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EFEITO DO EXTRATO AQUOSO DE CAVALINHA (*Equisetum giganteum* L.) EM MODELO DE MONOARTRITE INDUZIDA POR ANTÍGENO

Porto Alegre
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Trabalho de conclusão de curso de graduação apresentado ao Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul, como requisito parcial para obtenção do título de Bacharel em Biomedicina.

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LISTA DE ABREVIATURAS

ACPA	Anticorpos Anti-proteínas/peptídeos Citrulinados
AEGH	Extrato Aquoso de Cavalinha (Aqueous Extract of Giant Horsetail)
AIA	Artrite Induzida por Antígeno
AINEs	Antiinflamatórios Não-esteroidais
AR	Artrite Reumatoide
BCR	Receptor de Célula B
CFA	Adjuvante Completo de Freund
ConA	Concanavalina A
DMCDs	Drogas Anti-reumáticas Modificadoras da Doença
FLS	Fibroblastos Sinoviais
FR	Fator Reumatóide
HLA	Antígeno Leucocitário Humano
IA	Intra-articular
IFA	Adjuvante Incompleto de Freund
IFN	Inferferon
IL	Interleucina
LPS	Lipopolissacarídeo
mBSA	Albumina Bovina Sérica Metilada
MMPs	Metaloproteinases
RANKL	Ligante do Receptor Ativador do Fator Nuclear κB
Th1	Célula T auxiliar do tipo 1
Th17	Célula T auxiliar do tipo 17
TLR	Toll-like Receptor
TNF	Fator de Necrose Tumoral

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RESUMO

A artrite reumatoide (AR) é uma doença autoimune sistêmica onde a inflamação crônica da sinóvia articular e a subsequente erosão óssea e da cartilagem resultam em destruição articular, dor e incapacidade funcional. De etiologia pouco esclarecida, afeta cerca de 1% da população mundial adulta e 0,46% da brasileira. Apesar dos recentes progressos no tratamento da AR, estes ainda apresentam limitações e significativos efeitos adversos, salientando a necessidade de novas estratégias terapêuticas. *Equisetum giganteum* L. é uma planta usada na medicina popular das Américas Central e do Sul para o tratamento de doenças inflamatórias, dentre outras enfermidades. Apesar desse uso popular, estudos sobre sua eficácia antiinflamatória ainda não foram realizados. Com o objetivo de avaliar o efeito do extrato aquoso de cavalinha (AEGH), *Equisetum giganteum* L., como uma terapia imunomoduladora *in vivo,* foi empregado um modelo de artrite induzida por antígeno (AIA) em camundongos BALB/c com o antígeno albumina bovina sérica metilada (mBSA). Os camundongos foram tratados com AEGH (600 mg/kg em 100 µl) ou veículo (salina 5 mg/kg) em via oral por gavagem. A nocicepção do joelho foi avaliada em 0; 1; 3; 6 e 24 horas após a indução de artrite, e a migração de leucócitos em 24h foi mensurada na articulação do joelho. *In vitro*, a viabilidade celular em 24h (AEGH 20-140 µg/ml) e a proliferação linfocitária com concanavalina A (conA) e lipopolissacarídeo (LPS) (AEGH 80 µg/ml) foram realizadas. AEGH não apresentou toxicidade sobre linfócitos e inibiu a proliferação celular estimulada com conA e LPS em 23,56% e 31,77%, respectivamente ($p<0,05$). *In vivo*, o tratamento reduziu a nocicepção da pata em 3,6 e 24h após a injeção intraarticular de mBSA ($p<0.01$) e inibiu a migração de leucócitos totais para a articulação do joelho ($16,42 \pm 6,54 \times 10^4$ leucócitos/cavidade) quando comparado com veículo ($38,07 \pm 4,24 \times 10^4$ leucócitos/cavidade) ($p<0,015$). A partir desses dados, pode-se concluir que o AEGH possui potencial antiinflamatório em modelo de inflamação aguda, apresentando efeito tanto sofre linfócitos B quanto em T, com uma ação não dependente de toxicidade celular.

Palavras-chave: Artrite reumatoide, *Equisetum giganteum* L. (cavalinha), Extrato Aquoso de Cavalinha (AEGH), Artrite Induzida por Antígeno (AIA), imunomodulação.

1. INTRODUÇÃO

1.1. ARTRITE REUMATOIDE

A artrite reumatoide (AR) é uma doença poliarticular simétrica e crônica, caracterizada por inflamação sinovial persistente, produção de autoanticorpos, destruição óssea e de cartilagem, resultando em incapacidade funcional (Firestein, 2003). Atinge 0,5-1% da população adulta mundial (Scott et al., 2010) RA) e 0,46% da brasileira (Senna et al., 2004). A doença é três vezes mais freqüente nas mulheres e sua incidência aumenta com a idade, atingindo um pico na quinta década de vida (Alamanos and Drosos, 2005)

Existe uma grande heterogeneidade no desenvolvimento e prognóstico da doença, com alta variabilidade entre pacientes em relação ao ritmo de progressão da inflamação articular, desenvolvimento de manifestações extra-articulares e resposta a tratamentos (Worthington, 2005). O diagnóstico é feito através dos critérios estabelecidos em 2010 pelo Colégio Americano de Reumatologia juntamente com a Liga Européia Contra o Reumatismo, e combina extensão do envolvimento articular, presença de autoanticorpos, resposta de fase aguda e duração dos sintomas para a classificação dos pacientes (Tabela 1).

Embora tenha etiologia ainda desconhecida, sabe-se que uma tríade de fatores – predisposição genética, autoanticorpos e fatores ambientais – está relacionada à quebra da auto-tolerância e desenvolvimento da AR (Wegner et al., 2010). A associação de predisposição mais bem estabelecida é com os alelos do antígeno leucocitário humano (HLA)-DRB1, que contém um motivo comum, denominado de epitopo compartilhado (Scott et al., 2010). A localização molecular desse epitopo sugere um papel na apresentação de peptídeos possivelmente artritogênicos (Firestein, 2003), ou uma predisposição na seleção do repertório das células T (McInnes and Schett, 2011).

Dois tipos de autoanticorpos são importantes biomarcadores da doença. O fator reumatóide (FR) é o autoanticorpo clássico, direcionado contra a porção Fc da imunoglobulina G (Wegner et al., 2010). Os anticorpos anti-proteínas/peptídeos citrulinados (ACPA) aparecem como um marcador mais específico para o diagnóstico da doença (van der Linden et al., 2009). A citrulinização, uma modificação pós-translacional que converte resíduos de arginina em citrulina

(Wegner et al., 2010), permite que antígenos se encaixem nos alelos de HLA que possuem o epitopo compartilhado, gerando a formação de anticorpos contra esses antígenos citrulinados, que estão relacionados a um pior prognóstico da AR (Scott et al., 2010).

O estudo da influência de fatores ambientais no desenvolvimento da AR é relativamente escasso. O fumo é o principal e mais bem estabelecido fator de risco ambiental, mas sua associação é específica a pacientes com FR e/ou ACPA positivos. Dentre outros fatores associados com maior risco de desenvolvimento da doença estão alguns agentes infecciosos (como o Epstein-Barr vírus e citomegalovirus), doença periodontal e influência hormonal (estrogênios) (Liao et al., 2009; Scott et al., 2010).

Tabela 1. Novos critérios de classificação para artrite reumatoide (Aletaha et al., 2010; Scott et al., 2010).

1. Envolvimento articular (0-5)
    - 1 articulação média a grande (0)
    - 2-10 articulações médias a grande (1)
    - 1-3 articulações pequenas (não contando articulações grandes) (2)
    - 4-10 articulações pequenas (não contando articulações grandes) (3)
    - > 10 articulações (pelo menos uma articulação pequena) (5)
2. Sorologia (0-3)
    - Fator reumatóide (RF) e Anticorpo contra antígenos citrulinados (ACPA) negativo (0)
    - RF e ACPA fracamente positivos (2)
    - RF e ACPA fortemente positivos (3)
3. Reagentes de fase aguda (0-1)
    - Proteína C reativa e taxa de sedimentação eritrocitária normal (0)
    - Proteína C reativa *ou* taxa de sedimentação eritrocitária anormal (1)
4. Duração dos sintomas (0-1)
    - < 6 semanas (0)
    - 6 semanas ou mais (1)

Ponto de corte para artrite reumatoide: 6 ou mais pontos.

1.1.2. FISIOPATOGÊNESE DA ARTRITE REUMATOIDE

Embora, conforme discutido acima, a etiologia da AR ainda não esteja devidamente esclarecida, sabe-se que tipos celulares do sistema imune inato e do adaptativo estão envolvidos nos mecanismos que iniciam a patogênese da doença (Cascão et al., 2010) (Figura 1).

