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Efeito anti-inflamatório do extrato etanólico obtido da parte aérea do *Blutaparon portulacoides*

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Dourados - MS
2019

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Área do CNPq: Dor e inflamação

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Há pessoas que vêem as coisas como elas
são e que perguntam a si mesmas: “Porquê?
E há pessoas que sonham as coisas como
elas jamais foram e que perguntam a si
mesmas: “Por que não?”.

(George Bernand Shaw)

LISTA DE ILUSTRAÇÕES

Figura 1 - HPLC-MS chromatogram of EEBP 55

Figura 2 - Effects of oral administration of EEBP in the pleurisy induced by carrageenan 56

Figura 3 - Effects of oral administration of EEBP in the pleurisy and pleural exsudate induced by BCG 57

Figura 4 - Effect of oral administration of EEBP on the carrageenan induced paw edema And hyperalgesia mechanical in mice 58

Figura 5 - Effects of oral administration of EEBP on mechanical hyperalgesia induced by CFA 59

Figura 6 - Effects of oral administration of EEBP induced by CFA 60

Figura 7 – Effects of oral administration of EEBP on cold hyperalgesia induced by CFA 61

LISTA DE ABREVIATURAS E SÍMBOLOS

AA: Ácido Araquidônico

AIE: Anti-Inflamatório Esteroidal

AINE: Anti-Inflamatório não Esteroidal

AMPc: Adenosina monofosfato cíclico

ANOVA: Análise de Variância

ANVISA: Agência Nacional de Vigilância Sanitária

AR: Artrite reumatoide

Aδ: Fibra Nervosa do Tipo A-δ

BCG: *Mycobacterium bovis*

C: Fibra Nervosa do Tipo C

CFA: Adjuvante completo de Freund

CFU: Unidade formadora de colonia

COX: Ciclooxigenase

COX-1: Ciclooxigenase 1

COX-2: Ciclooxigenase 2

CEUA: Comitê de Ética no Uso de Animais

EDTA: Etilenodiaminotetracético

EEBP: Extrato etanólico de *Blutaparon portulacoides*

FLA_2: Fosfolipase A_2

GC: Glicocorticoide

IASP: International Association for the Study of Pain

i.a.: Intra-articular

i.p.: Intraperitoneal

IFN: Interferon

IFNγ: Interferon gama

IL: Interleucina

IL-1: Interleucina1

IL-1β: Interleucina 1β

IL-4: Interleucina 4

IL-6: Interleucina 6

IL-8: Interleucina 8

IL-10: Interleucina 10
MS: Ministério da Saúde
NF-kB: Fator Nuclear kappa B
NGF: Fator de crescimento nervoso
NMDA: Receptor N-metil D-Aspartato
NO: Óxido Nítrico
PAF: Fator de Ativação de Plaquetas
PBS: Tampão Fosfato-Salina
PG: Prostaglandina
PGE: Prostaglandina do tipo E
PMNs: Polimorfonucleares
PNPMF: Política Nacional de Plantas Medicinais e Fitoterápicos
RDC: Resolução da Diretoria Colegiada
RMN: Ressonância Magnética Nuclear
RENISUS: Relação Nacional de Plantas Medicinais de Interesse ao SUS
s.c.: Administração Via Subcutânea
SNC: Sistema Nervoso Central
STAT: Transdutores de sinal e ativadores da transcrição
SUS: Sistema Único de Saúde
TGF-β: Fator de transformação do crescimento β
TLR-1: Receptor toll-like do tipo 1
TLR-2: Receptor toll-like do tipo 2
TLR-6: Receptor toll-like do tipo 6
TNF: Fator de Necrose Tumoral
TX: Tromboxano
v.o.: Administração Via Oral
WHO: World Health Organization

Efeito anti-inflamatório do extrato etanólico obtido da partes aérea do *Blutaparon portulacoides*

RESUMO

Blutaparon portulacoides (capotiraguá)(Amaranthaceae) é encontrado nas regiões costeiras do Brasil e é usado popularmente para o tratamento de leucorréia e vulvovaginite. Ambas as doenças podem indicar a presença de condições inflamatórias e patogênicas. O objetivo deste estudo foi a caracterização química de extrato etanólico obtido de *B. portulacoides* (EEBP) e a investigação antimicrobiana, bem como seus efeitos contra o adjuvante completo de Freund (CFA) nas patas e na pleurisia induzida com carragenina. ou *Mycobacterium bovis* (BCG) de camundongos. A concentração inibitória mínima de EEBP foi determinada para microorganismos, incluindo *Mycobacterium tuberculosis*. Experimentos *in vivo* foram realizados para analisar os exsudatos pleurais onde camundongos *Swiss* receberam administração por via oral de EEBP, após 1 hora da administração dos extratos foram injectados carragenina e após 4 horas coletado exsudato na cavidade pleural. A inflamação da pata induzida por carragenina ou CFA foi tratada com EEBP previamente. O 3,5,3'-tri- hidroxi-4'-metoxi-6,7-metilenodioxiflavona, gomphrenol e ácidos ferúlico, vanílico e cafeico foram identificados usando espectrometria de massa – e ionização por eletrospray e HPLC-MS / MS. O EEBP apresentou atividade antibacteriana contra *M. tuberculosis*, *S. typhimurium* e *B. cepacia*. O EEBP apresentou efeitos anti-inflamatórios e foi capaz de inibir o aumento dos níveis de IL-1β resultantes da pleurisia induzida por BCG e carragenina. A EEBP reduziu o crescimento de *M. tuberculosis in vitro* e experimentos *ex vivo* em órgãos mostrados por ensaios de UFC. Na inflamação induzida por carragenina, a EEBP foi capaz de inibir o edema e a hiperalgesia mecânica. O tratamento persistente com EEBP inibiu a inflamação induzida por CFA. O presente estudo confirma as propriedades anti-inflamatórias e antibióticas da EEBP e esses resultados podem apresentar novas possibilidades para o desenvolvimento de novos antibióticos.

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Palavras-chave: Inflamação, infecção, Amaranthaceae, camundongos, *Mycobacterium tuberculosis*, *Blutaparon portulacoides*.

Anti-inflammatory effect of ethanolic extract from *Blutaparon portulacoides* stem

Astract

Blutaparon portulacoides (capotiraguá) is found in the coastal regions of Brazil and is used ethnopharmacologically to treat leukorrhea and vulvovaginitis. Both diseases could indicate the presence of inflammatory and pathogen conditions. The aim of this study was the chemical characterization of ethanolic extracts derived from B. portulacoides (EEBP) stems and investigation to kill microorganism, as well as its effects against carrageenan or Freund's complete adjuvant (CFA) into the paws and on pleurisy induced with carrageenan or Mycobacterium bovis (BCG) of mice. The minimum inhibitory concentration of EEBP was determined for microorganisms, including *Mycobacterium tuberculosis*. In vivo experiments were undertaken to analyze the pleural exudates; one hour before, Swiss mice were orally administered EEBP, and carrageenan or BCG was injected into the lung pleural cavity. Paw inflammation induced by carrageenan or CFA was treated with EEBP. 3,5,3'-Trihydroxy-4'-methoxy-6,7-methylenedioxy-flavone, gomphrenol, and ferulic, vanillic, and caffeic acids were identified using electrospray ionization–mass spectrometry and HPLC-MS/MS analysis. EEBP showed antibacterial activity against *M. tuberculosis*, *S. typhimurium*, and *B. cepacia*. EEBP has anti-inflammatory effects and is able to inhibit the increase in the IL-1β levels resulting from BCG- and carrageenan-induced pleurisy. EEBP reduced *M. tuberculosis* growth *in vitro* and *ex vivo* in organs shown by CFU assays. In the carrageenan-induced inflammation, EEBP was able to inhibit edema and mechanical hyperalgesia. Persistent EEBP treatment inhibited inflammation resulted from CFA-induced inflammation. The present study confirms the anti-inflammatory and antibiotic properties of EEBP and these results may present new possibilities for the development of novel antibiotic drugs.

Keywords**:** Inflammation, infeccion, Amaranthaceae, mice, *Mycobacterium tuberculosis*.

SUMÁRIO

1 INTRODUÇÃO 12

2 REVISÃO DE LITERATURA 13

2.1 Plantas medicinais 13

2.2 *Blutaparon portulacoides* 14

2.3 Resposta inflamatória 15

2.4 Modelos de inflamação 18

2.4.1 Pleurisia induzida por carragenina 18

2.4.2 Edema de pata induzido por carragenina 19

2.4.3 Pleurisia induzida por BCG 19

2.4.4 Inflamação de pata induzida por CFA 20

3 OBJETIVOS 20

4 REFERENCIAS BIBLIOGRÁFICAS 21

5 APÊNDICES 32

5.1 Artigo: ANALYSIS OF THE EFFECTS OF *Blutaparon portulacoides* ETHANOLIC EXTRACT ON THE INFLAMMATORY REACTION INDUCED BY THE *Mycobacterium complex* and CARRAGEENAN IN MICE. 33

6 ANEXOS 62

6.1 Aprovação do Comitê de ética no uso de animais (CEUA) 63

1 INTRODUÇÃO

As substâncias naturais oriundas de plantas medicinais, microorganismos e venenos têm atraído a atenção de pesquisadores de diversas áreas. Os produtos que mais se destacam são aqueles derivados do chamado “metabolismo secundário” nas plantas medicinais, por sua importância terapêutica ou por sua toxicidade (SIMÕES et al., 2004). O uso de terapia herbal ou medicina alternativa constitui uma importante abordagem para o tratamento de várias doenças inflamatórias.

A dor e a inflamação estão implicadas em praticamente todas as doenças humanas e animais e geralmente são produzidas por estímulos agressores físicos, químicos e biológicos, ou alguma combinação destes (NATHAN, 2008). As características típicas da inflamação são rubor, edema, calor, dor e perda da função (que pode ou não estar presente). Portanto, sempre há interações entre dor e inflamação.

