged, the animals were placed in individual boxes, and the abdominal constrictions were counted cumulatively over a period of 20 min. Nociception was expressed as the number of writhings.

The Formalin Test

The formalin test was used to analyze the licking behavior, which is characteristic of this nociceptive model. This procedure has been described by Hunskaar & Hole (1987). Briefly, 1 h after pre-treatment, Swiss mice injected with 20 μL of 2.5% formaldehyde (formalin solution) into the right hind paw. The animals were placed in individual boxes and observed from 0-5 min (neurogenic phase) and 15-30 min (inflammatory phase), and the total time spent licking the injected paw (licking time) was recorded with a chronometer and considered as a parameter of nociception.

Carrageenan-induced hyperalgesia

Hyperalgesia was analyzed using the hot plate test as previously described (Sammons *et al*., 2000). Briefly, mice were injected with 20 μL of carrageenan (25 μg/paw) into one hind paw1 h after the pre-treatments. The other hind paw was injected with an equal volume of PBS and was used as negative control. The animals were placed on a plate (IITC Life Science Inc., Woodland Hills, CA) at 52°C. The latency time of each paw was manually recorded with a chronometer for 30 s, and the following behaviors were considered: 1- jump (*i.e*., all paws raised from the surface of plate), 2- licking of a hind paw, 3- shaking of a hind paw, 4- lifting of a hind paw and spreading of the phalanxes or 5- rapid repeated lifting of the hind paws. Hyperalgesia was defined as a decrease in the withdrawal latency of the carrageenan-challenged paw compared with the PBS- challenged paw.

Peritoneal macrophage culture

Peritoneal macrophages from C57Bl/6 mice were obtained 4 days after an injection of 4% thioglycollate by washing the peritoneal cavity with RPMI 1640 medium supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin. The cells wereadjusted to a concentration of 2 x 10^6 /mL and were plated in 24-well culture plates (500 μL) at 37°C in 4% CO_2 atmosphere overnight. Following the incubation, the cells were pre-treated with curine (1 or 10μM) and were stimulated with LPS (500 ng/mL) l h later. Notably, 1 μM and 10 μM curine did not affect the cell viability.

COX-2 expression and PGE_2 production analyses

Twelve hours after LPS stimulus, the supernatants from the cell cultures described above were collected, and the PGE_2 production was analyzed using DuoSet kits, according to manufacturer's instructions (R&D Systems).Then, COX-2 expression was analyzed by Western blotting as follows. Briefly, the cells were washed with PBS buffer and homogenized with 10 mM Tris-HCl buffer (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, 10% glycerol (v/v), 0.1 mM EDTA, 1 mMDTT and a cocktail of protease inhibitors (Roche, Germany). The proteins from the cell homogenates were separated by polyacrylamide gels in the presence of 10% SDS at a

constant current of 16 mA. Full range rainbow (RPN800E, GE Healthcare Life Sciences, Piscataway, NJ) was used as a relative molecular mass standard. After gel separation, the samples were transferred at 200 mA for 120 min onto a nitrocellulose membrane using 25 mM Tris–HCl, pH 8.3 and 192 mM glycine at 4°C. The membranes were blocked with TBS-0.tTween 20 and 5% milk for 1 h, incubated with a polyclonal antibody (1:1000) raised against COX-2 (sc-1745, Santa Cruz Biotechnology, CA)for 18 h, incubated in a secondary antibody (anti-goat IgG-HRP, Santa Cruz Biotechnology, CA) for 1 h and developed using the Super Signal West Picochemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL).

Statistical analyses

A one-way ANOVA and a Dunnett test, as post-test, were used to determine the statistical significance in comparison to the control. Data are expressed as the mean ± standard error of the mean (SEM) of at least four independent experiments performed in duplicate or triplicate, as described above. P values of 0.05 or less were considered to be statistically significant. Statistical analyses were made using GraphPad Prism 5 Software (San Diego, CA, USA).

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DISCLOSURE OF CONFLICT OF INTEREST

The authors state that they have no conflict of interest.

REFERENCES

Allen, D.B., 2006. Effects of inhaled steroids on growth, bone metabolism, and adrenal function. Advances in Pediatrics. 53, 101-110.

Armstrong, R.A.,Marr, C.,Jones, R.L., 1995. Characterization of the EP-receptor mediating dilatation and potentiation of inflammation in rabbit skin. Prostaglandins. 49 (4), 205-224.

Barnes, P.J., 2006. How corticosteroids control inflammation, British Journal of Pharmacology**.** 148, 245-254.

Barnes, P.J., Greening, A.P., Crompton, G.K., 1995. Glucocorticoid resistance in asthma.American Journal of Respiratory and Critical Care Medicine. 152, 125 - 140.

Bentley, G.A., Newton, S.H., Starr, J., 1983. Studies on the Anti-nociceptive action of α-Agonist Drugs and their Interaction with Opioid Mechanisms.British Journal ofPharmacology. 79, 125-134.

Bezerra-Santos, C.R., Vieira-de-Abreu, A.,Barbosa-Filho, J. M. Piuvezam, M. R., Bozza, P.T., 2006. Anti-allergic properties of Cissampelos sympodialis and its isolated alkaloid warifteine. International Immunopharmacology. 6 (7), 1152-1160.

Bjarnason I.,Hayllar J.,MacPherson, A.J.,Russell, A.S., 1993. Side effects of nonsteroidal anti-inflammatory drugs on the small and large intestine in humans. Gastroenterology. 104(6), 1832-1847.

Boniface, k., Bak-Jensen, k.S., Li, Y.,Blumenschein, W.M., McGeachy, M.J., McClanahan, T.K., McKenzie, B.S., Kastelein, R.A., Cua, D.J., de Waal Malefyt, R., 2009. Prostaglandin E2 regulates Th17 cell differentiation and function through cyclic AMP and EP2/EP4 receptor signaling. The Journal of Experimental Medicine. 206 (3), 535-548.

Campos, M.M., Calixto, J.B.,2000. Neurokinin mediation of edema and inflammation. Neuropeptides. 34 (5), 314–322.

Castellheim, A., Brekke, O.L., Espevik, T., Harboe, M., Mollnes, T.E., 2009.Innate immune responses to danger signals in systemic inflammatory response syndrome and sepsis.Scandinavian Journal of Immunology. 69, 479-491.

Chen, G. Y.,Nunes, G.,2010. Sterile inflammation: sensing and reacting to Damage. Nature Reviews Immunology. 10, 826-837.

Claudino, R.F., Kassuya, C.A., Ferreira, J., Calixto J.B., 2006. Pharmacological and molecular characterization of the mechanisms involved in prostaglandin E2-induced mouse paw edema. The Journal of Pharmacology and Experimental Therapeutics. 318 (2), 611-618.

Collier, H.O.,Dinneen, L.C., Johnson, C.A., Schneider, C., 1968. The abdominal constriction response and its suppression by analgesic drugs in the mouse. British Journal of Pharmacology and Chemotherapy. 32, 295-310.

Corrêa, P.M., 1984. Dicionário das plantas úteis do Brasil e das exóticas cultivadas. Brasília: Ministério da Agricultura, Instituto Brasileiro de Desenvolvimento Florestal.

Dannhardt, G., Kiefer, W., 2001. Cyclooxygenase inhibitors-current status and future prospects. European Journal of Medicinal Chemistry. 36, 109-126.

Dias, C.S., Barbosa-Filho, J.M.; Lemos, V.S., Cortes, S.F., 2002. Mechanisms involved in the vasodilator effect of curine in rat resistance arteries. Planta Medica. 68, 1049-1051.

Guedes, D.N., Barbosa-Filho, J.M., Lemos, V.S., Côrtes, S,F., 2002. Mechanism of the vasodilator effect of 12-O-methylcurine in rat aortic rings.Journal of Pharmacy and Pharmacology.54, 853-858.

He, M., Lau, H.Y., NG, S.W., Bhatia, M., 2007. Chemokines in acute inflammation: regulation, function and therapeutic strategies. International Journal of Integrative Biology. 1, 18-27.

Hinson, R.M., Williams, J.A., 1996. Shacter, E., Elevated interleukin 6 is induced by prostaglandin E2 in a murine model of inflammation: possible role of cyclooxygenase-2. Proceedings of the National Academy of Sciences of the United States of America. 93, 4885-4890.

Hunskaar, S., Hole, K., 1987. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain. 30, 103-114.

Ivanovska, N., Georgieva, P., Barot-Ciorbaru, R.,1996. Correlation between inhibited alternative complement activity and the protective effect induced by Nocardia lysozyme digest (NLD) during Klebsiella pneumoniae infection in mice. International Journal of Immunopharmacology. 18, 515-519.

Kaplan, A.P., Joseph, K., Silvenberg, M., 2002. Pathways for bradykinin formation and inflammatory disease. Journal of Allergy and Clinical Immunology**.**109(2),195-209.
Kidd, B.L., Urban, L.A.,2001. Mechanisms of inflammatory pain. British Journal of Anaesthesia. 87, 3-11.

Kinghorn, A. D., 2001. Pharmacognosy in the 21st century. The Journal of Pharmacy and Pharmacology. 53, 135-148.

Kingston, W., P., Greaves, M. W.,1985. Actions of prostaglandin E2 metabolites on skin microcirculation. Agents and Actions. 16, 13-14.

Kondo, Y., Imai, Y., HOJO, H., Hashimoto, Y.,Nozoe, S., 1992. Selective inhibition of T-cell-dependent immune responses by bisbenzylisoquinoline alkaloids in vivo. International Journal of Immunopharmacology.14,1181-1186.

Kubota, T., Koike, R.,2010. Cryopyrin-associated periodic syndromes: backgroundand therapeutics. Modern Rheumatology. 20, 213-221.

Kumar, H., Kawai, T., Akira, S., 2011. Pathogen recognition by the innate immune system.International Reviews of Immunology. 30, 16-34.

Kupeli, E.,Kosar, M., Yesilada, E., Husnu, K.,Baser, C., 2002. A comparative study on the anti-inflammatory, antinociceptive and antipyretic effects of isoquinoline alkaloids from the roots of Turkish *Berberis*species. Life Sciences. 72, 645-657.

Lawrence, T., Willoughby, D.A., Gilroy, D.W., 2002. Anti-inflammatory lipid mediators and insights into the resolution of inflammation. Nature Review Immunology. 2, 787-795.

Lee, S. H., Soyoola, E., Chanmugam, P.,Hart, S., Sun, W., Zhong, H., Liou, S., Simmons, D., Hwang, D.,1992. Selective expression of mitogen-inducible cyclooxygenase in macrophages stimulated with lipopolysaccharide. The Journal of Biological Chemistry. 267, 25934-25938.

Lefkowitz, D.L., Gelderman, M.P., Fuhrmann, S.R.,Graham, S., Starnes, J.D., Lefkowitz, S.S., Bollen, A., Moguilevsky, N., 1999. Neutrophilicmyeloperoxidase-macrophage interactions perpetuate chronic inflammation associated with experimental arthritis. Clinical Immunology. 91,145-155.

Leite, D.F., Echevarria-Lima, J., Ferreira, S.C., Calixto, J.B., Rumjanek, V.M.2007. ABCC transporter inhibition reduces zymosan-induced peritonitis. Journal of Leukocyte Biology. 82, 630-637.

Luo C.N., Lin, X., Li, W.K.,Pu, F., Wang, L.W.,Xie, S.S., Xiao, P.G., 1998.Effect of berbamine on T-cell mediated immunity and the prevention of relection on skin transplants in mice. Journal of Ethnopharmacology. 59, 211-215.

McInnes, I. B., Schett, G., 2011. The Pathogenesis of Rheumatoid Arthritis. The New England Journal of Medicine. 365, 2205-2219.

Medeiros, M.A., Pinho, J.F.,de-Lira, D.P., Barbosa-Filho, J.M,, Araújo, D.A., Cortes, S.F., Lemos, V.S., Cruz, J.S., 2011. Curine, abisbenzylisoquinoline alkaloid, blocks L-type Ca(2) channels and decreases intracellular Ca(2) transients in A7r5 cells. European Journal of Pharmacology. 669, 100-107.

Medzhitov, R., 2010. Inflammation 2010: new adventures of an old flame. Cell. 140, 771-776.

Medzhitov, R., Horng, T.,2009. Transcriptional control of the inflammatory response. Nature Reviews Immunology. 9, 692-703.

Mima, S., Ushijima, H., Hwang, H.J., Tsutsumi, S., Makise, M., Yamaguchi, Y., Tsuchiya, T., Mizushima, T., 2007. Identification of the TPO1 gene in yeast, and its human orthologue TETRAN, which cause resistance to NSAIDs. FEBS Letters 581, 1457-1463.

Nakajima, H., Tanahashi, T., Yamada, J., Sun, S-J., Sugimoto, Y., 2011. Psycothropic agent and health food containing neferine. European Publication Server patent registration number: EP 1774968 B1.

Nardi, G.M., Dalbo, S., Monache, F.D., Pizzolatti, M.G., Ribeiro-do-Valle, R.M., 2006. Antinociceptive effect of Croton celtidifolius Baill (Euphorbiaceae). Journal of Ethnopharmacology.107, 73-78.

Pang, L., Pitt, A., Petkova, D., Knox, A.J., 1998. The COX-1/COX-2 balance in asthma. Clinical and Experimental Allergy. 28, 1050, 1058.

Posadas, I., Bucci, M., Roviezzo, F., Rossi, A., Parente, L., Sautebin, L., Cirino, G., 2004.Carrageenan-induced mouse paw oedema is biphasic, age-weight dependent and displays differential nitric oxide cyclooxygenase-2 expression. British Journal of Pharmacology. 142, 331-338.

Saad, B., Abouatta, B.S., Basha, W., Hmade, A., Kmail, A., Khasib, S., Said, O., 2011. Hypericum triquetrifolium-Derived Factors Downregulate the Production Levels of LPS-Induced Nitric Oxide and Tumor Necrosis Factor-alpha in THP-1 Cells. Evidence-based complementary and alternative medicine : eCAM2011, 1-7.

Sammons, M.J., Raval, P., Davey, P.T., Rogers, D., Parsons, A.A., Bingham, S., 2000. Carrageenan-induced thermal hyperalgesia in the mouse: role of nerve growth factor and the mitogen-activated protein kinase pathway. Brain Research. 876, 48-54.

Schmid-Schonbein, G.W., 2006. Analysis of inflammation. Annual Review of Biomedical Engineering. 8, 93-131.

Scrivo, R., Vasile, M., Bartosiewicz, I., Valesini, G., 2011. Inflammation as "common soil" of the multifactorial diseases.Autoimmunity Reviews.10, 369-374.

Sheibanie, A.F., Tadmori, I., JING, H., Vassiliou, E., Ganea, D.,2004. Prostaglandin E2 induces IL-23 production in bone marrow-derived dendritic cells. FASEB journal: official publication of the Federation of American Societies for Experimental Biology. 18, 1318-1320.

Shibata, M., Ohkubo, T., Takahashi, H., Inoki, R., 1989.Modified formalin test: characteristic biphasic pain response. Pain. 38, 347-352.

Solomon, L.M., Juhlin, L., Kirschenbaum, M.B.,1968. Prostaglandin on cutaneous vasculature. The Journal of Investigative Dermatology. 51, 280-282.

Souto, A.L., Tavares, J.F.,da Silva, M.S., Diniz, Mde F., de Athayde-Filho, P.F., Barbosa Filho, J.M.,2011. Anti-inflammatory activity of alkaloids: an update from 2000 to 2010. Molecules. 16, 8515-8534.

Tabas, I., Glass, C.K.,2013. Anti-inflammatory therapy in chronic disease: challenges and opportunities. Science.339, 166-172.

Tjølsen, A., Berge, O.G., Hunskaar, S.,Rosland, J.H., Hole, K.,1992. The formalin test: an evaluation of the method. Pain. 51,5-17.

Underhill, D.M.,2003. Macrophage recognition of zymosan particles. Journal of Endotoxin Research.9, 176-180.

Vane, J.R.,Botting R.M.,1996.Mechanism of action of anti-inflammatory drugs.Scandinavian Journal of Rheumatology. 25,9-21.

Whittle, B.A.,1964. The Use of Changes in Capillary Permeability in Mice to Distinguish between Narcotic and Nonnarcotic Alalgesics. British Journal of Pharmacology and Chemotherapy. 22, 246-253.

Williams, J.A., Shacter, E.,1997. Regulation of macrophage cytokine production by prostaglandin E2. Distinct roles of cyclooxygenase-1 and -2. The Journal of Biological Chemistry. 272, 25693-25699.

Williams, K.I., Higgs, G.A.,1988. Eicosanoids and inflammation. The Journal of Pathology. 156, 101-110.

Williams,C.M., Galli,S.J., 2000. The diverse potential effector andimmunoregulatory roles of mast cells in allergic disease.Journal of Allergy and Clinical Immunology. 105, 847-849.

Wu, S.J., Ng, L.T.,2007. Tetrandrine inhibits proinflammatory cytokines, iNOS and COX-2 expression in human monocytic cells. Biological &Pharmaceutical Bulletin. 30, 59-62.

Wu, Y., Li, J.Q., Kim, Y.J., Wu, J., Wang, Q., Hao, Y., 2011.*In vivo*and *in vitro* antiviral effects of berberine on influenza virus. Chinese Journal of Integrative Medicine. 17, 444-452.

LEGENDS

Figure 1.0 The effect of curine on mouse paw edema. Swiss mice were orally pre-treated with curine (2.5 mg/Kg) or indomethacin (10 mg/Kg). After 1 h, the animals were injected (s.c.) with 20 µL of carrageenan (500 µg/paw) or zymosan (200 µg/paw) solutions into the left hind paw, and 20 µL of PBS was injected into the right hind paw. The results are expressed as difference of volume between the left and the right paw. A) Carrageenan or B) zymosan-induced mouse paw edema. The results are expressed as the mean ± SEM from at least 6 animals. * Significantly different ($p< 0.05$) from non-treated group.

Figure 2.0 The effect of curine on acetic acid-induced vascular permeability. Swiss mice pre-treated (30 min before) with curine (2.5 mg/Kg) or indomethacin (10 mg/Kg)received an injection (ip) of 1% Evans blue solution (0.1 ml/10 g) followed (30 min later) by 0.6% acetic (ip). After 50 min, the animals were sacrificed, and the peritoneal fluid was collected. Vascular permeability was expressed as Evans blue dye O.D. at 610 nm. The results are expressed as the mean ± SEM from at least 6 animals. +Significantly different ($p< 0.05$) from non-stimulated group. * Significantly different from non-treated group.

Figure 3.0 The effect of curine on the acetic acid-induced writhing response. Swiss mice were treated (p.o.) with curine (2.5 mg/Kg) or indomethacin (10 mg/Kg) or morphine (10mg/kg). One hour later, the animals received an injection (i.p.) of 1.0% acetic acid (0.1 mL/10 g) and were placed in individual boxes for 20 min for analyses of the writhing response. The results are expressed as the mean ± SEM of the number of writhings from at least 6 animals. * Significantly different ($p< 0.05$) from non-treated group. ** Significantly different from non-treated or curine-treated group.

Figure 4.0 The effect of curine on formalin-induced licking behavior. Swiss mice were orally treated with curine (2.5 mg/Kg) or indomethacin (10 mg/Kg) or morphine (10 mg/Kg). After 1 h, 20µL of 2.5% formaldehyde solution (formalin) was injected into the right hind paw. The results are expressed as the licking time in the first 5 min (neurogenic phase;A)or between 15-30 min (inflammatory phase;B).The results are

expressed as the mean ± SEM of the number of writhings from at least 6 animals. * Significantly different ($p< 0.05$) from non-treated group. ** Significantly different from non-treated or curine treated group.

Figure 5.0 The effect of curine on carrageenan-induced hyperalgesia. Swiss mice pre- treated with curine (2.5 mg/kg) or indomethacin (10 mg/kg) were injected with carrageenan (25 µg/paw) into one hind paws. PBS was injected into the other paw as a negative control. Hyperalgesia was analyzed 4 h later using a hot-plate test and is expressed as a decrease in the latency timeof the carrageenan-challenged paw compared to PBS-challenged paw.The results are expressed as the mean ± SEM of at least 6 animals. * Significantly different ($p< 0.05$) from PBS-challenged group.

Figure 6.0 The effect of curine on COX-2 expression and PGE_2 production. Peritoneal macrophages (5×10^5 cells/well) were incubated for 1h with curine (1 or 10 µM) and stimulated with LPS (500 ng/mL) for 12h. PGE_2 concentrations were determined using DuoSet kits, and COX-2 expression was analyzed by Western blotting. A) COX-2 expression. The results are representative of two different experiments. B) PGE_2concentrations. The results are expressed as the mean ± SEM of two different experiments.

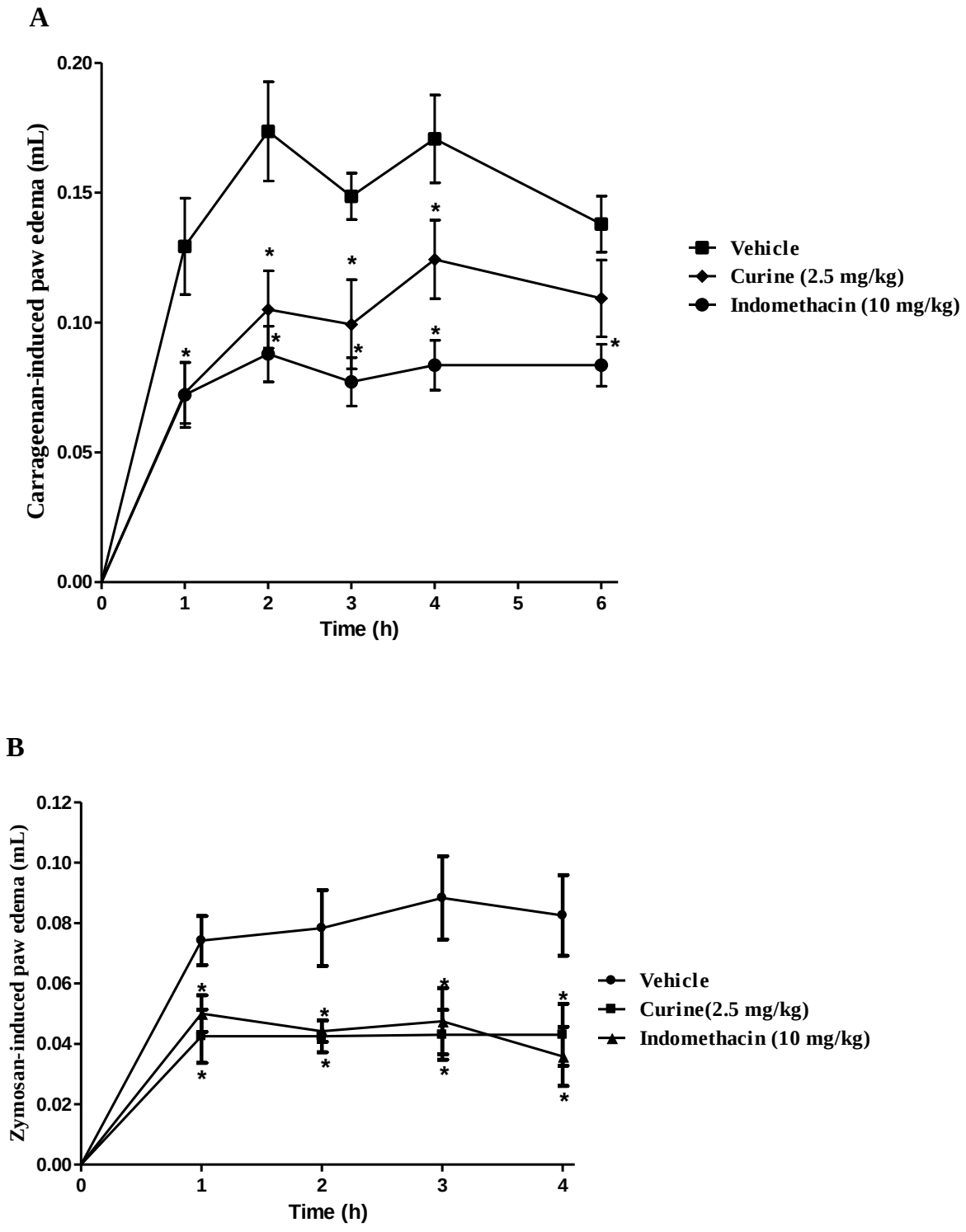
A
Carrageenan-induced paw edema (mL)
0.20
0.15
0.10
0.05
0.00
0
1
2
3
4
5
6
Time (h)
Vehicle
Curine (2.5 mg/kg)
Indomethacin (10 mg/kg)
B
Zymosan-induced paw edema (mL)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
1
2
3
4
Time (h)
Vehicle
Curine(2.5 mg/kg)
Indomethacin (10 mg/kg)