O evento inicial é um processo inflamatório na membrana sinovial, decorrente primeiramente da infiltração e acúmulo de leucócitos, principalmente neutrófilos atraídos por moléculas de adesão e quimiocinas expressas no endotélio de microvasos da sinóvia e, posteriormente da infiltração e/ou ativação local de macrófagos, células T (principalmente CD4+) e B, plasmócitos, mastócitos e células dendríticas, que comumente formam estruturas parecidas com folículos linfóides. (McInnes and Schett, 2011; Smolen and Steiner, 2003).

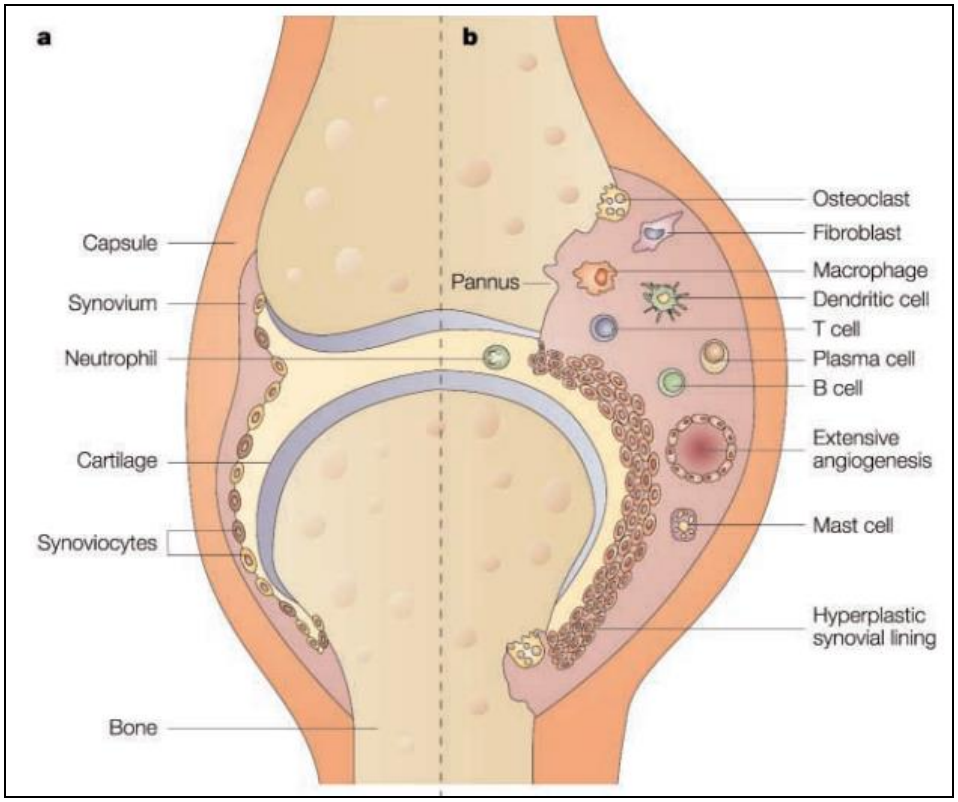

Figura 1. Alterações articulares na AR.
(a) Articulação saudável; (b) Articulação doente (Smolen and Steiner, 2003).

Os sinoviócitos (macrófagos e fibroblastos sinoviais) se tornam hipertróficos e aumentam sua proliferação, gerando hiperplasia da membrana sinovial. A membrana sinovial hiperplásica, juntamente com o infiltrado celular e a neoangiogênese, formam um tecido altamente invasivo conhecido como *pannus*,

que invade o osso e degrada a cartilagem, levando à destruição progressiva da articulação (McInnes and Schett, 2011; Smolen and Steiner, 2003).

Um grande número de células está envolvido na fisiopatogenia da AR (Figura 2). Os neutrófilos contribuem para a inflamação da membrana sinovial através da síntese de prostaglandinas, proteases e espécies reativas de oxigênio e nitrogênio, além de secretarem fator de necrose tumoral (TNF) (Cascão et al., 2010). Células B são precursoras de plasmócitos secretores de autoanticorpos, processam e apresentam antígenos promovendo a ativação de células T e secretam citocinas pro-inflamatórias, como interleucina (IL)-6 e TNF-α (Martinez-Gamboa et al., 2006). Macrófagos liberam TNF-α, IL-1β, IL-6, espécies reativas de oxigênio e nitrogênio e metaloproteinases (MMPs), além de realizarem fagocitose e apresentação de antígenos (McInnes and Schett, 2011).

Os fibroblastos sinoviais (FLS) em pacientes com AR assumem um fenótipo agressivo e tumoral, com inibição por contato diminuída, resistência à apoptose e migração aumentada. Essas células secretam MMPs, moléculas de adesão e o ligante do receptor ativador do fator nuclear κB (RANKL), que promove a diferenciação de osteoclastos, os quais promovem reabsorção óssea e dano ao osso subjacente à cartilagem articular (Bottini and Firestein, 2013). Recentemente, um estudo demonstrou que os FLS de pacientes com AR possuem a habilidade de migrar de uma articulação à outra, sugerindo como a poliartrite pode se desenvolver (Lefèvre et al., 2009).

As células T CD4+ que produzem IL-2 e IFN-γ apresentam uma polaridade de resposta Th1 (Smolen and Steiner, 2003). Embora a AR seja considerada uma doença mediada por células Th1, o papel das células Th17 no processo de destruição articular vem ganhando importância. Essas células produzem IL-17 e TNF-α que, sinergicamente, promovem a ativação de FLS e condrócitos (McInnes and Schett, 2011).

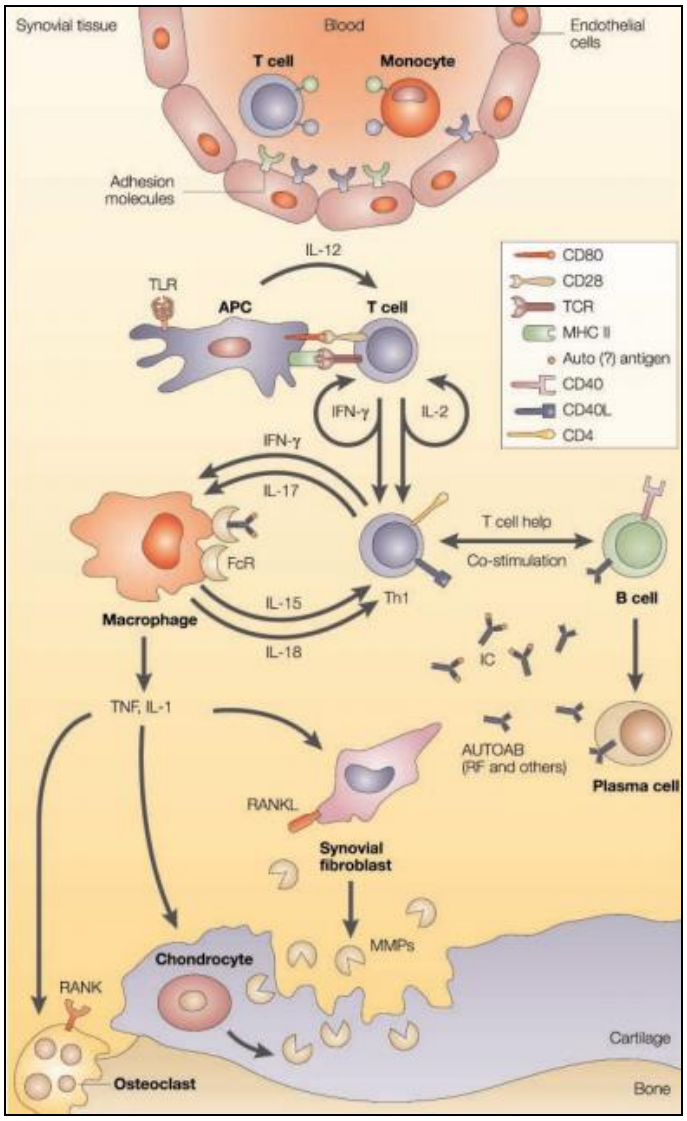

Figura 2. Fisiopatogênese da AR. (Smolen and Steiner, 2003).