Os analgésicos são medicamentos que podem aliviar a sensação de dor, mas o uso prolongado de analgésicos convencionais como opioides e anti-inflamatórios não esteroidais podem causar inúmeros efeitos adversos durante o uso da clínica (CAMPOS e GONZÁLEZ-et al., 2005). Do mesmo modo medicamentos como os glicocorticóides, os anti-inflamatórios esteroidais (AIEs) e antiinflamatórios não esteroidais (AINES) são a terapia primária para doenças com resposta inflamatória crônica, mas o uso prolongado destes também pode causar efeitos adversos graves, incluindo complicações cardiovasculares e gastrointestinais, que limitam o uso desses fármacos (ORLANDO e GONZALES, 2010; BURKE et al, 2006) Portanto, a pesquisa para novos agentes analgésicos e anti-inflamatórios é necessária.

Entre os vários distúrbios ginecológicos, a leucorrea, doenças inflamatórias pélvicas (PID) e sangramento uterino disfuncional (DUB) são condições muito comuns. Apesar da diferença patofisiológica dessas doenças, todas respondem a drogas antiinflamatórias. A vulvovaginite é geralmente considerada como problema ginecológico mais comum em meninas premenarcais (Tewiri e Neelam, 2001). Nesse contexto, existem poucos trabalhos científicos relatando a atividade anti-inflamatória ou analgésica da *Blutaparon portulacoides* apesar do uso popular ser para a leucorréia (corrimento vaginal).

2 REVISÃO DE LITERATURA

2.1 Plantas medicinais

As plantas medicinais são importantes para a pesquisa e desenvolvimento de novos medicamentos, muitos constituintes das plantas são usados diretamente como agentes terapêuticos, mas também como material de estudo para a síntese de novas moléculas (WHO, 1998). Cerca de 50% dos medicamentos aprovados durante os últimos 30 anos foram obtidos a partir de produtos naturais (NEWMAN E CRAGG, 2012).

O Brasil possui grande diversidade vegetal, com mais de 55.000 espécies catalogadas, sendo que apenas uma pequena parte já foi estudada quanto aos seus constituintes químicos e efeitos biológicos. Além disso, a população brasileira destaca-se pelo uso de plantas medicinais para tratamentos de enfermidades e esse conhecimento popular é de grande importância para a pesquisa científica, **p**ossibilitando unir o conhecimento popular e científico em pesquisas experimentais e clínicas, para comprovar a atividade biológica das plantas utilizadas na medicina popular e desvendar seus constituintes químicos e garantir a segurança de seu uso (MATIAS et al., 2013).

A utilização de plantas medicinais e fitoterápicos nunca deixou de acontecer em nosso pais, principalmente pela sua importância cultural, social e econômica. Boa parte das regiões brasileiras dispunham apenas da medicina popular como forma terapêutica no tratamento de doenças, situação que ainda persiste em algumas regiões, tanto pela falta de acesso ou escassez de medicamentos, como pela livre escolha de recursos terapêuticos (RIBEIRO, 2015).

Durante anos, importantes práticas de valorização da utilização de plantas medicinais foram desenvolvidas a nível mundial. No Brasil, a partir da década de 1990, houve um aumento progressivo de projetos de incorporação do uso de fitoterápicos e plantas medicinais no SUS (Sistema Único de Saúde), mas só em 2006 o Ministério da Saúde (MS) determinou a criação da Política nacional de Plantas Medicinais e Fitoterápicos (PNPMF) (Brasil, 2006). Após isso, foi instituída a Portaria Interministerial nº2.960, de 9 de dezembro de 2008, criando o Programa Nacional de Plantas Medicinais e Fitoterápicos e o Comitê Nacional de Plantas Medicinais e Fitoterápicos, objetivando "garantir à população brasileira o acesso seguro e uso racional de plantas medicinais e fitoterápicos, promovendo o uso sustentável da biodiversidade, o desenvolvimento da cadeia produtiva e da indústria nacional" (BRASIL, 2008).

No ano de 2009, foi lançada pelo Departamento de Assistência Farmacêutica a Relação Nacional de Plantas Medicinais de Interesse ao SUS (Renisus) contendo uma lista de 71

espécies vegetais, com o objetivo de priorizar recursos e pesquisas para o desenvolvimento de medicamentos fitoterápicos e uso no Sistema Único de Saúde (SUS).

A ANVISA (Agência Nacional de Vigilância Sanitária), através da Resolução da Diretoria Colegiada nº 26 (RDC 26/14), atualizou as normas para registro de fitoterápicos, contemplando testes clínicos padronizados, criando o registro e notificação da categoriade produtos tradicionais fitoterápicos. A partir de então, é permitido o registro de fitoterápicos que apresentem eficácia e segurança reconhecidos através do uso tradicional por um período determinado pela agência em 30 anos, mesmo não sendo objetos de estudos experimentais e clínicos (RIBEIRO, 2015).

Sendo assim, passam a ser reconhecidos pela Anvisa somente produtos fitoterápicos industrializados, não se aplicando o registro a formas tradicionais de terapia medicinal tradicional como "garrafadas, xaropes, misturas artesanais feitas por populações tradicionais (curandeiros, indígenas, quilombolas, ribeirinhos, entre outros).

2.2 *Blutaparon portulacoides e* família *Amaranthaceae*

A espécie *Blutaparon portulacoides*, conhecida popularmente como "capotiraguá" pertence à família Amaranthaceae. A família Amaranthaceae foi estabelecida por A. L. Jussieu em 1789 e está inserida na classe Magnoliopsida, ordem Caryophyllates. Essa família é composta de aproximadamente 1000 espécies e 65 gêneros dos quais 14 ocorrem no Brasil. Está dividida em quatro tribos: Celosieae, Amarantheae, Braylineae e Gomphreneae (SIQUEIRA e GUIMARÃES, 1984; SIQUEIRA, 1987). Muitas plantas dessa família têm sido empregadas na medicina tradicional para o tratamento de muitas doenças e como alimento, por seu valor nutritivo (SOUZA et al., 1998; GORINSTEIN et al., 1991; MACEDO et al., 1999; PATTERSON et al., 1991). A família Amaranthaceae tem importância econômica direta e indireta, no Brasil e no Mundo. Pertencem à família as espécies medicinais conhecidas como ginseng-brasileiro (*Hebanthe eriantha* (Poir.) Pedersen e *Pfaffia glomerata* (Spreng.) Pedersen), além de diversas outras citadas em levantamentos etnobotânicos e listas de espécies úteis e prioritárias para estudos (BARROS, 1981/1982; SIQUEIRA, 1981, 1987 e 1988; ALMEIDA et al., 1998; VIEIRA et al., 2002; AGRA et al., 2007). As espécies alimentícias beterraba (*Beta vulgaris*) e espinafre (*Spinacia oleracea*), as espécies ornamentais conhecidas como perpétua (*Gomphrena globosa* L.), crista-de-galo (*Celosia argentea*) e periquito (*Alternanthera tenella*), entre outras, também são membros da família Amaranthaceae. Há,

ainda, espécies não cultivadas de potencial ornamental inexplorado, como *G. arborescens* (ALMEIDA et al., 1998; FANK-DE-CARVALHO et al., 2009).

A *Blutaporon portulacoides* é uma planta de crescimento anual e perene, é encontrada principalmente em regiões temperadas, subtropicais e tropicais. No Brasil, ocorre principalmente na Mata Atlântica, restinga, nos cordões arenosos do litoral brasileiro (SIQUEIRA e GUIMARÃES, 1984; SIQUEIRA, 1987).

Foram isolados e identificados compostos com atividade biológica do extrato bruto etanólico preparado a partir das partes aéreas e raízes da *B. portulacoides.* Esses componentes apresentaram atividade antitripanicida e leishmanicida, *in vitro*, e também atividade antimicrobiana. Além disso, outros flavonoides como a Irisona B e Bisflavonoide, derivado do metiledioxiflavol também foram isolados desta espécie vegetal. (SALVADOR et al., 2002).

2.3 Resposta inflamatória

A resposta inflamatória pode estar envolvida em praticamente todas as doenças e também nas respostas de defesa do organismo. Os estímulos agressores endógenos e exógenos (LEE et al., 2017; JOHNSON et al., 2018) podem induzir a ativação desta resposta sendo que o término do processo inflamatório é realizada com mecanismos de resolução ativa da inflamação (BUCKLEY *et al.*, 2014, LESLIE, 2015).

Os agentes agressores exógenos podem ser agentes físicos; agentes químicos; agentes infecciosos (bactérias, fungos, parasitas); podem ser também agentes flogísticos, ou seja agente pró-inflamatório (carragenina ou zymosan, por exemplo são muito utilizados experimentalmente) dentre outros. Além dos estímulos exógenos, os estímulos endógenos liberados por trauma mecânico, isquemia, estresse (DAMPs (padrões moleculares associados a danos)) podem causar inflamação e ativação da resposta imune. Tanto os DAMPs (padrões moleculares associados a danos) como PAMPs (padrões moleculares associados a patógenos) estimulam os receptores de reconhecimento de padrões (PRRs) (WEST e SHADEL, 2017). Para o processo inflamatório e/ou imune ser ativado, o organismo dispõe de receptores como receptor do tipo Toll (TLRs), receptores tipo NOD (proteínas com domínio de ligação *a* nucleotídeos e oligomerização*).*

Vários mediadores inflamatórios como serotonina, bradicinina, histamina, prostaglandinas produzidas a partir das ciclooxigenases -1 e -2 (COX-1 e COX-2), leucotrienos produzidos a partir da lipoxigenase (LOX), citocinas, quimiocinas entre outros são constantemente produzidos a partir da ativação do processo inflamatório. Por exemplo, após

uma lesão a célula libera o DNA mitocondrial (mtDNA) que pode ser reconhecido por receptores celulares de leucócitos e ativar o TLR9, consequentemente a MYD88 e depois o fator nuclear Kappa B (NFκB) (WEST e SHADEL, 2017). O NFκB é um fator transcripcional fundamental para produção de mediadores inflamatórios como citocinas e ativação de ciclooxigenase-2 (COX-2) que por sua vez ativam outros processos para aumentos da liberação de mediadores inflamatórios. Vários fatores de transcrição podem ser constantemente ativados em doenças inflamatórias como ocorre na colite ulcerativa a modulação de NFκB, família dos transdutores de sinal e ativadores da transcrição (STAT) e proteína AP1 (JIN *et al.*, 2017).

