UNIVERSIDADE FEDERAL DA GRANDE DOURADOS

AVALIAÇÃO DA ATIVIDADE ANTI-INFLAMATÓRIA, ANTIHIPERALGÉSICA E HIPOTENSORA DO EXTRATO BRUTO ETANÓLICO E AMIDA OBTIDA DE *Piper amalago* L. (PIPERACEAE) EM ROEDORES

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Dissertação apresentada à Universidade Federal da Grande Dourados – Faculdade de Ciências da Saúde, como requisito parcial para obtenção do Título de Mestre em Ciências da Saúde.

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"Há mais pessoas que desistem do que pessoas que fracassam"

Henry Ford

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Sumário

Lista de Abreviaturas

AIES	Anti-inflamatório Esteroide
AINES	Anti-inflamatório Não-Esteroide
AVC	Acidente Vascular Cerebral
C5a	C5a do Complemento
Células Th	Células T helper
Cg	Carragenina
CI-1	Composto Isolado 1
DNA	Acido Desoxi-Ribonucleico
COX	Ciclooxigenase
DRG	Gânglio da Raiz Dorsal
EEPA	Extrato Etanólico de *Piper amalago*
IL-1	Interleucina 1
IL-6	Interleucina 6
IFN-γ	Interferon Gamma
LPS	Lipopolissacarídio bacteriano
LTB_4	Leucotrieno B_4
MPO	Mieloperoxidase
NF-kB	Fator Nuclear Kappa-B
NGF	Fator de Crescimento do Nervo
OMS	Organização Mundial da Saúde
PG	Prostaglandina
PA-1	Proteína Ativadora - 1
RMN	Ressonância Magnética Nuclear
SNC	Sistema Nervoso Central
TNF	Fator de Necrose Tumoral
TX	Tromboxano
V.I.	Via Intraperitoneal
V.O.	Via Oral
V.S.	Via Subcutânea

Resumo

Piper amalago L. é conhecida popularmente pelo nome "pariparoba" e tem sido utilizada na medicina popular como um agente anti-inflamatório, porém apenas um estudo demonstrou atividade anti-inflamatória tópica. Outras espécies do mesmo gênero apresentaram efeito anti-hiperalgésico e hipotensora na literatura. Assim sendo, o presente estudo teve como objetivo avaliar o efeito anti-inflamatório, anti-hiperalgésico e hipotensor do extrato etanólico de *P. amalago* (EEPA) e de seu composto isolado N-[7-(3',4'-metilenodioxifenil)-2(Z),4(Z)-heptadienoil]pirrolidina em modelos experimentais de inflamação (edema da pata e pleurisia induzidos por injeção de carragenina), hiperalgesia (von Frey eletrônico), vasorrelaxamento (*in vitro*) e hipotensor (análise de pressão arterial média - PAM - *in vivo)* em roedores. A administração oral de EEPA em doses de 30 e 100 mg/kg diminuiu significativamente o edema da pata, enquanto para a pleurisia a dose de 100 mg/kg diminuiu significativamente o número total de leucócitos no lavado pleural e a proteínas extravasadas pela carragenina. O composto N-[7-(3',4'-metilenodioxifenil)-2(Z),4(Z)-heptadienoil]pirrolidina foi identificado por estudos fitoquímicos e isolado a partir do EEPA. A administração oral de N-[7-(3',4'-metilenodioxifenil)-2(Z),4(Z)-heptadienoil]pirrolidina em doses de 1 e 3 mg/kg diminuiu significativamente o edema da pata, contagem total de leucócitos no lavado pleural e proteínas que sofreram extravazamento. O EEPA (100 mg/kg) e composto isolado (3 mg/kg) exibiram atividade antihiperalgésica no modelo de hiperalgesia mecânica induzida por aplicação de carragenina em camundongos. *In vitro* o EEPA exibiu atividade vasorrelaxante em aorta torácica isolada de ratos e, *in vivo,* reduziu a PAM de ratos SHR em cerca de 70%. Conclui-se que EEPA demonstra efeito anti-inflamatório, antihiperalgésico e hipotensor, sendo que estas duas primeiras atividades podem ser atribuídas parcialmente a N-[7-(3',4'-metilenodioxifenil)-2(Z),4(Z)-heptadienoil] pirrolidina, apoiando o uso terapêutico dessa planta pela medicina popular.

Palavras-chave: *Piper amalago;* inflamação; hiperalgesia; hipotensão; ratos; camundongos.

Abstract

Piper amalago L. is popularly known as "pariparoba" and has been used in folk medicine as an anti-inflammatory agent, however, there is only a study that showed the topical anti-inflammatory activity for such popular use. Other species from *Piper* also have anti-hiperalgesic and hypotension effect. Therefore, this study aimed to evaluate the anti-inflammatory and analgesic effects of *P. amalago* ethanolic extract (EEPA) and its isolated compound N-[7-(3′,4′-Methylenedioxyphenyl)-2(Z),4(Z)-heptadienoyl]pyrrolidine in experimental models of inflammation (paw edema and pleurisy induced by carrageenan injection), hyperalgesy (electronic von Frey), vasorelaxation (*in vitro)* and mean arterial pressure (MAP - *in vivo),* in rodents. EEPA orally administered at doses of 30 and 100 mg/kg significantly decreased paw edema. In pleurisy 100 mg/kg dose significantly decreased the total number of leukocytes in pleural lavage and protein extravasation both induced by carrageenan. N-[7-(3′,4′-Methylenedioxyphenyl)-2(Z),4(Z)-heptadienoyl]pyrrolidine was identified and isolated from EEPA. Oral administration of N-[7-(3′,4′-Methylenedioxyphenyl)-2(Z),4(Z)-heptadienoyl] pyrrolidine in doses of 1 and 3 mg/kg significantly decreased paw edema, total leukocytes counted in pleural lavage and protein quantification. Both EEPA (100 mg/kg) and its isolated compound (3 mg/kg) exhibited antihyperalgesic effect in the model of mechanical hyperalgesia induced by carrageenan injection in mice. *In vitro* EEPA exhibited vasorelaxant activity in thoracic aorta isolated from rats and *in vivo* EEPA reduced MAP to about 70% in SHR. We concluded that EEPA shows anti-inflammatory, analgesic and hypotensive properties. These two first effects are due to the presence of, at least in part, N-[7 - (3 ', 4'-methylenedioxyphenyl) -2 (Z) 4 (Z)-heptadienoyl] pyrrolidine, supporting the popular use of this plant in folk medicine.

Keywords: *Piper amalago;* inflammation; hyperalgesia; rats; hypotension; mice.