Adicionalmente a inflamação e dano articular, ocorre o dano à cartilagem e ao osso subjacente à cartilagem articular. O dano da cartilagem ocorre através da secreção de MMPs por macrófagos e FLS constituintes do *pannus* invasivo, que desorganiza a rede de colágeno do tipo II, alterando o conteúdo de glicosaminoglicanos e a retenção de água, levando a degeneração do tecido. Adicionalmente, o potencial de regeneração do tecido está limitado, uma vez que citocinas presentes no ambiente articular doente, principalmente IL-1 e IL-17, e espécies reativas de nitrogênio promovem a apoptose dos condrócitos, tipo celular que regula a formação e clivagem da matriz cartilaginosa (McInnes and Schett, 2011). O dano ao tecido ósseo é mediado pelos osteoclastos, que tem sua diferenciação ativada por RANKL, TNF-α, IL-1, IL-6 e IL-17 (Deal, 2012).

1.1.3. TRATAMENTO DA ARTRITE REUMATOIDE

O principal objetivo no tratamento da AR não é a cura da doença, mas sua remissão, com eliminação da inflamação articular ativa e deterioração funcional. De 10-50% dos pacientes com AR atingem a remissão. Outros objetivos de tratamento incluem a redução da dor, manutenção de função, controle das comorbidades e preservação de atividades recreativas e de trabalho (Scott et al., 2010).

O medicamento padrão de comparação no tratamento da doença é o metotrexato, que substituiu as drogas antiinflamatórias não esteroidais (AINEs) como primeira linha de escolha no tratamento. Metotrexato é uma droga anti-reumática modificadora da doença (DMCD). Seu mecanismo de ação ainda não está bem estabelecido, porém, esse agente reduz o inchaço e dor articular, diminui marcadores de fase aguda (proteína C reativa e taxa de sedimentação eritrocitária) e limita o dano articular progressivo, melhorando a funcionalidade. Seus efeitos adversos variam desde náuseas até hepatotoxicidade e doenças pulmonares intersticiais (O'Shea et al., 2013; Scott et al., 2010).

Avanços na compreensão da fisiopatogênese da AR, como o papel de citocinas pro-inflamatórias e o envolvimento de diferentes tipo celulares e suas moléculas de superfície no desenvolvimento da doença, permitiram o avanço de novas terapias nos últimos anos (Smolen and Steiner, 2003).

Dentre os agentes biológicos, os inibidores de TNF foram os primeiros a serem desenvolvidos e os mais bem estabelecidos na literatura. O infliximabe e o adalimumabe são anticorpos anti-TNF, enquanto o etanercepte é uma proteína de fusão do receptor II de TNF. Dentre os efeitos adversos está o risco aumentado de desenvolvimento de tuberculose, bem como de infecções bacterianas, virais e fúngicas. (Scott et al., 2010; Smolen and Steiner, 2003).

Outros alvos de agentes biológicos são as células B e T. O rituximabe é um anticorpo monoclonal anti-CD20, uma molécula de superfície encontrada na célula B, e o abatacepte é um inibidor de sinais coestimulatórios de células T (O'Shea et al., 2013).

Os agentes biológicos geralmente são utilizados em terapia combinada com metotrexato ou algum outro DMCD, que também podem ser combinados entre si (Smolen and Steiner, 2003).

O uso em longo prazo de glicocorticóides é evitado devido a seus efeitos adversos, porém pode-se usá-los por períodos curtos de tempo durante picos da doença para promover uma melhora rápida enquanto se aguarda a resposta aos DMCDs, que possuem um tempo de início de ação mais lento ou, ainda, de forma localizada, através de infiltração de glicocorticóides nas articulações (Scott et al., 2010).

Os últimos avanços nos estudos da fisiopatogenia e de novos agentes de tratamento para a AR levaram à ideia da existência de uma janela terapêutica, como a oportunidade de se atingir o objetivo de remissão ou, ainda, a cura nos estágios iniciais da doença. Novos estudos devem seguir neste sentido (O'Shea et al., 2013; Smolen and Steiner, 2003).

1.2. ARTRITE INDUZIDA POR ANTÍGENO (AIA) - ALBUMINA BOVINA SÉRICA METILADA (mBSA)

Modelos animais de doenças humanas são ferramentas importantes no estudo da patogenia dessas doenças, possibilitando um melhor entendimento dos mecanismos biológicos básicos e a identificação de novas vias e alvos moleculares envolvidos no curso e desenvolvimento da doença, além de permitir o estudo e avaliação de possíveis agentes terapêuticos e de prevenção (Kollias et al., 2011).

Dentro do estudo experimental da artrite, existe um bom número de modelos animais que em diferentes níveis mimetizam a artrite humana (Hegen et al., 2008; Kollias et al., 2011). A seleção de um bom modelo para trabalho deve seguir alguns critérios importantes, dentre eles a capacidade de predição da eficácia em humanos dos agentes estudados; um protocolo experimental de fácil execução e reprodutibilidade, com um período total de execução relativamente curto e, por fim, apresentar uma patologia e/ou patogenia similar ao da doença humana (Bendele, 2001).

Nesse contexto, a artrite induzida por antígeno se apresenta como um bom modelo de escolha para o estudo da doença, tendo a vantagem de uma reprodutibilidade da duração e curso da inflamação e uma incidência próxima a 100% (Schaible et al., 2010).

Adicionalmente, esse modelo é uma artrite imunomediada dependente de células T e apresenta características semelhantes à artrite humana, como hiperplasia sinovial, infiltração de células inflamatórias, neoangiogênese, formação de *pannus* e destruição da cartilagem (Ferraccioli et al., 2010). O antígeno de escolha costuma ser a mBSA, por ser uma molécula catiônica que é atraída à cartilagem negativamente carregada, ficando retida na articulação (Bendele, 2001; van don Berg et al., 1984).

De acordo com o protocolo experimental, inicialmente os animais são imunizados por injeção subcutânea de mBSA emulsificada em adjuvante completo de Freund (CFA), composto por óleo mineral e *Mycobacterium tuberculosis* inativado. A emulsificação com CFA promove a apresentação contínua do antígeno ao sistema imunológico, necessária para uma resposta imune forte e persistente. Adicionalmente, a presença de *M. tuberculosis* atrai macrófagos para o local da lesão, o que aumenta a resposta imunológica (Sigma-Aldrich, 2013). Imunizações de reforço são administradas 7 e 14 dias após a primeira injeção, com mBSA emulsificada em adjuvante incompleto de Freund (IFA), composto somente de óleo mineral, sem a adição de *M. tuberculosis*. O IFA é utilizado em injeções de reforço para minimizar efeitos adversos, como reação inflamatória exacerbada e fibrose no local da injeção (Sigma-Aldrich, 2013). No 21º dia, os animais previamente sensibilizados recebem uma injeção intra-articular (IA) do antígeno (Figura 3) (Grespan et al., 2008).

Essa injeção IA de mBSA induz acentuada migração de neutrófilos (células polimorfonucleares) em 6h, com um pico em 24h (Grespan et al., 2008). Além de elevada infiltração celular, durante a fase aguda de AIA a articulação apresenta acentuados níveis de mRNA de IL-1β, IL-6, IL-2 e IFN-γ (Ferraccioli et al., 2010). IL-17 também parece ter um papel importante na fase inicial da doença (Ebbinghaus et al., 2012).

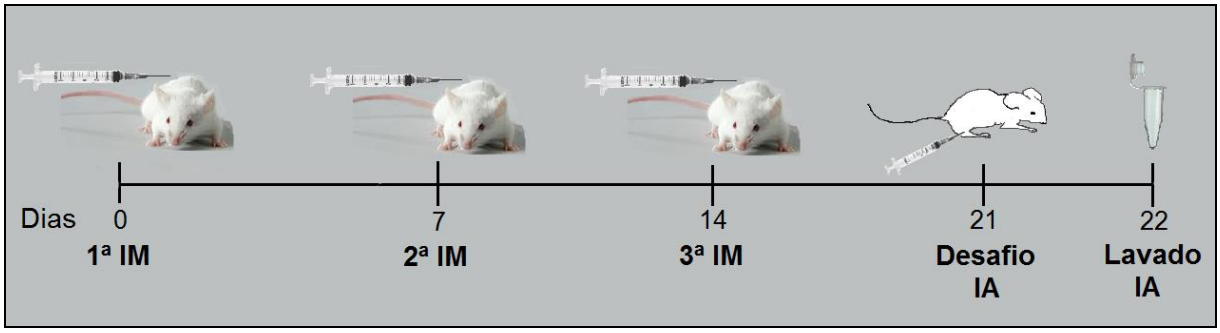

Figura 3. Esquema do protocolo experimental agudo de AIA. IM: Imunização; IA: Intra-articular.

Após 7 dias da injeção IA, a fase tardia de AIA apresenta características de doença crônica, apresentando infiltração de células mononucleares, fibrose periarticular, hiperplasia sinovial e destruição do osso e da cartilagem (Ebbinghaus et al., 2012; Schaible et al., 2010).