Eventos vasculares e celulares, mediadores derivados de células e da ativação plasmática contribuem para o desenvolvimento dos sinais clássicos da inflamação (dor, rubor, calor, edema e/ou perda da função). As alterações vasculares (vasodilatação, aumento do fluxo sanguíneo, aumento da permeabilidade vascular e exsudação de plasma) iniciam-se imediatamente e desenvolvem-se durante as primeiras horas após o estímulo inflamatório (WILLIAMS *et al.,* 1983). A microcirculação torna-se permeável a macromoléculas e fluídos vindos do sangue, causando edema tecidual (GILROY *et al.,* 2004). O evento celular principal é migração leucocitária dependente da expressão das moléculas de adesão nos leucócitos e células endoteliais além mediadores quimiotáticos (SPRINGER, 1994; WEBER, 2003) que contribuem para fagocitose (macrófagos/monócitos), clearance e resolução (BUCKLEY *et al.,* 2014). A mobilização adequada dos leucócitos circulantes para o sítio inflamado é fundamental para a defesa do organismo, já que estas células podem desenvolver suas ações de fagocitose e destruição de agentes patogênicos levando à resolução do processo. No processo inflamatório agudo, há acúmulo predominante de neutrófilos no tecido lesado, enquanto que os mononucleares são a maioria em processos crônicos. Algumas das células envolvidas já estão presentes no tecido afetado tais como: células endoteliais, células mesoteliais, mastócitos, eosinófilos, macrófagos e alguns linfócitos (BOYTON e OPENSHAW, 2002).

Os mecanismos de resolução ativa da inflamação envolvem neutrófilos, macrófagos, células endoteliais, neurônios e principalmente moléculas chamadas de pró-resolutoras do processo inflamatório como resolvinas, protectinas, lipoxinas, maresinas, (BUCKLEY *et al.,* 2014, LESLIE, 2015). Em modelos experimentais de asma, aterosclerose, Alzheimer essas moléculas bloquearam ou reduziram os parâmetros inflamatórios (LESLIE, 2015). A resolução da doença com inflamação crônica não depende somente da cessação do estímulo pro-inflamatório. A restauração da homeostasia é alcançada através da indução ativa imunossupressora das células T auxiliares, macrófagos e células residentes que pode ser

induzida pela resolvina, outros lipídeos e citocinas anti-inflamatórias como f*ator* de transformação do crescimento (TGF)-β, IL-10 entre outras (SERHAN, 2014).

Semelhante a inflamação, vários mediadores produzidos por nosso organismo induzem dor. A dor tem sido identificada com a maior prioridade no tratamento da artrite reumatoide e de diversas doenças (como espondilite anquilosante, dor nas costas, osteoartrite)(LEE *et al.,* 2011; TUBACH *et al.,* 2012). A dor é uma experiência sensorial e emocional que é substancialmente modulada por fatores psicológicos, sociais e contextuais (CARLINO *et al.,* 2014). Em termos de duração, a dor pode ser aguda ou crônica. A dor aguda está associada com uma lesão tecidual recente e ativação de nociceptores, podendo desaparecer até mesmo antes da cura do dano tecidual. Por outro lado, a dor crônica pode se perpetuar por meses ou anos e se caracteriza em relação à persistência de alterações, o que muitas vezes dificulta o tratamento (PARK e VASKO, 2005). A dor crônica por outro lado é uma doença neurológica comum que envolve adaptações duradouras, complexas, variando a modulação de epigenéticas não elucidadas e se distingue da dor aguda pelo aparecimento de alterações sinápticas pouco adaptadas e celulares em longo prazo, que envolvem processos de memória dinâmica e causam distúrbios emocionais característicos incluindo depressão, estresse e ansiedade (ZHANG *et al.,* 2011). A dor crônica está associada pela sensibilização central que é um estado de hiperresponsividade das vias dolorosas espinhais que envolvem dentre outros sítios a sinalização via receptor NMDA (LEWIS, 2013a). Esta dor caracteriza-se por dor evocada por estímulos normalmente inócuos (alodinia) ou por uma sensibilidade exacerbada a estímulos nocivos ou não nocivos (hiperalgesia)(KUNER, 2010). O termo hipernocicepção refere-se à sensibilização dos nociceptores, isto é, a diminuição do seu limiar de ativação, e tem sido empregado para designar alodinia ou hiperalgesia em animais de experimentação (CUNHA *et al.,* 2004).

A transmissão da dor aguda envolve uma interação complexa de estruturas centrais e periféricas desde a pele, vísceras ou outros tecidos até o córtex cerebral. Vários estímulos nocivos ativam as terminações nervosas livres e periféricas de fibras aferentes sensoriais delgadas do tipo C e Aδ, chamadas de nociceptores (JULIUS e BASBAUMN, 2001). Estas fibras são formadas por neurônios cujos corpos celulares encontram-se nos gânglios da raiz dorsal (DRG). Imediatamente, um reflexo de retirada mediado pela medula espinhal é desencadeado no intuito de remover a região do corpo ameaçada (WATKINS e MAIER, 2002). A dor crônica é um grande desafio para a prática clínica e ciência básica. As redes neurais periféricas e centrais que medeiam a nocicepção mostram extensa plasticidade nos estados de doença patológica. A plasticidade induzida por doença pode ocorrer em nível estrutural e

funcional e se manifesta como mudanças em moléculas individuais, sinapses e função celular (KUNER, 2010).

2.4. Modelo de inflamação

2.4.1 Pleurisia Induzida por Carragenina

O modelo de pleurisia induzida por carragenina é caracterizado por apresentar uma resposta inflamatória bifásica (4 e 48 h) após injeção intrapleural do agente flogístico, com pico máximo de infiltração leucocitária e formação de edema na quarta hora (COELHO, 2009).

TRACEY et al. (1995), demonstraram o envolvimento do óxido nítrico no processo inflamatório desencadeado neste modelo experimental, foi observado a atividade do óxido nítrico-sintase sobre as células inflamatórias na cavidade pleural. A importância do óxido nítrico no processo inflamatório também foi demostrada em outros modelos de inflamação por carragenina, como no caso do edema de pata (LALENTI et al., 1992).

Na primeira fase (4 h) também ocorre aumento dos níveis de mieloperoxidade, correlacionada com o aumento do número de leucócitos no exsudato inflamatório e (KRAWISZ et al., 1984), ocorre também uma resposta inflamatória mediada por bradicinina, que por meio de seu receptor B2 modula a resposta inflamatória, analgésica e o extravasamento plasmático (HALL, 1992; MARCEAU, 1995) além de aumentar a liberação de mieloperoxidase. Outro mediador envolvido no processo inflamatório na pleurisia induzida pela carragenina é a histamina, importante no processo de aumento da permeabilidade vascular, prostanóides, IL-1, IL-8 e TNF (FRODE, 2000).

2.4.2 Edema de pata induzido por carragenina

Dentre os modelos experimentais de inflamação *in vivo*, os induzidos por carragenina são utilizados com frequência. A carragenina é um polissacarídeo sulfatado, proveniente de algas vermelhas, principalmente da alga *Chondrus crispus*, encontrada em Carragheen (WATERFORD, Irlanda) (DI ROSA et al., 1972).

De modo particular, o edema de pata induzido por carragenina destaca-se pela possibilidade de mensurar o papel de diferentes mediadores presentes durante o processo inflamatório. O modelo experimental foi proposto por WINTER et al., (1962), utilizando-se

apenas ratos, porém LEVY (1969) demonstrou que o modelo poderia ser realizado também em camundongos.

Neste modelo, é possível avaliar não apenas o processo inflamatório, onde ocorre infiltração local de neutrófilos, aumentando o tamanho da pata (edema), mas também a nocicepção, visto que ocorre a sensibilização de nociceptores, que podem ser avaliados quando estimulados de forma mecânica ou térmica (ZHANG et al., 1997; PINHEIRO E CALIXTO 2002).

O modelo caracteriza-se pela liberação de histamina, serotonina e bradicinina na primeira fase (1-2 h, após indução com carragenina), e uma elevada produção de prostaglandinas e NO na segunda fase (3-6 h) (DI ROSA et al., 1971; OGONOWSKI et al., 1997). A formação do edema é oriundo do aumento da permeabilidade vascular, pela interação de substâncias como a histamina e a bradicinina por exemplo, com mediadores como as prostaglandinas E2, que causam principalmente vasodilatação, que por si só, aumentam a pressão de perfusão e o aporte sanguíneo nas vênulas da região inflamada (LAPA et al., 2007).

2.4.3 Pleurizia induzida por BCG

A inflamação exacerbada contribui para a patologia do *Micobacterium tuberculosis* (TB). A intensa migração de neutrófilos para o pulmão tem sido associada ao dano tecidual, aumento da carga bacteriana e redução na sobrevida de animais em modelos experimentais de TB. Em adição, eventos inflamatórios induzidos pela micobactéria, como a formação de corpúsculos lipídicos que contribuem para a persistência do patógeno no hospedeiro. Estes fatores evidenciam a necessidade de modular a resposta inflamatória durante a infecção micobacteriana.

A terapia direcionada ao hospedeiro para TB objetiva modular as vias inflamatórias a fim de reduzir a inflamação exacerbada e o dano tecidual; e também, contribuir com os mecanismos da resposta imune inata e adaptativa, enquanto os antibióticos mata a micobactéria. Neste contexto, estudos com abordagem farmacológica que visam investigar os efeitos de substâncias com potencial imunomodulador no processo inflamatório causado por micobactérias representam uma oportunidade de descoberta de moléculas que podem contribuir com a terapia convencional da TB.

2.4.4 Inflamação de pata induzido por CFA

Pesquisas realizadas em humanos são difíceis de serem controladas, seja por questões éticas, pela interferência dos hábitos diários na resposta individual observada, ou pela dificuldade de realizar testes repetidos, os quais se tornam desgastantes (GOMES et al., 2014). Modelos animais que apresentam semelhança com a fisiopatologia da doença humana são fundamentais para aprofundar o conhecimento sobre diversas doenças, possibilitando a descoberta de novos tratamentos através de diferentes modelos (CRYAN et al., 2002).

Diversos modelos animais são utilizados para avaliação de propriedades antinociceptivos e mecanismos de ação de novos fármacos analgésicos e antiinflamatórios (AOKI et al., 2014), dentre eles um dos mais frequentemente utilizados para mimetizar a dor causada por um processo inflamatório crônico é o modelo de artrite induzida por adjuvante completo de Freund (CFA, do inglês Complete Freund´s adjuvant) (SANTOS et al., 2013). O CFA é um agente indutor de inflamação crônica que é capaz de ativar a resposta imunológica do organismo, levando a produção e liberação de citocinas e mediadores inflamatórios (SILVA, 2009; AOKI et al., 2014).