Figure 1

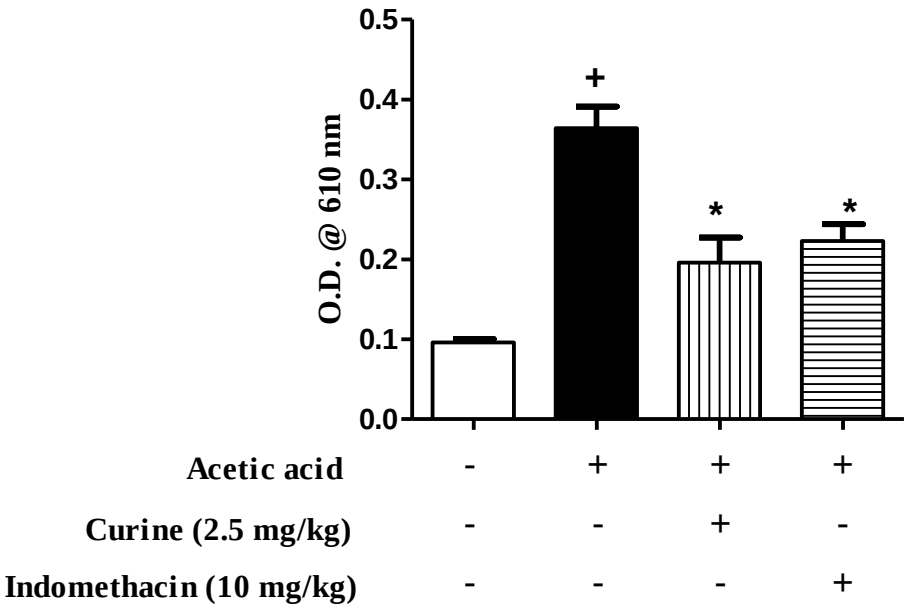
O.D. @ 610 nm
0.5
0.4
0.3
0.2
0.1
0.0
+
*
*
Acetic acid - + + +
Curine (2.5 mg/kg) - - + -
Indomethacin (10 mg/kg) - - - +

Figure 2

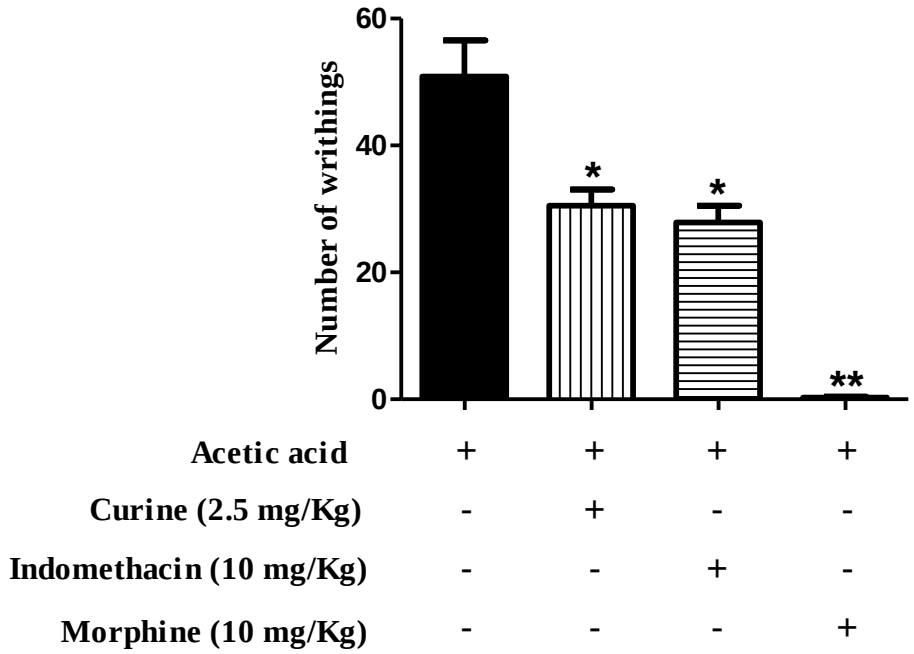
Number of writhings
60
40
20
0
*
*
**
Acetic acid + + + +
Curine (2.5 mg/Kg) - + - -
Indomethacin (10 mg/Kg) - - + -
Morphine (10 mg/Kg) - - - +

Figure 3

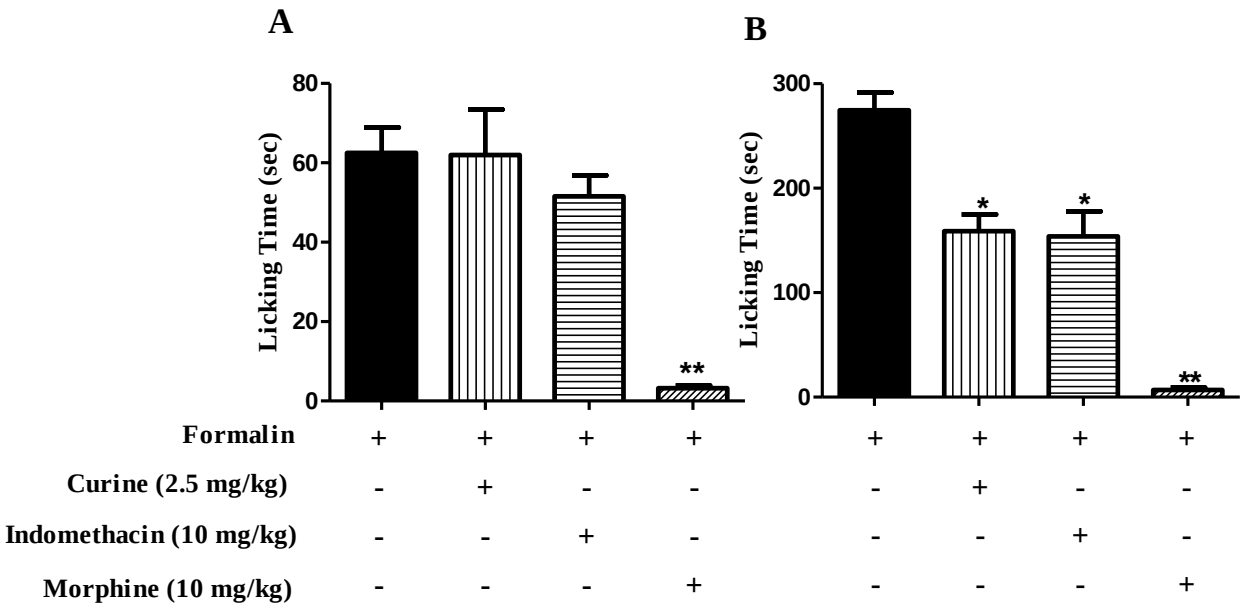
A
B
Licking Time (sec)
80
60
40
20
0
300
200
100
0
*
*
**
**
Formalin + + + + + + + +
Curine (2.5 mg/kg) - + - - - + - -
Indomethacin (10 mg/kg) - - + - - - + -
Morphine (10 mg/kg) - - - + - - - +

Figure 4

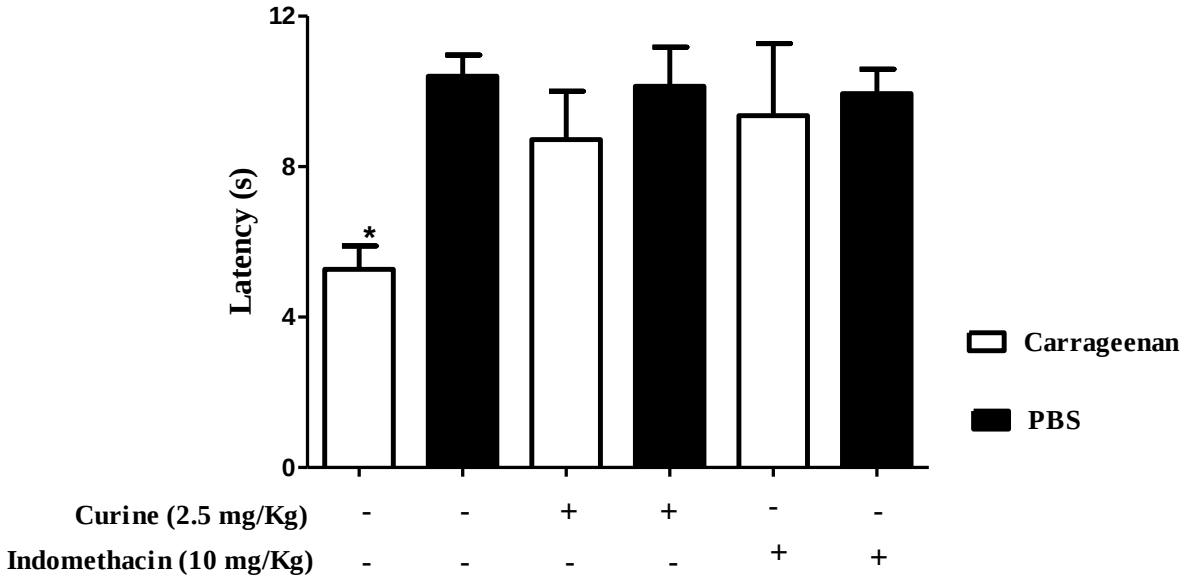
12
8
4
0
Latency (s)
*
Carrageenan
PBS
Curine (2.5 mg/Kg) - - + + - -
Indomethacin (10 mg/Kg) - - - - + +

Figure 5

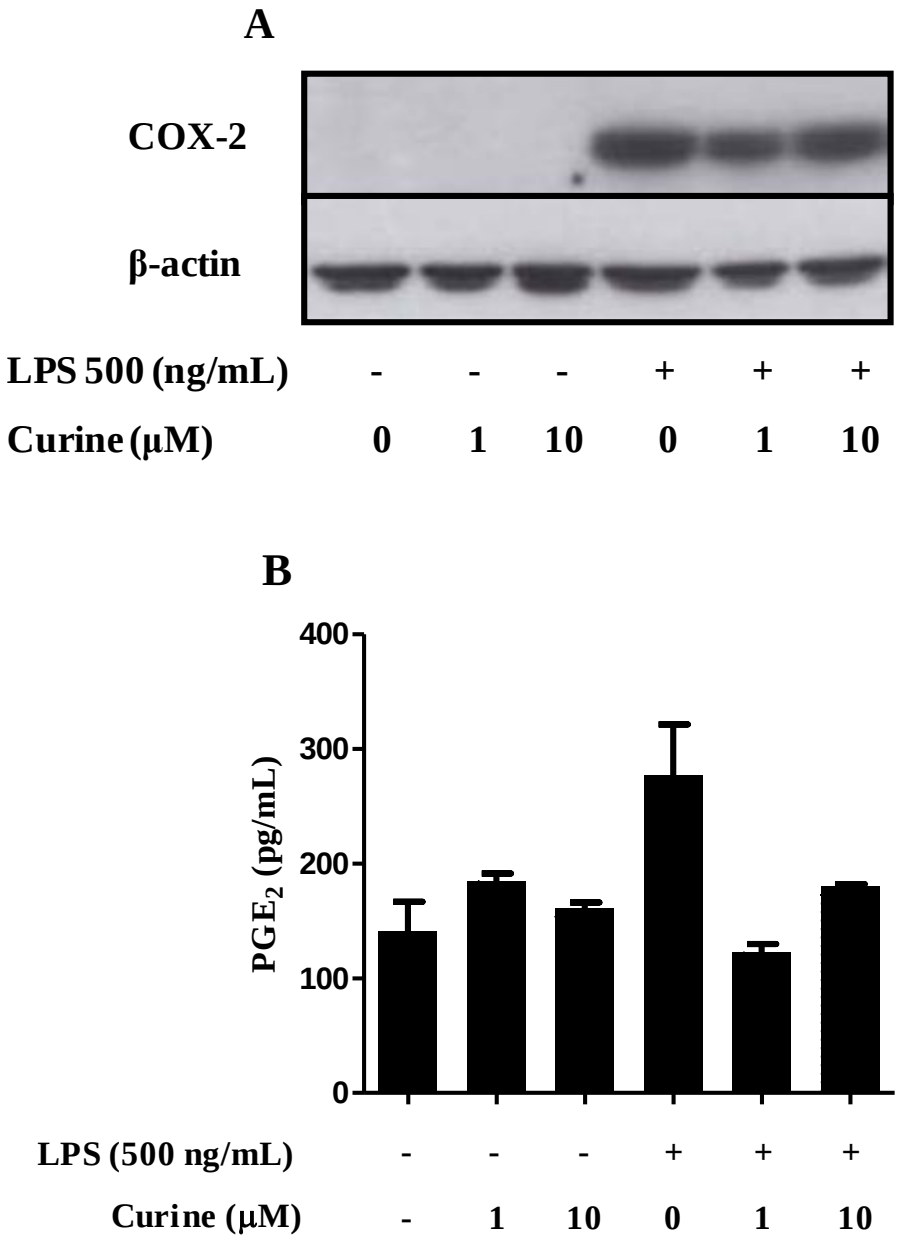
A
COX-2
β-actin
LPS 500 (ng/mL) - - - + + +
Curine (μM) 0 1 10 0 1 10
B
400
300
200
100
0
PGE2 (pg/mL)
LPS (500 ng/mL) - - - + + +
Curine (μM) - 1 10 0 1 10

Figure 6

3.3. Artigo III

CURINE INHIBITS MACROPHAGE ACTIVATION AND NEUTROPHIL RECRUITMENT IN A MOUSE MODEL OF LIPOPOLYSACCHARIDE-INDUCED INFLAMMATION. Ribeiro-Filho, J.; Leite, F. C.; Calheiros, A. S.; Carneiro, A. B.; Azeredo, J. A.; Assis, E. F.; Dias, C. S.; Piuvezam, M. R; Bozza, P. T. 2013. Em edição.

Nos artigos anteriores nós demonstramos os efeitos anti-alérgicos, analgésicos e anti - inflamatórios da curina administrada em doses não-tóxicas. Neste terceiro artigo, para melhor caracterizar a ação antiinflamatória deste alcaloide, nós investigamos os seus efeitos na ativação de macrófagos e no recrutamento de neutrófilos em um modelo experimental de pleurisia induzida por LPS. Nós demonstramos que o tratamento oral com a curina inibiu significativamente o recrutamento de neutrófilos para o lavado pleural, associado com a inibição da produção de citocinas inflamatórias tais como o TNF-α , a IL-6 , a MCP-1 e com a inibição de leucotrieno B_4 no lavado pleural. A curina também inibiu a produção de TNF-α, IL-1β, IL-6 e NO por macrófagos estimulados com LPS *in vitro*, e o verapamil, um antagonista de canal de cálcio apresentou efeitos inibitórios semelhantes na produção de TNF-α e IL- 1β por macrófagos, sugerindo que a curina desempenha um efeito inibitório sobre a ativação dos macrófagos, que podem estar associados com a inibição do influxo de cálcio. Para finalizar este artigo, nós estamos conduzindo experimentos para avaliar o efeito da curina na ativação de MAP quinases e do fator de transcrição NFκB, bem como na modulação do influxo de cálcio em macrófagos ativados com LPS, o que permitirá caracterizar os mecanismos moleculares de ação da curina neste modelo.

Curine inhibits macrophage activation and neutrophil recruitment in a mouse model of lipopolysaccharide-induced inflammation

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ABSTRACT

Macrophages play essential roles in immunity, working as initial detectors of inflammatory signals, such as bacterial derived lipopolysaccharide (LPS). Activated macrophages release mediators that cause neutrophil recruitment, and cross-talk between these two cells is associated with the development of inflammatory diseases. Curine is a bisbenzylisoquinoline alkaloid that is isolated from *Chondrodendron platyphyllum* (Menispermaceae). Recent reports have demonstrated the anti-allergic, analgesic and anti-inflammatory effects of curine at non-toxic doses. However, the mechanisms underlying this effects remain to be elucidated. This study investigated the effects of curine on macrophage activation and neutrophil recruitment in a mouse model of LPS-induced inflammation. Curine significantly inhibited the recruitment of neutrophils to the pleural lavage that was associated with the inhibition of the production of inflammatory cytokines such as TNF-α, IL-1β, IL-6, MCP-1 and inhibition of leukotriene B_4 in the pleural lavage. Curine inhibited the production of TNF-α, IL-1β, IL-6 and NO by LPS-stimulated macrophages *in vitro,* and verapamil, a calcium channel antagonist presented similar effects in the production of TNF-α, IL-1β by microphages, suggesting that curine plays an inhibitory effect on macrophage activation that result, at least in part, from calcium influx inhibition. In conclusion, curine presented anti-inflammatory effects that are associated with inhibition of macrophage activation through mechanisms that involve inhibition of inflammatory cytokines, LTB_4, NO and of Ca^{++} influx, confirming that it has the potential for use in anti-inflammatory drug development.

INTRODUCTION

Macrophages are innate cells that play essential roles in immunity. These cells work as initial detectors of inflammatory signals, including those produced by the host and the derived from microorganisms, such as lipopolysaccharide (LPS) (Borregaard, 2010). LPS signaling through TLR_4 induces macrophage activation by regulating intracellular pathways involved in cytokine, lipid mediator and oxygen reactive species (ROS) production, in a process that depends on mitogen-activated protein kinase (MAPK) and calcium influx (Dean *et al*, 1999). The mediators released by activated macrophages play key roles on neutrophil recruitment and activation, and there are evidences that cross-talk between these two cells is essential in the progress of immune responses as well as in the development of many inflammatory diseases (Amulic *et al*., 2012).

Curine (Figure 1A) is a bisbenzilisoquinoline alkaloid (BBA) that is the major constituent of the root bark of *Chondrodendron platyphyllum* (Menispermaceae). This plant is popularly known as "abútua", and it has been used in the Brazilian folk medicine to treat inflammatory conditions (Correa, 1984; Gotfredsen, 2013). The phytochemical analysis of the root demonstrated that *C. platyphyllum* contains at least three alkaloids, including curine, isocurine and 12-O-metilcurine (Dias *et al*., 2002). Earlier reports demonstrated that BBA are bioactive natural compounds, presenting interesting pharmacological properties, such as anti-inflammatory, anti-allergic and analgesic (Souto, 2011). Accordingly, previous studies demonstrated the pharmacological properties of the alkaloids extracted from *Chondrodendron platyphyllum* (Guedes *et al.,* 2002). Dias and collaborators (2002) have demonstrated that curine and isocurine presented vasodilator effect and suggested that this effect was associated with inhibition of calcium channels. Medeiros and collaborators (2011) demonstrated that curine decreased intracellular Ca^{2+} transients in A7r5 cells, possibly through a direct blockade of L-type Ca^{2+} channels.

Recently, we demonstrated the anti-allergic effects of an oral treatment with curine using a mouse model of allergic asthma. The oral administration of curine significantly inhibited eosinophilic inflammation, eosinophil lipid body formation, cytokine production and airway hyper-responsiveness (AHR) *in vivo*. Verapamil, a calcium channel antagonist, had similar anti-allergic properties, and curine pre-treatment inhibited the calcium-induced tracheal contractile response *ex-vivo*,

suggesting that the mechanism by which curine exerts effects is through the inhibition of a calcium-dependent response. Importantly, oral treatment with curine for 7 consecutive days in doses up to 10 fold higher than its median effective dose (ED_{50}) did not induce evident toxicity (Ribeiro-Filho *et al*., 2013).

We have provided additional data of curine properties in a work that investigated the anti-inflammatory and analgesic effects of this alkaloid. We have demonstrated that curine significantly inhibited the formation of paw edema induced by different inflammatory agents by decreasing vascular permeability. The analyses of the analgesic properties showed that curine inhibited acetic acid-induced writhing response, licking behavior in the inflammatory, but not in the neurogenic phase of the formalin test and carrageenan-induced hyperalgesia. Additionally curine inhibited PGE_2 production *in vitro*, without affect COX-2 expression. Importantly, the effects of curine treatment were similar to indomethacin (a non-steroidal anti-inflammatory drug), but different from morphine (a central acting analgesic drug), suggesting that the analgesic effects of curine do not result from a direct inhibitory effect on neuronal activation, but depend on anti-inflammatory mechanisms that, at least in part, result from inhibition of PGE_2 production (Leite *et al*., 2013).

Despite the consistent anti-inflammatory and analgesic properties of curine, the mechanisms underlying this effects remain to be elucidated. Therefore, this work aims to characterize the effects of curine on macrophage activation and neutrophil recruitment in LPS -induced inflammation.

METHODS

Curine Purification

Chondrodendron platyphyllum Hil St. (Miers) was collected in the municipality of Santa Rita, Paraíba, Brazil. The voucher specimen of this plant is deposited in the Herbarium Prof. Lauro Pires Xavier, number 3631-P, and was identified by Prof. Dr Maria de Fatima Agra. Spectroscopically pure curine was isolated from the root bark of *C. platyphyllum* as described by Mambu *et al.* (2000). The curine solution was prepared using 1 mg of the crystal in 50 µL of 1 N HCl and 500 µl of distilled water. The pH was

adjusted to 7-8 with 1 N NaOH. The volume was adjusted to 1000 µl, and the dilutions were made in phosphate-buffered saline (PBS).

Animals

Male C57Bl/6 mice weighing 20-30 g were obtained from the Oswaldo Cruz Foundation breeding unit. The animals were maintained with food and water *ad libitum* in a room with the temperature ranging from 22 to 24 ºC and a 12 h light/dark cycle. This study was carried out in accordance with the recommendations of the Brazilian National Council for the Control of Animal Experimentation (CONCEA). The protocols were approved by the Animal Welfare Committee of the Oswaldo Cruz Foundation (CEUA/FIOCRUZ protocol # L-002/08).

Treatments

For *in vivo* experiments, animals were orally (po) pre-treated with curine (2.5 mg/ kg) or dexamethasone (2.5 mg/Kg, po). PBS (po) was given as negative control. All treatments were performed 1h before PBS challenge. For *in vitro* experiments, cells were treated with curine (1 or 10 µM), 1h before the stimulus. Non-treated cells received supplemented RPMI medium as detailed below. The dose of curine (2.5 mg/Kg) was chosen based on the results obtained from the pre-treatment dose-response curve performed by Ribeiro-Filho and colleagues (2013) in a mouse model of allergic asthma.

LPS-induced pleurisy

Male C57Bl/6 mice orally pre-treated with curine or dexamethasone (2.5 mg/Kg) received an intrapleural injection of LPS (250 ng / cavity) dissolved in 100 µL of PBS. Four hour after the LPS injection, the animals were euthanized by exposure to an atmosphere of CO_2, and the pleura was surgically exposed. The pleural lavage (BAL) was collected from mice by washing the pleural cavity with 1 mL of PBS add with heparin (20 U/ml).

Leukocyte counts

Total leukocyte counts were performed using a Neubauer chamber under a light microscope after diluting the pleural lavage samples in Turk fluid (2% acetic acid). Differential counts were performed by microscopy with an objective lens at 100x magnification.

Peritoneal macrophage culture

Peritoneal macrophages from C57Bl/6 mice were obtained 4 days after the injection of 4% thioglycolate by washing the peritoneal cavity with RPMI 1640 medium supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin. Cells had the concentration adjusted to 2 x 10^6 /mL and were plated in 24-well culture plates (500 μL) at 37°C in 4% CO_2 atmosphere overnight Following incubation, cells were pre-treated with curine (1 or 10μM) and 1h later, stimulated with LPS (500 ng/mL) or LPS (100 ng/mL) + IFN-γ (10 U/ml). Of note, curine 1 or 10 μM did not affect cell viability.

Assay for nitrite and cytokine analyses

For NO determination, 100 μL of supernatants of macrophage culture were removed 24 h after LPS stimulus and incubated with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine hydrochloride in 2.5% $H3PO_4$), for 10 min at room temperature, and absorbance was measured at 540 nm in an ELISA reader (Titer tek Multiscan, Flow Laboratories, Finland). Nitrite concentration was calculated with reference to a standard curve obtained using $NaNO_2$ (1–200 μM in culture medium). The concentrations of cytokines in the supernatants of the pleural washes or macrophage culture were determined by ELISA 4 hours after stimulation with LPS according to the instructions of the manufacturer.