1.3. CAVALINHA (*Equisetum giganteum* L.)

Equisetum giganteum L., popularmente conhecida como cavalinha e rabo-de-cavalo, é uma planta nativa das Américas Central e do Sul. Da família Equisetaceae, pertence ao subgênero *Hippochaete* do gênero *Equisetum*, o qual compreende herbáceas que se reproduzem através de esporos e que são encontradas em regiões temperadas e tropicais (Wright et al., 2007).

A autenticidade da planta utilizada no presente trabalho foi avaliada e a ficha da espécie foi depositada no Herbário da Fundação Zoobotânica do Rio grande do Sul sob o número 88339.

1.3.2. CONSTITUIÇÃO QUÍMICA DA *E. giganteum* L.

Estudos da constituição química da *E. giganteum*, relatam a presença de alcalóides, saponinas e flavonóides como flavonas, isoflavonase e flavonóis nas partes aéreas da planta (Danielski et al., 2007). Nas partes aéreas secas são encontradas grandes quantidades de potássio, sílica e cinzas, bem como consideráveis quantidades de lítio, sódio, ferro, zinco e prata (Ovalles, 1996).

No extrato hidroetanólico da cavalinha foi relatada a presença de estirilpironas, derivados do ácido caféico e identificados os constituintes flavonóides

como derivados do canferol e quercetina, bem como um alto conteúdo de sílica e minerais (Francescato et al., 2013).

Na oleoresina extraída da planta são encontrados alcanos como *n*-heneicosano, ácidos graxos como ácido dodecanóico, metil ésteres e triterpenos esteroidais (Michielin, 2005).

1.3.3. ATIVIDADES BIOLÓGICAS DA *E. giganteum* L.

Para extratos das partes aéreas de *E. giganteum* é relatada atividade diurética (Cáceres et al., 1987; Pérez Gutiérrez et al., 1985), potencializadora do fator de crescimento neural (NGF) (Li et al., 1999), antimicrobiana (Kloucek et al., 2005), tripanocida frente tripomastigotas de Trypanosoma cruzi (Abe et al., 2005), hipoglicemiante, hipocolesterolêmica e hipotrigliceridêmica (Vieira et al., 2006).

Existem duas patentes relacionadas à cavalinha. Uma delas apresenta a *E. giganteum* como constituinte de um chá composto para o tratamento de hemorróidas (Garza, 1999), e a outra em um cosmético (Shiseido CO. LTD., 1997).

1.3.4. USO POPULAR DA CAVALINHA

O uso popular de plantas para o tratamento de diversas doenças, incluindo a AR, ocorre por diversas razões, como a falta de eficácia das terapias convencionais e seus efeitos adversos, a ampla e livre promoção das terapias não convencionais através da imprensa, internet e livros populares, a fácil distribuição dessas terapias e a tendência a ver essas terapias como naturais e consequentemente livres de riscos relacionados ao consumo e custo das terapias convencionais (Ernst, 2011).

O uso popular de plantas do gênero *Equisetum* é feito principalmente sob a forma de decoctos e infusões e amplamente difundido no mundo (Wright et al., 2007), em especial da *Equisetum arvense* na Europa (Cunha, 2003) e da *Equisetum giganteum* nas Américas (Tabela 2).

Tabela 2. Usos populares de *Equisetum giganteum* L.

Localidade	Indicações de uso / Ações terapêuticas	Referência
México	Diurética e adstringente, no tratamento de cálculos renais e para polir utensílios de cobre	(Pérez Gutiérrez et al., 1985)
Venezuela	Tratamento de doenças renais e por suas propriedades adstringentes, depurativas, diaforéticas, diuréticas, emenagoga, hemostática e remineralizante.	(Ovalles, 1996)
Argentina	Diurética e hemostática	(Leal, 1999)
oeste da Argentina	Tratamento de transtornos hepáticos	(Scarpa, 2002)
norte da Argentina	Utilizada para "urinar, menstruação, facilitar a digestão, limpar a pele, para os rins e fígado"	(Marinoff et al., 2006)
Jupi, PE	Tratamento de "problemas oftalmológicos e hemorroidais"	(Teixeira and Miranda de Melo, 2006)
Campo Grande, MS	"Rins, fígado, bexiga, infecções renais, na bexiga e olhos e depurativo do sangue"	(Nunes, 2003)
Índios Xokleng da Terra Indígena Ibirama, SC	Tratamento de "males dos rins e bexiga"	(Sens, 2002)
Porto Alegre, RS	"Depurativo do sangue, diabete, para urinar, prosta (próstata), purifica o sangue e reumatismo"	Vendruscuço & Mentz, 2006
Porto Alegre, RS	"Para perder peso"	(Dickel et al., 2007)
Não especificada	"Diurético, hemostático e adstringente, sendo também apropriado no tratamento da diarréia, gonorréia e cálculos renais, revitalização de unhas e cabelos sem vida, entre outras aplicações"	(Danielski et al., 2007)

É importante ressaltar que muitas vezes o uso de plantas está associado a efeitos adversos e a interação com drogas convencionais. A produção desses fitoterápicos costuma ser variável, principalmente quando na forma de infusões, dificultando a produção constante de uma droga com alta qualidade (Ernst, 2011).

2. TRABALHO EXPERIMENTAL NA FORMA DE ARTIGO CIENTÍFICO

Periódico: The Open Rheumatology Journal (TORJ)

TITLE: EFFECT OF AQUEOUS EXTRACT OF GIANT HORSETAIL (*Equisetum giganteum* L.) IN ANTIGEN-INDUCED ARTHRITIS

RUNNING TITLE: HORSETAIL AND ANTIGEN-INDUCED ARTHITIS

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ABSTRACT

Equisetum giganteum is a plant used in traditional medicine as diuretic. From our knowledge, this is the first time this plant is tested in an *in vivo* model of acute inflammation. To evaluate the effect of aqueous extract of giant horsetail (AEGH) as immunomodulatory therapy, antigen-induced arthritis (AIA) was generated in mice with methylated bovine serum albumin (mBSA). Inflammation was evaluated by articular nociception, leukocytes migration and lymphocyte proliferation. AEGH reduced nociception at 3, 6 and 24 h ($P < 0.01$), decreased leukocyte migration ($P < 0.015$), and inhibited lymphocyte proliferation stimulated with Concanavalin A and Lipopolysaccharide ($P < 0.05$). In conclusion, AEGH has an anti-inflammatory potential in acute model of inflammation, as well as immunomodulatory effect on both B and T lymphocytes, with an action independent of cytotoxicity.

Keywords: *Equisetum giganteum* L., aqueous extract of giant horsetail (AEGH), Antigen-Induced Arthritis (AIA), immunomodulatory therapy.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease characterized by inflammation of the synovium that leads to destruction of cartilage and bone [1]. Despite the significant progress in treatment strategies and new therapeutics for RA [2], significant limitations as well as side effects are found in their efficacy [3]. Thus, there is a great interest in studying new therapies.

Equisetum giganteum L., also known as “cavalinha”, “cola de caballo” or “giant horsetail”, is a native plant of Central and South America. Methods for the preparation of this species, mainly decoction and infusion, are widely used in traditional medicine as diuretic, hemostatic, urinary disorders, inflammatory conditions [4-7] and rheumatic diseases [8], among other applications. *E. giganteum* constitution includes flavonoids, styrylpyrones glycosides, hydroxycinnamic acid derivatives [9], metals, silica [10] and some apolar compounds in their oleoresin, as alkanes, fatty acids, methyl esters and steroid triterpenes [11].

Pharmacological studies revealed that the extracts of *E*. *giganteum* present antimicrobial effects [12], diuretic activity [4, 6] and no oral acute toxicity in mice [5]. Other *Equisetum* species have shown to present antioxidant, antinociceptive and anti-inflammatory activities [12,13]. These data together with the experience in traditional uses indicate the possibility of employing *E*. *giganteum* for the treatment of inflammatory diseases.

Antigen-induced arthritis (AIA) is a T-cell dependent immunological model in mice. Similar to other *in vivo* model of arthritis, AIA shows mimics with human rheumatoid arthritis [14]. As a consequence, this animal model may represent an excellent *in vivo* model for preclinical testing of new potential therapies RA and other inflammatory diseases. The aim of this study was to evaluate the effect of aqueous extract of *E*. *giganteum* as an immunomodulatory therapy.

MATERIALS AND METHODOLOGY

Animals

Male BALB/c wild-type mice (8-12 weeks, 20-25 g) were maintained in temperature-controlled rooms and were given water and food *ad libitum*. All experimental

procedures involving animals were performed in accordance with the National Institutes of Health Guide for Care and Use of Animals and with the approval of our institutional ethics committee under number 120004.