Por ser um modelo já bem conhecido para avaliar atividades antinociceptivas de novas drogas, e por já ter sido caracterizado como um modelo de comportamento tipo depressivo em roedores, o modelo de dor crônica induzido por CFA pode ser útil para avaliar efeitos de novas drogas que possam estar associadas com a dor crônica e depressão.

3 OBJETIVOS

GERAL

O objetivo geral do trabalho foi avaliar o efeito anti-inflamatório do extrato etanólico das partes aéreas de *Blutaparon portulacoides*.

ESPECÍFICOS

Realizar a análise fitoquímica das frações do extrato etanólico de *Blutaparon portulacoides*

Determinar a atividade anti-inflamatória em modelo de pleurisia induzida por carragenina;

Avaliar o processo inflamatório induzido por edema de pata induzido por carragenina.

Avaliar a atividade inflamatória induzido por BCG.

Avaliar a atividade antinociceptiva e anti-inflamatória através do teste de CFA.

Avaliar atividade antimicobacteriana.rônico.

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5 APÊNDICES

5.1 Artigo: Revista Journal of Ethnopharmacology (Qualis A2 – Medicina II)

Link com as normas da revista

https://www.elsevier.com/journals/journal-of-ethnopharmacology/0378-8741/guide-for-authors

ANALYSIS OF THE EFFECTS OF *Blutaparon portulacoides* ETHANOLIC EXTRACT ON THE INFLAMMATORY REACTION INDUCED BY THE *Mycobacterium complex* and CARRAGEENAN IN MICE

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Abbreviations:

EEBP, ethanolic extracts derived from *B. portulacoides*; DEX, dexamethasone; BCG, *Mycobacterium bovis*; CFA, Freund's complete adjuvant; CFU, Colony Forming Unity;

RENISUS, List of Medicinal Plants of Interest to the Unified Health System; MIC, minimum inhibitory concentration.

Abstract

Ethnopharmacological relevance: Blutaparon portulacoides, also known as capotiraguá, is found in the coastal regions of Brazil and is used ethnopharmacologically to treat leukorrhea and vulvovaginitis. Both diseases could indicate the presence of several pathological conditions that involve pathogen infection, leukocyte migration, and other inflammatory conditions.

Aim of study: The aim of this study was the chemical characterization of the ethanolic extracts derived from *B. portulacoides* (EEBP) aerial parts and investigation to kill microorganism, as well as its effects against carrageenan or Freund's complete adjuvant (CFA) into the paws and on pleurisy induced with carrageenan or *Mycobacterium bovis* (BCG) of mice.

Materials and methods: The minimum inhibitory concentration of EEBP was determined for microorganisms, including *Mycobacterium tuberculosis*. *In vivo* experiments were undertaken to analyze the pleural exudates; one hour before, *Swiss* mice and C57BL-6 were orally administered EEBP, and carrageenan or BCG was injected into the lung pleural cavity. Paw inflammation induced by carrageenan or CFA was treated with EEBP.

Results: 3,5,3'-Trihydroxy-4'-methoxy-6,7-methylenedioxy-flavone, gomphrenol, ferulic, vanillic, and caffeic acids were identified using electrospray ionization–mass spectrometry and HPLC-MS/MS analysis. EEBP showed antibacterial activity against *M. tuberculosis*, *S. typhimurium*, and *B. cepacia*. EEBP has anti-inflammatory effects and is able to inhibit the increase in the IL-1β levels resulting from BCG- and carrageenan-induced pleurisy. EEBP reduced *M. tuberculosis* growth *in vitro* and *ex vivo* in organs shown by CFU assays. In the

carrageenan-induced inflammation, EEBP was able to inhibit edema and mechanical hyperalgesia. Persistent EEBP treatment inhibited inflammation resulted from CFA-induced inflammation.

Conclusions: The present study confirms the anti-inflammatory and antibiotic properties of EEBP and these results may present new possibilities for the development of novel antibiotic drugs.

Key-words: *Blutaparon portulacoides*; inflammation; *Mycobacterium tuberculosis*; Amaranthaceae

Author contributions

VKN, and MJS, preparation and phytochemical analysis of the extract; CALK, JC, ACR, designed the study; RMK, JASR, RJO, LFM, and PRTL performed the anti-inflammatory assays. All authors participated in the design, interpretation, and analysis of the data and approved the final manuscript.

1. INTRODUCTION

Since time immemorial, medicinal plants have been widely investigated to validate their health benefits or to develop new products during drug discovery. Natural products are often secondary metabolites that are extracted from living organisms, such as plants, microbes, and animals; many of these medicinal compounds were first investigated due to their use in folk medicine (Mathur and Hoskins, 2017).

The Brazilian National List of Medicinal Plants of Interest to the Unified Health System (RENISUS) was developed in 2009 with the main goal of making herbal remedies derived from plants more widely available (Brasil, 2009; *Marmitt et al., 2016)*. Therefore, the investigation of the biological properties of plants used in folk medicine is important not only for drug discovery but also for the public health care system.

Mammals respond with inflammation and pain upon infection by pathogens or physical injury as a result of endogenous or exogenous stimuli, and a goal of treatment is to control cellular stress and the inflammatory process (Xiahou, 2017). Almost all of the medications currently used to treat chronic inflammation, including nonsteroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids, have a number of adverse side effects. Glucocorticoid drugs are an extraordinarily potent class of medications that are widely used to control inflammation in humans and animals; however, no glucocorticoid analogs have been developed that do not cause adverse effects when used for long periods of time (Cain and Cidlowski, 2017). Therefore, the investigation of novel anti-inflammatory and antibiotic drugs derived from folk medicine continues to be an important endeavor.

Blutaparon portulacoides, also known as “capotiraguá,” “pirrixiu” or “bredo-de-praia” (Siqueira, 1987; Bertier, et al., 2008), is found in coastal areas in northern and southern Brazil (Pio, et al., 2018). *B. portulacoides* is commonly used in folk medicine to treat *leukorrhea and v*ulvovaginitis. Methylenedioxyflavonol and a mixture of acyl steryl glycosides have been

identified in and isolated from extracts of *B. portulacoides* (Ferreira and Dias, 2000), and they were assayed for their activity against *Trypanosoma cruzi*, *Leishmania amazonensis*, gram-positive and gram-negative bacteria, and yeasts (Salvador, et al., 2002). The ethanolic extract from the aerial parts of *B. portulacoides* was also tested for its activity against the venom of the snake *Bothrops jararacussu,* and it was shown to inhibit edema and leukocyte influx resulting from exposure to the venom (Pereira, et al., 2009).

Leukorrhea and *v*ulvovaginitis could be indicative of the presence of several pathological conditions that involve infection, leukocyte migration, and inflammation. Our groups studied the ability of an extract from *B. portulacoides* to kill several different types of microorganisms (Salvador et al., 2002), and investigated the use of a crude ethanolic extract from *B. portulacoides* (EEBP) stems in the treatment of infections caused by *Mycobacterium sp.* and the prevention of leukocyte migration, inflammation, and pain. In the present study, a model of inflammation was developed based on the investigation of the biological actions of EEBP *in vivo*.

2. MATERIAL AND METHODS

2.1 Plant material, extraction and phytochemical analysis *Blutaparon portulacoides* (St. Hil) Mears was collected at Restinga de Marica, Rio de Janeiro (RJ, Brazil, in January 2015), and identified by Prof. J.C. de Siqueira (Pontifical Catholic University of Rio de Janeiro). A voucher specimen (SPFR-2962) was deposited in the herbarium of the Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo (FFCLRP/USP). The material vegetal (1800g) was dried at 40 °C for 72 hours, powdered and exhaustively extracted (maceration, at room temperature), with hexane and ethanol, successively, in the proportion powder mass plant/solvent 1:2 (w/v). The spent biomass was filtered, and the solvents were

removed in a rotatory evaporator under reduced pressure and temperature below 40ºC, yielding 4 g of hexanic extract and 88 g of ethanolic extract (EEBP). The chemical composition of EEBP was analyzed by ESI–MS and HPLC-MS. For ESI-MS fingerprints the extract (1 mg/mL) was diluted in a solution containing 50% (v/v) chromatographic grade methanol, 50% (v/v) deionized water, and 0.5% of ammonium hydroxide (Merck, Darmstadt, Germany). The fingerprinting ESI-MS analyses were performed according to De Santana Aquino et al. (2015), using UPLC-MS equipment, model ACQUITY TQD (Waters Corporation, Milford, MA, USA). The general conditions were: a source temperature of 100ºC, capillary voltage of 3.0 kV, and cone voltage of 30 V. ESI-MS was performed by direct infusion using a syringe pump, with a flow rate of 10 µL. min/mL. Structural analysis of single ions in the mass spectra from the extract was performed by ESI-MS/MS in negative ion mode. The ion with the *m/z* of interest was selected and submitted to 15–45 eV collisions with argon in the collision quadrupole. The collision gas pressure was optimized to produce extensive fragmentation of the ion under investigation. The compounds were identified by comparing their ESI-MS/MS fragmentation spectra to fragmentation spectra of authentic standard samples and literature data (Salvador et al., 2002; Ferreira and Dias, 2000). The HPLC-MS chromatographic analysis were conducted using an C-18 column (Kinetex, 1.7 µ, 100A, 50 x 2.1 mm). The mobile phase consisted of a linear gradient combining solvent A (water/formic acid, 99:1, v/v) and solvent B (methanol) as follows: 5-100% A (1-9 min), 100% A (9.1-10 min), and 5% A (10:1 min). The analyses were carried out in triplicate at a flow rate of 0.2 mL/min with the MS detector (ESI source) and an injection volume of 5 µL. The samples (1 mg/mL) were dissolved in methanol/water (1:1, v/v) and analyzed using the same chromatographic conditions as those for the standards. Identification of the chromatographic peak was based on the retention times of the single compound and confirmed by co-injection with an authentic standard, comparing their ESI-

MS/MS fragmentation spectra to fragmentation spectra of authentic standard samples and literature data (Ferreira and Dias, 2000; Salvador, et al., 2002; Salvador et al., 2012).