1 INTRODUÇÃO

Há uma necessidade de busca por substâncias ativas para o controle da inflamação e dor em várias doenças, uma vez que os atuais fármacos de referência, sejam eles anti-inflamatórios esteroides (AIES) ou não-esteroides (AINES), apresentam importantes e significativos efeitos colaterais devido a predisposição individual ou uso contínuo. Devido aos perfis de efeitos colaterais significativos desses medicamentos, existe atualmente um maior interesse em compostos naturais, como suplemento dietético e produtos naturais à base de plantas medicinais, que têm sido utilizados durante séculos como um meio de reduzir a dor e a inflamação [1]. Muitos compostos naturais funcionam através da inibição das vias inflamatórias de uma maneira similar aos AINES. Além das vias das ciclooxigenases (COXs), diversos compostos naturais atuam também inibindo a via associada à ativação do fator nuclear kappa-B (NF-kB), tendo como principais exemplos o chá verde (*Camellia sinensis*) e a curcumina (*Curcuma longa*) [2]. Além disso, os medicamentos à base de plantas estão se tornando cada vez mais populares em virtude dos efeitos colaterais geralmente reduzidos [3, 4].

Uma planta promissora para o controle da inflamação é *Piper amalago (Piperaceae)*. O gênero *Piper* inclui um vasto número de espécies conhecidas popularmente por Pariparoba e têm sido utilizados para a produção de óleos essenciais na indústria farmacêutica e também para a indústria de inseticidas [5, 6]. A *Piper* encontra-se distribuída nos dois hemisférios, em regiões tropicais e subtropicais, sendo conhecidas cerca de 700 espécies diferentes [7]. A *P. amalago* é nativa do México e é popularmente utilizada para o tratamento de dores estomacais e também utilizada como um anti-inflamatório [8].

Algumas plantas do gênero *Piper* são utilizadas pela medicina popular do Brasil objetivando a redução da pressão arterial e estudos científicos demonstraram tal efeito biológico utilizando-se extrato de *P. truncatum* e também alcaloide piperina extraído de *P. nigrum* e *P. officinarum [9]*.

Existem raros estudos envolvendo a *P. amalago*, sobretudo na inflamação e, desta forma, o presente estudo teve como objetivo a avaliação da atividade anti-inflamatória, antihiperalgésica e hipotensora do extrato bruto e do composto isolado N-[7-(3',4'-metilenodioxifenil)-2(Z),4(Z)-heptadieno]pirrolidina de *Piper amalago* L. em roedores.

2 REVISÃO DA LITERATURA

2.1 Inflamação

O processo inflamatório é um fenômeno complexo ativado durante alterações da homeostase, como infecção, lesão e exposição a contaminantes, sendo desencadeada por receptores da imunidade inata que reconhecem patógenos e células danificadas. A inflamação, portanto, é um processo protetor que objetiva eliminar a causa inicial da lesão e também as células e tecidos necróticos resultantes. A inflamação pode desempenhar sua função protetora destruindo, neutralizando ou até mesmo diluindo os agentes nocivos [10].

As reações inflamatórias ocorrem em três fases temporais, sendo: 1) fase aguda, havendo vasodilatação local transitória e também aumento da permeabilidade capilar; 2) fase subaguda, havendo migração de leucócitos e células fagocitárias; e 3) fase proliferativa crônica, onde há degeneração e fibrose do tecido [11].

Classicamente, a inflamação é constituída pelos seguintes sinais e sintomas: calor, rubor, edema e dor. Tais sinais foram descritos por Cornelius Celsus, um enciclopedista romano, em seu Livro III 'De Medicina': "*Notae vero inflammationis sunt quattuor: rubor et tumour cum calore et dolore"*. (Que significa: Agora, os sinais de uma inflamação são quatro: vermelhidão e inchaço com calor e dor) [12]. Rudolf Virchow mais tarde acrescentou o quinto sinal "functio laesa", ou seja, a perda da função [13] (Fig. 1).

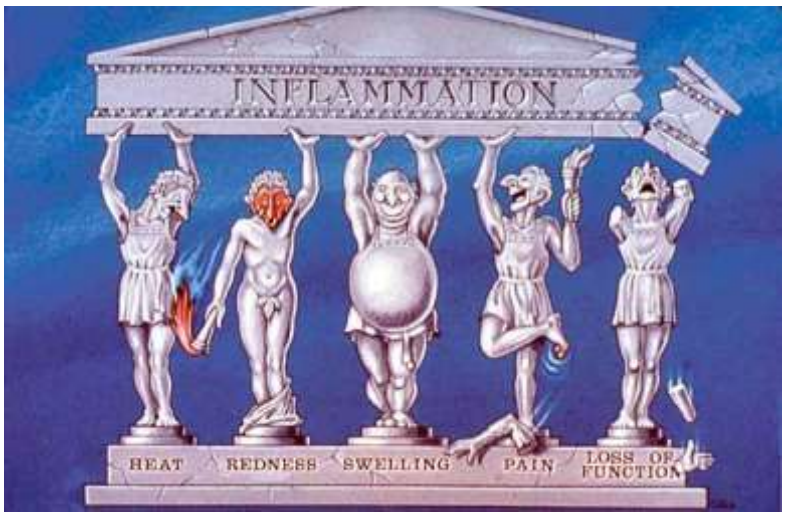

Figura 1 – Os 4 sinais cardinais da inflamação segundo Celsus (30 A.C. – 38 D.C.) e quinto sinal introduzido posteriormente por Virchow, no século XIX. **Fonte: [14].**

Calor e rubor são decorrentes do aumento do fluxo sanguíneo para área inflamada do corpo enquanto que o edema é devido ao acúmulo de líquido. A dor ocorre porque há liberação de substâncias químicas que estimulam terminações nervosas que ativam ou sensibilizam as terminações nervosas sensoriais no local inflamado. Por fim, a perda da função tem múltiplas causas e é caracterizada pelo impedimento da função fisiológica do local lesionado [15].

Existem diversos tipos de mediadores inflamatórios. Eles podem ser liberados por vários tipos celulares durante a lesão tecidual, química ou traumática, desempenhando diversas funções. Por exemplo, i) as prostaglandinas (PGs) induzem febre, inflamação e dor ao ativar respectivos receptores; ii) Os tromboxanos regulam o tônus do vaso sanguíneo, a agregação plaquetária e a formação de coágulos, visando o aumento da resposta inflamatória; iii) Os leucotrienos participam na formação do infiltrado de leucócitos e a subsequente formação de mediadores inflamatórios, além de promover o aumento da permeabilidade vascular, induzir a liberação de enzimas lisossômicas e estimular a produção de citocinas inflamatórias como, por exemplo, Fator de Necrose Tumoral (TNF), Interleucina-1 (IL-1) e Interleucina-6 (IL-6) [16-18].

Em relação às citocinas, o TNF, a IL-1 e a IL-6 desempenham papéis importantes como citocinas pró-inflamatórias, mediando a inflamação local e ativando outras células inflamatórias como os neutrófilos, monócitos e macrófagos. Há pelo menos 29 diferentes tipos de citocinas de baixo peso molecular que são secretadas por leucócitos ativados que, por sua vez, são os responsáveis por desencadear a resposta de fase aguda da inflamação, fase esta caracterizada por febre, leucocitose, aumento da secreção de hormônios adreno-corticotrópicos e produção de proteínas de fase aguda. As proteínas de fase aguda são produzidas no fígado sob a influência de citocinas e são translocadas pelo fluxo de sangue até o local da inflamação, onde irá eliminar os micro-organismos patogênicos por opsonização e também pela ativação das vias do sistema complemento. As três proteínas mais conhecidas de fase aguda são: proteína C-reativa, soro amiloide A e haptoglobina [10].