Statistical analyses

Data were analyzed by one-way ANOVA followed by Tukey's test using GraphPad Prism software (GraphPad, San Diego, CA). The values were expressed as the means ± S.E.M. Differences with $p < 0.05$ were considered significant.

RESULTS

Curine inhibits neutrophil recruitment in LPS- induced pleurisy

An intrapleural injection of LPS in mice induced an intense influx of neutrophils into the pleural cavity that was associated with the development of pleural inflammation (Figure 1B). To demonstrate the anti-inflammatory effects of pre-treatment with curine, the animals were pre-treated orally with curine (2.5 mg/Kg) or dexamethasone (2 mg/Kg) 1 h before the LPS challenge. This treatment significantly reduced the total number of neutrophil in the pleural lavage (Fig. 1B) compared with the untreated LPS-challenged group, demonstrating the inhibitory role that curine plays on neutrophil recruitment.

The effects of curine on cytokine production *in vivo*

Because we demonstrated that curine has a significant effect on neutrophil recruitment, we analyzed the effect of curine in the production of cytokines involved in neutrophil recruitment and inflammation. Supernatants of pleural lavages from mice challenged with LPS presented increased levels of inflammatory cytokines, including interleukin (IL) -1β, IL-6, tumor necrosis factor (TNF)-α, monocyte chemotactic protein (MCP)-1, KC and the eicosanoid leukotryene B_4 (LTB4) (Figure 2 A-F), compared to non-stimulated animals. The treatment with curine significantly inhibited the production of IL-6, TNF-α, MCP-1 and LTB_4, indicating that the inhibitory effect of curine on neutrophil recruitment is associated with inhibition of cytokine production.

Curine inhibits macrophage activation *in vitro*

Activated macrophages are importantly involved in the production of mediators in early inflammation. So, we investigated if curine has a direct inhibitory effect on macrophage activation, by analyzing the production of inflammatory cytokines and nitric oxide *in vitro.* As shown in Figure 3 (A-C), the addition of LPS in peritoneal macrophage culture induced an expressive production of IL- 1β, IL- 6 and TNF-α that was significantly inhibited by the pre-treatment with curine (Figure 3 A-C). Also, macrophages stimulated with LPS (Figure 3D) or LPS + IFN-γ (Figure 3E) presented increased levels of nitrite in the supernatants compared to non-stimulated cells, that were significantly decreased by curine pre-treatment (Figure 3 D-E). Together these data indicate that curine inhibits LPS-induced macrophage activation.

The effect of calcium influx inhibition on macrophage activation

We have recently demonstrated that curine and verapamil, a calcium channel antagonist, presented similar anti-allergic effects that were associated with calcium influx inhibition (Ribeiro-Filho *et al*., 2013). To evaluate the importance of calcium influx inhibition on macrophage activation as well its potential participation in curine anti-inflammatory mechanisms we compared the effects of verapamil and curine on macrophage activation. As shown in Figure 3, curine or verapamil pre-treatments at the same concentrations similarly inhibited the production of IL-1β (A) and TNF-α (B), suggesting that the inhibitory effect that curine plays on macrophage activation is at least in part dependent on calcium influx inhibition.

DISCUSSION

Inflammatory diseases are current important public health problems, with increasing prevalence, morbidity and mortality worldwide (Edwards *et al*., 2009). Such diseases frequently result from an inadequate immune response driven against infection or self components (Scrivo *et al*., 2011). Although inflammatory diseases can differ in various aspects, there are consistent evidences that macrophages and neutrophils play key roles in the initiation and development of many inflammatory conditions by

producing mediators that amplify the inflammatory response, including cytokines, lipid mediators and reactive oxygen species (Takeda & Akira, 2005).

In the world context, questions have raised concerning the efficacy and safety of the currently available medications (Cosendey *et al*., 2000). Classical anti-inflammatory drugs such as corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs) can be successfully used in the treatment of many inflammatory disorders, however there have been specific conditions on which they are not effective or can cause significant side effects (Rainsford, 2007). Therefore, the development of novel, safe and effective drugs has an important impact on anti-inflammatory therapy.

In this work we have demonstrated new roles as well as provided new mechanisms of curine as an anti-inflammatory compound. We demonstrated that curine oral pre-treatment significantly inhibited the recruitment of neutrophils to the pleural cavity in a mice model of pleurisy. Accordingly, curine significantly inhibited the production of various inflammatory cytokines including, IL-6, TNF-α, MCP-1 and the eicosanoid LTB_4, in the supernatants of pleural lavages from mice challenged with LPS, indicating that the inhibitory effect of curine on neutrophil recruitment is associated with inhibition of cytokine production. Neutrophils are the most abundant leukocytes in the blood, But under inflammatory conditions, they can rapidly leave the circulation and migrate to the inflammatory site (Amulic *et al*., 2013). Inflammatory signals such as chemokines and cytokines released by activated endothelial cells and macrophages play a direct effect on neutrophil recruitment (Borregaard *et al*., 2010). Previous studies have demonstrated that LTB_4 work as an important chemotactic agent to neutrophils (Afonso *et al*., 2012) stimulating the recruitment of this cells via BLT_1 receptor activation (Monteiro *et al*., 2011). Therefore, the inhibitory effect of curine on LTB_4 production might have a direct impact on neutrophil recruitment. Additionaly, LPS-induced cytokines such as IL-6, IL-β and TNF-α play central roles in inflammation. TNF-α and IL-β directly affect neutrophil recruitment by stimulating the expression of adhesion molecules, including selectins and integrins (Libby *et al*., 2007).

Activated macrophages are one of the most important sources of mediators in the early phase of inflammation (Fujiwara & Kobayashi, 2005). So, we hypothesized that curine might inhibit neutrophil recruitment and cytokine production in the pleural lavage by affecting macrophage activation. We demonstrated that curine significantly inhibited the production of IL-6, IL-β and TNF-α by murine macrophages stimulated with LPS *in vitro.* This suggest that the inhibitory effect of curine on cytokine

production by macrophage can result in inhibition of the phenotypic changes triggered by LPS challenge and thus is associated with the mechanisms by with curine regulates neutrophil recruitment. In the other hand, neutrophils can also affect macrophage biology by releasing products involved in monocyte / macropage influx and activation, such as MCP-1 (Soehnlein *et al*., 2009). In this work we demonstrated that the treatment with curine significantly inhibited the production of MCP-1 in the pleural lavage, suggesting that the cross-talk between neutrophils and macrophages might be impaired by curine treatment. Additionaly, curine significantly decreased nitrite concentrations in the supernatants of macrophages stimulated with LPS or LPS and IFN-γ, indicating that curine inhibit nitric oxide (NO) production *in vitro.* This finding bring additional information about the role of curine in macrophage activation, since NO production is a hallmark of activated macrophages as well as of inflammation (Chang *et al*., 1998). Importantly, recent reports have demonstrated NO in association with other ROS is critically involved in the formation of Neutrophil Extracellular Traps (NETs) (Patel *et al*., 2010).

We have recently reported that curine present ant-inflammatory and analgesic effects which mechanisms involve inhibition of PGE_2 production (Leite *et al*., 2013). Our data suggest that in addition to this mechanism, the inhibitory effect that curine plays in the production of cytokines such as IL-1β and TNF-α as well as in NO production significantly contribute to the ant-inflammatory and analgesic effects previously described, since several reports have demonstrated the role of these mediators on inflammatory pain (Kidd & Urban, 2001).

In previous works we have demonstrated that curine present an anti-allergic effect that, at least in part, result from inhibition of calcium influx (Ribeiro-Filho *et al*., 2013). In this work we hypothesized that calcium influx inhibition impair macrophage activation and this corroborate our previous data that highly suggest that the mechanism by which curine exerts its effects is through the inhibition of a calcium-dependent response. To confirm our hypothesis we compared the effect of verapamil to that of curine on the production IL-1β and TNF-α by LPS-stimulated macrophages and this was used as parameter of macrophage activation. When used under the same conditions (concentrations and time of pre-treatment), verapamil and curine showed similar inhibitory effects, suggesting that inhibition of calcium influx is significantly involved in the mechanisms by with curine inhibit inflammatory responses, corroborating with previously reported data (Ribeiro-Filho *et al*., 2013) .

In conclusion, curine presented anti-inflammatory effects that are associated with inhibition of macrophage activation through mechanisms that involve inhibition of inflammatory cytokines, LTB_4, NO and of Ca^{++} influx, confirming that it has the potential for use in anti-inflammatory drug development.

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REFERENCES

Afonso, P.V., Janka-Junttila, M., Lee, Y.J., McCann, C.P.; Oliver, C. M., Aamer, K. A., Losert, W., Cicerone, M.T., Parent, C.A., 2012. LTB_4 is a signal-relay molecule during neutrophil chemotaxis. Developmental Cell. 22 (5), 1079-1091.

Amulic, B., Cazalet, C., Hayes, G.L., Metzler, K.D., Zychlinsky, A., 2012. Neutrophil Function: From Mechanisms to Disease. Annual Review of Immunology. 30, 459-89.

Borregaard, N., 2010. Neutrophils, from marrow to microbes. Immunity. 33, 657-670.

Chang, C. I., Liao, J.C., Kuo, L., 1998. Arginase modulates nitric oxide production in activated macrophages. The American Journal of Physiology. 274 (1), 342-348.

Correa, P.M., 1984. Dicionário das plantas úteis do Brasil e das exóticas cultivadas. Brasília: Ministério da Agricultura, Instituto Brasileiro de Desenvolvimento Florestal.

Cosendey, M.A.E., Bermudez, J.A.Z., Reis, A.L.A., Silva, H.F., Oliveira, M.A., Luiza, V.L., 2000. Assistência farmacêutica na atenção básica de saúde: a experiência de três estados brasileiros. Cadernos Saúde Pública. 16, 171-182.

Dean, J.L.E., Brook, M., Clark, A.R., Saklatvala, J., 1999. p38 Mitogen-activated Protein Kinase Regulates Cyclooxygenase-2 mRNA Stability and Transcription in Lipopolysaccharide-treated Human Monocytes. The Journal of Biological Chemistry. 274, 264-269.

Dias, C.S., Barbosa-Filho, J.M., Lemos, V.S., Cortes, S.F., 2002. Mechanisms involved in the vasodilator effect of curine in rat resistance arteries. Planta Medica. 68, 1049-1051.

Edwards, M.R., Bartlett, N.W., Clarke, D., Birrell, M., Belvisi, M.. Johnston, S.L., 2009. Targeting the NF-κB pathway in asthma and chronic obstructive pulmonary disease. Pharmacology & Therapeutics. 121, 1-13.

Fujiwara, N., Kobayashi, K., 2005. Macrophages in inflammation. Current Drug Targets. Inflammation and Allergy. 4(3), 281-286.

Gotfredsen, E. Liber Herbarum II: Chondrodendron platyphyllum (A.St.Hil.) Miers. 2013. Retrieved August 8 from <http://www.liberherbarum.com/pn7625.HTM.

Guedes, D.N., Barbosa-Filho, J.M., Lemos, V.S., Côrtes, S,F., 2002. Mechanism of the vasodilator effect of 12-O-methylcurine in rat aortic rings.Journal of Pharmacy and Pharmacology. 54, 853-858.

Kidd, B. L., Urban, L. A. Mechanisms of inflammatory pain. 2001. British Journal of Anesthesia. 87 (1), 3-11.

Leite, F.C., Ribeiro-Filho, J., Costa, H.F., Salgado, P.R.R., Calheiros, A.S., Carneiro, A.B., Almeida, R.N., Dias, C.S., Bozza, P.T., Piuvezam, M.R., 2013. The anti-inflammatory and analgesic effects of curine, a bisbenzylisoquinoline alkaloid isolated from Chondrodendron platyphyllum (Menispermaceae). Journal of Natural Products, Submitted.

Libby, P. 2007., Inflammatory Mechanisms: The Molecular Basis of Inflammation and Disease. Nutrition Reviews. 65 (12), 140-146.

Mambu, L., Martin, M.T., Razafimahefa, D., Ramanitrahasimbola, D., Rasoanaivo, P., Frappier, F. 2000. Spectral characterisation and antiplasmodial activity of bisbenzylisoquinolines from Isolona ghesquiereina. Planta Medica. 66, 537-540.

Medeiros, M.A., Pinho, J.F., de-Lira, D.P., Barbosa-Filho, J.M,, Araújo, D.A., Cortes, S.F., Lemos, V.S., Cruz, J.S., 2011. Curine, abisbenzylisoquinoline alkaloid, blocks L-type Ca^{2+} channels and decreases intracellular Ca^{2+} transients in A7r5 cells. European Journal of Pharmacology. 669, 100-107.

Monteiro, A,T., Pinheiro, C.S., Luna-Gomes, T., Alves, L.R., Maya-Monteiro, C.M., Porto, B.N., Barja-Fidalgo, C., Benjamim, C.F., Peters-Golden, M., Bandeira-Melo, C., Bozza, M.T., Canetti, C., 2011. Leukotriene B_4 mediates neutrophil migration induced by heme. The Journal of Immunology. 186 (11), 6562-6567.

Patel, S., Kumar, S., Jyoti, A., Srinag, B.S.S., Keshari, R.S., Saluja, R., Verma, A., Mitra, K., Barthwal, M.K., Krishnamurthy, H., Bajpai, V.K., Dikshit, M., 2010. Nitric oxide donors release extracellular traps from human neutrophils by augmenting free radical generation. Nitric Oxide. 22, 226-234.

Rainsford, K.D., 2007. Anti-inflammatory drugs in the 21st century. Sub-cellular Biochemistry. 42, 3-27.

Scrivo, R., Vasile, M. Bartosiewicz, I., Valesini, G., 2011. Inflammation as "common soil" of the multifactorial diseases. Autoimmunity Reviews. 10, 369-374.

Soehnlein, O., Weber, C., Lindbom, L. 2009. Neutrophil granule proteins tune monocytic cell function. Trends in Immunology. 30 (11), 538-546.

Souto,A.L., Tavares, J.F.,Silva, M.S., Diniz, M. F., Athayde-Filho, P.F., Barbosa- Filho, J.M., 2011. Anti-inflammatory activity of alkaloids: an update from 2000 to 2010. Molecules. 16, 8515-8534.

Takeda, K, Akira, S., 2005. Toll-like receptors in innate immunity. Int Immunol.17, 1-14.

LEGENDS

Figure 1. The effect of curine on neutrophil recruitment in LPS-induced Pleurisy. C57Bl/6 mice (n = 6 - 7) were treated orally with increasing doses of curine (2.5 mg/Kg), or dexamethasone (2.5 mg/kg) 1 h before LPS challenge. Four hours after challenge, the pleural lavage was collected and the leukocytes were counted using a light microscope. A) the chemical structure of curine; B) the number of total leukocytes; C) the number of eosinophils; These results are expressed as the mean ± SEM of at least 6 animals. + Significantly different ($p < 0.05$) from the unchallenged group; * significantly different from the untreated, LPS-challenged group.

Figure 2. The effects of curine on cytokine production *in vivo*. C57Bl/6 mice (n = 6 - 7) were treated orally with increasing doses of curine (2.5 mg/Kg), or dexamethasone (2.5 mg/kg) 1 h before LPS challenge. Four hours after challenge, the pleural lavage was collected and the concentration of cytokines in the supernatants were analyzed by ELISA. A) The concentration of IL-1β; B) The concentrations of IL-6; C) The concentrations of TNF-α; D) The concentrations of MCP-1; E) The concentrations of KC / CXCL-1; F) The concentration of LTB_4 ; These results are expressed as the mean ± SEM of at least 6 animals. + Significantly different ($p < 0.05$) from the unchallenged group; * significantly different from the untreated, LPS-challenged group.

Figure 3. The effects of curine on macrophage activation. Peritoneal macrophages (5 x 10^5 cells/well) were incubated for 1h with curine (1 or 10 μM) and stimulated with LPS (500 ng/mL) or. The concentrations of IL-1β (A), IL-6 (B) and TNF-α (C) in the supernatants were determined by ELISA 4h after the LPS stimulus. The concentrations of nitrite were analyzed using the method of Griess 24h after the stimulus with LPS (500 ng/mL) (A) or LPS (100 ng/mL) + IFN-γ (10 U/ml) (B). These results are expressed as the mean ± SEM of at least 2 experiments made in triplicate. + Significantly different ($p < 0.05$) from non-stimulated cells; * significantly different from the untreated, LPS-stimulated cells.

Figure 4. The effect of calcium influx inhibition on macrophage activation. Peritoneal macrophages (5 x 10^5 cells/well) were incubated for 1h with curine (1 or 10 μM) or verapamil (1 or 10 μM) and stimulated with LPS (500 ng/mL). The concentrations of IL-1β (A), and TNF-α (B) in the supernatants were determined by ELISA 4h after the LPS stimulus. These results are expressed as the mean ± SEM of at least 2 experiments made in triplicate. + Significantly different ($p < 0.05$) from non-stimulated cells; * significantly different from the untreated, LPS-stimulated cells.

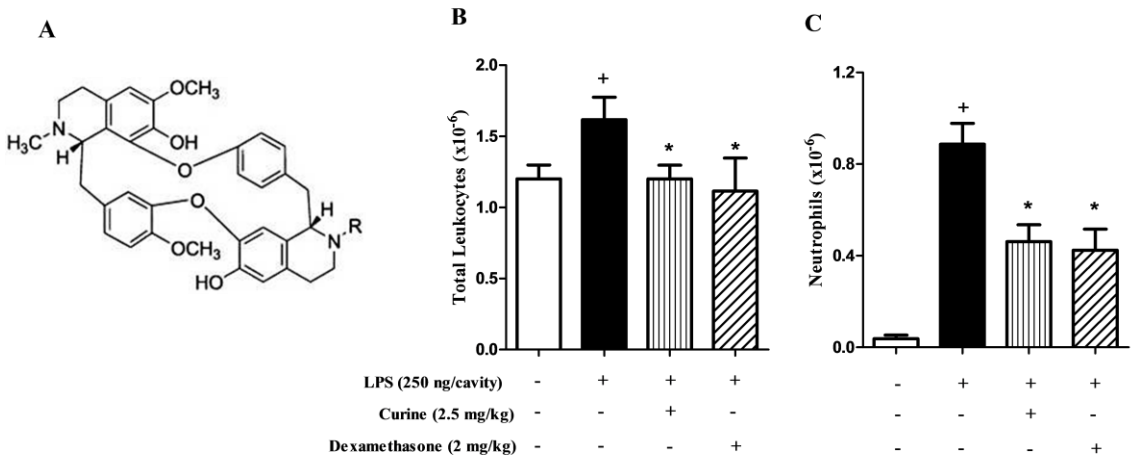
A
B
C
OCH3
OH
H3C
N
H
O
H
N
R
HO
Total Leukocytes (x10-6)
Neutrophils (x10-6)
2.0
1.5
1.0
0.5
0.0
1.2
0.8
0.4
0.0
+
*
LPS (250 ng/cavity) - + + +
Curine (2.5 mg/kg) - - + -
Dexamethasone (2 mg/kg) - - - +

Figure 1

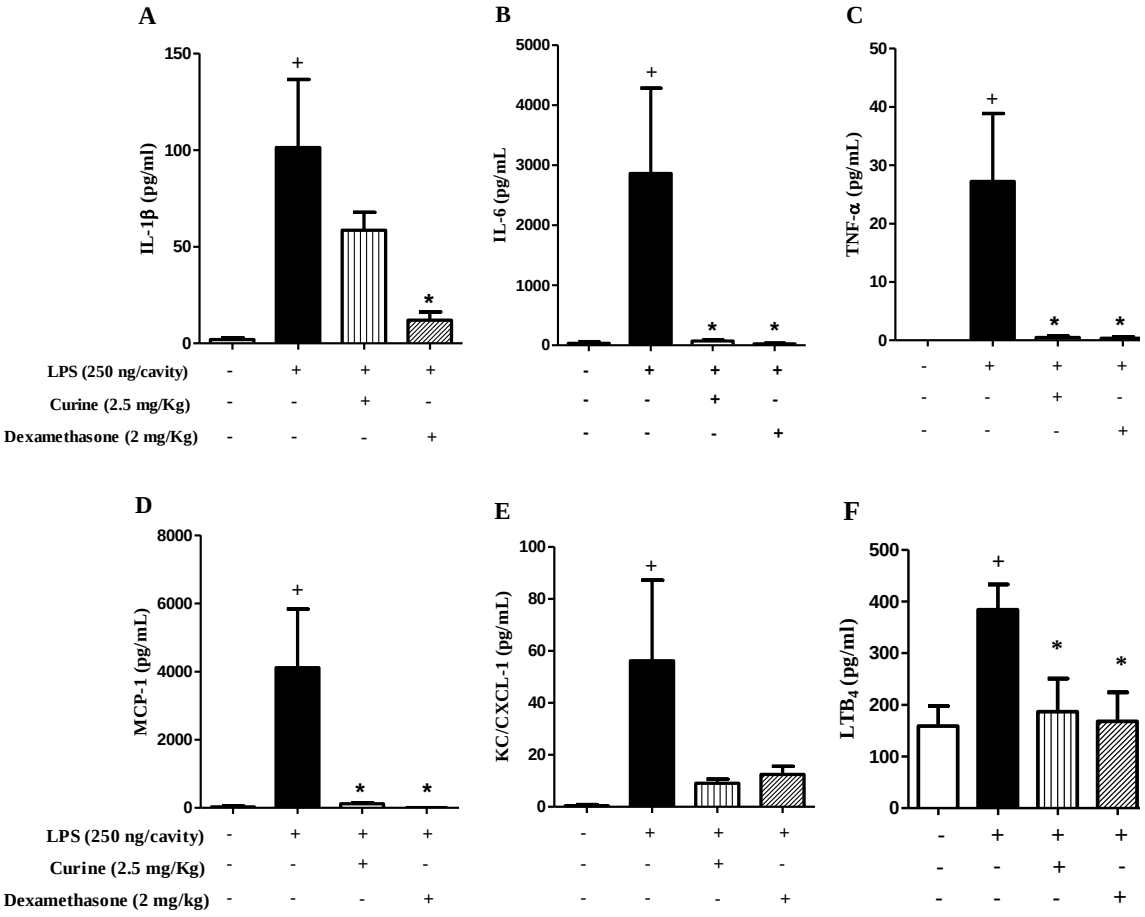
A
IL-1β (pg/ml)
150
100
50
0
B
IL-6 (pg/mL)
5000
4000
3000
2000
1000
0
C
TNF-α (pg/mL)
50
40
30
20
10
0
D
MCP-1 (pg/mL)
8000
6000
4000
2000
0
E
KC/CXCL-1 (pg/mL)
100
80
60
40
20
0
F
LTB4 (pg/ml)
500
400
300
200
100
0
LPS (250 ng/cavity) - + + +
Curine (2.5 mg/Kg) - - + -
Dexamethasone (2 mg/Kg) - - - +
LPS (250 ng/cavity) - + + +
Curine (2.5 mg/Kg) - - + -
Dexamethasone (2 mg/kg) - - - +