Preparation of Aqueous Extract

The *E. giganteum* sterile stems collected in May of 2011, in Santo Antônio da Patrulha, Rio Grande do Sul, Brazil (S 29° 52.374', W 50° 25.265', 12 m) were air-dried and grounded in a hammer mill. The plant authenticity was evaluated and the voucher specimen (number 88339) was deposited in the Herbarium at the Fundação Zoobotânica do Rio Grande do Sul (Porto Alegre, RS, Brazil). A decoction (plant: solvent ratio of 1:11.7, 95°C for 15 min) was prepared and filtered after cooled. The decoct was spray-dried using a Niro Production Minor atomizer (GEA, Copenhagen, Denmark).

The AEGH was prepared under decoction based on the traditional use of the plant and the good yield of the extraction process. Aqueous extractive solution obtained was then spray-dried. This technique is widely used in the herbal processing industries for thermally-sensitive materials, obtaining a dry product with good technological characteristics. The chemical profile of the AEGH was not assessed in this study. However, the chemical and physicochemical profile of the plant has been previously established by our group [9], where we can verify high content of silica and minerals, as well as the presence of caffeic acid derivatives, flavonoids and styrylpyrones. The AEGH obtained was employed in the experiments.

Induction of AIA

AIA was induced according to *Grespan et al.* (2008) [16]. Fourteen BALB/c mice were sensitized by subcutaneous (s.c.) injected with 500 μg of mBSA (Sigma Aldrich, St. Louis/EUA) dissolved in 0.2 ml of an emulsion containing 0.1 ml of 0.9% saline and 0.1 ml of complete Freund's adjuvant (Sigma Aldrich, St. Louis/USA) administered on day 0. Booster injections were administered for almost 2 weeks (7-14 days) using incomplete Freund's adjuvant (Sigma Aldrich, St. Louis/USA). Treatment started at day 19 and mice received AEGH (600 mg/kg) or vehicle (0.9% saline) orally in 100 μl, twice a day. On day 21, arthritis was induced in pre-immunized animals by intra-articular (i.a.) injection with 30 μg of mBSA dissolved in

10 μl of saline into the left tibiofemoral joint. As a negative control 10 μl of saline without mBSA was injected into the right tibiofemoral joint (contralateral joint) of vehicle group, consisting in the saline group.

Evaluation of Articular Nociception

Articular nociception was evaluated on 0, 1, 3, 6 and 24 h after i.a. injection of mBSA according to *Oliveira et al.* (2011) [17]. Mice were placed in a quiet room in acrylic cages with a wire-grid floor for 15-30 minutes before testing for environmental adaptation. An electronic pressure meter was used, consisting of a hand-held force transducer fitted with a polypropylene tip (Insight Instruments, Ribeirão Preto, São Paulo/BR). An increasing perpendicular force was applied to the central area of the plantar surface of the hind paw to induce flexion of the tibiofemoral joint, followed by paw withdrawal. The electronic pressure meter automatically recorded the intensity of the force applied when the paw was withdrawn, with results expressed as the flexion-elicited withdrawal threshold in grams (g).

Evaluation of *in vivo* Total Leucocytes Migration

After death, articular cavities of mice were washed twice with 5 μl phosphate buffered saline (0.15 M NaCL, 6.5 mM phosphate and 1 mM EDTA) in a final volume of 100 μl to evaluate leukocyte migration at 24 h after i.a. injection of mBSA. The total number of leukocytes was determined in a Neubauer chamber under optical microscopy.

Cell Viability Assay

Cell viability was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide] assay [18]. Lymphocytes from non-sensitized BALB/c lymph nodes were removed aseptically and cultured in triplicate (5 X 10^5 cells/well - 96-well plate) for 48 h at 37°C in 5% CO_2 in RPMI medium, treated or not with AEGH (20, 40 and 80 μg/ml). Twenty microliters of MTT (0.5 mg/ml) was added in each well and after 4 h of incubation the supernatants were removed and 100 μl of DMSO (Sigma Aldrich, St. Louis/EUA) was added to dissolve the MTT formazan crystals. After shaking the plate, the absorbance of each well was read at 570 nm.

Lymphocyte Proliferation Assay

Lymphocyte proliferation assay was performed using MTT assay [18]. Lymph nodes of non-sensitized BALB/c were removed aseptically. Lymphocytes were extracted, plated and cultured in triplicate (5 X 10^5 cells/well in a 96-well plate) for 48 h at 37°C in 5% CO_2 with RPMI medium stimulated with concanavalin A (ConA) (5 μg/ml) or lipopolysaccharide (LPS) (10 μg/ml) and treated or not with AEGH (80 μg/ml). Twenty microliters of MTT (0.5 mg/ml) was added in each well and after 4 h of incubation the supernatants were removed and 100 μl of DMSO (Sigma Aldrich, St. Louis/EUA) was added to dissolve the MTT formazan crystals. After shaking the plate, the absorbance of each well was read at 570 nm.

Statistical Analysis

Data are presented as mean ± SEM. Groups were compared by the analysis of variance with Tukey's adjustment for multiple comparisons or by Student's t-test using GraphPad Prism 5.0. Statistical differences were considered to be significant when $P < 0.05$.

RESULTS

Treatment with AEGH Reduced Inflammatory Parameters of Acute AIA

In AIA model, mBSA injection into tibiofemoral joint of immunized mice induces inflammation which causes significant increased nociception and leukocyte migration into the articular cavity, as compared to the negative control contralateral joint in the same mice [17].

Nociception (Figure 1A) was first noted 1 h after the antigenic challenge and became more intense in 3 h and 6 h, then remaining relatively stable till 24 h. Treatment with AEGH significantly reduced nociception on 3, 6 and 24 h ($P < 0.01$).

Leukocyte infiltration triggered by i.a. injection plays an essential role in this experimental model and contributes to the articular damage [16], being an important marker of local inflammatory activity. Treatment with AEGH significantly reduced leukocyte recruitment (43.13%) to the site of inflammation (16.42 ± 6.54 x 10^4

leukocytes/cavity) as compared with vehicle (38.07 $\pm$ 4.24 x 10^4 leukocytes/cavity) ($P < 0.015$) (Figure 1B).

AEGH Inhibited *in vitro* Lymphoproliferation

Lymphocytes proliferation was induced with ConA, which present nonspecific action, stimulating both T and B lymphocytes, and with LPS, that acts primarily on B lymphocytes, via B Cell Receptor and Toll-like 4 Receptor. AEGH inhibited lymphoproliferation induced by both mitogens in 23.56% and 31.77%, respectively ($P < 0.05$) (Figures 2B and 2C).

In addition, AEGH showed no cytotoxicity on lymphocytes (Figure 2A) at the treatment doses, maintaining cells viability similar to the unexposed control cells.

DISCUSSION

In order to evaluate the effect of AEGH on inflammation we chose an arthritis immunomediated T cell-dependent model. This model produces an acute inflammation and similar pathological aspects of RA [15]. In this model, the i.a. injection of mBSA induces accentuated migration of leucocytes after 6 h, with a highest influx on 24 h [16]. Additionally, the inflammatory process with cell influx and circulation of proinflammatory cytokines such as interleukin (IL)-6, IL-1β, IL-2 and interferon γ generate accentuated nociception.

In our study, treatment with AEGH markedly reduced nociception induced by AIA. This finding corroborates with the study of *Do Monte et. al*. (2004) [14], where treatments with hydroalcoholic extract of *E. arvense* reduced nociception in mouse experimental models of abdominal constriction and formalin-injected paw. Also, leukocytes migration locally was reduced with treatment. This finding confirms again the study of *Do Monte et al. (2004)* [14], where treatment with hydroalcoholic extract of *E. arvense* reduced mice paw edema caused by injection of carrageenan.

Regarding the pharmacological activity, previous studies have shown that *Equisetum* species presents antioxidant, antinociceptive and anti-inflammatory activities [12, 14], however this is the first study to evaluate the anti-inflammatory effect of this specie (*E. giganteum*) on a model of arthritis and on lymphocytes *in vitro*. Our results agree with the previously reported anti-inflammatory and antinociceptive effects of

flavonoids [19], the major secondary metabolites present in *E. giganteum* [9]. One of the important mechanisms of flavonoids in these processes can be an inhibition of eicosanoid generating enzymes including phospholipase A2, cyclooxygenases, and lipoxygenases, thereby reducing the concentrations of prostanoids and leukotrienes [20]. For other compounds, such as styrylpyrones and hydroxycinnamic acid derivatives, antioxidant [21, 22] and NF-κB inhibitory activity [23, 24] have been reported.