A inflamação pode ser classificada em aguda ou crônica. A inflamação aguda é uma resposta de curto prazo, que geralmente resulta na cura, ou seja, é autolimitada

provavelmente pelo fenômeno de auto-resolução do processo inflamatório. Este tipo de inflamação é caracterizado pela formação de infiltrado de leucócitos na região inflamada, removendo o estímulo, o que culmina na reparação do tecido. Diferentemente, a inflamação crônica já é caracterizada como sendo uma resposta prolongada, desregulada e inadequada que envolve a inflamação ativa, destruições teciduais e tentativas de reparação tecidual. Essa inflamação crônica, ou seja, persistente, é associada a diversas doenças como alergia, aterosclerose, artrite e doenças auto-imunes [19-21].

Estudos têm indicado que, nas últimas três décadas, houve aumento drástico na quantidade de pessoas no mundo que sofrem de doenças crônicas, como doenças cardiovasculares, diabetes, doenças respiratórias, doenças mentais, doenças auto-imunes e câncer. O crescimento das taxas de doenças sistêmicas crônicas sugere que a inflamação causada por excessiva e inadequada atividade do sistema imune inato, é incapaz de responder adequadamente aos sinais de perigo, o que culmina na ativação inflamatória crônica ou não resolvida do organismo [22-24]. Tumor tem sido associado à inflamação desde 1863, quando Rudolf Virchow descobriu leucócitos em tecidos neoplásicos e fez a primeira ligação entre a inflamação e câncer [25]. As respostas inflamatórias desempenham papéis decisivos em diferentes estágios de desenvolvimento do tumor, incluindo a iniciação, promoção e transformação maligna, invasão e metástase. A inflamação também afeta a vigilância imunológica e respostas à terapia [26].

Existem descritas na literatura científica, as famílias de mediadores químicos locais da classe dos resolutores do processo inflamatório denominadas resolvinas, protectinas e neuroprotectinas que são derivadas de ácidos graxos essenciais (ômega). Tais mediadores químicos são agonistas estereosseletivos potentes responsáveis pelo controle da duração e também da magnitude da inflamação. Já as lipoxinas, substâncias derivadas do ácido araquidônico e biossintetizadas principalmente nas mucosas e vasos sanguíneos, são responsáveis por diversas ações pró-resolução: participam na redução da quantidade de neutrófilos infiltrados no local inflamado, na diminuição da produção de superóxido e citocinas, ativam células fagocitárias e apoptóticas. As lipoxinas também exibem efeito antinociceptivo direto [27, 28].

2.1.1 Dor e Inflamação

Como dito anteriormente, uma das características fundamentais de estados inflamatórios é que os estímulos normalmente inócuos produzem dor [29].

Alguns parâmetros são utilizados para identificar processos associados com a dor. São exemplos a nocicepção, percepção da dor e uma série de conseqüências secundárias, incluindo sofrimento e comportamento de dor. Nesse contexto, a nocicepção pode ser definida como a detecção de estímulos nocivos e a subsequente transmissão de informação codificada ao cérebro. Em contraste, a dor é um processo essencialmente perceptual que surge em resposta frente a um estímulo [30].

Como alguns tipos de dor pode-se citar: i) dor nociceptiva onde há responsividade dolorosa a um estímulo nocivo de frio, calor ou mecânico que causa dor; ii) dor inflamatória que está associada a danos nos tecidos e a infiltração de células imunitárias, iii) e dor patológica que é comumente associada aos danos no sistema nervoso - dor neuropática - ou pelo seu funcionamento alterado - fibromialgia, síndrome do intestino irritável, cefaléia do tipo tensional [31].

Os estímulos nocivos que evocam dor são detectados pelas terminações nervosas de neurônios sensoriais primários (nociceptores), cujos corpos celulares são encontrados nos gânglios sensoriais, tais como gânglios da raiz dorsal (DRG). Sob a forma de potenciais de ação, o sinal é então transmitido ao longo das fibras nervosas sensoriais primárias até o corno dorsal da medula espinhal e, então, para alguns receptores localizados no cérebro, onde finalmente é interpretado como dor. Os nociceptores podem ser ativados por estímulos mecânicos, químicos e térmicos de alta ou baixa intensidade, sendo uma característica em comum o fato de que o fenomeno de sensibilização, com redução do limiar de ativação pode ser induzido através dos mediadores inflamatórios, tais como prostaglandinas (PGs), bradicinina, adenosina trifosfato (ATP), fator de crescimento do nervo (NGF), que são substâncias liberadas durante a lesão de tecidos, estresse metabólico e também no processo inflamatório. Os efeitos dos mediadores inflamatórios sobre os nociceptores são comumente decorrentes da alteração da sensibilidade dos canais iônicos de membranas nas terminações nervosas [32-36].

2.2 Hipertensão Arterial (HA)

A HA é definida como sendo a elevação da pressão arterial além de 140/90 mmHg e está fortemente correlacionada com eventos como acidente vascular cerebral (AVC),

doença cardíaca isquêmica, insuficiência cardíaca e doença renal. Trata-se de uma doença que afeta uma grande população heterogênea de pacientes que somará por volta de 1.6 bilhões no ano de 2025 [37].

O Sistema Nervoso Autônomo (SNA) desempenha um papel central na manutenção da homeostase cardiovascular através dos sinais de pressão, volume e quimiorreceptores. Tal controle acontece através da modificação da vasculatura periférica e da função dos rins, o que modifica os parâmetros de débito cardíaco, resistência vascular e de retenção de líquidos. Anormalidades no SNA como o excesso de atividade do Sistema Nervoso Simpático (SNP) resultam no aumento da pressão sanguínea, contribuindo com o desenvolvimento da HA [38, 39].

Outro componente importante para o controle da homeostase hemodinâmica é o Sistema Renina-Angiotensina-Aldosterona (S-RAA). No S-RAA circulante, o angiotensinogênio é produzido pelo fígado e a renina, que é liberada pelas células justaglomerulares dos rins, hidrolisa o angiotensinogênio em angiotensinogênio I. O angiotensinogênio I é então clivado pela Enzima Conversora de Angiotensinogênio (ECA) localizada no endotélio vascular da circulação pulmonar, produzindo angiotensinogênio II, que é o peptídeo mais vasoativo e possui uma atividade constritora potente sobre todos os vasos sanguíneos [40].

O endotélio de vasos sanguíneos é responsável pela produção de uma variedade de substâncias capazes de modificar os parâmetros da circulação sanguínea. Dentre essas substâncias, destaca-se o óxido nítrico (NO). O NO é biosintetizado a partir da L-arginina/oxigênio/NADPH pelas enzimas Óxido Nítrico Sintases (NOS) e secretado pelo endotélio vascular [41].