Figure 2

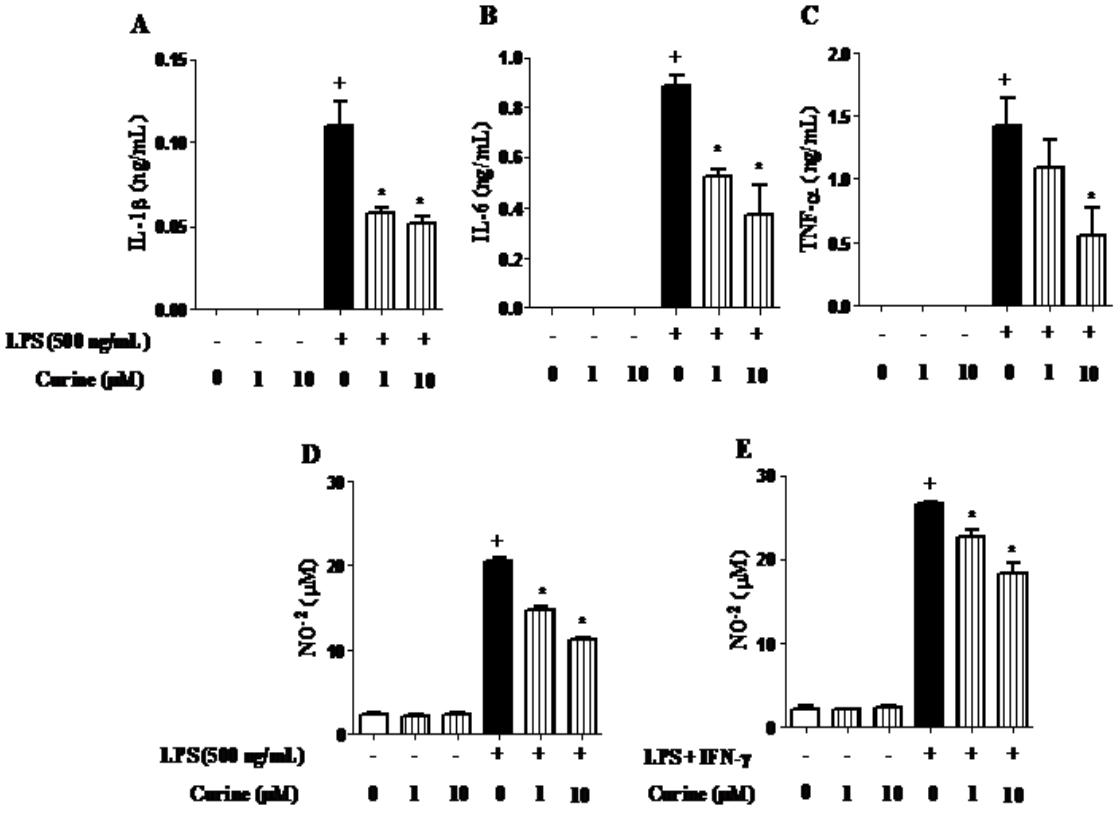
A
B
C
D
E
IL-1β (ng/mL)
IL-6 (ng/mL)
TNF-α (ng/mL)
NO-2 (μM)
LPS (500 ng/mL)
LPS+IFN-γ
Curine (μM)
0 1 10 0 1 10

Figure 3

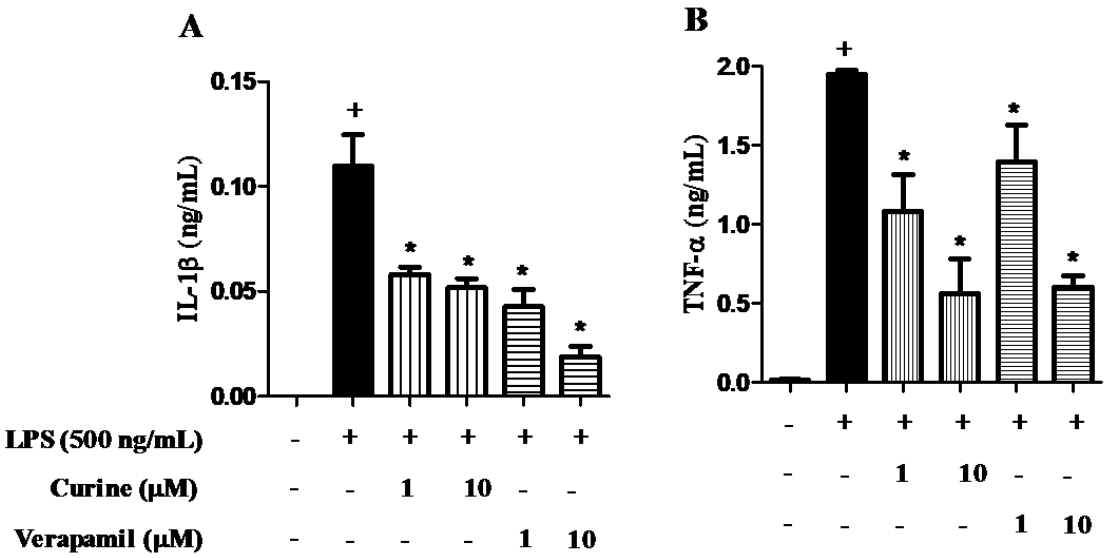
A
IL-1β (ng/mL)
0.15
0.10
0.05
0.00
B
TNF-α (ng/mL)
2.0
1.5
1.0
0.5
0.0
LPS (500 ng/mL) - + + + + +
Curine (μM) - - 1 10 - -
Verapamil (μM) - - - - 1 10

Figure 4

4. DISCUSSÃO

As doenças inflamatórias atualmente representam importantes problemas de saúde pública em termos de prevalência, morbidade e mortalidade. Várias destas doenças, incluindo a asma, são crônicas, e como tais, necessitam de terapia farmacológica a longo prazo (Edwards *et al*., 2009). A asma alérgica é uma das doenças mais prevalentes em todo o mundo (SBPT, 2012). Estas doença geralmente é tratada com uma combinação de medicamentos antiinflamatórios e broncodilatadores. Os corticosteróides inalatórios são atualmente os medicamentos mais importantes utilizados para controlar a inflamação das vias aéreas. Devido aos seus efeitos inibitórios potentes na ativação celular, produção de citocinas e mediadores lipídicos e expressão de moléculas de adesão e receptores inflamatórios, eles são capazes de bloquear simultaneamente vários passos essenciais da cascata inflamatória (Barnes, 2011, 2006; Barnes & Adcock, 2009; Rhen & Cidlowski, 2005). Contudo, a corticoterapia provoca efeitos colaterais significativos (Schacke *et al*., 2004), e alguns pacientes são completamente resistentes corticóides, ou seja, eles não apresentam melhora clínica após tratamento com altas doses de glicocorticóides orais (Barnes *et al*., 1995). Esta situação pode limitar o uso de corticosteróides e prejudicar a qualidade de vida dos pacientes. Alguns estudos sugerem que a ineficácia de corticosteróides em algumas formas graves de asma é devida à sua incapacidade de diminuir o nível de citocinas do perfil Th1, tais como o TNF (Truyen *et al*., 2006). Os medicamentos broncodilatadores clássicos, incluindo os agonistas β_2-adrenérgicos e os inibidores de fosfodiesterases têm sido muito utilizados para controlar a falta de ar, que é uma característica marcante de ataques de asma, no entanto, estes fármacos também não são isentos de efeitos colaterais (Boswell-Smith *et al*., 2006). Atualmente, os antagonistas de leucotrienos têm sido empregadas com sucesso na terapia da asma. No entanto, eles não são eficazes como monoterapia e, portanto, são utilizados principalmente como terapia complementar aos corticosteróides inalatórios (Polosa, 2007). Os inibidores de mastócitos, os anticorpos monoclonais, os inibidores da 5-lipoxigenase e a imunoterapia específica para alérgenos também são úteis no tratamento da asma (Edwards & Howell, 2000; Holgate & Polosa, 2008). No entanto, medicamentos inovadores, seguros e eficazes para o tratamento da asma são necessários, e a avaliação de novos compostos com mecanismos de ação estabelecidos continua a ser uma importante área de pesquisa .

Neste trabalho, nós demonstramos pela primeira vez as propriedades anti- alérgicas e a caracterização toxicológica de curina, um alcaloide bisbenzilsisoquinolínico ativo por via oral. Para investigar as propriedades anti- alérgicas da curina , foi utilizado um modelo experimental de asma induzida pela sensibilização alérgica e desafio com OVA. A inflamação

eosinofílica das vias aéreas é uma importante característica deste modelo. O desafio alérgico induz a maturação e diferenciação de eosinófilos a partir de precursores da medula óssea e sua migração para o sítio inflamatório em resposta a mediadores, tais como a eotaxina e a IL -5 (Gleich *et al.*, 2000). Portanto, foi realizado um experimento para avaliar a relação dose-resposta do tratamento oral com curina neste modelo, e observou-se que tal tratamento reduziu significativamente o número de eosinófilos no BAL, demonstrando que a curina desempenha uma função inibitória no recrutamento de eosinófilos.

Para entender melhor os efeitos da curina, nós analisamos a sua ação na formação de corpúsculos lipídicos em eosinófilos. O aumento destas organelas em eosinófilos está associada com a ativação celular e a produção de CisLTs na resposta alérgica e portanto, desempenha importantes funções em estados inflamatórios, podendo ser utilizados como um marcador da ativação de eosinófilos (Mesquita-Santos *et al*., 2006; Vieira-de-Abreu *et al*., 2005). Nós não apenas demonstramos que os animais tratados com a curina não apenas apresentaram uma redução do número de eosinófilos no BAL, mas também demonstramos que estes eosinófilos possuíam menos corpúsculos lipídicos citoplasmáticos. Além disso os sobrenadantes do BAL de animais alérgicos tratados com a curina, apresentaram concentrações reduzidas de CisLTs, comparados aos de animais não tratados (dados não mostrados), indicando que a curina desempenha um papel inibitório tanto na migração como na ativação de eosinófilos. O nosso grupo tem demonstrado que a inibição de corpúsculos lipídicos é um parâmetro que pode ser utilizado para avaliar os efeitos antiinflamatórios de drogas, além disso, a avaliação da inibição dos corpos de lipídicos pode ser útil na compreensão da farmacologia e eficácia de novas intervenções terapêuticas para inúmeras doenças, tais como a asma, o câncer, a aterosclerose, o diabetes e outras doenças em que a formação destas organelas é um parâmetro clínico negativo (Bozza *et al*., 2009). Observou-se que o tratamento com a curina, bem como a dexametasona, reduziu significativamente a produção de eotaxina no modelo utilizado. Tem sido demonstrado que a eotaxina desempenha um papel-chave na migração e ativação de eosinófilos (Vieira-de-Abreu *et al*., 2005), o que corrobora com os efeitos de curina aqui descritos. AHR é uma característica particular da asma, resultante de interações complexas entre células inflamatórias e do músculo liso das vias aéreas (ASM), levando a episódios recorrentes de falta de ar, chiado e tosse (Lauzon *et al*., 2012). Neste trabalho, nós demonstramos que a curina inibiu a AHR, diminuindo os valores de Penh (pausa respiratória; *do inglês:* enhanced pause) observadas para níveis semelhantes aos observados nos animais tratados com dexametasona. Para elucidar os mecanismos envolvidos nos efeitos antialérgicos da curina, analisou-se a produção de mediadores com funções significativas nas respostas alérgicas, tais como a IL-13. Estudos

anteriores demonstraram que o bloqueio da IL-13 em camundongos impede o desenvolvimento de AHR (Wills-Karp *et al*., 1998). Além disso, Kuperman e colaboradores (2005, 2002) demonstraram que a super-expressão de IL-13 em camundongos foi suficiente para provocar a AHR, possivelmente através de um efeito direto no epitélio das vias aéreas. Além disto, a IL-13 também está envolvida na modulação de vários aspectos fisiopatológicos da asma, tais como: a mudança de isotipo para a produção de IgE, a inflamação pulmonar, o remodelamento das vias aéreas, a produção de muco e a contração do ASM, através de uma interação com receptores específicos na superfície da célula (Bloemen *et al*., 2007).

Os mecanismos alérgicos envolvem a ativação de muitos tipos de células, incluindo leucócitos e células musculares e epiteliais das vias aéreas (Paul & Zhu, 2010). A Inflamação eosinofílica e AHR são características importantes da asma , e ambos os processos requerem mecanismos dependentes de cálcio. Um trabalho recente demonstrou que o verapamil, um antagonista de canais de cálcio, inibiu significativamente a hiperplasia das células caliciformes, a hipersecreção de muco e a inflamação num modelo de asma alérgica (Khakzad *et al*., 2012). Medeiros e colaboradores (2011) demonstraram que a curina pode ter um efeito inibitório direto sobre canais de Ca^{2+} do tipo-L em células do músculo liso vascular. No presente trabalho, nós investigamos o envolvimento de mecanismos dependentes de cálcio na ação antialérgica de curina. Nós demonstramos que o pré-tratamento com a curina inibiu significativamente a resposta contrátil de traquéia *ex vivo*, sugerindo que curina inibe o influxo de cálcio através do bloqueio de canais dependentes da voltagem de Ca^{2+} no músculo liso da traqueia. Curiosamente, em camundongos não-alérgicos, a curina não alterou a broncoconstrição induzida por metacolina (*in vivo*), a qual é mediada pela liberação de cálcio a partir de reservatórios intracelulares (Foster *et al*., 1984). Estes dados suportam a hipótese de que a curina inibe os canais de cálcio na membrana da célula, ao invés de impedir o efluxo de Ca^{2+} a partir do retículo endoplasmático. Além disso, em tratamentos *in vivo* utilizando a curina ou o verapamil, na mesma dose, tempo e via de administração tiveram efeitos semelhantes na AHR e na inflamação eosinofílica, sugerindo que os efeitos anti-alérgicos da curina podem ser, pelo menos em parte, dependentes da inibição do influxo de cálcio a partir do meio extracelular. De fato, a sinalização dependente de cálcio desempenha papéis importantes tanto na produção como na função de citocinas relacionadas com a asma. A expressão de citocinas e quimiocinas na resposta alérgica é em grande parte dependente da ativação do NFAT, um fator de transcrição que requer Ca^{2+} para sua ativação (Macian, 2005). Rothenberg e colaboradores (1996) demonstraram que a sinalização mediada pela eotaxina através do seu receptor, CCR3 , induziu eficazmente um influxo de cálcio nas eosinófilos estimulados *in vitro*. Além disso, Moynihan e colaboradores (2008) demonstraram que a IL-

13 modulou a AHR por induzir um influxo de cálcio nas células do músculo liso das vias respiratórias humanas.

Ainda neste trabalho, foram investigados os possíveis efeitos tóxicos decorrentes do tratamento oral com a curina, uma vez que tais efeitos não houveram sido previamente investigados experimentalmente. Para cumprir este objetivo, camundongos Swiss foram tratados diariamente durante sete dias com a curina em doses de 2,5 e 8 mg/Kg . A dose de 2,5 mg/kg foi escolhida porque ela teve um efeito anti-alérgico significativo ao longo dos experimentos e a dose de 8 mg/Kg é 10 vezes maior do que a DE_{50} da curina. Neste experimento também incluímos um grupo tratado por via oral com a dexametasona (2 mg/Kg), como fizemos para os outros experimentos. Todos os tratamentos foram comparados com o tratamento oral com PBS adicionado com HCl e NaOH (pH 7), o qual foi utilizado como veículo para a diluição da curina. A avaliação toxicológica demonstrou que o tratamento com a curina não induziu alterações nos parâmetros hematológicos (tais como: o número de leucócitos, plaquetas e glóbulos vermelhos, e os valores de hematócrito e hemoglobina) ou bioquímicos (incluindo as concentrações de: fosfatase alcalina, alanina transaminase, aspartato transaminase, bilirrubina, creatinina , creatinina quinase, colesterol, glicose, proteínas totais e ácido úrico). Além disso, o tratamento com a curina não induziu a formação de úlceras gástricas, e não foram observadas alterações físicas ou comportamentais, o que indica que, nestas condições, a curina não apresentou toxicidade evidente.

Com o intuito de fornecer informações terapêuticas adicionais sobre as propriedades antialérgicas da curina, nós investigamos o efeito do pós-tratamento com a curina e demonstramos que tal tratamento foi eficaz na inibição do recrutamento e da ativação de eosinófilos. Esta observação realça o potencial da curina como um composto antialérgico, pois o pós-tratamento reproduz com mais precisão o que acontece em situações clínicas.

Tendo demonstrado que a curina possui uma atividade antialérgica significante, apresentando efeitos como a inibição da produção de citocinas inflamatórias e da migração celular e sabendo que a planta da qual ela é o principal constituinte é popularmente utilizada para tratar sintomas inflamatórios, nós investigamos as propriedades antiinflamatórias e analgésicas deste composto. Nós demonstramos que o pré-tratamento oral com a curina inibiu significativamente o edema da pata induzido por carragenina ou zimosan e a permeabilidade vascular, o que indica que a curina desempenha um papel inibitório na formação de edema que está associado com uma diminuição da permeabilidade vascular. A administração de ácido acético, carragenina ou zimosan é conhecida por desencadear uma reação inflamatória local, com a liberação de mediadores inflamatórios que induzem alterações vasculares que causam o extravasamento de plasma e a formação de edema (Campos & Calixto, 2000).

Então, nós hipotetizamos que a curina inibe a formação de edema através da modulação da reação inflamatória, baseados tanto no seu efeito modulador da resposta alérgica (Ribeiro-Filho *et al*., 2013) como em uma grande variedade de estudos que demonstraram previamente que os BBA possuem propriedades inflamatórias, inclusive neste modelo (Ivanovska *et al*., 1996; Kondo *et al*., 1992.; Luo *et al*., 1998; Kupeli *et al*., 2002; Wong *et al*., 1991). Além disso, o nosso grupo tem demonstrado as propriedades antialérgicas e antiinflamatórias da warifteína, um BBA isolado de *Cissampelos sympodialis* (Menispermaceae) (Bezerra-Santos *et al*., 2006; 2012; Costa *et al*., 2008).

Demonstrou-se que o pré-tratamento com a curina inibiu tanto a resposta de contorção abdominal induzida por ácido acético e o comportamento de lambida de pata (dois parâmetros importantes na avaliação da nocicepção) na fase inflamatória do teste de formalina, demonstrando as propriedades analgésicas da curina. No entanto, a curina não inibiu a fase neurogênica do teste de formalina, o que indica que o efeito analgésico da curina está associada com mecanismos de antiinflamatórios e não é um resultado da inibição direta da ativação neuronal. De fato, a injeção de formalina induz uma resposta nociceptiva bifásica. A primeira fase é desencadeada nos primeiros 5 minutos após o estímulo, provocando ativação neural direta e causando dor. A segunda fase ocorre no intervalo de 15 a 30 minutos após o estímulo e é provocada pela ação de mediadores liberados na reação inflamatória. Mecanismos inibitórios suprimem a dor entre estas fases e portanto nenhuma resposta nociceptiva é observada (Hunskaar & Hole, 1987). Adicionalmente, os nossos experimentos demonstraram que a curina apresentou resultados fenotípicos semelhantes aos observados para a indometacina, um AINE, mas diferente da morfina, um analgésico de ação central. Além disso, a curina inibiu significativamente a hiperalgesia provocada por carragenina, que é uma resposta altamente dependente de mecanismos de antiinflamatórios (Tjølsen *et al*., 1992), principalmente dependente da ação de PGE_2 (Hunskaar & Hole, 1987). Deste modo, considerando que a PGE_2 está significativamente envolvida no desenvolvimento das respostas inflamatórias e nociceptivas que foram avaliadas no presente estudo e que a curina e a indometacina (cujo efeito analgésico é altamente dependente da inibição da produção de PGE_2) apresentaram efeitos analgésicos e antiinflamatórios semelhantes, nós demonstramos que a curina inibiu a produção de PGE_2 em macrófagos ativados por LPS, sem alterar a expressão da enzima COX-2, sugerindo que a ação da curina provavelmente resulta na inibição de outras enzimas na via de metabolismo do ácido araquidônico, como por exemplo a PLA_2 e a PGE sintase. Estes dados também indicam que curina possui efeitos antiinflamatórios e analgésicos que são, pelo menos parcialmente, dependentes da inibição da produção de PGE_2. Em macrófagos ativados, a PLA_2 promove a liberação do ácido

araquidônico a partir de fosfolipídios da membrana que, associado com a indução da expressão de COX-2 por estímulos inflamtórios, conduz a um aumento da produção de PGE_2 (Tabas & Glass, 2013; Williams & Galli, 2000). Os AINEs clássicos inibem a síntese de PGE_2 por inibir a atividade da COX sem no entanto alterar a expressão desta enzima (Vane & Botting, 1996). Os efeitos de muitos produtos naturais na produção de PGE_2 foram descritos previamente, no entanto, devido à grande diversidade de estruturas, foram encontrados diversos mecanismos, incluindo a inibição da expressão da COX-2 (Bae *et al*., 2012; Kim *et al*., 2012; Yun *et al*., 2009), inibição da atividade da COX-2 (Dannhardt *et al.*, 2000; Estevão *et al*., 2012; Yang *et al*., 2012) e inibição da PLA_2 (Folmer *et al*., 2010).

Os dados obtidos com o estudo dos efeitos antialérgicos da curina (Ribeiro-Filho *et al*., 2013), assim como os trabalhos anteriores desenvolvidos em outros modelos farmacológicos (Medeiros *et al*., 2011) sugerem que a ação da curina envolve a inibição do influxo de cálcio. Isto indica que a curina poderia modular muitas vias de sinalização inflamatórias, incluindo aquelas que estão envolvidas na produção de PGE_2 (van Rossum e Petterson, 2009). É importante enfatizar que o tratamento oral com a curina durante 7 dias consecutivos, em doses até 10 vezes superiores a sua DE_{50} não alterou diversos parâmetros toxicológicos, incluindo parâmetros celulares e bioquímicos, que são frequentemente alterados por antiinflamatórios esteroidais, nem induziu alterações gastrointestinais, que caracterizam um dos principais efeitos adversos dos AINEs, indicando que a curina pode ser uma importante alternativa para o desenvolvimento de medicamentos antiinflamatórios inovadores, seguros e eficazes.

As doenças inflamatórias frequentemente resultam de uma resposta imune inadequada dirigido contra componentes agentes infecciosos (Scrivo *et al*., 2011). Embora as doenças inflamatórias possam diferir em vários aspectos, há evidências consistentes de que os macrófagos e neutrófilos desempenham um papel-chave na iniciação e desenvolvimento de muitas doenças inflamatórias através da produção de mediadores que tanto iniciam, como amplificam a resposta inflamatória, incluindo as citocinas, os mediadores lipídicos e as espécies reativas de oxigênio (Takeda & Akira, 2005). Entretanto, o papel da curina na modulação deste tipo de resposta não havia sido previamente estabelecido. Deste modo, para melhor caracterizar as ações antiinflamatórias da curina, nós avaliamos o efeito do tratmento com esta substância na ativação de macrófagos e no recrutamento de neutrófilos em um modelo experimental de pleurisia induzida por LPS.

Nós demonstramos que o pré-tratamento oral com a curina inibiu significativamente o recrutamento dos neutrófilos para a cavidade pleural de camundongos, além de inibir significativamente a produção de várias citocinas pró-inflamatórias, incluindo a IL-6, o TNF-

α e MCP-1, além de inibir a produção de LTB_4 nos sobrenadantes do lavado pleural de camundongos com LPS, indicando que a curina tem um papel inibitório no recrutamento de neutrófilos, que pode estar associado com a inibição da produção de mediadores inflamatórios. Os neutrófilos são os leucócitos mais abundante no sangue, mas em condições inflamatórias, que podem deixar rapidamente a circulação e migrar para o local da inflamação (Amulic *et al*., 2013). Os sinais inflamatórios, como as quimiocinas e citocinas liberadas pelas células endoteliais ativadas e macrófagos exercem um efeito direto no recrutamento de neutrófilos (Borregaard *et al*., 2010). Estudos anteriores demonstraram que o LTB_4 funciona como um importante agente quimiotático para neutrófilos (Afonso *et al*., 2012) estimulando o recrutamento destas células através da ativação do receptor BLT_1 (Monteiro *et al*., 2011). Portanto, o efeito inibitório da curina na produção de LTB_4 pode ser um importante mecanismo pelo qual ela modula o recrutamento destas células. Em termos de mecanismos, considerando que a curina também inibiu a produção de outros mediadores, como a PGE_2 e os CisLTs é possível que tais efeitos sejam resultantes da inibição de um alvo comum envolvido na síntese destes mediadores, como por exemplo, a PLA_2. Adicionalmente, as citocinas induzida pela sinalização do LPS, tais como a IL-6, a IL- β e o TNF-α desempenham um papel central na inflamação. O TNF-α e a IL-β afetam diretamente o recrutamento de neutrófilos, estimulando a expressão de moléculas de adesão, incluindo as selectinas e as integrinas (Libby *et al*., 2007).