In conclusion, our study shows that AEGH has an interesting anti-inflammatory potential in an acute model of inflammation, as well as immunomodulatory effect on both B and T lymphocytes, with an action independent of cytotoxicity. Further studies are needed to better explore the immuno-inflammatory pathways affected by AEGH, as well as its potential clinical use.

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REFERENCES

[1] Scott DL, Wolfe F, Huizinga TW. Rheumatoid arthritis. Lancet 2010; 376: 1094-108.

[2] Smolen JS, Steiner G. Therapeutic strategies for rheumatoid arthritis. Nat Rev Drug Discov 2003; 2: 473-88.

[3] Doran MF, Crowson CS, Pond GR, O'Fallon WM, Gabriel SE. Predictors of infection in rheumatoid arthritis. Arthritis Rheum 2002; 46: 2294-300.

[4] Leal DP, Isla MI, Vattuone MA, Sampietro AR. A hysteretic invertase from Equisetum giganteum L. Phytochemistry 1999; 52: 1009-16.

[5] Cáceres A, Girón LM, Martínez AM. Diuretic activity of plants used for the treatment of urinary ailments in Guatemala. J Ethnopharmacol 1987; 19: 233-45.

[6] Pérez Gutiérrez RM, Laguna GY, Walkowski A. Diuretic activity of Mexican equisetum. J Ethnopharmacol 1985; 14: 269-72.

[7] Gorzalczany S, Rojo A, Rondina RVD, Debenedetti SL, Acevedo MCD. Estudio de toxicidade aguda por via oral de plantas medicinais argentinas. Acta Farmaceutica Bonaerense 1999; 18: 221-4.

[8] Vendruscolo GS, Simões CMO, Mentz L. Etnobotanica do Rio Grande do Sul: Análise comparativa entre o conhecimento original e atual sobre plantas medicinais nativas. Pesquisa botânica, São Leopoldo: Instituto Anchietano de Pesquisas 2005; 56: 585-320.

[9] Francescato LN, Debenedetti SL, Schwanz TG, Bassani VL, Henriques AT. Identification of phenolic compounds in Equisetum giganteum by LC-ESI-MS/MS and a new approach to total flavonoid quantification. Talanta 2013; 105: 192-203.

[10] Ovalles J, Fuller J, Spinetti M. Metals, silica and ahs content of Equisetum bogotense H.B.K. and Equisetum giganteum L.(Horsetail). Rev Fac de Farm 1996; 32: 2-4.

[11] Michielin EMZ, Bresciani LFV, Danielski L, Yunes RA, Ferreira SRS. Composition profile f horsetail (Equisetum giganteum L.) oleoresin: comparing SFE and organic solvents extraction. J Supercrit Fluids 2005; 33: 131-8.

[12] Kloucek P, Polesny Z, Svobodova B, Vlkova E, Kokoska L. Antibacterial screening of some Peruvian medicinal plants used in Callería District. J Ethnopharmacol 2005; 99: 309-12.

[13] Stajner D, Popović BM, Canadanović-Brunet J, Anackov G. Exploring Equisetum arvense L., Equisetum ramosissimum L. and Equisetum telmateia L. as sources of natural antioxidants. Phytother Res 2009; 23: 546-50.

[14] Do Monte FH, dos Santos JG, Russi M, Lanziotti VM, Leal LK, Cunha GM. Antinociceptive and anti-inflammatory properties of the hydroalcoholic extract of stems from Equisetum arvense L. in mice. Pharmacol Res 2004; 49: 239-43.

[15] Ferraccioli G, Bracci-Laudiero L, Alivernini S, Gremese E, Tolusso B, De Benedetti F. Interleukin-1β and interleukin-6 in arthritis animal models: roles in the early phase of transition from acute to chronic inflammation and relevance for human rheumatoid arthritis. Mol Med 2010; 16:552-7.

[16] Grespan R, Fukada SY, Lemos HP, Vieira SM, Napimoga MH, Teixeira MM, et al. CXCR2-specific chemokines mediate leukotriene B4-dependent recruitment of neutrophils to inflamed joints in mice with antigen-induced arthritis. Arthritis Rheum 2008; 58: 2030-40.

[17] Oliveira PG, Grespan R, Pinto LG, Meurer L, Brenol JC, Roesler R, et al. Protective effect of RC-3095, an antagonist of the gastrin-releasing peptide receptor, in experimental arthritis. Arthritis Rheum 2011; 63: 2956-65.

[18] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65: 55-63.

[19] De Melo GO, Malvar DoC, Vanderlinde FA, Rocha FF, Pires PA, Costa EA, et al. Antinociceptive and anti-inflammatory kaempferol glycosides from Sedum dendroideum. J Ethnopharmacol 2009; 124: 228-32.

[20] Kim HP, Son KH, Chang HW, Kang SS. Anti-inflammatory plant flavonoids and cellular action mechanisms. J Pharmacol Sci 2004; 96: 229-45.

[21] Jung JY, Lee IK, Seok SJ, Lee HJ, Kim YH, Yun BS. Antioxidant polyphenols from the mycelial culture of the medicinal fungi Inonotus xeranticus and Phellinus linteus. J Appl Microbiol 2008; 104: 1824-32.

[22] Maurya DK, Devasagayam TP. Antioxidant and prooxidant nature of hydroxycinnamic acid derivatives ferulic and caffeic acids. Food Chem Toxicol 2010; 48: 3369-73.

[23] Wu CS, Lin ZM, Wang LN, Guo DX, Wang SQ, Liu YQ, et al. Phenolic compounds with NF-κB inhibitory effects from the fungus Phellinus baumii. Bioorg Med Chem Lett 2011; 21: 3261-7.

[24] Nagasaka R, Chotimarkorn C, Shafiqul IM, Hori M, Ozaki H, Ushio H. Anti-inflammatory effects of hydroxycinnamic acid derivatives. Biochem Biophys Res Commun 2007; 358: 615-9.

APPENDIX

Figure legends:

Figure 1. *In vivo* experiments.

1A. Nociception, evaluated at 0, 1, 3, 6 and 24 hours after i.a. injection. Each bars represents mean ± SEM (n= 7). *P < 0.05 on time 1, **P < 0.01 on times 3, 6 and 24 h versus vehicle treatment, by two-way analysis of variance (ANOVA) followed by the Tukey's post hoc test.

1B. Leucocyte migration into the articular cavity of the knee joint, assessed 48 hours after treatment with vehicle or AEGH [600 mg/kg orally, twice a day]. Injection of saline alone was used as negative control. Each bar represents mean ± SEM (n= 7). *P = 0.015 versus vehicle treatment, by Student's unpaired t-test.

Figure 2. *In vitro* assay.

2A. Effect of AEGH on lymphocyte cell viability. Lymphocytes were incubated with AEGH at indicated concentrations for 48 h. Each bar represents mean ± SEM. Treatment with AEGH did not affect cell viability.

2B. Effect of AEGH on lymphocyte proliferation induced by ConA [5 μg/ml] and treated with or without AEGH [80 μg/ml] for 48 h. Each bar represents mean ± SEM (n=6). Cells incubated only with RPMI medium were used as unstimulated controls. *P < 0.05 versus ConA stimulated but non-treated cells, by Student's unpaired t-test.

2C. Effect of AEGH on lymphocyte proliferation induced by LPS. Lymphocytes were incubated with LPS [10 μg/ml] and treated with or without AEGH [80 μg/ml] for 48 h. Each bars represents mean ± SEM (n=6). Cells incubated only with RPMI medium was used as unstimulated control. *P < 0.05 versus LPS stimulated but non-treated cells, by Student's unpaired t-test.