A ação do NO pode ser direta ou indireta. A ação direta é mediada pela ativação da Guanilil Ciclase Solúvel (GCs) e com a produção do segundo mensageiro Monofosfato Cíclico de Guanosina (GMPc) ou ativação direta dos canais iônicos. A ação direta é geralmente mediada pela produção de espécies reativas de oxigênio (EROs). O NO liberado pelas células se liga ao Fe^{2+} do grupamento heme da GCs, ativando esta enzima. A GCs transforma a Guanosina Trifosfato (GTP) em GMPc através da ativação das proteínas quinases dependentes de GMPc (PKG). Estas últimas catalizam a fosforilação de diferentes proteínas, podendo também ativar ou inibir alguns canais iônicos e regular a atividade das fosfodiesterases (PDEs). Todos esses processos podem resultar em diferentes respostas

biológicas no organismo, como relaxamento da musculatura lisa, alteração da permeabilidade endotelial, entre outros [42].

2.3 Farmacologia dos Anti-inflamatórios

2.3.1 Anti-inflamatório Não-Esteroide (AINE)

Os AINEs são uma classe de medicamentos mais comumente utilizados no tratamento de doenças, uma vez que são eficazes no tratamento da dor, febre (rubor) e edema, sinais que surgem como consequência da liberação dos mediadores inflamatórios [43].

Tanto os AINEs tradicionais não-seletivos quanto os da subclasse dos inibidores seletivos da ciclo-oxigenase tipo 2 (COX-2), são anti-inflamatórios, antipiréticos e analgésicos. A exceção é o paracetamol, que é antipirético e analgésico, mas praticamente desprovida de atividade anti-inflamatória. Os AINEs atuam principalmente por inibição das enzimas das ciclo-oxigenases (COX-1 e COX-2) que catalisam o primeiro passo na biossíntese de prostaglandinas (PGs) e tromboxano (TX) a partir do ácido araquidônico. Isto leva à diminuição da síntese de PGs resultando em efeitos benéficos e também em efeitos indesejados [44] [11].

Os AINEs reduzem a dor e inflamação através do bloqueio das COXs, enzimas essas que são necessárias na produção de PGs. A maioria dos AINEs disponíveis atuam bloqueando a COX-1 e COX-2 enquanto os inibidores seletivos de COX-2 tem inibição predominante da atividade da COX2. A COX-2 é encontrada nas articulações e nos músculos, contribuindo com o desenvolvimento da dor e inflamação [45].

A utilização de AINEs não seletivos está ligada ao desenvolvimento de hemorragia digestiva. Isso porque tal classe de fármacos atua bloqueando além da enzima COX-2, também a enzima COX-1 conhecida como sendo “constitutiva”, presente no organismo humano e desempenhando funções fisiológicas importantes como, por exemplo, de proteção estomacal à ação do suco gástrico (ácido clorídrico). Nos Estados Unidos, estima-se que as complicações decorrentes do uso dos AINEs possam ser a causa de cerca de 6 óbitos a cada 100.000 habitantes que é uma taxa de mortalidade significativa [43].

Já a utilização de inibidores COX-2 seletivo está ligada ao desenvolvimento de eventos cardiovasculares como infarto do miocárdio e acidente vascular cerebral (AVC). A

causa do desenvolvimento dos problemas cardiovasculares se tornou e continua sendo um objeto de estudo. No ano de 2012, os resultados de uma pesquisa convergiram para a hipótese de que a inibição de enzimas COX-2 nos vasos sanguíneos leva a um desbalanço entre a prostaciclina e o tromboxano, onde há decréscimo relativo na produção de prostaciclina e a elevação de tromboxano. A prostaciclina está correlacionada com a agregação plaquetária e também a vasoconstrição, ou seja, a inibição da COX-2 poderia resultar em excesso de formação de coágulo e elevação da pressão arterial sistêmica [46, 47].

2.3.2 Anti-inflamatório Esteroide (AIE)

Os AIEs, também chamados de glicocorticoides, são altamente eficazes no controle da inflamação, mas seu uso torna-se limitado, devido aos seus efeitos indesejáveis. Os glicocorticoides são imunossupressores, atuando de forma a restringir a proliferação clonal de células T *helper* (Th), através da redução da transcrição do gene da interleucina-2 (IL-2). No entanto, os glicocorticoides também diminuem a transcrição de muitos outros genes de citocinas (como TNF, interferon-γ (IFN-γ), IL-1, entre outras interleucinas) tanto na fase de indução quanto na fase efetora da resposta imune. A síntese e liberação de proteínas anti-inflamatórias (como a anexina-1, inibidores de proteases, etc.) também se encontram aumentadas. Todos esses efeitos sobre a transcrição são mediados através da inibição de fatores de transcrição que produzem agentes inflamatórios (por exemplo, PA-1 e NF-kB) e através da ativação de fatores de transcrição de agentes anti-inflamatórios [48]. Efeitos indesejados ocorrem com doses elevadas ou devido à administração prolongada. Alguns dos principais efeitos colaterais são: supressão da resposta do organismo frente à infecção ou lesão; síndrome de *Cushing* (hipercortisolismo ou hiperadrenocorticismo); osteoporose, uma vez que os glicocorticoides suprimem a absorção intestinal de Ca^{2+}, inibem a formação de osso e diminui a síntese de hormônio sexual; hiperglicemia produzida por glicocorticoides exógenos, com possibilidade de desenvolvimento do diabetes; perda de massa e força muscular; efeitos sobre o SNC como psicose, depressão e euforia; glaucoma; aumento de pressão intracraniana; e em crianças, inibição de crescimento [44].

O efeito anti-inflamatório dos glicocorticóides inicia-se com sua ligação a receptores específicos no citoplasma de células alvo. O complexo formado “receptor-

esteroide" então migra para o núcleo, onde se liga ao DNA e altera a síntese genética das proteínas. Essa alteração atinge inúmeras funções celulares, incluindo as enzimas que regulam processos metabólicos e a síntese de citocinas inflamatórias [49].

Os glicocorticoides são amplamente utilizados no tratamento de várias doenças de caráter inflamatório ou não. Ao contrário dos AINEs esses agentes não aliviam a dor, mas reduzem a inflamação através da inibição do funcionamento dos leucócitos. No entanto, para se obter uma ação terapêutica, os glicocorticoides necessitam ser administrados a níveis supra-fisiológicos, sendo inevitáveis as reações adversas por exemplo a supressão da medula adrenal e não produção de corticoide endógeno. Muitos destes efeitos adversos podem ser evitados administrando-se glicocorticoide por via tópica, fato que abriu caminho para o desenvolvimento de glicocorticoides inalatórios para o tratamento de doenças inflamatórias do sistema respiratório e também cremes contendo esteroides para o tratamento da inflamação da pele. No entanto, para o tratamento de artrite reumatoide implicaria a utilização de injeção intra-articular da substância. Ou seja, existe uma necessidade de um medicamento que proporcione alívio dos sintomas de inflamação, mas que possa ser administrada sistemicamente e com uma quantidade menor possível de reações adversas [50].