Os macrófagos ativados são uma das principais fontes de mediadores na fase inicial de inflamação (Fujiwara & Kobayashi, 2005). Assim, a nós elaboramos a hipótese de que a curina poderia inibir o recrutamento de neutrófilos e a produção de citocinas no lavado pleural, devido a uma ação inibitória na ativação dos macrófagos. Nós demonstramos que a curina inibiu significativamente a produção de IL-6, IL-β e TNF-α por macrófagos peritoneais murinos estimulados com LPS *in vitro*. Isto sugere que o efeito inibitório que a curina exerce na produção de citocinas por macrófagos, pode ser um dos mecanismos pelos quais esta substância modula a inflamação induzida por LPS *in vivo* e, portanto, inibe o recrutamento de neutrófilos no modelo estudado. Por outro lado, os neutrófilos também podem alterar a biologia dos macrófagos através da liberação de produtos envolvidos no recrutamento e ativação de monócitos/macrófogos (Soehnlein *et al*., 2009). No presente trabalho, nós demonstramos que o tratamento com a curina reduziu significativamente a produção de MCP-1 no lavado pleura , sugerindo que a comunicação entre os neutrófilos e os macrófagos na inflamação pleural induzida por LPS pode ter sido prejudicada pelo tratamento com a curina . Adicionalmente, a curina diminuiu significativamente as concentrações de nitrito nos sobrenadantes de macrófagos estimulados com LPS ou LPS e IFN-γ , indicando que a curina

inibiu a produção óxido nítrico (NO) *in vitro*. Estes dados acrescentam informações importantes sobre o papel da curina na ativação dos macrófagos, uma vez que a produção de NO é uma característica chave de macrófagos ativados, e da resposta inflamatória como um todo (Chang *et al*., 1998). De modo importante, estudos recentes demonstraram que o NO em associação com outras ROS está criticamente envolvida na formação NETs, com consequências relevantes para o processo inflamatório e para a patogênese de doenças inflamatórias (Patel *et al*., 2010).

No artigo onde abordamos os efeitos antiinflamatórios e analgésicos da curina, nós sugerimos que os mecanismos relacionados a tais efeitos envolvem a inibição da produção de PGE_2 (Leite *et al*., 2013). Entretanto, a partir destes dados mais recentes é possível sugerir , em adição aos mecanismos previamente propostos, o efeito inibitório que a curina desempenha na produção de citocinas, tais como a IL-1β e o TNF- α, bem como na produção de NO pode contribuir significativamente para os efeitos antiinflamatórios e analgésicos anteriormente descritos, uma vez que muitos estudos tem demonstrado o papel destes mediadores na dor inflamatória (Kidd & Urban, 2001).

Como previamente discutido, os estudos anteriores sugerem fortemente que o mecanismo pelo qual curina exerce os seus efeitos é através da inibição de uma resposta dependente de cálcio. Deste modo, sabendo que muitas vias de sinalização que estão envolvidas na ativação de macrófagos são dependentes do influxo de cálcio e que trabalhos prévios demonstraram que o uso de antagonistas de canais de cálcio inibe a ativação de macrófagos ativados por diferentes estímulos inflamatórios (Wright *et al*., 1985), nós comparamos o efeito da curina e do verapamil na produção de IL-1β e TNF-α por macrófagos estimulados LPS, que foi utilizada como parâmetro da ativação nestes macrófagos. Foi demonstrado que quando utilizado sob as mesmas condições (concentrações e tempos de pré - tratamento), o verapamil e curina apresentaram efeitos inibitórios semelhantes na produção destas citocinas, sugerindo que a inibição do influxo de cálcio também está envolvido nos mecanismos pelos com a curina inibe a ativação celular e portanto, a resposta inflamatória, neste modelo, corroborando com os dados previamente relatados (Ribeiro-Filho *et al*., 2013).
Este conjunto de dados indicam que a curina apresentou um efeito antiinflamatório caracterizado pela inibição do recrutamento de neutrófilos que está associado com a inibição da ativação de macrófagos através de mecanismos que envolvem a inibição da produção de citocinas pró-inflamatórias, LTB_4 e NO, que pode ser dependente da inibição do influxo de Ca^{2+}.

No contexto internacional tem surgido problemas relacionados com a eficácia e segurança dos medicamentos atualmente disponíveis (Cosendey *et al*., 2000). Os

medicamentos mais amplamente empregados na terapia antiinflamatória, tais como os corticosteróides e os AINEs podem ser utilizados com sucesso no tratamento de muitas doenças, tem havido condições específicas em que eles ou não são eficazes ou podem causar efeitos colaterais significativos (Rainsford, 2007). Portanto, o desenvolvimento de novos medicamentos, seguro e eficaz tem um impacto importante na terapia antiinflamatória, e neste sentido, a curina administrada oralmente apresentou atividades antialérgicas, antiinflamatórias e analgésicas muito interessantes. Contudo, embora os nossos resultados indiquem que, nas condições testadas, a curina não apresenta efeitos tóxicos evidentes, uma avaliação toxicológica mais aprofundada pode contribuir significativamente para estabelecer a segurança do uso desta substância. Isto inclui a avaliação dos efeitos toxicológicos do tratamento com a curina a longo prazo, uma vez que os medicamentos antiinflamatórios são, muitas vezes, utilizados de forma crônica. Embora alguns alcaloides sejam principalmente conhecidos por suas propriedades tóxicas, isto varia de muito de uma substância para outra. Kaneko e colaboradores (1989) demonstraram que os BBA tetrandrina e berbamina, duas substâncias com estruturas químicas relativamente semelhantes a da curina e cujas propriedades antiinflamatórias descritas na literatura incluem alguns dos efeitos demonstrados para a curina no presente trabalho, não apresentaram efeitos tóxicos significativos quando utilizados na dose de 75 mg/Kg durante 8 dias por via intraperitoneal. Como demonstramos, esta dose é quase 100 vezes superior a DE_{50} da curina para a inibição da migração de eosinófilos, corroborando com os nossos dados que indicam que a curina exerce efeitos farmacológicos em doses não tóxicas. Contudo, também é importante que sejam realizados estudos posteriores que avaliem os efeitos tóxicos da curina no sistema cardiovascular, uma vez que, tendo apresentado efeitos semelhantes aos do verapamil, um fármaco utilizado para o tratamento da hipertensão, é fundamental investigar possíveis alterações na pressão arterial de animais normotensos.

Ainda no que diz respeito a identificação dos mecanismos moleculares de ação da curina, atualmente o nosso grupo tem conduzido alguns experimentos para caracterizar o efeito da curina na ativação de fatores de transcrição, tais como o NFκB e o NFAT, e de proteinas quinases que medeiam vias de sinalização importantes tanto na inflamação como no câncer, tais como a ERK, a JNK e a P38. De fato, em dados não apresentados neste trabalho, nós demonstramos que a curina inibiu a proliferação de células da linhagem de carcinoma de cólon Caco2 em concentrações nas quais ela não alterou a viabilidade de macrófagos murinos, indicando que a curina é um candidato interessante para a pesquisa de fármacos com atividade antitumoral. Em concordância, tem sido demonstrado que BBAs com atividade antiinflamatória também podem apresentar propriedades antitumorais significativas (Liu *et*

al., 2008). Nós também estamos preparando um outro manuscrito (não incluido neste trabalho), no qual demonstramos que a curina apresenta uma inibição significativa do *scratching behavior* (do inglês: *comportamento de coceira*) que parece estar associado com a inibição da ativação de mastócitos corroborando com os efeitos demonstrados em modelo de asma alérgica e indicando que os efeitos antialérgicos da curina também podem ser aplicáveis a outros tipo de doenças alérgicas, incluindo as alergias cutâneas (Garibyan *et al*., 2013).

Em conclusão, a curina apresentou efeitos antialérgicos, antiinflamatórios e analgésicos que estão associados com a inibição da ativação de leucócitos e da produção de mediadores inflamatórios, cujos mecanismos envolvem uma inibição de uma resposta dependente de cálcio, na ausência de toxicidade evidente, e portanto tem potencial para o desenvolvimento de fármacos.

5. CONCLUSÕES

A caracterização dos efeitos antialérgicos da curina em modelo experimental de asma alérgica indicou que este alcaloide inibe o recrutamento e a ativação de eosinófilos *in vivo*, bem a como a AHR vias aéreas, cujos mecanismos envolvem a inibição da produção de eotaxina e IL-13 e a inibição do influxo de cálcio.

A análise os efeitos tóxicos induzidos pelo tratamento oral com a curina em doses até 10 vezes superiores a sua DE_{50} relacionada aos efeitos antialérgicos, demonstrou que nestas condições a curina não apresenta efeitos tóxicos significativos.

Em adição, a análise dos efeitos antiinflamatório e analgésico da curina em camundongos, demonstrou que esta substância inibe as respostas nociceptivas na fase inflamatória, mas não inibe a fase neurogênica do teste de formalina, além de inibir a produção de PGE_2 por macrófagos, indicando que os efeitos analgésicos da curina, estão associados a mecanismos antiinflamatórios, que, pelo menos em parte dependem a da inibição de PGE_2.

A investigação das ações da curina na ativação de macrófagos e no recrutamento de neutrófilos em modelo experimental de inflamação induzida por LPS, revelou que a curina inibe o recrutamento de neutrófilos e a produção de mediadores inflamatórios no lavado pleural, associado com a inibição da ativação de macrófagos através de mecanismos que envolvem a inibição da produção de citocinas pró-inflamatórias, LTB_4 e NO, que podem ser dependentes da inibição do influxo de Ca^{2+}.

Em conclusão, a curina apresentou efeitos antialérgicos, antiinflamatórios e analgésicos na ausência de toxicidade evidente, e portanto tem potencial para o desenvolvimento de fármacos inovadores.

6. REFERÊNCIAS

Afonso, P. V.; Janka-Junttila, M.; Lee, Y. J.; McCann, C. P.; Oliver, C. M.; Aamer, K. A.; Losert, W.; Cicerone, M. T.; Parent, C. A. LTB_4 is a signal-relay molecule during neutrophil chemotaxis. Developmental Cell, v. 22 (5), p. 1079-1091, 2012.

Allen, D. B. Effects of inhaled steroids on growth, bone metabolism, and adrenal function. Advances in Pediatrics, v. 53, p.101-110, 2006.

Amulic, B.; Cazalet, C.; Hayes, G. L.; Metzler, K. D.; Zychlinsky, A. Neutrophil Function: From Mechanisms to Disease. Annual Review of Immunology, v. 30, p. 459-89, 2012.

Austen, K. F. The cysteinyl leukotrienes: where do they come from? What are they? Where are they going? Nature Immunology, v, 9 (2), p.113-115, 2008.

Bae, D. S.; Kim, Y. H.; Pan, C.; Nho, C. W.; Samdan, J.; Yansan, J.; Lee, J. K. Protopine reduces the inflammatory activity of lipopolysaccharide-stimulated murine macrophages. BMB Reports, v. 45(2), p. 108-113, 2012.

Barbosa-Filho, J. M.; Piuvezam, M. R.; Moura, M. D.; Silva, M. S.; Batista-Lima, K. V.; Leitão-da-Cunha, E. V.; Fechine, I. M.; Takemura, O. S. Anti-inflammatory activity of alkaloids: A twenty-century review. Revista Brasileira de Farmacognosia, v. 16 (1), p.109-139, 2006.

Barnes, P. J. Glucocorticosteroids: current and future directions. British Journal of Pharmacology, v. 163, p. 29-43, 2011.

Barnes, P. J. Immunology of asthma and chronic obstructive pulmonary disease. Nature Reviews Immunology, v. 8, p. 183- 92, 2008.

Barnes, P. J. How corticosteroids control inflammation. British Journal of Pharmacology, v. 148, p. 245-254, 2006.

Barnes, P. J.; Adcock, I. M. Glucocorticoid resistance in inflammatory diseases. Lancet, v. 373, p. 1905-1917, 2009.

Barnes, P. J.; Greening, A. P.; Crompton, G. K. Glucocorticoid resistance in asthma. American Journal of Respiratory and Critical Care Medicine, v. 152, p. 125- 140, 1995.

Barnes, P. J. Immunology of asthma and chronic obstructive pulmonary disease. Nature Reviews Immunology, v. 8, p. 183-192, 2008.

Basbaum, A. I.; Woolf, C. J. Pain. Current Biology. v. 9, R429–R431,1999.

Besedovsky, H.; Del Rey, A.; Sorkin, E.; Dinarello, C. A. Immunoregulatory feedback between interleukin-1 and glucocorticoid hormones. Science, v. 233, p. 652-654, 1986.

Bevan, S. Textbook of Pain (ed. Wall, P. D. & Melzack, R.). Churchill-Livingstone, New York, p. 85-103, 1999.

Bezerra-Santos, C. R.; Balestieri, F. M.; Rossi-Bergmann, B.; Peçanha, L. M.; Piuvezam, M. R.; *Cissampelos sympodialis* Eichl. (Menispermaceae): oral treatment decreases IgE levels and induces a Th1-skewed cytokine production in ovalbumin-sensitized mice. Journal of Ethnopharmacology, v. 95, p. 191- 97, 2004.

Bezerra-Santos, C. R.; Peçanha, L. M. T.; Piuvezam, M. R. *Cissampelos sympodialis* Eichl. (Menispermaceae) inhibits anaphylactic shock reaction in murine allergic model. Brazilian Journal Pharmacognosy, v. 15, p. 287-291, 2005.

Bezerra-Santos, C.R.; Vieira-de-Abreu, A.; Barbosa-Filho, J. M.; Bandeira-Melo, C.; Piuvezam, M. R.; Bozza, P. T. Anti-allergic properties of *Cissampelos sympodialis* and its isolated alkaloid warifteine. International Immunopharmacology, v. 6, p. 1152-1160, 2006.

Bezerra-Santos, C. R.; Vieira-de-Abreu, A.; Vieira, G. C.; Ribeiro-Filho, J.; Barbosa-Filho, J. M., Pires, A. L., Martins, M. A.; Souza, H. S.; Bandeira-Melo, C.; Bozza, P. T.; Piuvezam, M. R. Effectiveness of *Cissampelos sympodialis* and its isolated alkaloid warifteine in airway hyperreactivity and lung remodeling in a mouse model of asthma. International Immunopharmacology, v.13, p. 148-155. 2012.

Bloemen, K.; Verstraelen, S.; Van Den Heuvel, R.; Witters, H.; Nelissen, I.; Schoeters, G. The allergic cascade: review of the most important molecules in the asthmatic lung. Immunology Letters, v. 113(1), p. 6-18, 2007.

Borregaard, N. Neutrophils, from marrow to microbes. Immunity, v. 33, p. 657-670, 2010.

Boswell-Smith, V.; Cazzola, M.; Page, C. P.; Are phosphodiesterase- 4 inhibitors just more theophylline? Journal of Allergy and Clinical Immunology, v. 117, p. 1237-1243, 2006.

Bozza, P. T.; Magalhães, K.G.; Weller, P. F.; Leukocyte lipid bodies - biogenesis and functions in inflammation. Biochemistry and Biophysics Acta, v.1791(6), p. 540-551, 2009.

Brierley, M. M.; Fish, E. N. Stats: multifaceted regulators of transcription. Journal of Interferon & Cytokine Research, v. 25, p. 733-744, 2005.

Buddenkotte, J.; Steinhoff, M. Pathophysiology and therapy of pruritus in allergic and atopic diseases. Allergy, v. 65, p. 805-821, 2010.

Butler, M. S. The role of natural product chemistry in drug discovery. Journal of Natural Products, v. 67, p. 2141-2153, 2004.

Campos, M. M.; Calixto, J. B. Neurokinin mediation of edema and inflammation. Neuropeptides. v. 34 (5), p. 314-322, 2000.

Capra, V.; Thompson, M. D.; Sala, A.; Cole, D. E.; Folco, G.; Rovati, G. E. Cysteinyl-leukotrienes and their receptors in asthma and other inflammatory diseases: Critical update and emerging trends. Medicinal Research Reviews, v. 27 (4), p. 469-527, 2007.

Chang, C. I.; Liao, J. C.; Kuo, L. Arginase modulates nitric oxide production in activated macrophages. The American Journal of Physiology, v. 274 (1), p. 342-348, 1998.

Chen, H.; Jiang, Z. The essential adaptors of innate immune signaling. Protein Cell, v. 4(1), p. 27-39, 2013.

Chomarat, P.; Dantin, C.; Bennett, L.; Banchereau, J.; Palucka, A. K. TNF skews monocyte differentiation from macrophages to dendritic cells. Journal of Immunology, v.171, p. 2262-2269, 2003.

Cockcroft, D. W.; Hargreave, F. E.; O'byrne, P. M.; Boulet, L. P. Understanding allergic asthma from allergen inhalation tests. Canadian Respiratory Journal, v. 14, p. 414-418, 2007.

Correa, P. M. Dicionário das plantas úteis do Brasil e das exóticas cultivadas. Brasília: Ministério da Agricultura, Instituto Brasileiro de Desenvolvimento Florestal. 1984.

Cosendey, M. A. E.; Bermudez, J. A. Z.; Reis, A. L. A.; Silva, H. F.; Oliveira, M. A.; Luiza, V. L. Assistência farmacêutica na atenção básica de saúde: a experiência de três estados brasileiros. Cadernos Saúde Pública, v. 16, p. 171-182, 2000.

Costa, H. F.; Bezerra-Santos, C. R.; Barbosa-Filho J. M.; Martins, M. A., Piuvezam, M. R. Warifteine, a bisbenzylisoquinoline alkaloid, decreases immediate allergic and thermal hyperalgesic reactions in sensitized animals. International Immunopharmacology, v. 8, p. 519-525. 2008.

Cowburn, A. S.; Sladek, K.; Soja, J.; Adamek, L.; Nizankowska, E.; Szczeklik, A.; Lam, B. K.; Penrose, J. F.; Austen, F. K.; Holgate, S. T.; Sampson, A. P. Overexpression of leukotriene C_4 synthase in bronchial biopsies from patients with aspirin-intolerant asthma. Journal of Clinical Investigation,v. 101, p. 834-846, 1998.

Dannhardt, G.; Kiefer, W. Cyclooxygenase inhibitors-current status and future prospects. European Journal of Medicinal Chemistry, v. 36, p. 109-126, 2001.

Dias, C. S.; Barbosa-Filho, J. M.; Lemos, V. S.; Cortes, S. F. Mechanisms involved in the vasodilator effect of curine in rat resistance arteries. Planta Medica, v. 68, p. 1049-1051, 2002.

Edwards, M. R.; Bartlett, N. W.; Clarke, D. Birrell, M.; Belvisi, M.; Johnston, S. L.; Targeting the NF-κB pathway in asthma and chronic obstructive pulmonary disease. Pharmacology and Therapeutics, v. 121, p. 1-13, 2009.

Edwards, A.M.; Howell, J. B. The chromones: history, chemistry and clinical development. A tribute to the work of Dr R. E. C. Altounyan. Clinical and Experimental Allergy, v. 30, p. 756-774, 2000.

Estevão, M.S.; Carvalho, L.C.R.; Freitas, M.; Gomes, A.; Viegas, A.; Manso, J.; Erhardt, S.; Fernandes, E.; Cabrita, E. J.; Marques, M. B. Indole based cyclooxygenase inhibitors: Synthesis, biological evaluation, docking and NMR screening. European Journal of Medicinal Chemistry, v. 54, p. 823-833, 2012.

Fanning, L. B. ; Boyce, J. A. Lipid mediators and allergic diseases. Annals of Allergy, Asthma & Immunology, v. 111(3), p.155-62, 2013.

Feghali, C. A.; Wright, T. M. Cytokines in acute and chronic inflammation. Frontiers in Bioscience, v. 2, p. 12-26, 1997.

Folmer, F.; Jaspars, M.; Schumacher, M.; Dicatoa, M.; Diederich, M. Marine natural products targeting phospholipases A_2. Biochemical Pharmacology, v. 80, p. 1793-1800, 2010.

Foster, R. W.; Okpalugo, B. I.; Small, R. C. Antagonism of Ca^{2+} and other actions of verapamil in guinea-pig isolated tracheas. British Journal of Pharmacology, v. 81, p. 499-507, 1984.

Fraser, I. P.; Koziel, H.; Ezekowitz, R. A. The serum mannose-binding protein and the macrophage mannose receptor are pattern recognition molecules that link innate and adaptive immunity. Seminars in Immunology, v.10, p. 363-372, 1998.

Galley, H. F.; Webster, N. R. The immuno-inflammatory cascade. British journal of anaesthesia, v. 77, p. 11-16, 1996.

Galli, S. J.; Maurer, M.; Lantz, C. S. Mast cells as sentinels of innate immunity. Current Opinion in Immunology, v. 11, p. 53-59, 1999.

Garibyan, L.; Rheingold, C. G.; Lerner, E. A. Understanding the pathophysiology of itch. Dermatologic Therapy, v. 26 (2), 84-91, 2013.

Gleich, G. J.; Adolphson, C. R.; Leiferman, K. M. The biology of the eosinophilic leukocyte. Annual Review of medicine, v. 44, p. 85-101, 1993.

Gleich, G. J. Mechanisms of eosinophil-associated inflammation. Journal of Allergy and Clinical Immunology, v. 105, 651-663, 2000.

Gilfillan, A. M.; Tkaczyk, C. Integrated signalling pathways for mast-cell activation. Nature Reviews Immunology, v. 6(3), p. 218-230, 2006.

Gotfredsen, E. Liber Herbarum II: *Chondrodendron platyphyllum* (A.St.Hil.) Miers. 2013. Disponível em: <http://www.liberherbarum.com/pn7625.HTM>. Acesso em agosto de 2013.

Gould, H. J.; Sutton, B. J. IgE in allergy and asthma today. Nature Reviews Immunology, v. 8, p. 205-217, 2008.

Greaves, M. W. Pathogenesis and Treatment of Pruritus. Current Allergy and Asthma Reports, v. 10, p. 236-242, 2010.

Guedes, D. N.; Barbosa-Filho, J. M.; Lemos, V. S.; Côrtes, S. F. Mechanism of the vasodilator effect of 12-O-methylcurine in rat aortic rings. Journal of Pharmacy and Pharmacology, v. 54, p. 853-858, 2002.

Hamilton, G. R.; Baskett, T. F. In the arms of Morpheus: the development of morphine for postoperative pain relief. Canadian Journal of Anesthesia, v. 47, p. 367-374, 2000.

Handwerker, H. O.; Kobal, G. Psychophysiology of experimentally induced pain. Physiological Reviews, v. 73, 639-671, 1993.

Harborne, J. B. Phytochemical methods. 2ª ed. Chapman & Hill. 288p. 1991.

Harrison, T. W.; Oborne, J.; Newton, S.; Tattersfield, A. E. Doubling the dose of inhaled corticosteroid to prevent asthma exacerbations: randomised controlled trial. Lancet, v. 363, p. 271-275, 2004.

Harvey, A. L. Natural products in drug discovery. Drug Discovery Today, v. 13, p. 894-901, 2008.

Hodges, M. K.; Weller, P. F.; Gerard, N. P.; Ackerman, S. J.; Drazen, J. M. Heterogeneity of leukotriene C_4 production by eosinophils from asthmatic and from normal subjects. American Review of Respiratory Diseases, v.138, p.799-804, 1988.

Holgate S, T. The role of mast cells and basophils in inflammation. Clinical and Experimental Allergy, v.30, p. 28-32, 2000.

Holgate, S. T.; Polosa, R. Treatment strategies for allergy and asthma. Nature Reviews Immunology, v. 8, p. 116-124, 2008.

Howe, L.R. Inflammation and breast cancer. Cyclooxygenase/Prostaglandin signalling and breast cancer. Breast cancer research, v. 9 (210), p. 1-9, 2007.

Huber, M.; Hughes, M. R.; Krystal, G. Thapsigargin-induced degranulation of mast cells is dependent on transient activation of phosphatidylinositol-3 kinase. The Journal of immunology, v.165, p.124-133, 2000.

Hunskaar, S.; Hole, K. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain, v. 30, p. 103-114, 1987.

Inotai, A.; Hankó, B.; Mészáros, Á. Trends in the non-steroidal anti-inflammatory drug market in six Central - Eastern European countries based on retail information. Parmacoepidemiology and Drug Safety, v.19 (2), p. 183-190, 2010.

Ivancsó, I.; Böcskei, R.; Müller, V.; Tamási, L. Extrafine inhaled corticosteroid therapyin the control of asthma. Journal of Asthma and Allergy, v. 6, p. 69-80, 2013.

Ivanovska, N.; Georgieva, P.; Barot-Ciorbaru, R. Correlation between inhibited alternative complement activity and the protective effect induced by Nocardia lysozyme digest (NLD) during *Klebsiella pneumoniae* infection in mice. International Journal of Immunopharmacology, v.18, p. 515-519, 1996.

Iwasaki, A.; Medzhitov, R. Regulation of Adaptive Immunity by the Innate Immune System. Science, v. 327 (5963), p. 291-295. 2010.