2.2 *In vitro* antibacterial Activity

The MIC values for EEBP (0.98 – 250 µg/ml) and isoniazid (0.004 – 1 µg/ml) in the presence of the *M. tuberculosis* strain H37Rv (ATCC27294) were determined according to the method described in Palomino, et al.,(2002).

MIC values were determined in the presence of *Enterobacter aerogenes* (ATCC13048), *Salmonella typhimurium* (ATCC14028), *Staphylococcus saprophyticus* (ATCC15305), *and Burkholderia cepacia* (ATCC25416). Each bacterial strain was incubated in brain-heart infusion broth at 37°C for 24 h. Subsequently, the cultures were seeded on Mueller-Hinton agar plates and incubated for 24 h. Each strain was then standardized in a solution of sterile saline (0.9%) according to the McFarland turbidity scale and diluted 1:10. Then, 10 µL was pipetted into a microplate well containing a variable concentration of EEBP in 100 µL and incubated at 37°C for 24 h, and the optical density was measured at 580 nm (NCCLS, 2002; Campos et al., 2014).

2.3. Animals

Female and male *Swiss* and C57BL/6 mice weighing 20 – 30 g were provided by the central vivarium at the Federal University of Grande Dourados (UFGD). The mice were kept in polypropylene boxes in a room at the UFGD vivarium that was maintained at 22°C in the presence of a 12 hour light/dark cycle, with food and water provided *ad libitum.* Approval of the animal protocol was granted by the UFGD ethics committee (32/2015).

2.4 Carrageenan-induced pleurisy

One hour prior to the administration of carrageenan, 42 female *Swiss* mice were divided into eight groups of six; the first group received vehicle (saline 0.9%) orally (p.o.) (control group), while the second, third, fourth and fifth groups received EEBP p.o. at a dose of 30, 100, 300, and 1000 mg/kg, respectively. The naïve group received 0.9% saline p.o. but did not receive carrageenan via intrathoracic (i.t) injection, while another group received dexamethasone (1 mg/kg) via s.c. Next, 100 µL of either 1% carrageenan or sterile saline (naïve mice only) (Vinegar et al., 1973) was injected i.t.; 4 h later, the animals were euthanized, and the thorax of each was opened in order to determine the total leukocyte number and the level of protein in the pleural exudate.

2.5 BCG-induced pleurisy

One hour prior to the induction of pleurisy, groups of six female C57BL/6 mice were treated orally with either EEBP (30 or 100 mg/kg), isoniazid (25 mg/kg) or saline solution (0.9%; control group). Pleurisy was induced with 0.1 ml of a suspension of BCG (4 X 10^5 CFU; Fundação Ataulpho de Paiva, Rio de Janeiro-Brazil) that was injected into the right pleural cavity. The animals were treated once daily for 7 days. The animals were then euthanized via an overdose of sodium thiopental, and the pleural cavity was washed with 1 ml of sterile phosphate buffered saline. A 50 µl aliquot of the wash buffer was diluted with Evans blue to determine the total number of leukocytes. The sample was then centrifuged, and the supernatant was stored for the later determination of the cytokine levels (including Il-1β) using ELISA. The precipitate was resuspended in 0.5 mL of sterile ultrapure water and 0.1 mL of Ogawa Kudu, and 0.1 mL of the suspension was plated onto 7H11 agar. The spleen and liver were macerated with 1 mL of sterile saline, and 0.1 mL of the suspension was plated on 7H11 agar. The cells were cultured for 60 days at 37°C in 5% CO2.

2.6 Carrageenan Paw inflammation model

EEBP was given orally to fifteen male *Swiss* mice at a dose of 30, 100, or 300 mg/kg 60 minutes prior to the administration of carrageenan (300 μg /paw) subcutaneously in the right paw. The negative control group was administered a 0.9% saline solution p.o., while the positive control group was treated with subcutaneous (s.c.) dexamethasone (1 mg/kg). The left paw of each of the mice was injected with 100 μL of 0.9% saline. The measurement of edema was made with a plethysmometer (Panlab) at 1, 2, and 4 h after the injection of carrageenan, and the mechanical hyperalgesia (Vivancos, et al., 2004) and cold sensitivity (Decosterd and Woolf, 2000) were assessed at 3 and 4 h after carrageenan injection using a digital analgesimeter (Insight) and an acetone test, respectively.

2.7 CFA test

The experiments were conducted over a 22 day period using 24 male C57BL/6 mice that received 30 μl of Freund's complete adjuvant (CFA) in an oil suspension containing inactive *Mycobacterium tuberculosis* via intraplantar injection into the right paw. The mice were divided into a control group (saline 0.9%, p.o.), EEBP treatment groups (30 and 100 mg/kg, p.o.), and a positive control group (dexamethasone 1 mg/kg, s.c.) every day, once a day. Mechanical sensitivity, heat and cold sensitivity and paw edema were measured 6, 11, 16 and 22 days after the injection of FCA.

2.8 Statistical analyses

The data are presented as the mean ± standard error (SEM). The determination of significant differences among 3 or more groups was made via an analysis of variance (ANOVA) followed by Dunnett's test or the Newman-Keuls test (GraphPad Prism Software). Differences were considered to be significant when $P < 0.05$.

3. RESULTS

3.1 Phytochemical analysis

The ESI-MS fingerprints of the EEBP in the negative mode showed characteristic distributions of the flavonoids, which were identified by comparison of their ESI–MS/MS fragmentation spectra with data found in the literature (Ferreira and Dias, 2000). These results were confirmed by HPLC-MS analysis using isolated standards, and the structures were confirmed by co-injection of standards and identified by retention values. The acids ferulic, vanillic and caffeic and flavonoids (aglycones and glycosides) were identified in EEBP (Figure 1). The flavonoid 3,5,3'-Trihydroxy-4'-methoxy-6,7-methylenedioxy-flavone was isolated previously from ethanolic extract from aerial parts of the *B. portulacoides* and gomphrenol from Amaranthaceae species (Ferreira and Dias, 2000; Salvador, et al., 2012). The ESI-MS fingerprints technique and HPLC-MS/MS analysis were used to characterize the chemical profile of EEBP in the negative ion mode. This method is for the identification of polar organic compounds with acidic sites, such as the phenolic organic acids. Deprotonated forms of the compounds of interest were then selected and dissociated and their ESI-MS/MS were compared to those which were considered standard (Figure 1).

3.2 EEBP antibacterial activity

The MIC value of EEBP in the presence of the *M. tuberculosis* strain was 123.4 µg/mL, while in the presence of isoniazid, it was 0.030 µg/mL. The extract of *B. portulacoides* was effective against *S. typhimurium* and *B. cepacia* (MIC = 1000 µg/mL). However, EEBP was not effective against *S. saprophyticus* and *E. aerogenes.*

3.3 ***EEBP inhibited pleurisy induced by carrageenan and inflammation induced by BCG***

At a dose of 1000 mg/kg, EEBP significantly reduced the invasion of pleural spaces by leukocytes (Figure 2A), with inhibition at 55%, while dexamethasone led to 87% inhibition. Protein exudation was significantly decreased at EEBP doses of 300 (41% inhibition) and 1000 mg/kg (54% inhibition) (Figure 2B), demonstrating the dose-dependent effects of the extract.

Doses of 30 and 100 mg/kg of EEBP did not decrease the invasion of leukocytes into the pleural spaces after intrathoracic injection of BCG (Figure 3A). Administration of IL-1β (30 mg/kg) via pleural lavage led to a significant reduction of 47% compared to that of the control group, while administration of 100 mg/kg EEBP or isoniazid reduced invasion by 70% or 83%, respectively. In serum, in relation to that of the control group, the reduction in the number of leukocytes was observed to be 60%, 61% and 67% for the administration of 30 and 100 mg/kg EEBP and isoniazid, respectively (Figure 3B and 3C).

During cell culture of the pleural lavage samples, there was a decrease in the development of CFUs from those taken from the groups treated with EEBP, with few or no colonies observed after 60 days. For samples from the 100 mg/kg EEBP group, only 1 plate had 3 CFU, and for the 30 mg/kg EEBP group, 3 plates had a mean of 5.2 UFC. The samples from the negative control group presented mycobacterial growth in all cultivated plaques, with a mean of 54 CFU after 60 days of culture, while the isoniazid group samples did not show any growth after 60 days, and all plaques were found to be positive for mycobacteria in the presence of Ziehl-Neelsen stain and Ogawa Kudu culture medium. Samples of macerated liver and spleen presented similar levels of mycobacterial growth in both the negative control and EEBP treatment groups (data not shown).

3.4 *EEBP inhibited carrageenan*- and CFA**-induced paw** *inflammation* **in a mouse**

model

Edema was reduced at all doses of EEBP at 2 h (with 33%, 33%, and 42% reduction at doses of 30, 100 and 300 mg/kg, respectively) and 4 h (50%, 58%, and 67% reduction at 30, 100 and 300 mg/kg, respectively) after the injection of carrageenan (Figures 4A-B); however, no edema reduction was observed 1 h after carrageenan administration (results not shown). Additionally, after the intraplantar injection of carrageenan, 30 mg/kg EEBP decreased the sensitivity to mechanical stimuli 3 h after administration, and a sensitivity decrease was observed at all doses after 4 hours. All doses of EEBP reduced the ability of carrageenan to induce mechanical hyperalgesia (Figures 4C-D). No significant effect of EEBP in the reduction in cold hyperalgesia was detected (results not shown). As expected, carrageenan-induced inflammation was significantly reduced by treatment with dexamethasone, as shown by the measurement of all inflammatory parameters (Figures 4A-D).

Six days after the administration of CFA, sensitivity to mechanical stimuli was inhibited by approximately 60% and 63% by treatment with EEBP at doses of 30 and 100 mg/kg, respectively. After 11 days, the inhibition observed with 30 mg/kg EEBP was 76%; after 16 days, the inhibition observed with 30 mg/kg EEBP was 90%; and 22 days after CFA, the inhibition observed with 30 mg/kg EEBP was 77% (Figure 5).

Six days after the administration of CFA, the edema in the injected paw was not significantly reduced by treatment with EEBP. However, after 11 days, a reduction of 63 ± 3% was observed at a dose of 30 mg/kg EEBP compared with that of the control, and a reduction of 70 ± 9% was observed with 100 mg/kg of EEBP. After 16 and 22 days, the reduction in edema was observed to be 65 ± 9% and 80 ± 3%, respectively, at 30 mg/kg EEBP, and 69 ± 3% and 80 ± 6% at 100 mg/kg EEBP (Figure 6).