FIGURES

Figure 1

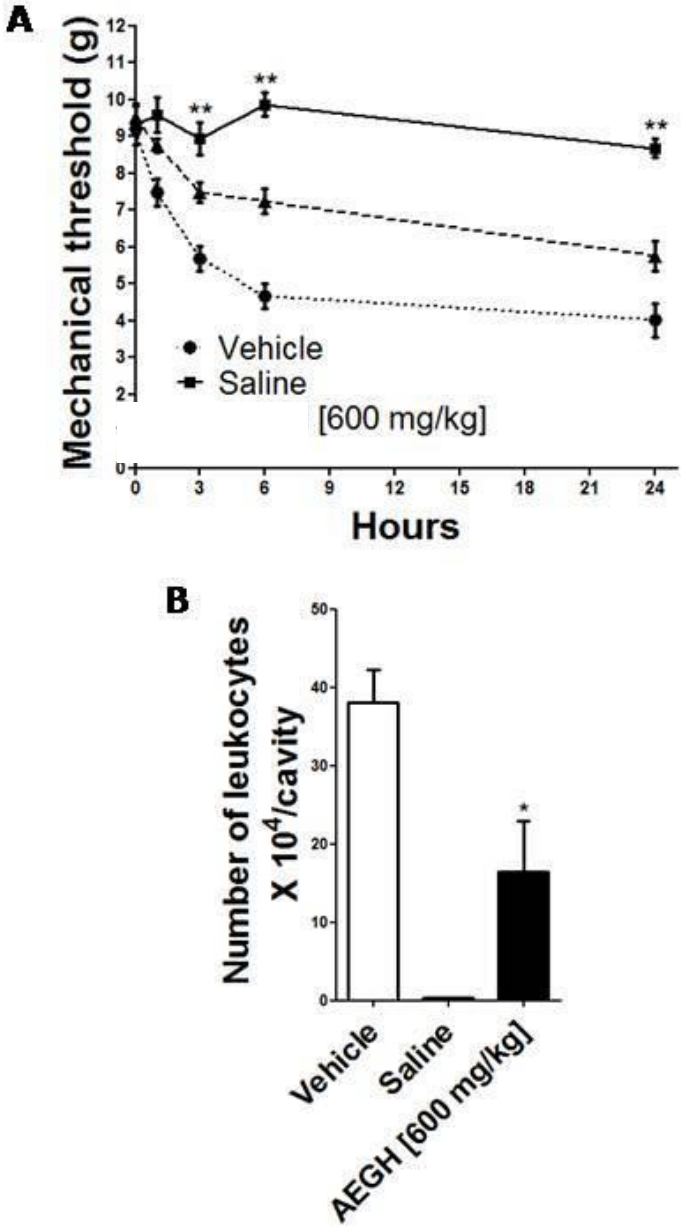
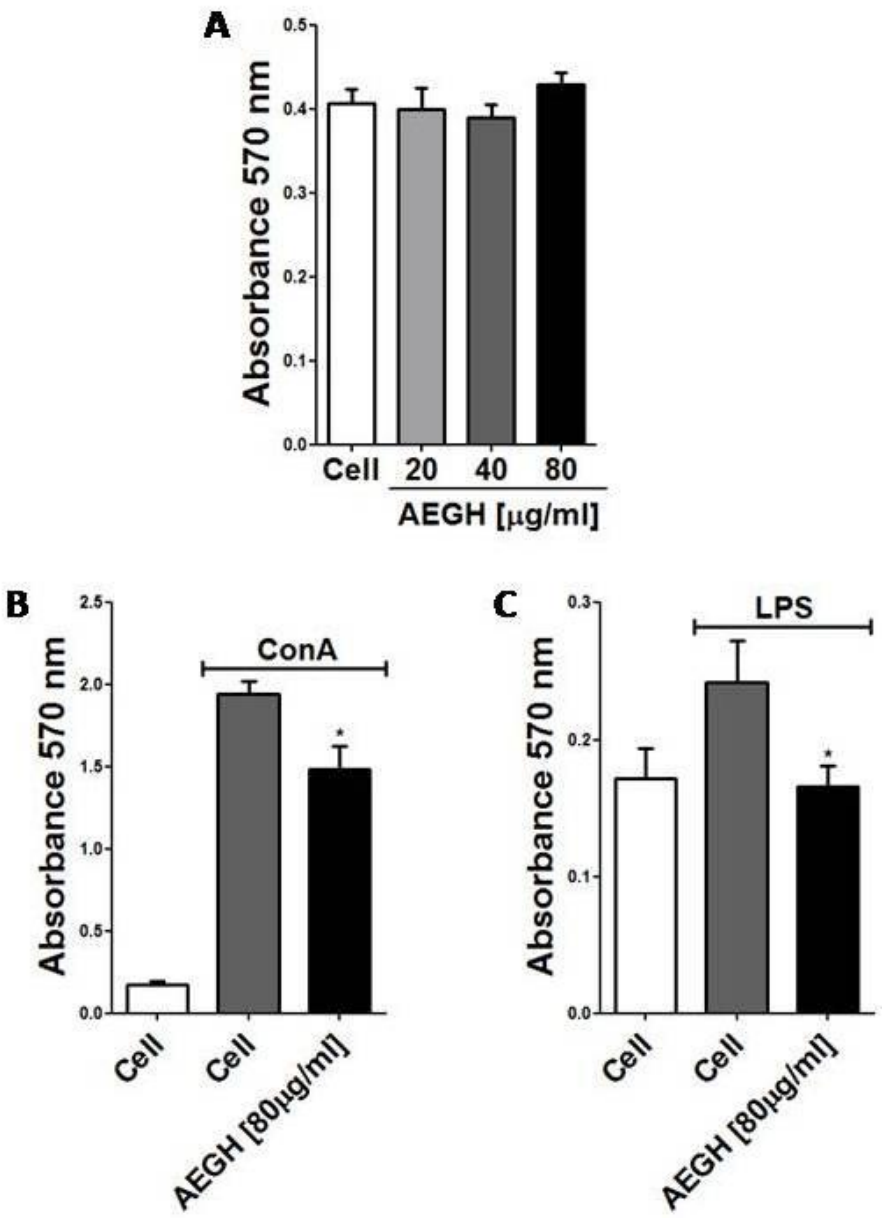


Figure 2


A
Absorbance 570 nm
0.5
0.4
0.3
0.2
0.1
0.0
Cell
20
40
80
AEGH [μg/ml]
B
Absorbance 570 nm
2.5
2.0
1.5
1.0
0.5
0.0
ConA
*
Cell
Cell
AEGH [80μg/ml]
C
Absorbance 570 nm
0.3
0.2
0.1
0.0
LPS
*
Cell
Cell
AEGH [80μg/ml]

3. CONCLUSÕES E PERSPECTIVAS

A partir dos resultados obtidos no presente trabalho, pode-se concluir que:

- A cavalinha apresenta um potencial antiinflamatório e analgésico em inflamação aguda, uma vez que o tratamento com AEGH diminuiu a nocicepção e a migração intra-articular de leucócitos para o local da lesão;
- A cavalinha apresenta um potencial de imunomodulação sobre linfócitos T e B, uma vez que inibiu a proliferação dessas células, apresentando ação possivelmente sobre receptores glicoprotéicos (ligantes da conA – estímulo à proliferação de linfócitos predominantemente T), Toll-like receptor 4 (TLR4) e/ou receptor de célula B (BCR) (ligantes do LPS – estímulo utilizado para a proliferação de linfócitos B);
- A ação relatada não depende de toxicidade sobre linfócitos;
- Os dados iniciais apresentados corroboram o uso da cavalinha na medicina popular, ressaltando que seus benefícios, assim como de qualquer terapêutico, estão ligados a sua dose de tratamento, que é amplamente variável durante o uso popular.

Perspectivas:

- Avaliar a ação do tratamento com AEGH em modelo de AIA crônico;
- Estudo de rotas intracelulares para a compreensão dos mecanismos de ação da *E. giganteum* L..

4. BIBLIOGRAFIA

Alamanos Y. and Drosos A. A., 2005. Epidemiology of adult rheumatoid arthritis. Autoimmun Rev 4, 130-6.

Aletaha D., Neogi T., Silman A. J., Funovits J., Felson D. T., Bingham C. O., Birnbaum N. S., Burmester G. R., Bykerk V. P., Cohen M. D., Combe B., Costenbader K. H., Dougados M., Emery P., Ferraccioli G., Hazes J. M., Hobbs K., Huizinga T. W., Kavanaugh A., Kay J., Kvien T. K., Laing T., Mease P., Ménard H. A., Moreland L. W., Naden R. L., Pincus T., Smolen J. S., Stanislawska-Biernat E., Symmons D., Tak P. P., Upchurch K. S., Vencovský J., Wolfe F. and Hawker G., 2010. 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum 62, 2569-81.

Bendele A., 2001. Animal models of rheumatoid arthritis. J Musculoskelet Neuronal Interact 1, 377-85.

Bottini N. and Firestein G., 2013. Duality of fibroblast-like synoviocytes in RA: passive responders and imprinted aggressors. Nat Rev Rheumatol 9, 10.

Cascão R., Rosário H. S., Souto-Carneiro M. M. and Fonseca J. E., 2010. Neutrophils in rheumatoid arthritis: More than simple final effectors. Autoimmun Rev 9, 531-5.

Cáceres A., Girón L. M. and Martínez A. M., 1987. Diuretic activity of plants used for the treatment of urinary ailments in Guatemala. J Ethnopharmacol 19, 233-45.