2.4 Farmacologia dos Anti-hipertensivos

As principais classes de medicamentos utilizados para tratamento da hipertensão incluem os diuréticos, β -bloqueadores, inibidores da Enzima Conversora de Angiotensina (IECA), bloqueadores de canais de cálcio e bloqueadores do receptor de angiotensina II [51].

Quimicamente, os diuréticos formam um grupo heterogêneo de substâncias que estimulam os inibem uma variedade de hormônios naturalmente presentes no organismo humano que regulam a produção de urina pelos rins. Com exceção do manitol e dos antagonistas de receptor da vasopressina, todos os diuréticos exercem sua função inicialmente bloqueando a reabsorção de sódio dentro dos túbulos renais [52]

Os β -bloqueadores bloqueiam a ação de catecolaminas endógenas (noradrenalina e adrenalina) sobre os receptores β-adrenérgicos. Os receptores beta-adrenérgicos são encontrados no coração (receptores β1) e no músculo liso (vasos sanguíneos, pulmões e outros órgãos - receptores β2). A ativação desses receptores resulta em inotropismo e

cronotropismo positivos, pela contração das artérias e, consequentemente, aumenta a resistência vascular periférica. Da mesma forma, quando esses receptores são bloqueados, a frequência cardíaca diminui (cronotropismo negativo) e há queda da pressão arterial. Estudos recentes mostram que o tratamento de pacientes com o uso de β-bloqueadores leva a uma redução modesta de doenças cardiovasculares e efeitos insignificantes sobre a mortalidade desses pacientes [53].

Os IECA são os fármacos que bloqueiam a produção de angiotensina II pelo corpo. A angiotensina II é um hormônio circulante no sangue e promove a constrição de vasos sanguíneos, o que leva ao aumento da resistência vascular periférica e, portanto, ao aumento da pressão sanguínea. Desta forma, utilizando-se um medicamento da classes dos IECA, não haverá produção de angiotensina II e, consequentemente, não haverá vasoconstrição promovida por este hormônio [54].

Os bloqueadores de receptor de angiotensina II são antagonistas de receptor AT_1, ou seja, tais fármacos bloqueiam a ação da angiotensina II no seu receptor. Esse bloqueio dos receptores AT_1 resulta em vasodilatação direta, reduz a secreção de vasopressina e reduz tanto a produção quanto a secreção de aldosterona, efeitos que culminam na redução da pressão sanguínea [55].

Os bloqueadores de canais de cálcio atuam inibindo o movimento dos íons de cálcio através da membrana celular pelo bloqueio dos canais de iônicos de cálcio do tipo L. Este bloqueio reduz tanto a contração do músculo liso e do músculo cardíaco quanto das células do nodos sinoatrial (SA) e atrioventricular (AV). As principais ações dos destes fármacos incluem dilatação da vasculatura arterial coronariana e periférica, inotropismo negativo, cronotropismo negativo e diminuição da condução AV [56].

2.5 Plantas Medicinais

Desde o início da existência humana, diferentes culturas e populações têm utilizado as plantas como um meio de cura ou alívio para diversos tipos de doenças [57].

A OMS - Organização Mundial da Saúde [58] define as plantas medicinais como sendo aquelas que contêm propriedades ou compostos que podem ser utilizados para fins terapêuticos ou mesmo aqueles que sintetizam metabólitos para produzir medicamentos úteis.

As plantas medicinais são importantes para a pesquisa farmacológica e também para o desenvolvimento de novos medicamentos, não só quando constituintes das plantas são usados diretamente como agentes terapêuticos, mas também como material para a síntese de medicamentos, ou até mesmo como protótipos para a síntese de compostos farmacologicamente ativos [59].

Em alguns países da África e da Ásia, 80% da população dependem da medicina tradicional para cuidados primários em saúde. Entre os países desenvolvidos, de 70% a 80% da população tem usado algum tipo de medicamento alternativo ou complementar. Os tratamentos à base de plantas são a forma mais popular de medicina tradicional, e é um negócio altamente lucrativo no mercado internacional, por exemplo, na Europa Ocidental o faturamento atingiu 5 bilhões de dólares durante o período 2003-2004. Na China, as vendas de produtos totalizaram 14 bilhões de dólares em 2005. No Brasil, o faturamento com a fitoterapia somou 160 milhões de dólares no ano de 2007 [60].

Doenças infecciosas e crônicas podem ser tratadas utilizando-se da medicina tradicional como prática alternativa e complementar no Brasil. Temos, por exemplo, os antimaláricos que foram desenvolvidos a partir da descoberta e isolamento de artemisinina, uma substância componente de *Artemisia annua* L., uma planta usada na China por quase 2000 anos [60].

A ciência têm explorado os produtos naturais como fontes de novas estruturas químicas farmacológicas. Cerca de 50% dos medicamentos aprovados durante os últimos 30 anos foram, de forma direta ou indireta, obtidos a partir de produtos naturais [61].

É notável o crescimento da química sintética como um meio de descoberta e fabricação de medicamentos, contudo, a contribuição de plantas para o tratamento e prevenção de doenças ainda é significativo. Atualmente, 11% dos 252 medicamentos considerados básicos e essenciais pela OMS são de origem vegetal [62].

2.6 *Piper amalago* L.

2.6.1 Aspectos Botânicos e Etnofarmacológicos

A família Piperaceae é constituída de uma diversidade de plantas que são utilizadas na medicina popular. Essa família possui 5 gêneros e 1400 espécies. O gênero *Piper* é o mais representativo, com mais de 700 espécies encontradas em todo o mundo [63].

O gênero *Piper* (Piperaceae) foi recentemente revisado e incluem cerca de 700 espécies, representadas por ervas, arbustos e árvores, sendo que 170 são nativas do Brasil. Encontra-se distribuída nos dois hemisférios, em regiões tropicais e subtropicais [7]. A *Piper amalago* L. (Fig. 2) é nativa do México, é popularmente conhecida pelo nome "pariparoba". É utilizado na medicina popular para o tratamento de dores estomacais e também como um anti-inflamatório [8].

Figura 2 – Arbusto de *Piper amalago* L. **Fonte:** http://www.backyardnature.net/yucatan/piper-am.htm. Acesso em: 05 Jun. de 2013.

2.6.2 Descrição Fitoquímica

Muitas espécies de *Piper* são aromáticas e, como consequência, a composição química dos óleos essenciais de várias espécies foi estudada em detalhe. Tais estudos revelaram uma diversidade de substâncias constituindo o óleo essencial, como monoterpenos, sesquiterpenos, aldeídos, cetonas, arilpropanóides, fenilpropanóides e álcoois de cadeia longa [64-67]. A análise fitoquímica das folhas sugere presença de óleo essencial, amidas, flavonoides e compostos fenólicos [68].

As raízes de *P. amalago* L. foram estudadas quimicamente e foram identificadas a presença de sesquiterpenos, pirrolidina e isobutilamidas [69-74].