Six days after the intraplantar administration of CFA, cold sensitivity was observed to be inhibited by 80 ± 8% and 51 ± 8% at dose of 30 and 100 mg/kg EEBP, respectively, when compared to that of the control group (CFA only). After 11, 16 and 22 days, cold sensitivity was observed to be inhibited by 85 ± 3%, 60% and 50 ± 9%, respectively, at an EEBP dose of 30 mg/kg, while a reduction of 45 ± 3%, 57 ± 7% and 55 ± 3% was observed at 100 mg/kg EEBP (Figure 7).

4. DISCUSSION

B. portulacoides is commonly used in folk medicine in the treatment of leukorrhea and other infections; however, its use for these types of infections has not been previously subject to scientific investigation. The activities of *B. portulacoides* against trypanosomiasis, leishmaniasis, *B. jararacussu* snake venom-induced edema, and bacteria have been studied in the past *(*Salvador, et al., 2002; Pereira, et al., 2009). These infections stimulate a number of inflammatory and pain-related processes, and the present study may support the medicinal use of *B. portulacoides* in their treatment by demonstrating its anti-inflammatory activity, including its inhibition of leukocyte migration and protein extravasation and its antihyperalgesic and antibacterial effects *in vivo* and *in vitro*. The use of *B. portulacoides* against infection is supported by the fact that NSAIDs and glucocorticoids decrease inflammation and fight infection in a similar manner to that of *B. portulacoides*.

The folk medicinal use of *B. portulacoides* extracts in the treatment of vaginitis spurred us to test its effects against other pathogens. *S*alvador et al. (2002), demonstrated the efficacy of ethanolic extracts derived from both the aerial parts and roots of *B. portulacoides,* as well as the compounds contained in these extracts, against several microorganisms, especially *S. aureus, S. mutans,* and *S. sobrinus*. In the present study, it was verified that EEBP was effective

in inhibiting the growth of *S. typhimurium, B. cepacia,* and *M. tuberculosis.* The fact that EEBP was not effective against *S. saprophyticus* and *E. aerogenes* showed that EEBP has specific potency against prokaryotic bacteria. These three pathogens are of great medical importance and are therefore relevant to the testing of EEBP in the treatment of infections. The results of Salvador et al. (2002) corroborate the fact that EEBP has antibacterial activity.

EEBP has the most potent effects against *M. tuberculosis*; however, the development of a treatment model using this agent is difficult, so *M. bovis* was chosen for study instead since it is also a component of the Mycobacterium complex that causes tuberculosis (Spargo, et al., 1993). Using the BCG model, we observed the expected systemic inflammatory reaction (acute-phase) that resulted from infection with mycobacteria. The IL-1β levels in the negative control group (infected) significantly differed from those of the naïve group (uninfected) or isoniazid treatment group after 7 days. Indeed, there was a statistically significant increase in serum IL-1β levels during the course of the infection, and this increase was abrogated in the EEPB (30 and 100 mg/kg) treatment groups, demonstrating that EEBP has anti-inflammatory properties. The reduction in IL-1β levels may be correlated with mycobacterial growth, as previous studies have indicated that mice deficient in either IL-1α or IL-1β showed some reduction in bacterial load 35 days postinfection, with a limited increase in bacterial load 2–3 months postinfection (Bourigault, et al., 2013). According to Krishnan and colleagues (Krishnan, et al., 2013), IL-1β secretion is dependent on the presence of viable mycobacteria, which was demonstrated using ELISA and by the growth of CFU in plaques. In addition to its effects on BCG growth in pleural, liver, and spleen tissues *ex vivo*, EEBP was able to inhibit the formation of CFUs, demonstrating its antibacterial and/or bacteriostatic effects (Table 1 and Figure 3). However, EEBP was not able to reduce the leukocyte infiltration that was induced by BCG. An analysis of the composition of EEBP revealed the presence of caffeic acid, ferulic acid, vanillic acid, gomphrenol, gomphrenol-3-glucoside, and 3,5,3'-trihydroxy-4'-methoxy-6,7-

methylenedioxyflavone (as well as its glycosylated form). Caffeic acid (Maresca, et al 2013; Dey, et al., 2015) and ferulic acid (Maresca, et al., 2013) are effective against *M. tuberculosis*. To study the general effects of EEPB on inflammation, carrageenan was injected in the pleural cavity of the lung, and it was shown that EEBP inhibited the infiltration of total leukocytes and the extravasations of protein. This result demonstrated the anti-inflammatory effects of EEPB and revealed a possible effect on vasodilation. Studies performed by Pereira, et al. (2009) showed that the essential oil derived from *B. portulacoides* significantly reduced edema induced by a myotoxin. Other studies conducted by our group demonstrated the antiedematogenic activity of topically applied EEBP in response to inflammation induced in mice using croton oil (Marson, et al., 2009). These results are of interest, as they demonstrate that EEBP has anti-inflammatory effects and is able to inhibit the growth of BCG or *M. tuberculosis in vitro* and *ex vivo* in the lung pleura, liver, and spleen, with results similar to those of isoniazid.

Using a model of acute inflammation induced by the injection of carrageenan into the paw, EEBP was shown to inhibit edema and mechanical hyperalgesia at doses of 30, 100 and 300 mg/kg by way of a single oral administration. Edema is characterized by the abnormal accumulation of interstitial fluid in the extracellular compartment and can be analyzed as an inflammatory parameter. EEBP is more potent in its inhibition of edema formation than it is for inhibiting leukocyte accumulation at sites of inflammation (Figure 2, 3, and 4). EEBP also inhibited mechanical hyperalgesia at all doses tested (30, 100, and 300 mg/kg), and its maximal efficacy was achieved. Mechanical hyperalgesia is an indicator of inflammation-induced pain arising from peripheral sensitization (Andrew and Greenspan, 1999). After tissue injury, the process of peripheral sensitization is triggered by mediators that include cytokines TNF and IL-1β, which promotes the synthesis of other cytokines such as NO, chemokines, and kinins. Increased pain sensitivity is a common feature of the inflammatory response, which occurs via the inhibition of type C nerve fibers that relay mechanical stimuli, thereby promoting allodynia

(Zhang and An, 2007; Curfs, et al., 1997). EEBP contains caffeic acid (Choudhary, et al., 2016; Choi, et al., 2017) and ferulic acid (Liu, et al, 2017; Lampiasi and Montana, 2018), both of which have effects on the interruption of IKKβ-inducible NF-κB activation and the NF-κB-regulated expression of TNF-α and IL-1α (Choi, et al., 2017). Yrbas et al. (2015) showed that vanillic acid, another component of EEBP, may play a role in controlling pain by interfering with the activity of ASICs (acid-sensing ion channels) and TPRV1, TRPA1, and TRPM8 receptors. Carrageenan induces the typical symptoms of inflammation (Morris, 2003) by increasing the levels of several inflammatory mediators, such as histamine, serotonin, kinins, and prostaglandins, and by increasing NO release (Di Rosa, et al., 1971; Posadas, et al., 2004). According to the results of this study, EEBP reduced the formation of edema after 4 h, which could suggest that the treatment acts on components involved in the production of prostaglandins. Therefore, the existing studies of the mechanisms underlying the control of inflammation or pain by compounds found in EEBP are difficult to interpret, but it is possible to conclude that EEBP is effective against edema and peripheral sensitization induced by inflammation.

Finally, to verify the effects of EEBP treatment after 21 days, persistent paw inflammation was induced by the injection killed *M. tuberculosis* (CFA model). Using this model, it was possible to evaluate the effects of EEBP on edema, mechanical sensitivity, and hyperalgesia. The persistent oral administration of EEBP inhibited edema, mechanical hyperalgesia and cold sensitivity induced by CFA. These observations showed that EEBP did not induce tolerance and that its benefits persisted throughout the course of treatment. These results demonstrated that EEBP has anti-inflammatory and antihyperalgesic effects against inflammation induced by *M. tuberculosis*.

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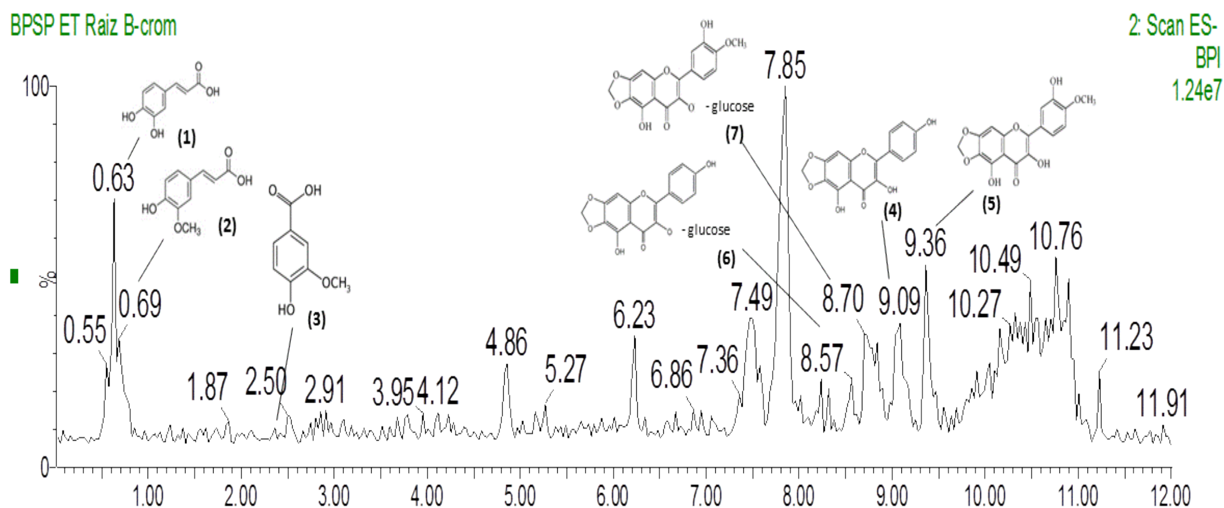

Figure 1. HPLC-MS chromatogram of EEBP. (**1**) caffeic acid – RT = 0.63 min, ESI-MS: m/z = 179 $[M-H]^-$; (**2**) ferulic acid – RT = 0.69 min, ESI-MS: m/z = 195 $[M-H]^-$; (**3**) vanillic acid – RT = 2.36 min, ESI-MS: m/z = 167 $[M-H]^-$; (**4**) Gomphrenol – RT = 9.09 min, ESI-MS: m/z = 313 $[M-H]^-$; (**5**) 3,5,3'-Trihydroxy-4'-methoxy-6,7-methylenedioxy-flavone – RT = 9.36 min, ESI-MS: m/z = 343 $[M-H]^-$; (**6**) Gomphrenol-3-glucoside – RT = 8.57 min, ESI-MS: m/z = 475 $[M-H]^-$; (**7**) 3,5,3'-Trihydroxy-4'-methoxy-6,7-methylenedioxy-flavone-glucosilated – RT 8.70 min, ESI-MS: m/z = 505 $[M-H]^-$.