Jackson, K. J.; Kidd, M. J.; Wang, Y.; Collins, A. M. The Shape of the Lymphocyte Receptor Repertoire: Lessons from the B Cell Receptor. Frontiers in Immunology, v. 4 (263), p. 1-12, 2013.

Janeway-Jr, C. A. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harbor Symposia on Quantitative Biology, v. 54, p.1-13, 1989.

Janeway-Jr, C.A; Medzhitov, R. Innate Immune Recognition. Annual Review of Immunology, v. 20, p.197-216, 2002.

Jensen, S.; Thomsen, A. R. Sensing of RNA Viruses: a Review of Innate Immune Receptors Involved in Recognizing RNA Virus Invasion. Journal of Virology, v.86 (6), 2900-2910, 2012.

Johnson, M. M.; Vaughan, B.; Triggiani, M.; Swan, D. D.; Fonteh, A. N.; Chilton, FH. Role of arachidonyl triglycerides within lipid bodies in eicosanoid formation by human polymorphonuclear cells. American Journal of Respiratory Cell and Molecular Biology, v. 21, p. 253-258, 1999.

Julius, D.; Basbaum, A. I. Molecular mechanisms of nociception. Nature, v.413, p. 203-210, 2001.

Kaminska, B. MAPK signalling pathways as molecular targets for anti-inflammatory therapy: from molecular mechanisms to therapeutic benefits. Biochimica *et* Biophysica Acta, v. 1754 (1-2), p. 253-262, 2005.

Kaneko, J. J. Clinical Biochemistry of Domestic Animals, 4th Ed. Academic Press, London. 1989.

Kansas, G. S. Selectins and their ligands: current concepts and controversies. Blood, v. 88, p. 3259–3287, 1996.

Kariyawasam, H. H.; Robinson, D. S. The eosinophil: the cell and its weapons, the cytokines, its locations. Seminars in Respiratory and Critical Care Medicine, v. 27, p. 117-127, 2006.

Khakzad, M. R.; Mirsadraee, M.; Mohammadpour, A.; Ghafarzadegan, K.; Hadi, R.; Saghari, M.; Meshkat, M. Effect of verapamil on bronchial goblet cells of asthma: An experimental study on sensitized animals. Pulmonary Pharmacology and Therapeutics, v. 25, p. 163-168, 2012.

Kemp, S. F.; Lockey, R. F. Anaphylaxis: a review of causes and mechanisms. Journal of Allergy and Clinical Immunology, v.110, p. 341-348, 2002.

Kendall, A. C.; Nicolaou, A. Bioactive lipid mediators in skin inflammation and immunity. Progress in Lipid Research, v. 52 (1), p. 141-64, 2013.

Kidd, B. L.; Urban, L. A. Mechanisms of inflammatory pain. British journal of anesthesia, v. 87 (1), p. 3-11, 2001.

Kim, H. G.; Han, E. H.; Jang, W.; Choi, J. H.; Khanal, T.; Park, B. H.; Tran, T. P.; Chung, Y. C.; Jeong, H. Piperine inhibits PMA-induced cyclooxygenase-2 expression through downregulating NF-κB, C/EBP and AP-1 signaling pathways in murine macrophages.Food and Chemical Toxicology, v. 50, p. 2342-2348, 2012.

Koehn, F. E.; Carter, G. T. The evolving role of natural products in drug discovery. Nature Reviews Drug Discovery, v. 4, p. 206-220, 2005.

Kondo, Y.; Imai, Y.; Hojo, H.; Hashimoto, Y.; Nozoe, S. Selective inhibition of T-cell-dependent immune responses by bisbenzylisoquinoline alkaloids in vivo. International Journal of Immunopharmacology, v. 14, p.1181-1186, 1992.

Kreek, M. J.; Levran, O.; Reed, B.; Schlussman, S. D.; Zhou, Y.; Butelman, E. R. Opiate addiction and cocaine addiction: underlying molecular neurobiology and genetics. Journal of Clinical Investigation, v. 122(10), p. 3387-3393, 2012.

Kupeli, E.; Kosar, M.; Yesilada, E.; Husnu, K.;Baser, C. A comparative study on the anti-inflammatory, antinociceptive and antipyretic effects of isoquinoline alkaloids from the roots of Turkish Berberisspecies. Life Sciences, v. 72, p. 645-657, 2002.

Kuperman, D. A.; Lewis, C. C.; Woodruff, P. G.; Rodriguez, M. W.; Yang, Y. H.; Dolganov, G. M.; Fahy, J. V.; Erle, D. J. Dissecting asthma using focused transgenic modeling and functional genomics. Journal of Allergy and Clinical Immunology, v. 116, p. 305-311, 2005.

Kuperman, D. A.; Huang, X.; Koth, L. L.; Chang, G. H.; Dolganov, G. M.; Zhu, Z.; Elias, J. A.; Sheppard, D.; Erle, D. J. Direct effects of interleukin-13 on epithelial cells cause airway hyper-reactivity and mucus overproduction in asthma. Nature Medicine, v. 8, p. 885-889, 2002.

Laitinen, L. A.; Laitinen, A.; Haahtela, T.; Vilkka, V.; Spur, B. W.; Lee, T. H. Leukotriene E_4 and granulocytic infiltration into asthmatic airways. Lancet, v. 341, p. 989-990, 1993.

Lambrecht, B. N.; Salomon, B.; Klatzmann, D.; Pauwels, R. A. Dendritic cells are required for the development of chronic eosinophilic airway inflammation in response to inhaled antigen in sensitized mice. Journal of Immunology, v. 160, p. 4090-4097, 1998.

Lauzon, A. M.; Bates, H. T.; Donovan, G.; Tawhai, M.; Sneid, J.; Sanderson, M. J. A multi-scale approach to airway hyperresponsiveness: from molecule to organ. Frontiers in Physiology, v. 3, p. 1-25, 2012.

Lawren, T.; Willowghby, D. A.; Gilroy, D. W. Anti-inflammatory lipid mediators and insights into the resolution of inflammation. Nature Reviews Immunology, v. 2 (10), p. 787-795, 2002.

Lee, I.; Yang, C. Inflammatory Signalings Involved in Airway and Pulmonary Diseases. Mediators of Inflammation, v 2013, p. 1-12, 2013.

Leite, F. C.; Ribeiro-Filho, J.; Costa, H. F.; Salgado, P. R. R.; Calheiros, A. S.; Carneiro, A. B.; Almeida, R. N.; Dias, C. S.; Bozza, P. T.; Piuvezam, M. R. The anti-inflammatory and analgesic effects of curine, a bisbenzylisoquinoline alkaloid isolated from *Chondrodendron platyphyllum* (Menispermaceae). Journal of Natural Products. 2013. Submitted.

Leonard, W. J.; Lin, J. X. Cytokine receptor signaling pathways. Journal of Allergy and Clinical Immunology, v.105 (5), 877-888, 2000.

Lewis, R. A; Austen, K. F.; Soberman, R. J. Leukotrienes and other products of the 5-lipoxygenase pathway. Biochemistry and relation to pathobiology in human diseases. New England Journal of Medicine, v. 323, p. 645-655, 1990.

Li, J. W. H.; Vederas, J. C. Drug Discovery and Natural Products: End of an Era or an Endless Frontier? Science, v. 325, p. 161-165, 2009.

Libby, P. Inflammatory Mechanisms: The Molecular Basis of Inflammation and Disease. Nutrition Reviews, v. 65(12), S 140-146, 2007.

Liu, B.; Wanga, T.; Qian, X.; Liu, G.; Yu, L.; Ding, Y. Anticancer effect of tetrandrine on primary cancer cells isolated from ascites and pleural fluids. Cancer Letters, v. 268 (1), p. 166-175, 2008.

Lu, Y. C.; Yeh, W. C.; Ohashi, P. S. LPS/TLR4 signal transduction pathway. Cytokine, v. 42(2),145-151, 2008.

Luo, C. N.; Lin, X.; Li, W. K.; Pu, F. Wang, L. W.; Xie, S. S.; Xiao, P. G. Effect of berbamine on T-cell mediated immunity and the prevention of relection on skin transplants in mice. Journal of Ethnopharmacology, v. 59, p. 211-215, 1998.

Macian, F. NFAT proteins: key regulators of T-cell development and function. Nature Reviews Immunology, v. 5, p. 472-484, 2005.

Malik, N. N. Drug discovery: past, present and future. Drug Discovery Today, v. 13(21-22), 909-912, 2008.

Markenson, J. A. Mechanisms of chronic pain. American Journal of Medicine, v. 101(S.1A), 6S–18S, 1996.

Merskey, H. The definition of pain. European Journal of Psychiatry, v. 6, p. 153-159, 1991.

Moncada, S.; Palmer, R. M. J.; Higgs, E. A. "Nitric oxide: physiology, pathophysiology, and pharmacology". Pharmacological Reviews, v. 43 (2), p. 109-142, 1991.

Moncada, S.; Higgs, A. "The L-arginine-nitric oxide pathway," The New England Journal of Medicine, v. 329 (27), p. 2002-2012, 1993.

Majno, G.; Joris, I. Cells, Tissues, and Disease, 2nd ed. Oxford: Oxford University Press, 2004.

McEver, R. P.; Cummings, R. D. Role of PSGL-1 binding to selectins in leukocyte recruitment. Journal of Clinical Investigation, v. 100, :S 97-103, 1997.

Medeiros, M. A.; Pinho, J. F.; de-Lira, D. P.; Barbosa-Filho, J. M.; Araújo, D. A.; Cortes, S. F.; Lemos, V. S.; Cruz, J. S. Curine, a bisbenzylisoquinoline alkaloid, blocks L-type Ca^{2+} channels and decreases intracellular Ca^{2+} transients in A7r5 cells. European Journal of Pharmacology, v. 669, p. 100-107, 2011.

Medzhitov, R. Origin and physiological roles of inflammation. Nature, v. 454 (24), 482-435, 2008.

Medzhitov, R. Inflammation 2010: New adventures for an old flame. Cell, v. 140 (6), p. 771-776, 2010.

Medzhitov, R.; Janeway-Jr, C.A. Innate immune recognition: mechanisms and pathways. Immunological Reviews, v. 173, p. 89-97, 2000.

Mesquita-Santos, F. P.; Vieira-de-Abreu, A.; Calheiros, A. S. Figueiredo, I. H.; Castro-Faria-Neto, H. C.; Weller, P. F.; Bozza, P. T.; Diaz, B. L.; Bandeira-Melo, C. Cutting edge:

prostaglandin D_2 enhances leukotriene C_4 synthesis by eosinophils during allergic inflammation: synergistic *in vivo* role of endogenous eotaxin. Journal of Immunology, v. 176 (3), p. 1326–1330, 2006.

Molina-Holgado, E.; Ortiz, S.; Molina-Holgado, F.; Guaza, C. Induction of COX-2 and PGE(2) biosynthesis by IL-1beta is mediated by PKC and mitogen-activated protein kinases in murine astrocytes. British Journal of Pharmacology, v. 131(1), p. 152-159, 2000.

Moynihan, B .J.; Tolloczko, B.; Bassam, S. E.; Ferraro, P.; Michoud, M.; Martin, J. G.; Laberge, S. IFN-γ, IL-4 and IL-13 modulate responsiveness of human airway smooth muscle cells to IL-13. Respiratory Research, v. 9, p. 1-16, 2008.

Mueller, H.; Stadtmann, A.; Van Aken, H.; Hirsch, E.; Wang, D.; Ley, K.; Zarbock, A. Tyrosine kinase Btk regulates E-selectin-mediated integrin activation and neutrophil recruitment by controlling phospholipase C (PLC) γ2 and PI3Kγ pathways. Blood, v. 115, p. 3118-3127, 2010.

Murakami M. Lipid mediators in life science. Experimental Animals, v. 60(1), p. 7-20, 2011.

Murdoch, J. R.; Lloyd, C. R. Chronic inflammation and asthma. Mutation Research, v. 690, p. 24-39, 2010.

Murphy, D. J. The biogenesis and functions of lipid bodies in animals, plants and microorganisms. Progress in Lipid Research, v.40, p.325-338, 2001.

Narumiya, S.; FitzGerald, G. A. Genetic and pharmacological analysis of prostanoid receptor function. Journal of Clinical Investigation, v. 108 (1), p. 25-30, 2001.

Nathan, C.; Ding, A. Non-resolving inflammation. Cell, v. 140, p.871-882, 2010.

O'Connor-Jr.; W.; Zenewicz, L. A.; Flavell, R. A. The dual nature of TH17 cells: shifting the focus to function. Nature Immunology, v. 11 (6), p. 471-476, 2010.

Oguma, T.; Asano, K.; Ishizaka, A. Role of prostaglandin D(2) and its receptors in the pathophysiology of asthma. Allergology International, v. 57 (4), 307-312, 2008.

Okamoto, T.; Iwata, S.; Ohnuma, K.; Dang, N. H.; Morimoto, C. Histamine H1-receptor antagonists with immunomodulating activities: potential use for modulating T helper type 1 (Th1)/Th2 cytokine imbalance and inflammatory responses in allergic diseases. Clinical and Experimental Immunology, v. 157 (1), p. 27-34, 2009.

Patel, S.; Kumar, S.; Jyoti, A.; Srinag, B. S. S.; Keshari, R. S.; Saluja, R.; Verma, A.; Mitra, K.; Barthwal, M. K.; Krishnamurthy, H.; Bajpai, V. K.; Dikshit, M. Nitric oxide donors release extracellular traps from human neutrophils by augmenting free radical generation. Nitric Oxide, v. 22, p. 226-234, 2010.

Patrono, C.; Rocca, B. Nonsteroidal antiinflammatory drugs: Past, present and future. Pharmacological Research, v. 59, p. 285-289, 2009.

Paul, W.; Zhu, J. How are TH2-type immune responses initiated and amplified. Nature Reviews Immunology, v.10, p.225-235, 2010.

Pecchi, E.; Dallaporta, M.; Jean, A.; Thirion, S.; Troadec, J. D. Prostaglandins and sickness behavior: old story, new insights. Physiological Behavior, v. 97, p. 279-292, 2009.

Piuvezam, M. R.; Peçanha, L. M.; Alexander, J.; Thomas, G. *Cissampelos sympodialis* Eichl. leaf extract increases the production of IL-10 by concanavalin-A treated BALB/c spleen cells. Journal of Ethnopharmacology, v. 67, p. 93-101, 1999.

Polosa, R. Critical appraisal of antileukotriene use in asthma management. Current Opinion in Pulmonary Medicine, v. 13, p. 24-30, 2007.

Prado, C. M.; Martins, M. A.; Tibério, I. F. L. C. Nitric Oxide in Asthma. Physiopathology. Allergy. v. 2011, p. 1-13, 2011.

Rainsford, K. D. Anti-inflammatory drugs in the 21st century. Sub-cellular Biochemistry, v. 42, p. 3-27, 2007.

Ryan, B.; Majno, G. Acute Inflammation. American Journal of Pathology, v.86(1), p. 183-276, 1977.

Puri, N.; Kruhlak, M. J.; Whiteheart, S. W.; Roche, P. A. Mast cell degranulation requires N-ethylmaleimide-sensitive factor-mediated SNARE disassembly. The Journal of Immunology, v. 17, p. 5345-5352, 2003.

Ribeiro-Filho, J.; Calheiros, A. S.; Vieira-de-Abreu, A.; Carvalho, K. I. M.; Mendes, D. S.; Bandeira-Melo, C.; Martins, M. A.; Dias, C. S.; Piuvezam, M. R. Bozza, P. T. Curine inibits eosinophil activation and airway hyper-responsiveness in a mouse model of allergic asthma. Toxicology and Applied Pharmacology, v. 273, p. 19-26, 2013.

Riedel, W.; Neeck, G. Nociception, pain, and antinociception: current concepts. Zeitschrift für Rheumatologie, v. 60, p. 404-415, 2001.

Rhen, T.; Cidlowski, J.A. Anti-inflammatory action of glucocorticoids - new mechanisms for old drugs. New England Journal of Medicine, v. 353, p. 1711- 1723, 2005.

Rodriguez-Palmero, M.; Hara, T.; Thumbs, A.; Hunig, T. Triggering of T cell proliferation through CD28 induces Gata-3 and promotes T helper type 2 differentiation in vitro and in vivo. European Journal of Immunology, v. 29, p. 3914-3924, 1999.

Rönnberg. E.; Calounova, G.; Guss, B.; Lundequist, A.; Pejler, G. Granzyme D is a novel murine mast cell protease that is highly induced by multiple pathways of mast cell activation. Infection and Immunity, v. 81(6), 2085-2094, 2013.

Rothenberg, M. E. Eosinophilia. New England Journal of Medicine, v. 28, p. 1592, 1998.

Rothenberg, M. E.; Robert-Ownbey, T.; Mehlhop, P. D.; Loiselle, P. M.; Van de Rijn, M.; Bonventre, J.V.; Oettgen, H. C.; Leder, P.; Luster, A. D. Eotaxin triggers eosinophil-selective chemotaxis and calcium flux via a distinct receptor and induces pulmonary eosinophilia in the presence of interleukin 5 in Mice. Molecular Medicine, v. 2 (3), p. 334-348, 1996.

Schacke, H.; Schottelius, A.; Docke, W.D.; Strehlke, P.; Jaroch, S.; Schmees, N.; Rehwinkel, H.; Hennekes, H.; Asadullah, K. Dissociation of transactivation from transrepression by a selective glucocorticoid receptor agonist leads to separation of therapeutic effects from side effects. Proceedings of the National Academy of Sciences. USA, v. 101, p. 227-232, 2004.

Scrivo, R.; Vasile, M.; Bartosiewicz, I.; Valesini, G.; Inflammation as "common soil" of the multifactorial diseases. Autoimmunity Reviews, v.10, p. 369-374, 2011.

Seow, W. K.; Li, S. T. Y.; Thong, Y. H.; Inhibitory effect of tetrandrine on human neutrophil and monocyte adherence. Immunology Letters, v. 13, p. 83-88, 1986.

Serhan, C. N.; Savill, J. Resolution of inflammation: the beginning programs the end. Nature Immunology, v. 6, p. 1191-1197, 2005.

Shacter, E.; Weitzman, S. A. Chronic inflammation and cancer. Oncology, v. 16 (2), p. 217-226, 2002.

Sicherer, S. H.; Sampson, H. A. Peanut allergy: emerging concepts and approaches for an apparent epidemic. Journal of Allergy and Clinical Immunology, v. 120, p. 491-503, 2007.

Silva, C. V. Alcaloides Benzofenantridínicos e outros metabólitos do caule e do fruto de *Zanthoxylum Tingoassuiba* St. Hill. 2006. Dissertação, UFBA. 123 p. disponível em: < https://repositorio.ufba.br/ri/bitstream/ri/9993/1/Cinara.pdf>.

Smith-Garvin, J. E.; Koretzky, G. A.; Jordan, M. S. T cell activation. Annual Review of Immunology, v. 27, p. 591-619, 2009.

Sociedade Brasileira de Pneumologia e Tisiologia (SBPT). Diretrizes da Sociedade Brasileira de Pneumologia e Tisiologia para o Manejo da Asma - 2012. J Bras Pneumol. v.38, Suplemento 1, p.S1-S46, 2012.

Soehnlein, O.; Weber, C.; Lindbom, L. Neutrophil granule proteins tune monocytic cell function. Trends in Immunology, v. 30 (11), p. 538-546, 2009.

Souto, A. L.; Tavares, J.F.,Silva; M. S.; Diniz, M. F.; Athayde-Filho, P. F.; Barbosa- Filho, J. M.; Anti-inflammatory activity of alkaloids: an update from 2000 to 2010. Molecules, v. 16, p. 8515-8534, 2011.

Takeda, K.; Akira, S.; Toll-like receptors in innate immunity. International Immunology, v. 17, p. 1-14, 2005.

Soroosh, P.; Doherty, T. A. Th9 and Allergic Disease.Immunology.v.127, p. 450-458, 2009.

Stockinger, B.; Veldhoen, M. Differentiation and function of Th17 cells. Current Opinion in Immunology, v. 19, p. 281-286, 2007.

Szefler, S. J. Advancing asthma care: the glass is only half full. Journal of Allergy and Clinical Immunology, v. 128(3), p. 485-494, 2011.

Tabas, I.; Glass, C. K. Anti-inflammatory therapy in chronic disease: challenges and opportunities. Science, 339, 166-172, 2013.

Tam, S. W.; Demissie, S.; Thomas, D.; Daeron, M. A bispecific antibody against human IgE and human FcεRII that inhibits antigeninduced histamine release by human mast cells and basophils. Allergy, v.59, p. 772-80, 2004.

Tatemoto, K.; Nozaki, Y.; Tsuda, R. Konno, S.; Tomura, K.; Furuno, M.; Ogasawara, H.; Edamura, K.; Takagi, H.; Iwamura, H.; Noguchi, M.; Naito, T. Immunoglobulin E-independent activation of mast cell is mediated by Mrg receptors. Biochemical and Biophysical Research Communications, v. 349, p. 1322-1328, 2006.

Tauber, A. I. Metchnikoff and the phagocytosis theory. Nature Reviews Molecular and Cell Biology, v. 4, p. 897-901, 2003.

Teh, B. S.; Seow, W. K.; Li, S.Y.; Thong, Y. H. Inhibition of prostaglandin and leukotriene generation by the plant alkaloids tetrandrine and berbamine. International Journal of Immunopharmacology, v.12, 321-326, 1990.

Teske, M.; Trentine, A. M. Herbarium: Compêndio de Fitoterapia. Curitiba, 1994.

Tjølsen, A.; Berge, O. G.; Hunskaar, S.; Rosland, J. H.; Hole, K. The formalin test: an evaluation of the method. Pain, v. 51, p. 5-17, 1992.

Tracey, K. J. The inflammatory reflex. Nature, v. 420, p.853-859, 2002.

Truyen, E.; Coteur, L.; Dilissen, E.; Overbergh, L.; Dupont, L. J.; Ceuppens, J. L.; Bullens, D. M. Evaluation of airway inflammation by quantitative TH1/TH2 cytokine mRNA measurement in sputum of asthma patients. Thorax, v. 61, p. 202-208, 2006.

Turner, H.; Kinet, J. P. Signalling through the high-affinity IgE receptor Fc epsilonRI. Nature, v. 25, p. 402(6760 S)B24-30, 1999.

Ueda, H.; Ueda, M. Mechanisms underlying morphine analgesic tolerance and dependence. Frontiers in Bioscience (Landmark Ed). v. 4, p. 5260-5272, 2009.

Vane, J. R.; Botting, R. M. Mechanism of action of anti-inflammatory drugs. Scandinavian Journal of Rheumatology, v. 25, p. 9-21, 1996.

van Rossum, D. B.; Patterson, R. L. PKC and PLA_2: Probing the complexities of the calcium network. Cell Calcium. v. 45, p. 535-545, 2009.

Vieira-de-Abreu A.; Assis, E. F.; Gomes, G. S.; Castro-Faria-Neto, H. C.; Weller, P. F.; Bandeira-Melo, C.; Bozza, P. T. Allergic Challenge-Elicited Lipid Bodies Compartmentalize In Vivo Leukotriene C_4 Synthesis within Eosinophils. American Journal of Respiratory Cell and Molecular Biology, v. 33, p. 254-261, 2005.

Wade, J. P.; Liang, M. H.; Sheffer, A. L. Exercise-induced anaphylaxis: epidemiologic observations. Progress in Clinical and Biological Research, v. 297, p. 175-182, 1989.

Wahlgren, C. F. Itch and atopic dermatitis: clinical and experimental studies. Acta DermatoVenereologica, v. 165, p. 1-53. 1991.

Weller, P.F. Human eosinophils. Journal of allergy and clinical immunology, v. 100, p. 283, 1997.

Weller, P. F.; Lee, C. W.; Foster, D. W.; Corey, E. J.; Austen, K. F.; Lewis, R. A. Generation and metabolism of 5-lipoxygenase pathway leukotrienes by human eosinophils: predominant production of leukotriene C_4. PNAS USA v. 80, p. 7626-7630, 1983.

Whittle, B. A. The Use of Changes in Capillary Permeability in Mice to Distinguish between Narcotic and Non narcotic Analgesics. British Journal of Pharmacology and Chemotherapy, v. 22, p. 246-253, 1964.

Willart, M. A. M.; Hammad, H. Alarming Dendritic Cells for Allergic Sensitization. Allergology International, v.59, p. 95-103, 2010.