2.6.3 Estudos Biológicos

Os estudos farmacológicos envolvendo a espécie *P. amalago* são escassos. Não existem estudos avaliando *P. amalago* e pressão arterial e encontra-se na literatura científica internacional apenas um estudo [75] relacionando *P. amalago* e inflamação. Todavia, nesse estudo, o extrato foi produzido utilizando-se hexano, clorofórmio e metanol, solventes diferentes do que foi empregado para a produção do extrato no presente trabalho. Além disso, o efeito anti-inflamatório foi avaliado apenas de forma tópica, utilizando-se o modelo experimental de edema de orelha induzido pela aplicação de óleo de cróton, em camundongos. Entretanto, o presente trabalho avaliou o composto químico isolado da planta na inflamação, buscando identificar o composto responsável, pelo menos em parte, pela atividade farmacológica.

Os estudos farmacológicos publicados envolvem outras espécies do gênero *Piper* e tais pesquisas demonstraram efeito esquistossomicida *in vitro* [76], efeito ansiolítico, ausência de efeito mutagênico ou genotóxico [68], atividade acaricida [67], inseticida [77], antifúngica [78], antimalárica [79], antidiabética e antihiperlipidémica [80], tripanocida [81], efeito anti-inflamatório para neuroinflamação [82], anti-ulceroso [83], antituberculoso [84], anti-HBV [85], entre muitos outros.

A substância N-[7-(3',4'-metilenodioxifenil)-2(Z),4(Z)-heptadieno]pirrolidina foi ainda menos estudada. Existem alguns estudos avaliando sua atividade anti-fúngica [86], anti-leishmania [87], anti-esquistossomose [76].

3 OBJETIVOS

Objetivo Geral

Avaliar o efeito anti-inflamatório, anti-hiperalgésico e hipotensor do extrato bruto e composto isolado de *Piper amalago* em modelos de inflamação aguda, hiperalgesia e vasorelaxamento, através do emprego de técnicas farmacológicas e avaliações bioquímicas.

Objetivos Específicos

Avaliar o efeito anti-inflamatório do EEPA e de N-[7-(3',4'-metilenodioxifenil)-2(Z),4(Z)-heptadieno]pirrolidina em modelo de edema de pata induzido por aplicação de carragenina, em camundongos.

Avaliar o efeito anti-inflamatório do EEPA e de N-[7-(3',4'-metilenodioxifenil)-2(Z),4(Z)-heptadieno]pirrolidina em modelo de pleurisia induzida por aplicação de carragenina, em camundongos, através da contagem de leucócitos totais e dosagem de proteínas.

Avaliar o efeito do EEPA e de N-[7-(3',4'-metilenodioxifenil)-2(Z),4(Z)-heptadieno]pirrolidina sobre a alodinia mecânica induzida por aplicação de carragenina, em camundongos.

Avaliar *in vitro* o efeito vasorrelaxante do EEPA sobre as aortas torácicas isoladas de ratos.

Avaliar *in vivo* o efeito hipotensor do EEPA no modelo de pressão arterial média (PAM) em ratos.

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5 ARTIGOS CIENTÍFICOS

5.1 Artigo Científico 1 – Journal of Inflammation

ANTI-INFLAMMATORY AND ANTI-HYPERALGESIC ACTIVITY OF ETHANOLIC EXTRACT AND N-[7-(3',4'-METHYLENEDIOXYPHENYL)-2(Z),4(Z)HEPTADIENOYL]PYRROLIDINE FROM *Piper amalago* L. (PIPERACEAE) IN MICE

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ABSTRACT

Piper amalago L. is popularly known as "pariparoba" and has been used in folk medicine as an anti-inflammatory agent, however there is few studies to support this popular use. In this way, our study aimed to evaluate the anti-inflammatory and antihyperalgesic effect of ethanolic extract of *Piper amalago* (EEPA) and its isolated compound N-[7-(3′,4′-Methylenedioxyphenyl)-2(Z),4(Z)-heptadienoyl]pyrrolidine in experimental models of inflammation (paw oedema and pleurisy induced by carrageenan injection) and nociception (Electronic Von Frey), performed in mice. The oral administration of EEPA at doses of 30 and 100 mg/kg significantly decreased paw oedema formation, while in pleurisy the dose of 100 mg/kg significantly decrease the total leucocytes number in pleural lavage and also the protein leakage. The compound N-[7-(3′,4′-Methylenedioxyphenyl)-2(Z),4(Z)-heptadienoyl]pyrrolidine was identified by phytochemical studies and isolated from EEPA. The oral administration of N-[7-(3′,4′-Methylenedioxyphenyl)-2(Z),4(Z)-heptadienoyl]pyrrolidine at doses of 1 and 3 mg/kg significantly decreased the total

leucocytes number in pleural lavage, protein extravasation and paw edema. Both EEPA (100 mg/kg) and isolated compound (3 mg/kg) exhibited antihyperalgesic activity in carrageenan induced mechanical hyperalgesic in mice. In conclusion, the present study showed that EEPA is an anti-inflammatory and analgesic natural agent, supporting, at least in part, the popular use of this plant in folk medicine. The results lead us to conclude that N-[7-(3′,4′-Methylenedioxyphenyl)-2(Z),4(Z)-heptadienoyl]pyrrolidine is a compound involved in the anti-inflammatory and antihyperalgesic properties of EEPA.

Keywords: *Piper amalago;* inflammation; hyperalgesia; mice.

Background

Inflammation takes part in the non-specific immune response that occurs in reaction noxious agents such as irritants, pathogens, or damaged cells. The signs of inflammation (heat, redness, swelling, pain and loss of function) can be explained by vasodilatation, increased blood flow, elevated cellular metabolism, extravasation of fluids, release and production of soluble mediators and cellular influx [1, 2].

Inflammatory acute response is a protective against harmful agents; however the use of substances and drugs with anti-inflammatory effects can be a helpful tool in the therapeutic treatment of the several diseases, eliminating or reducing the symptoms from inflammation. Non-steroidal anti-inflammatory drugs (NSAIDs) have analgesic, anti-inflammatory and antipyretic activity [3].

In the other hand, the chronic use of NSAIDs, including selective COX-2 inhibitors (coxibs) and traditional non-steroidal anti-inflammatory drugs (tNSAIDs), is connected to

the increased risk in the development of peptic ulcers [4] and vascular problems [5, 6].

The side effects associated with synthetic drugs are a great problem that increases the interest in traditional systems of medicine [7]. In this way, we investigated the anti-inflammatory and analgesic effect of the ethanolic extract of a medicinal plant used in folk medicine called *Piper amalago* L. popularly known as "pariparoba" and its isolated compound N-[7-(3′,4′-Methylenedioxyphenyl)-2(Z),4(Z)-heptadienoyl] pyrrolidine (IC-1) in two *in vivo* acute models of inflammation – pleurisy and paw oedema induced by carrageenan – and in a *in vivo* model of inflammatory pain also induced by carrageenan application, all in mice.

Methods

Plant Material

The leaves of *Piper amalago* were collected in Dourados – MS - Brazil, in August 2008, and identified by Elsie Franklin Guimarães (Instituto de Pesquisas Jardim Botânico, Rio de Janeiro, RJ, Brazil). A voucher specimen of the species *P. amalago* (DDMS 4410) is deposited in the herbarium of the Universidade Federal da Grande Dourados, MS, Brazil. The species was collected in the following geographical coordinates: *P. amalago* (S22012'42, 9 ", WO54054'55, 6").