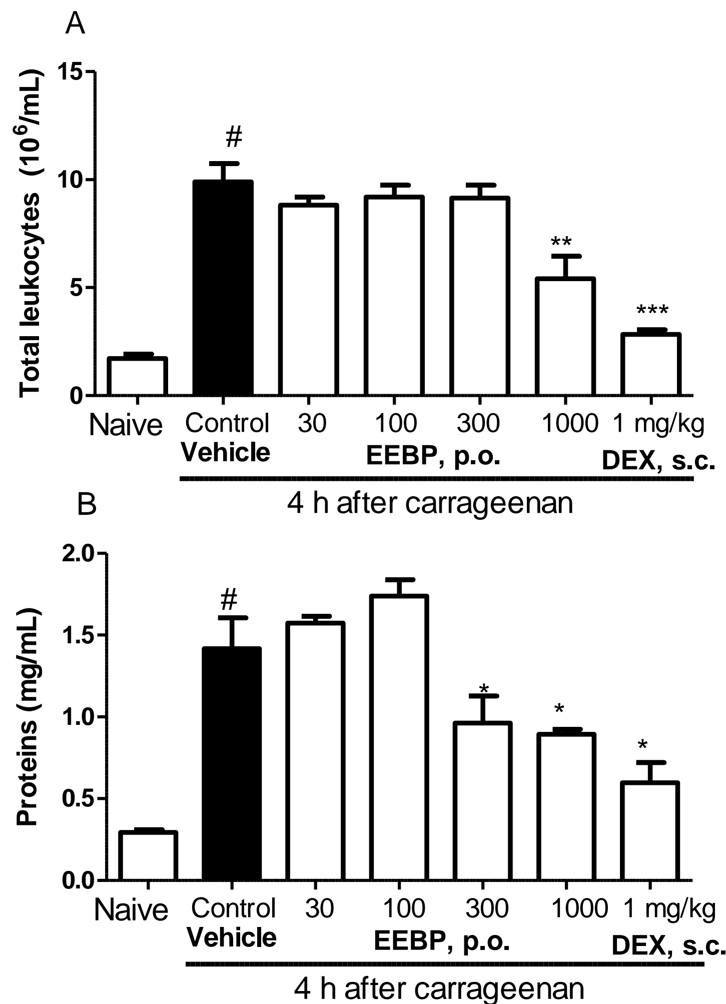

Figure 2: Effects of oral administration of EEBP on leukocyte migration (A) and protein leakage (B) in the pleurisy induced by carrageenan (cg). The animals received EEBP (30, 100 or 300 mg/kg, p.o.), vehicle (control) or dexamethasone (DEX, 1 mg/kg, s.c.), and 1 h later, an intrathoracic injection of Cg was administered. The naïve group (# indicates a statistically significant difference from the vehicle group) received an intrapleural injection of sterile saline instead of Cg and was also treated with saline solution. Each bar represents the mean ± SEM of 6 animals. **P*<0.05, ***P*<0.01, ****P*<0.001, #*P*< 0.001 compared with the control group. One-way ANOVA followed by the Newman-Keuls test.

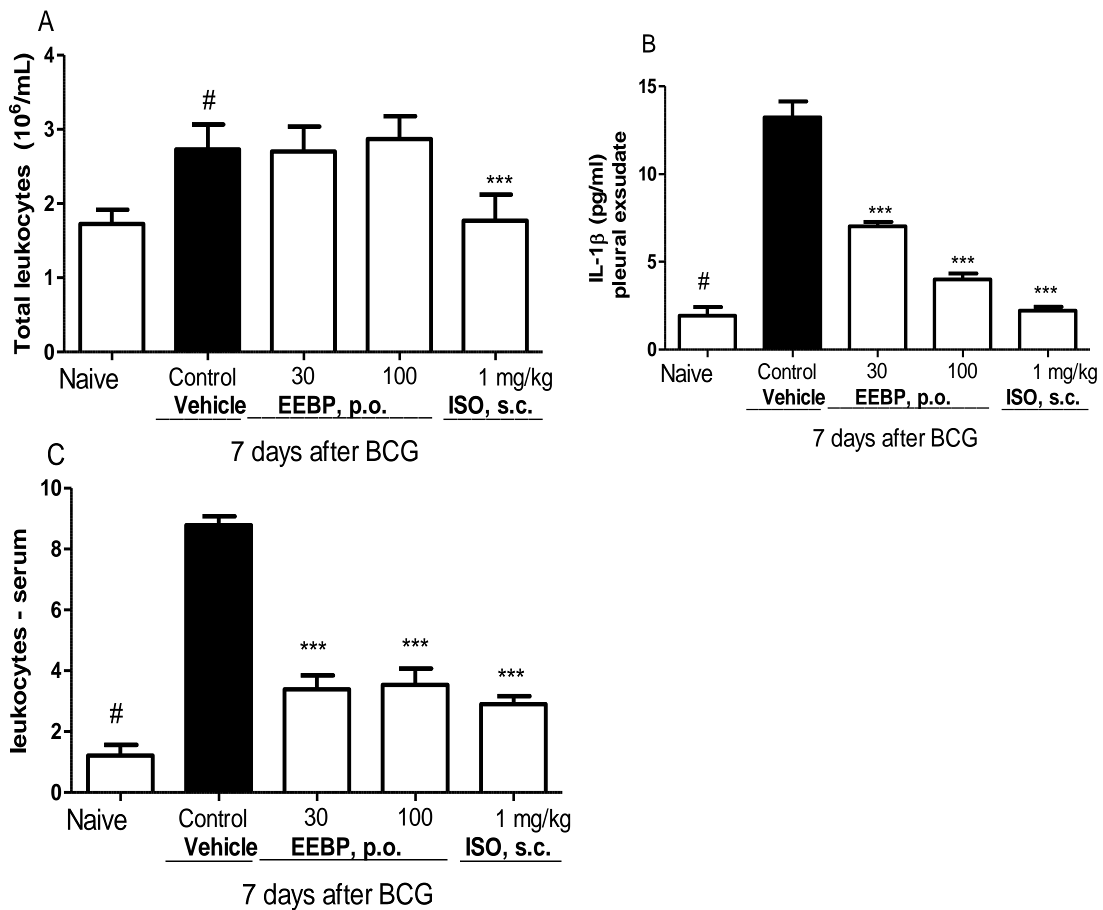

Figure 3: Effects of oral administration of EEBP on leukocyte (A) in the pleurisy, Il-1β levels in blood (B), and pleural exsudate (C) induced by BCG. The animals received EEBP (30, or 100, p.o.), vehicle (control) or Isonizade (ISO, 25 mg/kg, p.o.) for 7 days and an intrathoracic injection of BCG was administered since the first day. The naïve group (# indicates a statistically significant difference from the vehicle group) received an intrapleural injection of sterile saline instead of BCG and was also treated with saline solution. Each bar represents the mean ± SEM of 6 animals. **P*<0.05, ***P*<0.01, ****P*<0.001, #*P*< 0.001 compared with the control group. One-way ANOVA followed by the Newman-Keuls test.

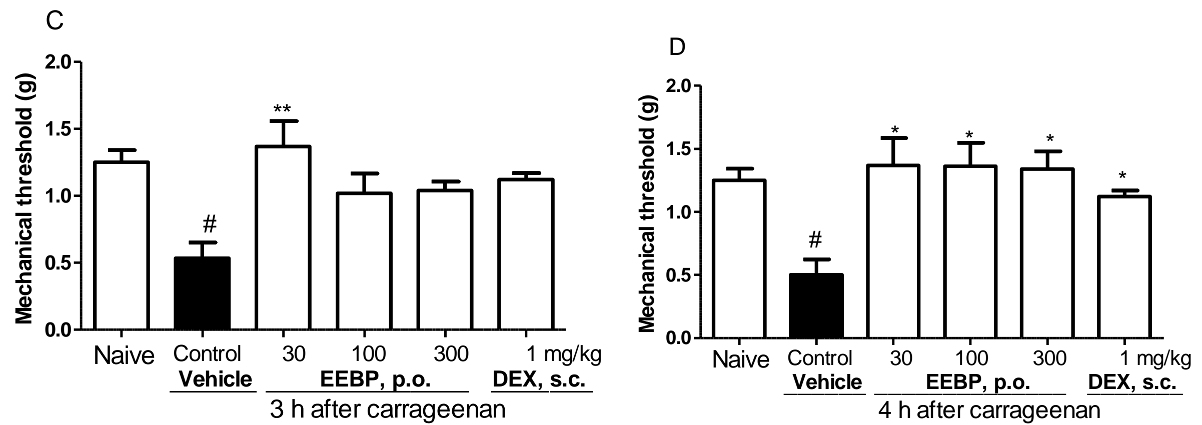

Figure 4 - Effect of oral administration of EEBP on the carrageenan (Cg)-induced paw edema and mechanical hyperalgesia in mice. The animals received EEBP (Naïve, 30, 100, and 300 mg/kg, p.o.), vehicle (control) or dexamethasone (DEX, 1 mg/kg, s.c.), and 1 h later, an intraplantar injection of Cg (300 μg/paw) was administered. Graphs (A), and (B) represent the evaluation of the paw edema at 2, and 4 h, respectively, after carrageenan injection while graphs (C), and (D) represent the evaluation of the mechanical hyperalgesia. Each bar represents the mean ± SEM of 6 animals. *P<0.05, **P<0.01 compared with the control group. One-way ANOVA followed by the Newman-Keuls test.