Williams, J. A.; Shacter, E. Regulation of macrophage cytokine production by prostaglandin E_2. Distinct roles of cyclooxygenase-1 and -2. The Journal of Biological Chemistry, v. 272, p. 25693-25699, 1997.

Wills-Karp, M.; Luyimbazi, J.; Xu, X.; Schofield, B.; Neben, T. Y.; Karp, C. L.; Donaldson, D. D. 1998. Interleukin - 13: central mediator of allergic asthma. Science, v. 282, p. 2258- 2261.

Wong, C. W.; Seow, W. K.; Zeng, T. S.; Halliday, J. J.; Thong, Y. H. Comparative immunopharmacology and toxicology of the bisbenzylisoquinoline alkaloids tetrandrine and berbamine. International Journal of Immunopharmacology, v. 13, p. 579-585, 1991.

Woolf, C. J.; Salter, M. W. Neuronal plasticity: increasing the gain in pain. Science, v. 288, p. 1765-1769, 2000.

Wright, B.; Zeidman, I.; Greig, R.; Poste, G. Inhibition of macrophage activation by calcium channel blockers and calmodulin antagonists. Cellular Immunology, v. 95 (1), p. 46-53, 1985.

Yadav, R.; Zammit, D. J.; Lefrancois, L.; Vella, A. T. Effects of LPS-mediated bystander activation in the innate immune system. Journal of Leukocyte Biology, v. 80, p.1251-1261, 2006.

Yago, T.; Shao, B.; Miner, J. J.; Yao, L.; Klopocki, A. G, Maeda, K.; Coggeshall, K. M.; McEver, R. P. E-selectin engages PSGL-1 and CD44 through a common signaling pathway to induce integrin αLβ2-mediated slow leukocyte rolling. Blood, v. 116, p. 485-94, 2010.

Yang, Z.; Lu, W.; Ma, X.; Song, D.; Bioassay-guided isolation of an alkaloid with antiangiogenic and antitumoractivities from the extract of *Fissistigma cavaleriei* root. Phytomedicine, v. 19, p. 301-305, 2012.

Yun, K.; Shin, J.; Choi, J.; Back, N.; Chung, H.; Lee, K. Quaternary alkaloid, pseudocoptisine isolated from tubers of *Corydalis turtschaninovi* inhibits LPS-induced nitric oxide, PGE2, and pro-inflammatory cytokines production via the down-regulation of NF-κB in RAW 264.7 murine macrophage cells. International Immunopharmacology, v. 9, p. 1323-1331, 2009.

Zhu, J., Yamane, H., Paul, W.E. Differentiation of Effector CD4 T Cell Populations. Annual Review of Immunology, v. 28, p. 445-489, 2010.

7. ANEXO I- Outras publicações

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Effectiveness of *Cissampelos sympodialis* and its isolated alkaloid warifteine in airway hyperreactivity and lung remodeling in a mouse model of asthma

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ABSTRACT

Background: Cissampelos sympodialis Eichl. (Menispermaceae) is a plant found in Northeastern and Southeast of Brazil and hot water infusion of *C. sympodialis* root bark is largely used in the indigenous and folk medicine to treat several inflammatory disorders, including asthma. Asthma is a chronic inflammatory allergic disease characterized by airway hyperreactivity (AHR), eosinophil tissue infiltration and lung remodeling. The aim of this study was to evaluate the therapeutic effect of *C. sympodialis* and its isolated alkaloid warifteine on allergen triggered airway hyperreactivity (AHR) and lung remodeling in murine model of asthma.
Methodology/principal findings: The oral pre-treatment with *C. sympodialis* or warifteine inhibited allergen-induced AHR to inhaled methacholine and IL-13 levels in the bronchoalveolar lavage (BAL). In order to investigate the therapeutic potential of *C. sympodialis* and warifteine, animals were treated 1 h after the last ovalbumin (OVA) challenge in sensitized animals. Similarly to the pre-treatment, post-treatment with warifteine was effective to inhibit significantly AHR to inhaled methacholine and to reduce IL-13 levels in the BAL. In addition, oral pre- or post-treatments with *C. sympodialis* or warifteine reduced OVA-induced eosinophil tissue infiltration, mucus production and subepithelial fibrosis to values similar to nonallergic controls.
Conclusions: Our data show the anti-allergic and immunoregulatory properties of *C. sympodialis*, acting mostly through the active compound warifteine, to inhibit the airway hyperreactivity and lung remodeling through a mechanism at least partially dependent of IL-13 and eosinophil inhibition. Therefore placing warifteine as an interesting therapeutic candidate in allergic inflammation and corroborating the folk medicine use of *C. sympodialis* as anti-allergic plant.

1. Introduction

Asthma is a chronic inflammatory disease characterized by eosinophil tissue infiltration, airway hyperreactivity (AHR) and lung remodeling [1]. Anti-inflammatory treatment with inhaled glucocorticoid alone or combined therapy of corticosteroids with long-acting β2-agonist bronchodilators is the current preconized therapy for asthma. Although, most asthmatics respond to these treatments, 5–10% of patients are entirely insensitive to corticosteroids. In addition, some patients require additional oral glucocorticoids, and long-term use of corticosteroids has been strongly associated with a number of adverse effects [2,3]. Thus, there is a need for new anti-asthmatic drug development.

Medicinal plants and their bioactive molecules are alternative options to conventional therapies for many diseases. *Cissampelos sympodialis* Eichl. (Menispermaceae) is a plant species found in Northeastern and Southeast of Brazil. A hot water infusion of *C. sympodialis* root bark is largely used in the indigenous and folk medicine to treat several inflammatory disorders, including asthma [4]. Phytochemical analysis of *C. sympodialis* root extracts leads to isolation of several alkaloids, of which warifteine has shown pharmacological effects [5–7]. In order to investigate the effect of *C. sympodialis* in immunological responses, previous studies showed that this plant enhanced IL-10 and IFN-γ levels in spleen cells from allergic mice and IL-10 levels in macrophage cultures [8,9]. This modulatory effect of *C. sympodialis* was correlated with decreased Ig-E production.

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Pertinent to the potential anti-asthmatic effects of *C. sympodialis*, recent published data showed that *C. sympodialis* and warifteine strongly reduced the eosinophilic inflammation into the bronchoalveolar lavage and pleural cavities in ovalbumin (OVA) sensitized mice [10]. This effect was dependent of cysteinyl leukotrienes generation and lipid body formation observed in activated eosinophils. *C. sympodialis* and warifteine were also capable of inhibiting the generation of the eosinophil chemoattractant factor, eotaxin. In addition, recent study showed in an experimental model of respiratory allergy to *Blomia tropicalis* which is the most important indoor allergen associated with asthma and rhinitis that the hydroalcoolic extract of *C. sympodialis* leaves and warifteine significantly reduced eosinophil migration and modulated Th2 cytokine production inducing IL-5 levels reduction in the bronchoalveolar lavage, while presenting high levels of the anti-inflammatory cytokine IL-10 [11].

Among the effects observed on inflammatory leukocytes, mast cell activation was also modified by warifteine. *In vitro* studies showed that warifteine inhibited mast cell degranulation induced by immunological or pharmacological challenge [12]. In these previous studies, it was also observed that warifteine decreased the immediate allergic reactions such as anaphylactic shock by IgE dependent mechanisms and thermal hyperalgesia.

The effects of *C. sympodialis* and warifteine on airway hyperreactivity and lung tissue pathological changes in OVA-induced allergic airway disease have not been addressed. The aim of this study was to evaluate the effectiveness of *C. sympodialis* on airway hyperactivity and collagen fibers and mucus production. For this purpose we used either the standardized hydroalcoholic extract of the *C. sympodialis* leaves, as well as, the *C. sympodialis*-derived alkaloid warifteine in OVA-induced lung inflammation in actively sensitized mice.

2. Materials and methods

2.1. Preparation of *C. sympodialis* extract and warifteine purification

Leaves from *C. sympodialis* were obtained from the Botanical Garden of the Laboratório de Tecnologia Farmacêutica/Universidade Federal da Paraíba (voucher specimen Agra 1456). The leaves were dried at 50 °C in an oven and pulverized. The powder was extracted with 70% ethanol in water at 70 °C for 5 days. The dried extract was dissolved in water; filtered and known volumes were dried to determine the final concentration of the water-soluble components. All doses are expressed in terms of the concentration of the soluble components (mg/kg of body weight). The yield was 22% on average, based on solid residues present [13]. *C. sympodialis* extract was dissolved in sterile water immediately before use. The extract used in all experiments described in this paper had a nominal concentration of 0.95% of warifteine [11].

The extract of *C. sympodialis* was submitted to procedures aimed to isolate the alkaloids, using column and thin layer chromatography (TLC). Briefly, the extract was dissolved in 3% HCl and extracted several times with $CHCl_3$. The aqueous fraction was basified with NH_4OH to pH 9 and again extracted with $CHCl_3$. The $CHCl_3$ extract was washed with H_2O, dried ($MgSO_4$) and the solvent evaporated to afford the total tertiary alkaloid fraction (TTA). The TTA was subjected to column over alumina, eluting with hexane containing increasing amounts of $CHCl_3$, $CHCl_3$ with increasing amounts of MeOH and finally with MeOH. The fraction eluted with $CHCl_3$–MeOH (49:1), after further purification by TLC (1.0 mm layer), yielded the isolation of the bisbenzylisoquinoline alkaloid warifteine (0.031%). The identification of the warifteine was performed by analyzing 1H and 13C NMR spectral data compared with those published in the literature (Fig. 1) [11]. Warifteine was quantified in the leaf extract (CsE) by means of High Performance Liquid Chromatography (HPLC) with ultraviolet detection and it was calculated at 96.4% pure.

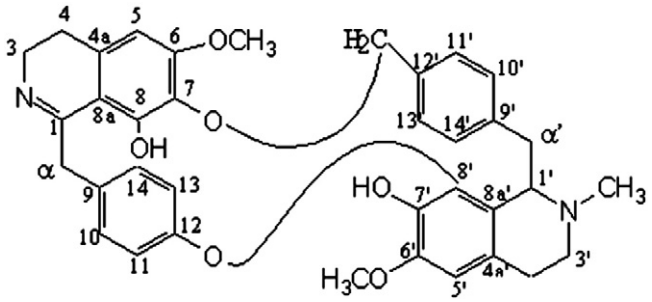

Fig. 1. Structure of warifteine.

2.2. Animals

Female BALB/c mice weighing 16–20 g were used. They were obtained from Oswaldo Cruz Foundation breeding unit. Animals were maintained with food and water *ad libitum* in a room with temperature ranging from 22 to 24 °C and a 12 h light/dark cycle. This study was carried out in accordance with the recommendations of the Brazilian National Council of Control of Animal Experimentation (CONCEA). The protocols were approved by the Animal Welfare Committee of the Oswaldo Cruz Foundation (CEUA/FIOCRUZ protocol # L-002/08).

2.3. Asthma model in actively sensitized mice

OVA-induced allergic airway disease was generated in mice as described previously [14]. Briefly, mice (n = 8) were sensitized with intraperitoneal injection of OVA (10 μg/mouse) and $Al(OH)_3$ (10 mg/mL) in 0.9% NaCl solution (saline; 0.2 mL) on days 1 and 10. From day 19 to day 24 after sensitization, mice were challenged daily for 20 min with OVA (5%) in phosphate buffered saline (PBS) by aerosol. Aerosolized PBS was administered to sensitized mice as a negative control. These procedures were performed in a 30 × 20 × 10 cm acrylic chamber and the aerosol was generated with an ultrasonic nebulizer.

2.4. Treatments

To evaluate the effect of *C. sympodialis* and warifteine in airway hyperreactivity and lung histopathological changes, mice were treated 1 h before (pre-treatment) or 1 h after (post-treatment) the last aerosol OVA challenge. *C. sympodialis* extract (40 mg/kg), warifteine (2 mg/kg) or dexamethasone (2 mg/kg) was administered via oral route (p.o). Of note, the *C. sympodialis* extract and warifteine doses were used according to previous reports [9–11].

2.5. Airway hyperreactivity analysis

Airway hyperreactivity (AHR) was analyzed in mice using non-invasive whole-body plethysmography (Buxco, Sharon, CT) one day after the last OVA challenge. AHR was measured following aerosolization of PBS followed by increasing concentrations of methacholine (0, 6, 25 mg/mL; Sigma-Aldrich) for 2 min into the chamber. AHR was expressed as an average enhanced pause (Penh). There was an interval of 10 min between each aerosol exposure and within this period of time the Penh values had returned to baseline.

2.6. Bronchoalveolar lavage (BAL) and quantification of IL-13

Animals were euthanized by CO_2 and the trachea was surgically exposed and cannulated at 6 or 24 h after the last allergen challenge. BAL was collected from the mice by washing the lungs with 1 mL PBS. Samples of BAL were centrifuged at 500 *g* for 8 min at 4 °C to obtain the supernatants. Then levels of IL-13 were measured according to

the manufacturer's instructions by the mouse IL-13 DuoSet kit (R&D Systems).

2.7. Lung histology

Lungs were inflated by injecting 1.0 mL of 4% buffered formalin through the catheter used to perform BAL. Lungs were then removed, fixed in the same solution, and embedded in paraffin. Lung sections of 5-μm thickness were stained with Sirius red, Mason's trichrome, and periodic acid-Schiff (PAS) according to standard protocols and examined under light microscopy. Analysis of tissue sections and captured images was performed by using a computer-assisted image analyzer (Image-Pro Plus Version 4.1 for Windows, Media Cybernetics, LP, Silver Spring, MD, USA). One observer who was unaware of the experimental setting examined all tissue sections randomly. Digital photographs of at least 10 bronchovascular bundles per tissue section (with bronchioles cross-sectional diameters ranging from 120 to 250 μm) were obtained under light microscopy at ×400 magnifications. For quantifying eosinophil infiltration of the pulmonary tissue, the total number of eosinophils was counted in the ten areas, and the mean was expressed as eosinophils/high power field (HPF). Mucus secretion from goblet cells of the bronchial epithelium was quantified by the staining of sections with PAS. Goblet cells were recognized by the intense dark red staining of their mucus content with PAS, and their characteristic distended lateral border and basal nucleus. The number of goblet cells was expressed as the percentage of positive cells in at least 500 cells of the bronchial epithelium. The Mason's trichrome dye was used to stain collagen fibers in tissue. Density of collagen fibers was defined by the positively stained area in relation to total tissue in the area of the bronchovascular bundles per millimeter squared, at a magnification of ×100.

2.8. Statistical analysis

Statistical analysis was carried out using the statistical software SPSS for Windows (Version 10.0.1, SPSS Inc., 1989–1999, USA) or GraphPad Prism statistical analysis and graphing software (GraphPad, San Diego, CA). Data were analyzed by ANOVA followed by Newman Keuls *t* test or Dunnett's test. Correlations between the densities of positive cells and the collagen fibers were assessed using the Spearman rank correlation coefficient. Values are expressed as means ± SEM. The level of significance was set at $P<0.05$.

3. Results

3.1. C. sympodialis and its alkaloid warifteine inhibit AHR and IL-13 production

OVA sensitization and airway challenge led to the development of AHR in mice illustrated by significant increases in Penh values compared with PBS-challenged control mice. As shown in Fig. 2A, the pre-treatment with *C. sympodialis* reduced AHR to inhaled methacholine (25 mg/mL) observed 24 h after allergen challenge. This effect was similar to the glucocorticoid dexamethasone a potent anti-inflammatory drug. The pre-treatment with *C. sympodialis* derived-alkaloid warifteine was also effective in blocking AHR (Fig. 3A).

The involvement of IL-13 in AHR development is well described. OVA-induced allergic airway inflammation leads to elevated levels of IL-13 when compared to PBS challenged animals (Figs. 2B and 3B). As illustrated in the same figures, OVA-sensitized and challenged mice pre-treated with either *C. sympodialis* or warifteine showed a significant reduction in IL-13 levels, comparable to dexamethasone treated animals.

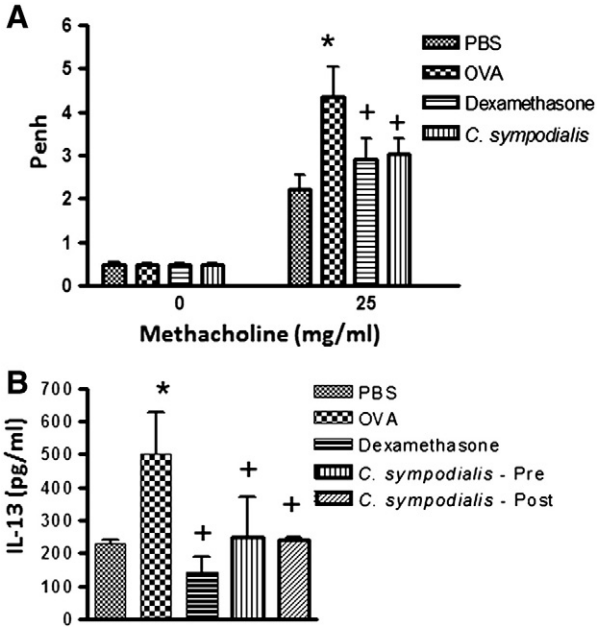

Fig. 2. Effect of *C. sympodialis* treatment on airway hyperreactivity (AHR) development and IL-13 production. OVA-sensitized mice were treated with a single oral dose of dexamethasone (2 mg/kg) 1 h before, or *C. sympodialis* extract (40 mg/kg) 1 h before (pre-) or 1 h after (post-) the last OVA challenge. PBS aerosolized mice were used as control. (A) AHR was measured by aerosolization of PBS followed by increasing concentrations of methacholine. AHR was analyzed 24 h after the last allergic challenge and expressed as an average enhanced pause (Penh). (B) IL-13 production was measured in BAL 24 h after the last allergic challenge. Results are expressed as the mean ± SEM from at least six animals. *, significantly different from PBS-challenged group ($P\leq0.05$). +, significantly different from allergen-challenged group ($P\leq0.05$).

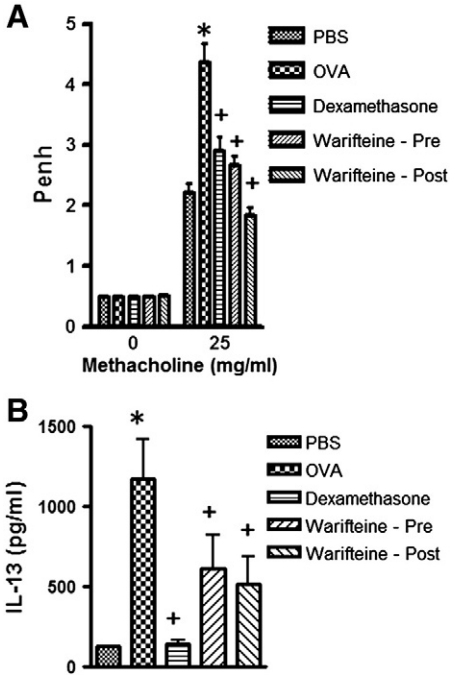

Fig. 3. Effect of warifteine treatment on airway hyperreactivity (AHR) and IL-13 production. OVA-sensitized mice were treated with a single oral dose of dexamethasone (2 mg/kg) 1 h before, or warifteine (2 mg/kg) 1 h before (pre-) or 1 h after (post-) the last OVA challenge. (A) AHR was measured by aerosolization of PBS followed by increasing concentrations of methacholine. AHR was analyzed 24 h after the last allergic challenge and expressed as an average enhanced pause (Penh). (B) IL-13 production was measured in BAL 6 h after the last allergic challenge. Results are expressed as the mean ± SEM from at least six animals. *, significantly different from PBS-challenged group ($P\leq0.05$). +, significantly different from allergen-challenged group ($P\leq0.05$).

In order to investigate the potential therapeutic effect of *C. sympodialis* and its active derivative warifteine, animals were treated 1 h after the last OVA challenge in sensitized animals. Similarly to the pre-treatment, oral post-treatment with warifteine but not with *C. sympodialis* extract (data not shown) was able to inhibit AHR in a significant manner. In addition, post-treatment with warifteine reduced IL-13 BAL levels detected 6 h after the last OVA challenge in the asthma model (Fig. 3B). These data indicate that *C. sympodialis*, mostly through the active compound warifteine, is capable to ameliorate an established inflammatory allergic reaction.

3.2. Effects of *C. sympodialis* and warifteine in mucus accumulation

The evaluation of airway mucins assessed by PAS staining is shown in Fig. 4A. The analysis of airway mucins demonstrated a significant metaplasia of goblet cells and mucus accumulation 24 h after the last OVA challenge in actively sensitized mice. Oral pre- or post-treatment with *C. sympodialis* or pre-treatment with warifteine reduced OVA-induced mucus accumulation and the percentage of mucus producing cells to values similar to nonallergic controls (Fig. 4B). Pre-treatment with dexamethasone showed decreased mucus production as expected.

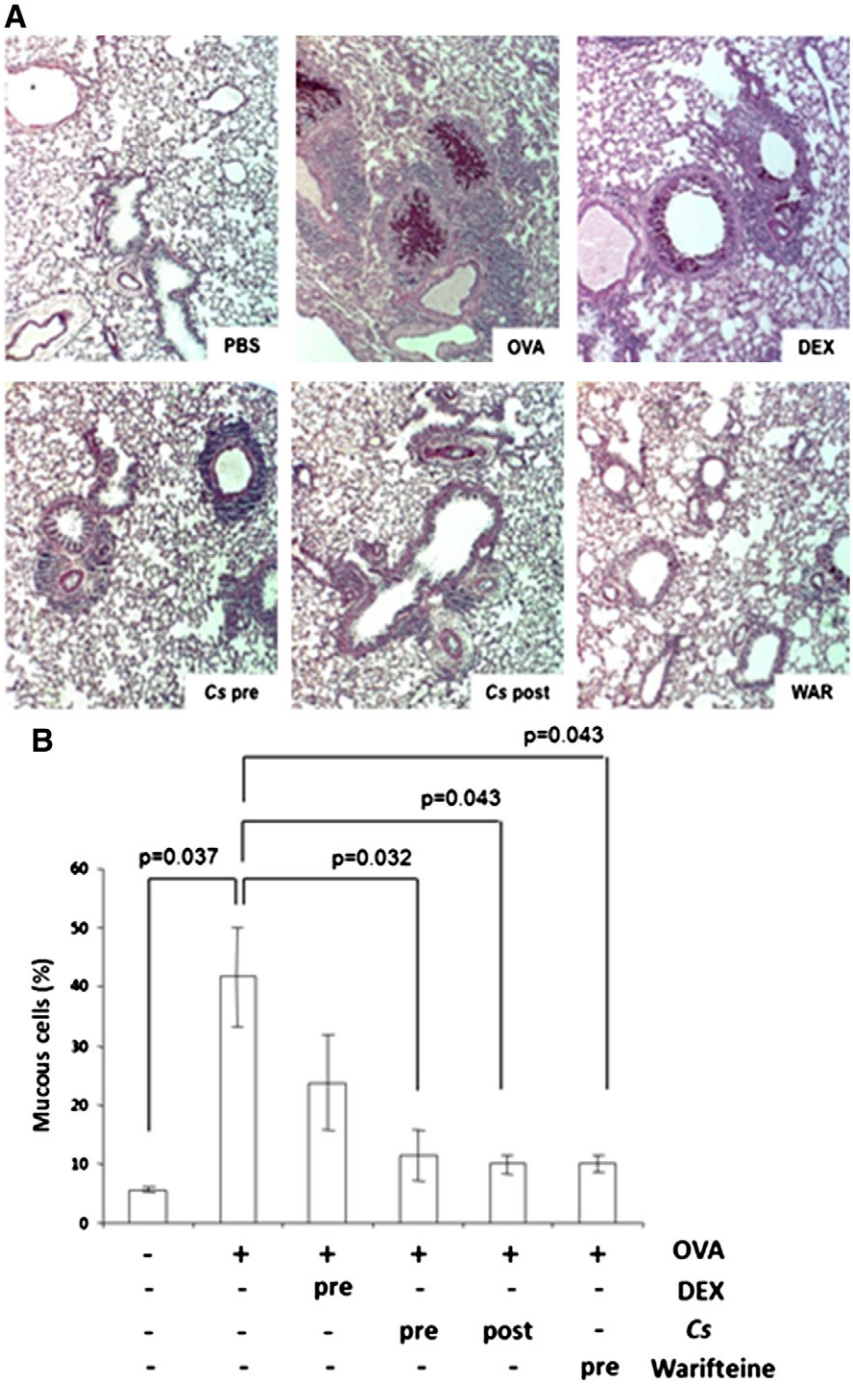

Fig. 4. Effects of *C. sympodialis* and warifteine treatments in airway mucus deposition. Twenty-four hours after the last day of challenge, lungs were inflation-fixed in 4% formalin, embedded in paraffin, and sectioned. The tissue section slides were stained with periodic acid-Schiff (PAS) for evaluation of airway mucins and number of mucus-producing cells. OVA-sensitized mice were treated with a single oral dose of dexamethasone or warifteine (2 mg/kg) 1 h before, or *C. sympodialis* extract (40 mg/kg) 1 h before (pre-) or 1 h after (post-) the last OVA challenge. PBS aerosolized mice were used as control. (A) Microscopic analysis of stained tissue sections. (B) Quantitative analysis of tissue sections stained with PAS. Data are representative of two independent experiments ($n=6$). *, significantly different from PBS-challenged group ($P \leq 0.05$). $^{+}$, significantly different from allergen-challenged group ($P \leq 0.05$).