Preparation of the ethanol extract

The preparation of the ethanolic extract and isolation of the compound N-[7-(3',4'-Methylenedioxyphenyl)-2 (Z), 4 (Z)-heptadienoyl] pyrrolidine (Figure 1) were previously conducted by our research group [8].

Animals

To the experimental procedures were used adult male Swiss mice (n=5 per group) with approximately weight of 20 g and 50 days age at the beginning of the experiments. These animals were provided from the Federal University of Grande Dourados – UFGD - biotherium. The animals were kept in collective cages (20 animals per cage) under

controlled temperature (23 ± 1 º C) and light cycle (12 h light / dark), treated with water and commercial diet *ad libitum*.

The project of this work was submitted to the Ethics Committee on Animal Use – CEUA/UFGD. It was approved and protocolled with the number 025/2013. These procedures were carried out following the Guide for the Care and Use of Laboratory Animals [9].

Reagents

λ-Carrageenan, phosphate-buffered saline (PBS), Bradford reagent, and dexamethasone were purchased from Sigma-Aldrich ® Co. LLC (St. Louis, MO, USA).

Model of Carrageenan-induced Paw Oedema

Each experimental methodology was conducted in two stages. In the first stage we tested the crude ethanolic extract at 3 different doses and in the second stage we tested the isolated compound, also in 3 different doses.

Animals were divided into experimental groups (n=5 animals/group), with group 1 (control) was treated orally with vehicle (1.0 mL/kg of saline 0.9% saline). Different groups of mice received by oral route increasing doses of the crude ethanolic extract of *P. amalago* (EEPA) (10, 30 and 100 mg/kg) or IC-1 (0.3, 1 and 3 mg) dissolved in 0.9% saline solution. The positive control group received dexamethasone at the dose of 1 mg/kg by subcutaneous route. 1 hour after the treatments, animals received, in the right hind paw, 50 μL of 0.9% saline solution containing 300 μg of carrageenan. The same volume of saline solution was administered in the left hind paws which were used as control. Edema was measured with a paw plethysmometer at times 0.5, 1, 2 and 4 hours after carrageenan application [10-12].

Model of Carrageenan-induced Pleurisy

The EEPA was tested orally at 3 different doses (10, 30, and 100 mg/kg) and the isolated compound IC-1 was tested orally (gavage) at two different doses (1 and 3 mg/kg) one hour before carrageenan injection. Positive control group received 1 mg/kg of

dexamethasone, subcutaneously, 30 minutes before carrageenan application. Pleurisy was induced applying 0.25 mL suspension containing 200 µg of carrageenan (dilution in phosphate buffered saline PBS, pH = 7.4) in the mice pleural cavity according to the technique previously described [13]. Four hours after induction of pleurisy, animals were euthanized and the pleural inflammatory exudate was collected through pleural lavage with 1 mL of sterile saline. The exudate volume was measured, an aliquot of 50 µL was diluted with Turk's solution (1:20). Total leukocytes were counted in a Neubauer chamber, considering the four external quadrants, in a light microscope.

In each ELISA microwell plate were added 10 µL of pleural lavage and 300 µL of Bradford. Protein measurement was performed colorimetrically (TP-photometer Reader / Thermo Plate ®).

Model of Carrageenan induced Hyperalgesia

One day earlier the basal measurement was performed in all animals. Mice were housed in containment boxes (WxDxH 230 x 200 x 180 mm - Insight ®) in a steel mesh with 1 cm diameter spacing for a period of 30 minutes and the digital analgesymeter (Insight ® - EFF 301 - Digital analgesymeter - von Frey) was used in order to determine the similar mean baseline of mechanical stimulus in the right hind paw.

At the following day, each group of male mice (n=6/group) received orally (gavage) 0.9% saline solution or EEPA 100 mg/kg or IC-1 3 mg/kg. One hour after oral treatment, animals received 300 µg of carrageenan injection, subcutaneously (λ-carrageenan - Sigma-Aldrich ®), in the right hind paw. Then, each animal was housed in the same containment boxes under the same steel mesh. The mechanical nociception was measured at the times 3 and 4 hours after carrageenan injection using the digital analgesymeter previously described.

Statistical Analysis

Values are expressed as mean ± standard error of the mean (SEM). Analysis of variance - ANOVA followed by Student-Newman-Keuls post-test were used to evaluate possible differences between the groups. Differences were considered significant with $p<0.05$.

Results

Identification of compound N-[7-(3', 4'-Methylenedioxyphenyl)-2(Z), 4(Z)-heptadienoyl] pyrrolidine (IC-1)

The IC-1 was identified by nuclear magnetic resonance spectroscopy (NMR) (1H, 13C, DEPT, gHSQC and gHMBC). The NMR spectroscopy data were compared with the literature [14-16]. The NMR spectra were obtained on a Bruker Ac 400 spectrometer operating between 400 MHz and 100 MHz (^{1}H and ^{13}C). The experiments were performed in the Department of Chemistry, Polytechnic Center, Federal University of Paraná, Curitiba, PR, Brazil.

^{1}H and ^{13}C NMR data for compound N-[7-(3′,4′-Methylenedioxyphenyl)-2(Z),4(Z)-heptadienoyl]pyrrolidine (IC-1): δ: 1.84–1.95 (m, H2″, H3″; 4H); 2.42–2.46 (m, H6; 2H); 2.64–2.67 (m, H7; 2H); 3.44 (t, *J* = 6.6 Hz, H1″; 2H); 3.52 (t, *J* = 6.6 Hz, H4″; 2H); 5.78 (d, *J* = 10.0 Hz, H2; 1H); 5.91 (s, H1‴; 2H); 5.92–5.99 (m, H5; 1H); 6.38 (t, *J* = 10Hz, H3; 1H); 6.61(dd, *J* = 1.6; 8.0 Hz, H6′; 1H); 6.66 (d, *J* = 1.8 Hz, H2′; 1H); 6.71 (d, *J* = 8.0 Hz, H5′; 1H); 7.33–7.38 (m, H4; 1H). 13C-NMR (CDCl3) δ: 24.37 (C3″); 26.21 (C2″); 34.91 (C6); 35.07 (C7); 45.56 (C1″); 46.90 (C4″); 100.73 (C1‴); 108.14 (C2′); 108.87 (C5′); 118.08 (C2); 121.13 (C6′); 127.87 (C4); 135.42 (C1′); 140.51 (C3); 141.67 (C5); 145.65 (C3′); 147.53 (C4′); 165.66 (C1).