Danielski L., MICHIELIN E. M. Z. and FERREIRA S. R. S., 2007. Horsetail (*Equisetum giganteum* L.) oleoresin and supercritical CO_2: Experimental solubility and empirical data correlation. **J**ournal of Food Engineering 78, 6.

Deal C., 2012. Bone loss in rheumatoid arthritis: systemic, periarticular, and focal. Curr Rheumatol Rep 14, 231-7.

Dickel M. L., Rates S. M. and Ritter M. R., 2007. Plants popularly used for loosing weight purposes in Porto Alegre, South Brazil. J Ethnopharmacol 109, 60-71.

Ebbinghaus M., Gajda M., Boettger M. K., Schaible H. G. and Bräuer R., 2012. The anti-inflammatory effects of sympathectomy in murine antigen-induced arthritis are associated with a reduction of Th1 and Th17 responses. Ann Rheum Dis 71, 253-61.

Ernst E., 2011. Herbal medicine in the treatment of rheumatic diseases. Rheum Dis Clin North Am 37, 95-102.

Ferraccioli G., Bracci-Laudiero L., Alivernini S., Gremese E., Tolusso B. and De Benedetti F., 2010. Interleukin-1β and interleukin-6 in arthritis animal models: roles in the early phase of transition from acute to chronic inflammation and relevance for human rheumatoid arthritis. Mol Med 16, 552-7.

Firestein G. S., 2003. Evolving concepts of rheumatoid arthritis. Nature 423, 356-61.

Francescato L. N., Debenedetti S. L., Schwanz T. G., Bassani V. L. and Henriques A. T., 2013. Identification of phenolic compounds in Equisetum giganteum by LC-ESI-MS/MS and a new approach to total flavonoid quantification. Talanta 105, 192-203.

Garza M. F., 1999. MON'S TEA PARTNERSHIP. Herbal composition for hemorrhoid treatment, Vol. United States Patent 5869059.

Grespan R., Fukada S. Y., Lemos H. P., Vieira S. M., Napimoga M. H., Teixeira M. M., Fraser A. R., Liew F. Y., McInnes I. B. and Cunha F. Q., 2008. CXCR2-specific chemokines mediate leukotriene B4-dependent recruitment of

neutrophils to inflamed joints in mice with antigen-induced arthritis. Arthritis Rheum 58, 2030-40.

Hegen M., Keith J. C., Collins M. and Nickerson-Nutter C. L., 2008. Utility of animal models for identification of potential therapeutics for rheumatoid arthritis. Ann Rheum Dis 67, 1505-15.

Kloucek P., Polesny Z., Svobodova B., Vlkova E. and Kokoska L., 2005. Antibacterial screening of some Peruvian medicinal plants used in Callería District. J Ethnopharmacol 99, 309-12.

Kollias G., Papadaki P., Apparailly F., Vervoordeldonk M. J., Holmdahl R., Baumans V., Desaintes C., Di Santo J., Distler J., Garside P., Hegen M., Huizinga T. W., Jüngel A., Klareskog L., McInnes I., Ragoussis I., Schett G., Hart B., Tak P. P., Toes R., van den Berg W., Wurst W. and Gay S., 2011. Animal models for arthritis: innovative tools for prevention and treatment. Ann Rheum Dis 70, 1357-62.

Leal D., 1999. A hysteretic invertase from Equisetum giganteum L. . Phytochemistry 52, 7.

Lefèvre S., Knedla A., Tennie C., Kampmann A., Wunrau C., Dinser R., Korb A., Schnäker E. M., Tarner I. H., Robbins P. D., Evans C. H., Stürz H., Steinmeyer J., Gay S., Schölmerich J., Pap T., Müller-Ladner U. and Neumann E., 2009. Synovial fibroblasts spread rheumatoid arthritis to unaffected joints. Nat Med 15, 1414-20.

Li P., Matsunaga K. and Ohizumi Y., 1999. Enhancement of the nerve growth factor-mediated neurite outgrowth from PC12D cells by Chinese and Paraguayan medicinal plants. Biol Pharm Bull 22, 752-5.

Liao K. P., Alfredsson L. and Karlson E. W., 2009. Environmental influences on risk for rheumatoid arthritis. Curr Opin Rheumatol 21, 279-83.

Marinoff M. A., CHIFA C. and RICCIARDI A. I. A., 2006. Especies hidrófitas y palustres utilizadas como medicinales por los habitantes del norte y nordeste de la provincia del Chaco. Dominguezia 22.

Martinez-Gamboa L., Brezinschek H. P., Burmester G. R. and Dörner T., 2006. Immunopathologic role of B lymphocytes in rheumatoid arthritis: rationale of B cell-directed therapy. Autoimmun Rev 5, 437-42.

McInnes I. B. and Schett G., 2011. The pathogenesis of rheumatoid arthritis. N Engl J Med 365, 2205-19.

Michielin E., 2005. Composition profile f horsetail (Equisetum giganteum L.) oleoresin: comparing SFE and organic solvents extraction. Journal of Supercritical Fluids 33, 8.

Nunes G. P., 2003. Plantas medicinais comercializadas por raizeiros no Centro de Campo Grande. Revista Brasileira de Farmacognosia 13.

O'Shea J. J., Laurence A. and McInnes I. B., 2013. Back to the future: oral targeted therapy for RA and other autoimmune diseases. Nat Rev Rheumatol 9, 173-82.

Ovalles J., 1996. Metals, silica and ahs content of Equisetum bogotense H.B.K. and Equisetum giganteum L.(Horsetail). Revista de la Faculdad de Farmacia 32, 2.

Pérez Gutiérrez R. M., Laguna G. Y. and Walkowski A., 1985. Diuretic activity of Mexican equisetum. J Ethnopharmacol 14, 269-72.

Schaible H. G., von Banchet G. S., Boettger M. K., Bräuer R., Gajda M., Richter F., Hensellek S., Brenn D. and Natura G., 2010. The role of proinflammatory cytokines in the generation and maintenance of joint pain. Ann N Y Acad Sci 1193, 60-9.

Scott D. L., Wolfe F. and Huizinga T. W., 2010. Rheumatoid arthritis. Lancet 376, 1094-108.

Senna E. R., De Barros A. L., Silva E. O., Costa I. F., Pereira L. V., Ciconelli R. M. and Ferraz M. B., 2004. Prevalence of rheumatic diseases in Brazil: a study using the COPCORD approach. J Rheumatol 31, 594-7.

Sens S. L., 2002. Alternativas para a Auto-Sustentabilidade dos Xokleng da Terra Indígena Ibirama, Vol. Mestrado, Universidade Federal de Santa Catarina, Programa de Pós-graduação em Engenharia de Produção.

Sigma-Aldrich, 2013. Freund's Adjuvant, Complete and Incomplete.

Smolen J. S. and Steiner G., 2003. Therapeutic strategies for rheumatoid arthritis. Nat Rev Drug Discov 2, 473-88.

Teixeira S. A. and Miranda de Melo J. I., 2006. Plantas medicinais utilizadas no município de Jupi. IHERINGIA - Série Botânica 61, 6.

van der Linden M. P., van der Woude D., Ioan-Facsinay A., Levarht E. W., Stoeken-Rijsbergen G., Huizinga T. W., Toes R. E. and van der Helm-van Mil A. H., 2009. Value of anti-modified citrullinated vimentin and third-generation anti-cyclic citrullinated peptide compared with second-generation anti-cyclic citrullinated peptide and rheumatoid factor in predicting disease outcome in undifferentiated arthritis and rheumatoid arthritis. Arthritis Rheum 60, 2232-41.

van don Berg W. B., van de Putte L. B. A., Zwarts W. A. and Joosten L. A. B., 1984. Electrical Charge of the Antigen Determines Intraarticular Antigen Handling and Chronicity of Arthritis in Mice J. Clin. Invest. 74, 9.

Wegner N., Lundberg K., Kinloch A., Fisher B., Malmström V., Feldmann M. and Venables P. J., 2010. Autoimmunity to specific citrullinated proteins gives the first clues to the etiology of rheumatoid arthritis. Immunol Rev 233, 34-54.

Worthington J., 2005. Investigating the genetic basis of susceptibility to rheumatoid arthritis. J Autoimmun 25 Suppl, 16-20.

Wright C. I., Van-Buren L., Kroner C. I. and Koning M. M., 2007. Herbal medicines as diuretics: a review of the scientific evidence. J Ethnopharmacol 114, 1-31.

5. ANEXO

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View Journal Articles

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[7] Suresh E, Lambert CM. Combination treatment strategies in early rheumatoid arthritis. BMJ [serial on the Internet]. 2005 April 28; [cited 2005 April 30]; 64: [about 12 screen]. Available from http://ard.bmj.com/cgi/content/full/64/9/1252

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