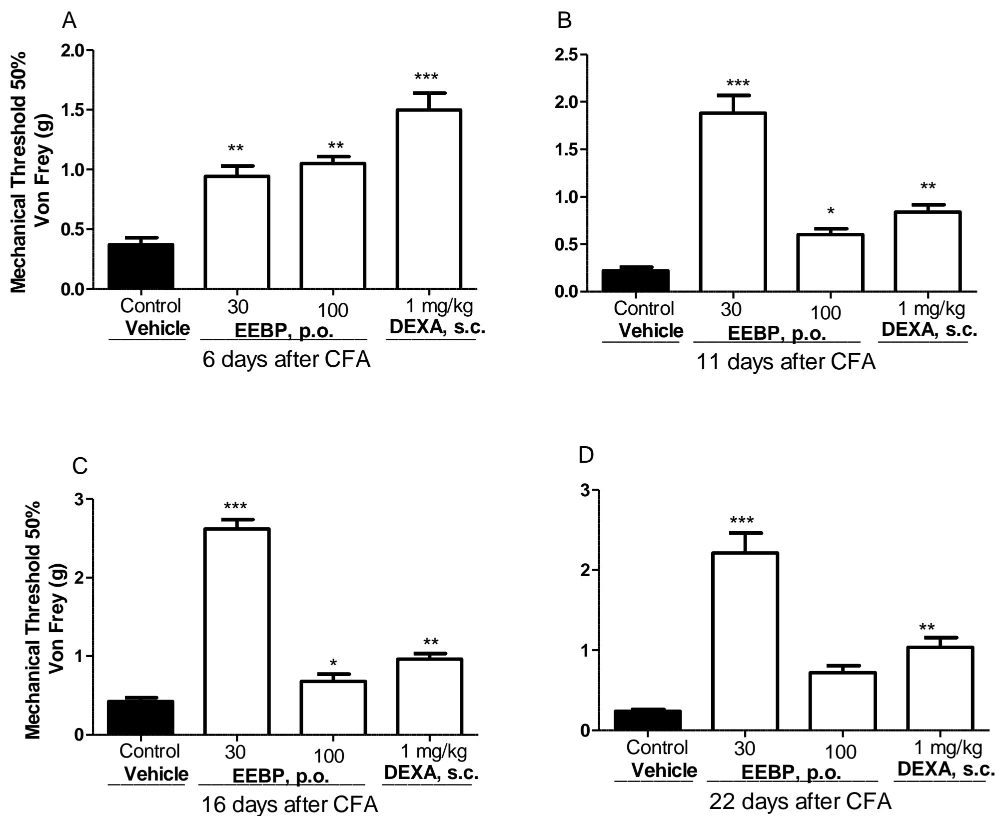

Figure 5: Effects of oral administration of EEBP on mechanical hyperalgesia induced by CFA. The animals received EEBP (30, or 100, p.o., everyday, once a day), vehicle (control) or dexametasona (DEXA, 1 mg/kg, s.c., everyday, once a day) for 6 (A), 11(B), 16 (C), and 22 (D) days. The CFA injection was perfomed in the first day. Each bar represents the mean ± SEM of 6 animals. *P<0.05, **P<0.01, ***P<0.001, compared with the control group. One-way ANOVA followed by the Newman-Keuls test.

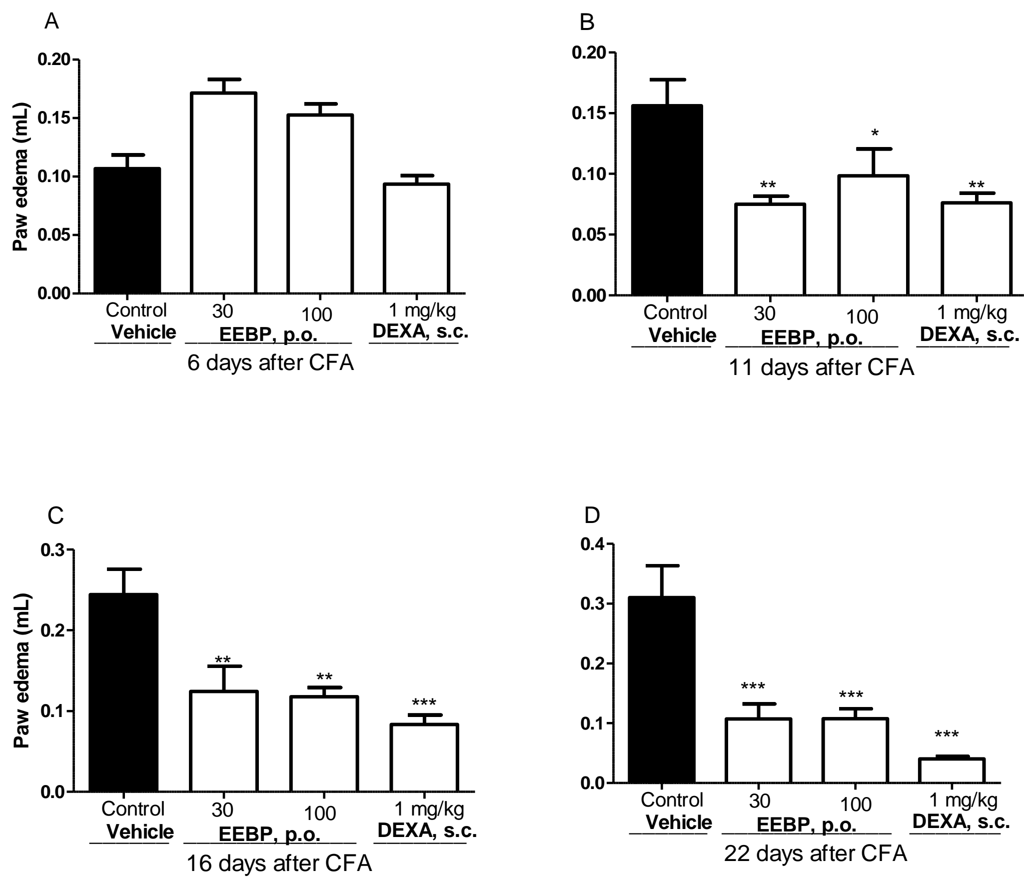

Figure 6: Effects of oral administration of EEBP on Oedema induced by CFA. The animals received EEBP (30, or 100, p.o., everyday, once a day), vehicle (control) or dexametasona (DEXA, 1 mg/kg, s.c., everyday, once a day) for 6 (A), 11(B), 16 (C), and 22 (D) days. The CFA injection was perfomed in the first day. Each bar represents the mean ± SEM of 6 animals. **P*<0.05, ***P*<0.01, ****P*<0.001, compared with the control group. One-way ANOVA followed by the Newman-Keuls test.

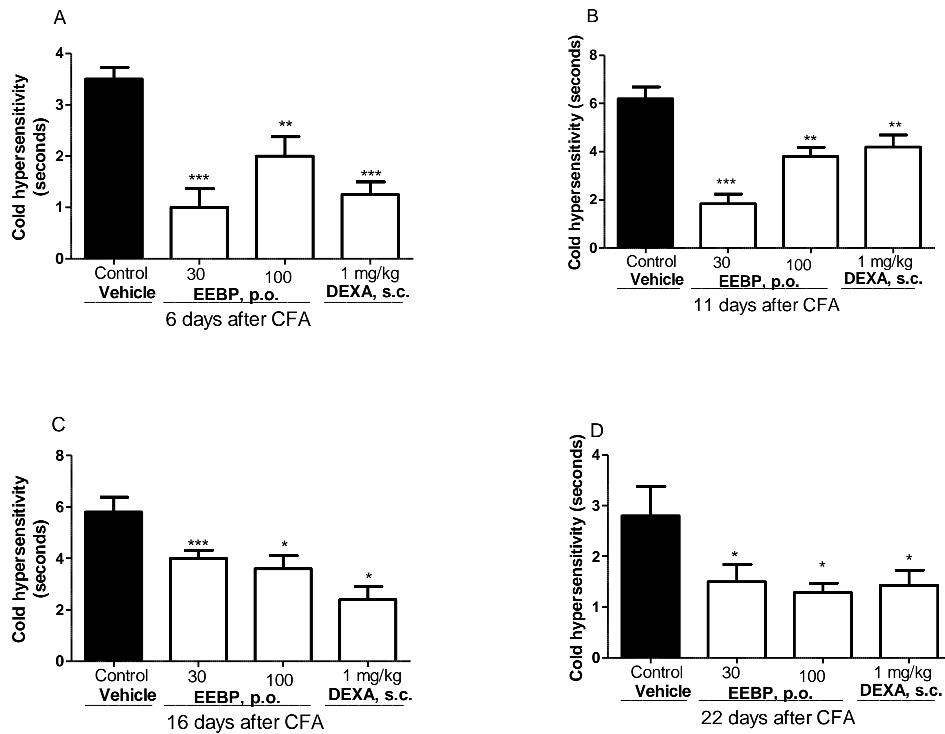

Figure 7: Effects of oral administration of EEBP on cold hyperalgesia induced by CFA. The animals received EEBP (30, or 100, p.o., everyday, once a day), vehicle (control) or dexametasona (DEXA, 1 mg/kg, s.c., everyday, once a day) for 6 (A), 11(B), 16 (C), and 22 (D) days. The CFA injection was perfomed in the first day. Each bar represents the mean ± SEM of 6 animals. **P*<0.05, ***P*<0.01, ****P*<0.001, compared with the control group. One-way ANOVA followed by the Newman-Keuls test.

Table 1. Mean number of colony forming units (CFU) and number of plaques with micobacterial growth of pleural wash.

	CFU (number of culture plates)			
Groups	**20 days**	**30 days**	**45 days**	**60 days**
Negative control	6.33 ± 3.8 (3)	12.60 ± 3.47 (6)	23.20 ± 3.69 (6)	54.6 ± 6.86 (6)
Positive control	-	-	-	-
EEBP (30mg/kg)	-	2 (1)	5 (1)	5.2 ± 3.1 (3)
EEBP(100mg/kg)	-	-	-	3 (1)

6 ANEXOS

6.1 PARECER DE APROVAÇÃO DO COMITÊ DE ÉTICA