3.3. Treatment with C. sympodialis or warifteine inhibits tissue eosinophil infiltration in OVA-induced airway inflammation

Eosinophil activation and recruitment are hallmarks of allergic disease and tissue eosinophil infiltration has been associated with collagen deposition and lung remodeling. The effect of *C. sympodialis* or warifteine oral treatment on OVA-induced eosinophil tissue infiltration was investigated. As shown in Fig. 5A, Sirius red stained lung sections revealed an increased leukocyte infiltration comprised mostly by a marked perivascular eosinophilia in OVA-challenged allergic mice when compared to nonallergic mice. Oral pre-treatment with *C. sympodialis* or warifteine significantly inhibited OVA-induced eosinophil tissue infiltration similar to pre-treatment with dexamethasone (Fig. 5B). Post-treatment with *C. sympodialis* extract was also able to significantly inhibit eosinophil tissue infiltration observed after 24 h (Fig. 5A and B).

3.4. Inhibition of collagen fiber deposition by C. sympodialis and warifteine

Airway remodeling is associated with clinical outcomes in asthmatic patients and contributes to thickening of airway walls leading to hyperreactivity and narrowing of airways. As shown in Fig. 6A, OVA induced significant subepithelial fibrosis with collagen fiber

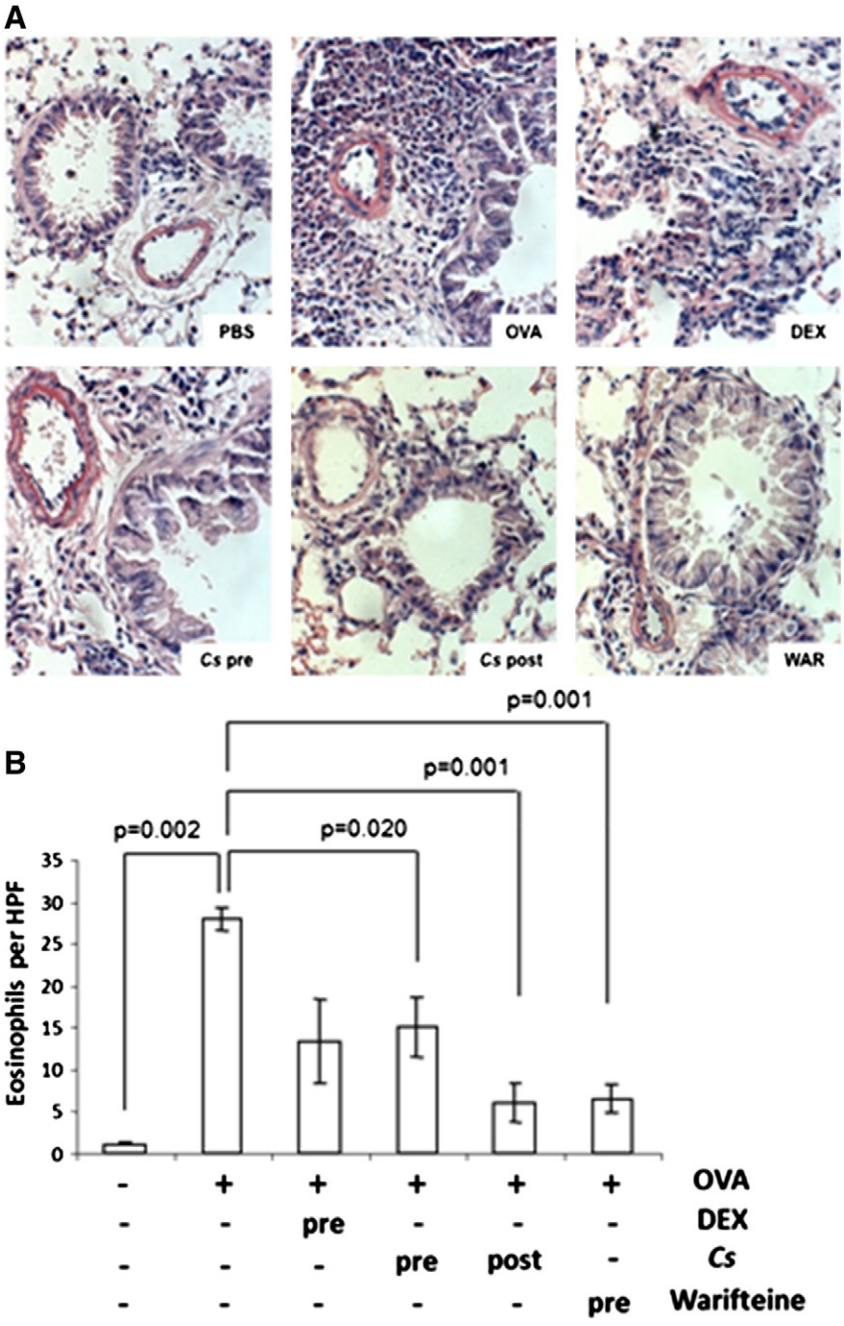

Fig. 5. Effect of *C. sympodialis* and warifteine treatment in eosinophil tissue infiltration following OVA-induced allergic airway disease. Twenty-four hours after the last day of challenge, lungs were inflation-fixed in 4% formalin, embedded in paraffin, and sectioned. The tissue section slides were stained with Sirius red for eosinophil tissue evaluation. OVA-sensitized mice were treated with a single oral dose of dexamethasone or warifteine (2 mg/kg) 1 h before, or *C. sympodialis* extract (40 mg/kg) 1 h before (pre-) or 1 h after (post-) the last OVA challenge. PBS aerosolized mice were used as control. (A) Microscopic analysis of stained tissue sections. (B) Quantitative analysis of tissue sections stained with Sirius red. *, significantly different from PBS-challenged group ($P \leq 0.05$). $^{+}$, significantly different from allergen-challenged group ($P \leq 0.05$).

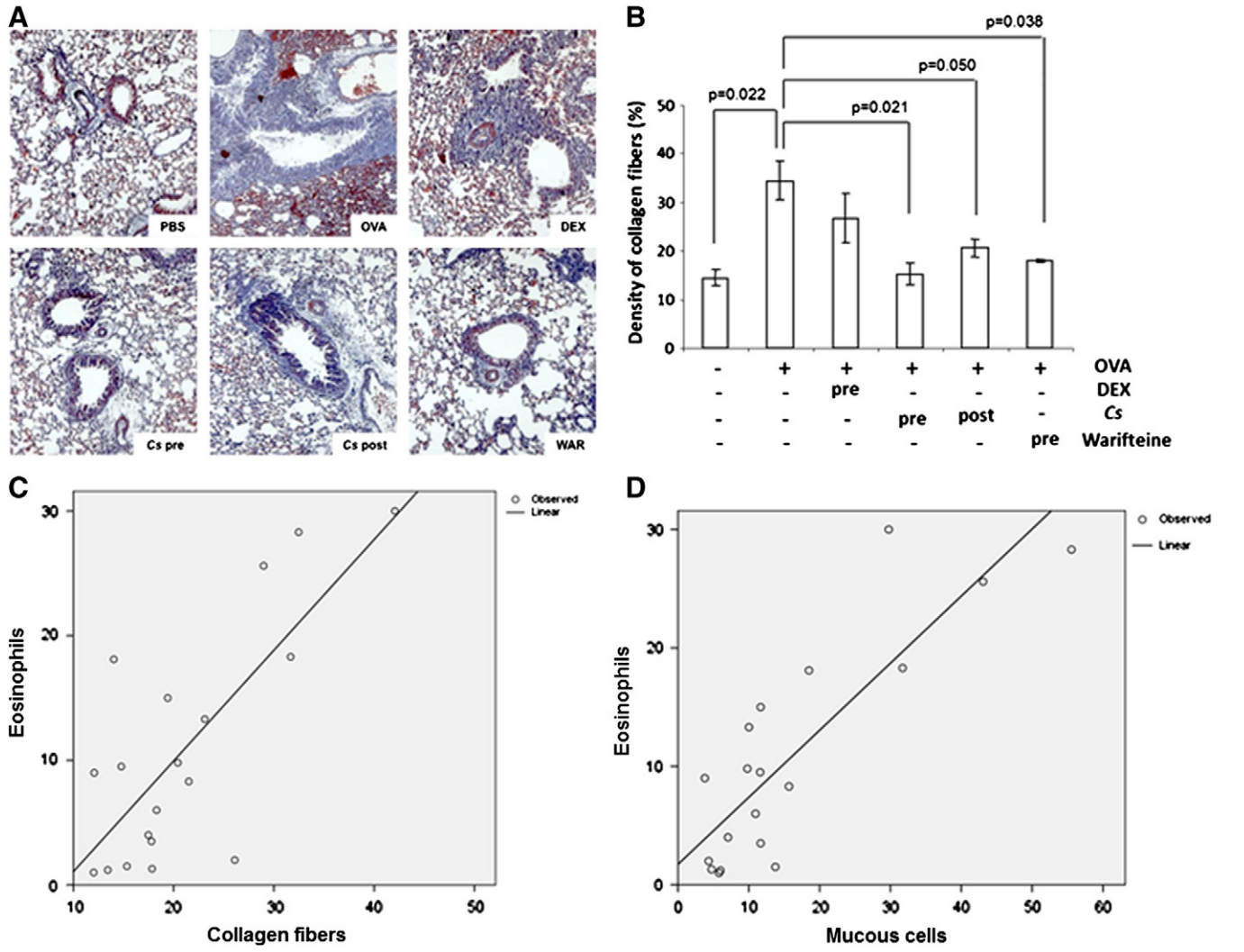

Fig. 6. Effects of *C. sympodialis* and warifteine treatments in collagen fiber deposition. (A) Lung serial sections were embedded in paraffin by routine methods and stained with Mason's trichrome stain to collagen fiber observations. OVA-sensitized mice were treated with a single oral dose of dexamethasone or warifteine (2 mg/kg) 1 h before or *C. sympodialis* extract (40 mg/kg) 1 h before (pre-) or 1 h after (post-) the last OVA challenge. PBS aerosolized mice were used as control. (A) Microscopic analysis of stained tissue sections. (B) Quantitative analysis of collagen fiber density was expressed in percentage. (C) Correlation between eosinophil tissue infiltration and collagen fiber deposition and (D) correlation between eosinophil tissue infiltration and mucus-producing cells. *, significantly different from PBS-challenged group ($P \leq 0.05$). $^{+}$, significantly different from allergen-challenged group ($P \leq 0.05$).

deposition assessed by Gomoris staining in allergic animals when compared to nonallergic controls. As observed in Fig. 6A and quantified in Fig. 6B, oral treatment with *C. sympodialis* (pre or post) or warifteine (pre) reduced OVA-induced collagen deposition to values similar to nonallergic controls (Fig. 6A, B). In accordance to a role for tissue eosinophils in collagen and mucus deposition and lung remodeling, there is a strong correlation between eosinophil infiltration and collagen deposition (correlation coefficient of 0.651, $P<0.003$) and mucus (correlation coefficient of 0.723, $P<0.001$) in the lungs (Fig. 6C, D).

4. Discussion

In this study we evaluate the effect of *Cissampelos sympodialis* and its isolated alkaloid warifteine in a murine asthma model. We provide novel evidence related to the anti-allergic and immunoregulatory properties of *C. sympodialis*, acting mostly through the active compound warifteine, to inhibit the airway hyperreactivity and lung remodeling via a mechanism at least partially dependent of IL-13 and eosinophil inhibition.

In order to test the hypothesis that *C. sympodialis* has anti-allergic properties and potential for anti-asthmatic use, the effect of ethanolic extract of *C. sympodialis* leaves and its derived-alkaloid warifteine to prevent or ameliorate AHR and lung remodeling were investigated. Oral treatment with *C. sympodialis* 1 h before the last ovalbumin challenge significantly reduced AHR. Development of AHR depends on airway inflammation and smooth muscle lung contraction induced by different stimuli including IL-13, IL-4 and cysteinyl leukotrienes [15–17]. Accumulating evidence has placed IL-13 as a key mediator of AHR due to the protective effect demonstrated by different strategies of disruption of IL-13 production and/or effects [16]. To gain insights on the mechanism of action of *C. sympodialis* its role in IL-13 production was analyzed. In parallel to the AHR inhibition, we observed a reduced level of IL-13 in bronchoalveolar lavage of orally treated with *C. sympodialis* and challenged mice. Thus suggesting that, at least in part, the mechanism involved in *C. sympodialis* inhibitory effect on OVA-induced AHR may occur through inhibition of IL-13 production. In order to investigate candidate compounds to anti-asthmatic effect from *C. sympodialis* the isolation of one bisbenzylisoquinoline alkaloid warifteine was performed [6]. Similar to the one observed with *C. sympodialis*, oral treatment with warifteine inhibited

allergen-induced AHR and IL-13 production, suggesting that warifteine represents the main active component responsible for the effects of *C. sympodialis*. However, other constituents mainly alkaloids such as methylwarifteine, milonine and roraimine may also contribute to the anti-allergic effects of *C. sympodialis* extract.

Of note, warifteine also has an additional property that may explain its inhibitory effects on AHR. Previous *in vitro* and *in vivo* studies demonstrated direct spasmolytic effects and tracheal muscle contraction inhibition of warifteine [6,7,13,15]. In addition, warifteine treatment was very effective impairing *in vivo* lipid body biogenesis and BAL levels of LTC_4 [10] which is produced within eosinophil lipid bodies recruited to BAL in the asthma model [18].

As key components in asthma, lung eosinophils influx during inflammatory response has been demonstrated as important players of progressive tissue damage and lung remodeling [19–21]. Eosinophils are multifunctional leukocytes capable of producing immunoregulatory cytokines and lipid mediators and are actively involved in allergic disease through regulation of Th2-type immune responses [22]. *C. sympodialis* and warifteine also significantly reduced the eosinophil influx into the lung tissue of OVA-induced allergic inflammatory disease in mice. Accordingly, we have showed that *C. sympodialis* and its isolated alkaloid warifteine reduced eosinophil recruitment and activation in broncoalveolar lavage as well as into pleural cavity of OVA sensitized mice in part through mechanisms dependent of eotaxin [10].

Allergic lung inflammation also induces changes in tissue phenotype named hyperplasia of goblet cells leading to excessive mucus production causing airway obstruction [23] and collagen deposition leading to remodeling of the airways. An array of molecules might trigger mucus production but IL-13 has been mentioned as a pivotal stimulus [24]. Mice orally treated with *C. sympodialis* and warifteine showed a diminished mucus production in lung tissue. Airway remodeling is characterized by altered composition, content, and organization of cellular and molecular constituents of the airway wall [1]. Oral treatment with *C. sympodialis* also preserved structural components of lung tissue such as airway smooth muscle (ASM) mass, distance between epithelium and ASM cells, proliferation of blood vessels, airway edema, goblet cell numbers, collagen fiber deposition and mucus production. Deposition of collagen fibers and mucus has been described as airway change able to cause narrowing of airway, rigidness, loss of elastic recoil and reversible obstruction [1]. *C. sympodialis* and warifteine reduced collagen fiber deposition and mucus in lung tissue. These data correlated to decrease of eosinophil numbers in the tissue. Previous studies related that different stimuli can initiate airway remodeling before the appearance of asthma symptoms suggesting remodeling as contributive phenomena to lung inflammation and AHR development, and there is compelling evidence that tissue eosinophil accumulation contributes to lung remodeling in OVA-induced airway allergic disease since animals genetically ablated in eosinophils were significantly protected from peribronchiolar collagen deposition and increases in airway smooth muscle [25].

In this work we demonstrated for the first time that *C. sympodialis* and its isolated alkaloid warifteine inhibits airway hyperreactivity and lung remodeling known as the main asthma features and we provide evidence that the protective effect occurs partially by an IL-13 dependent mechanism. These findings, together with previous reports of this plant and warifteine inhibiting anaphylactic shock reaction in OVA sensitized mice, allergic pleurisy and pulmonary inflammation; suggest their therapeutic potential as anti-allergic and anti-asthmatic [9,26,10]. This alkaloid also reduced mast cell degranulation and hyperalgesia response in rats [12]. Of interest for potential therapeutic applications, both *C. sympodialis* extract and purified warifteine showed unique post-treatment properties via oral administration maintaining the anti-allergic inhibition of AHR, IL-13 production and lung eosinophil infiltration even when administered after the installed allergic process.

5. Conclusion

Indigenous and other people living in non-developed countries use *C. sympodialis* as a preventive method to asthma condition. Our data corroborate the folk anti-allergic medicine use of *C. sympodialis*. In addition, our findings indicate that the anti-allergic and immunoregulatory properties of *C. sympodialis* occur mostly through the active compound warifteine, to inhibit the airway hyperreactivity and lung remodeling through a mechanism at least partially dependent of IL-13 and eosinophil inhibition. Therefore, warifteine is suggested as an interesting therapeutic candidate in allergic inflammation.

Acknowledgments

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References

[1] Sumi Y, Hamid Q. Airway remodeling in asthma. Allergol Int 2007;56:341–8.
[2] Barnes PJ. Inhaled glucocorticoids for asthma. N Engl J Med 1995;332:868–75.
[3] Jarjour NN, Wilson SJ, Koenig SM, Laviolette M, Moore WC, et al. Control of airway inflammation maintained at a lower steroid dose with 100/50 μg fluticasone propionate/salmeterol. J Allergy Clin Immunol 2006;118:44–52.
[4] Correa M. Dicionário de plantas úteis do Brasil e exóticas cultivadas (Dictionary of Brasilian useful plants and exotics cultivated). Ministério da Agricultura; 1984. p. 320.
[5] Barbosa-Filho JM, Agra M. Botanical, chemical and pharmacological investigation on *Cissampelos* species from Paraiba (Brasil). Cienc Cult 1997;49:386–94.
[6] Cortes SF, Alencar JL, Thomas G, Barbosa Filho JM. Spasmolytic actions of warifteine, a bisbenzylisoquinoline alkaloid isolated from the root bark of *Cissampelos sympodialis* Eichl. Phytother Res 1995;9:579–83.
[7] Freitas MR, Cortes SF, Thomas G, Barbosa-Filho JM. Modification of Ca^{+} metabolism in the rabbit aorta as a mechanism of spasmolytic action of warifteine, a bisbenzylisoquinoline alkaloid isolated from the leaves of *Cissampelos sympodialis* Eichl. (Menisperamaceae). J Pharm Pharmacol 1996;48:332–6.
[8] Piuvezam MR, Peçanha LM, Alexander J, Thomas G. *Cissampelos sympodialis* Eichl. leaf extract increases the production of IL-10 by concanavalin-A-treated BALB/c spleen cells. J Ethnopharmacol 1999;67:93–101.
[9] Bezerra-Santos CR, Balestieri FM, Rossi-Bergmann B, Peçanha LM, Piuvezam MR. *Cissampelos sympodialis* Eichl. (Menispermaceae): oral treatment decreases IgE levels and induces a Th1-skewed cytokine production in ovalbumin-sensitized mice. J Ethnopharmacol 2004;95:191–7.
[10] Bezerra-Santos CR, Vieira-de-Abreu A, Barbosa-Filho JM, Bandeira-Melo C, Piuvezam MR, Bozza PT. Anti-allergic properties of *Cissampelos sympodialis* and its isolated alkaloid warifteine. Int Immunopharmacol 2006;6:1152–60.
[11] Cerqueira-Lima AT, Alcântara-Neves NM, Carvalho LCP, Costa RS, Barbosa-Filho, Barbosa-Filho, et al. Effects of *Cissampelos sympodialis* Eichl. and its alkaloid, warifteine, in an experimental model of respiratory allergy to *Blomia tropicalis*. Curr Drug Targets 2010;11:1458–67.
[12] Costa HF, Bezerra-Santos CR, Barbosa Filho JM, Martins MA, Piuvezam MR. Warifteine, a bisbenzylisoquinoline alkaloid, decreases immediate allergic and thermal hyperalgesic reactions in sensitized animals. Int Immunopharmacol 2008;8:519–25.
[13] Thomas G, Araujo SS, Duarte JCA, De Souza DP. Bronchodilatory activity of on aqueous fraction of the ethanolic extract of the leaves of *Cissampelos sympodialis* Eichl. (Menispermaceae). Phytomedicine 1997;4:233–8.
[14] Lloyd CM, Gonzalo JA, Nguyen T, Delaney T, Tian J, et al. Resolution of bronchial hyperresponsiveness and pulmonary inflammation is associated with IL-3 and tissue leukocyte apoptosis. J Immunol 2001;166:2033–40.
[15] Bucchioni E, Czoma Z, Allegra L, Chung KF, Barnes PJ, et al. Adenosine 5′-monophosphate increases levels of leukotrienes in breath condensate asthma. Respir Med 2004;98:651–5.
[16] Yang G, LI L, Volk A, Emmell E, Petley T, et al. Therapeutic dosing with anti-interleukin-13 monoclonal antibody inhibits asthma progression in mice. J Pharmacol Exp Ther 2005;313:8–15.
[17] Cowden JM, Riley JP, Ma JY, Thurmond RL, Dunford PJ. Histamine H4 receptor antagonism diminishes existing airway inflammation and dysfunction via modulation of Th2 cytokines. Respir Res 2010;11:1–12.
[18] Vieira-de-Abreu A, Assis EF, Gomes GS, Castro-Faria-Neto HC, Weller PF, et al. Allergic challenge-elicited lipid bodies compartmentalize *in vivo* leukotriene C4 synthesis within eosinophils. Am J Respir Cell Mol Biol 2005;33:254–61 Epub 2005 Jun 9.
[19] Rothenberg ME, Hogan SP. The eosinophil. Annu Rev Immunol 2006;24:147–74.
[20] Lee JJ, Dimina D, Macias MP, Ochkur SI, McGarry MP, et al. Defining a link with asthma in mice congenitally deficient in eosinophils. Science 2004;305:1773–6.
[21] Humbles AA, Lloyd CM, McMillan SJ, Friend DS, Xanthou G, Kharitonov SA. A critical role for eosinophils in allergic airways remodeling. Science 2004;305:1776–9.

[22] Akuthota P, Xenakis JJ, Weller PF. Eosinophils: offenders or general bystanders in allergic airway disease and pulmonary immunity? J Innate Immun 2011;3: 113–9.
[23] Curran DR, Cohn L. Advances in mucous cell metaplasia a plug for mucus as a therapeutic focus in chronic airway disease. Am J Respir Cell Mol Biol 2010;42: 268–75.
[24] Chung KF, Adcock IM. Pathophysiological mechanisms of asthma. Application of cell and molecular biology techniques. Mol Biotechnol 2001;18:213–32.
[25] Brightling CE. Eosinophils, bronchitis and asthma: pathogenesis of cough and airflow obstruction. Pulm Pharmacol Ther 2011;24(3):324–7 (Jun).
[26] Bezerra-Santos CR, Peçanha LMT, Piuvezam MR. *Cissampelos sympodialis* Eichl. (Menispermaceae) inhibits anaphylactic shock reaction in murine allergic model. Braz J Pharmacogn 2005;15:287–91.

Glossary

CS: Cissampelos sympodialis
DEX: Dexamethasone
WAR: Warifteine
OVA: Ovalbumin
AHR: Airway hyperreactivity
IL-13: Interleukin-13
Penh: Enhanced pause
BAL: Bronchoalveolar lavage
PAS: Periodic acid-Schiff
PBS: Phosphate buffered saline
Pre/Post: Pre-treatment/Post-treatment