Effects of EEPA and IC-1 against carrageenan induced oedema

The Cg injection in the right hind paw of the animals induced edema, peaking at 2 h (Figure 2C). Oral treatment with EEPA was able to significantly inhibit the edema formation in a dose-dependent manner. The maximum inhibitions were: 0.5 h) 37 ± 4 % (EEPA 30 mg/kg) 46 ± 2 % (EEPA 100 mg/kg) and 67 ± 5 % (dexamethasone 1 mg/kg). 1 h) 22 ± 3 % (EEPA 10 mg/kg), 35 ± 2 % (EEPA 30 mg/kg), 53 ± 2 % (EEPA 100 mg/kg) and 57 ± 10 % (dexamethasone 1 mg/kg). 2 h) 28 ± 2 % (EEPA 10mg/kg), 39 ± 1 % (EEPA 30mg/kg) and 57 ± 1 % (EEPA 100 mg/kg) and 59 ± 4 % (dexamethasone 1 mg/kg). 4 h) 24 ± 1 % (EEPA 10 mg/kg), 35 ± 2 % (EEPA 30 mg/kg), 58 ± 1 % (EEPA 100 mg/kg) and 67 ± 9 % (dexamethasone 1 mg/kg) (Figure 2). The animals treated by

positive control dexamethasone presented a significant reduction in all times points observed (Figure 2).

We can also observe in the graph that administration of IC-1 significantly decreased paw edema in mice at doses of 1 and 3 mg/kg (Figure 3). The maximum inhibitions were: 1 h) 36 ± 6 % (IC-1 1 mg/kg) and 53 ± 6 % (IC-1 3 mg/kg). 2 h) 70 ± 2 % (IC-1 1 mg/kg) and 71 ± 3 % (IC-1 3 mg/kg). 4 h) 52 ± 4 % (IC-1 1 mg/kg) and 61 ± 2 % (IC-1 3 mg/kg) (Figure 3).

The anti-inflammatory activity of EEPA on carrageenan induced leukocyte migration and protein extravasation in pleural cavity

In the pleurisy test, the number of total leukocyte counted in a Neubauer chamber significantly decreased in groups EEPA 30 and 100mg/kg (Figure 4), when compared to the control group. The maximum inhibitions were respectively: 43 ± 2 % and 41 ± 8 % (Figure 4).

There was also a significant decrease in protein dosage of pleural lavage for the group EEPA 100 mg/kg (24 ± 3), compared to the control group (Figure 4). The steroidal anti-inflammatory group had a decrease in leukocyte migration and also in protein extravasation (maximum inhibitions of 42 ± 5 and 32 ± 6, respectively) (Figure 4A and 4B).

The anti-inflammatory activity of N-[7-(3′,4′-Methylenedioxyphenyl)-2(Z),4(Z)-heptadienoyl] pyrrolidine *on carrageenan induced leukocyte migration and protein extravasation in pleural cavity*

In the evaluation of IC-1 in the model of pleurisy, the two tested doses (1 and 3 mg/kg) showed significant effects, decreasing the total leukocyte count in a dose-dependent manner compared to the control group (Figure 5A).

The amount of protein measured in pleural lavage was also significantly lower for groups IC-1 (1 and 3 mg) when compared to the control group (Figure 5B).

The antihyperalgesic activity of EEPA and N-[7-(3′,4′-Methylenedioxyphenyl)-2(Z),4(Z)-heptadienoyl] pyrrolidine *on carrageenan induced leukocyte migration and*

protein extravasation in pleural cavity

Carrageenan injection decreased significantly the basal values of measure by electronic von frey mechanical stimulation. It is possible to see in Figure 6 that the substances tested (EEPA 100 mg/kg and IC-1 3 mg/kg) relieved the pain, so that the animals withstood more strength applied to the von Frey filaments. Maximum inhibitions were: 3 h) 49 ± 10 % (EEPA 100 mg/kg) and 19 ± 3 % (IC-1 3 mg/kg). 4h) 64 ± 5 % (EEPA 100 mg/kg) and 32 ± 10 % (IC-1 3 mg/kg).

Discussion

The results presented in this study revealed for the first time that the ethanolic extract of *P. amalago* exhibited anti-inflammatory and antihyperalgesic activity after oral treatment in mice corroborating with popular use of this species by population. Several compounds could be responsible by EEPA activity therefore this work focus in the potential anti-inflammatory and analgesic activity of compound N-[7-(3′,4′-Methylenedioxyphenyl)-2(Z),4(Z)-heptadienoyl] pyrrolidine. Both EEPA and the cited isolated compound are in lines to exhibit the same activity leading us to report that IC-1 is important, at least in part, by EEPA activity.

One recent review study about the phytochemistry and pharmacology of *Piper* species [17] reported some biological activity of its alkaloids like antirheumatic, diuretic, stimulant, anti-inflammatory, abortifacient, anxiogenic/anxiolytic, antibacterial, antifungal and antidermatophytic. Another study suggested that *P. amalago* acts on the central nervous system (anxiogenic effect) without inducing genetic toxicity [18]. The essential oil of *P. longum* Linn. reduced the paw edema by 65.95% at a dose of 0.5 mL/kg and 72.34% at a dose of 1 mL/kg, when administered orally to rats [19]. In another study [20], the ethanolic extract of *P. interruptum* Opiz. at doses of 300, 600, and 1.200 mg/kg significantly reduced the paw edema in rats. Ethanolic extract of *P. chaba* Linn. also significantly inhibited edema, but only at a dose of 1.200 mg/kg. Previous study with *P. amalago* showed the topical anti-inflammatory activity and the present original study showed, for the first time, that the ethanolic extract of *P. amalago* significantly reduced paw edema by 28% at dose of 10 mg/kg, 39% at dose of 30 mg/kg and 57% at dose of 100 mg/kg. Our study extends and corroborates with anti-inflammatory description of *Piper*

genus in literature showing also that *P. amalago* exhibit this biological properties. Indeed it can be suggested that the ethnopharmacological observations on anti-inflammatory activity may be due to presence of anti-inflammatory compounds in the *P. amalago*.

P. amalago L. roots have been studied phytochemically and results showed the presence of pyrrolidine, sesquiterpenes and isobutylamides [15, 21-25]. The leaves also has been studied [16] and showed that pyrrolidine amides isolated from the leaves of *P. amalago* and their analogs [26] were effective against promastigotes and intracellular amastigotes of *Leishmania amazonensis*. Our study showed the presence of compound N-[7-(3′,4′-Methylenedioxyphenyl)-2(Z),4(Z)-heptadienoyl] pyrrolidine that was chemically isolated from *P. amalago* leaves, characterized by NMR spectroscopy and data were compared to the literature [8]

The *in vivo* model of paw edema induced by carrageenan injection is used to investigate potential anti-inflammatory drugs, evaluating substances in acute inflammation [10]. This model is known to be sensitive to COX inhibitors and it was used before to evaluate the effects of NSAIDs that are responsible for the inhibition of prostaglandin synthesis [27].

It is described in the scientific literature [27] that there is an ordinal temporal release of chemical mediators in paw edema model . The first phase, which lasts until the beginning of the edema after about 3 hours, is characterized by the release of bradykinin, histamine and serotonin. From the third hour until 6 o'clock also happens to have a release of prostaglandins. In the final phase of inflammation there is an acute vascular maximal response with leukocyte migration into the inflamed area [28]. In this context, since inhibition of edema by EEPA and IC-1 occurred after the firsts measurements (0.5 h for EEPA and 1 h for IC-1) we suggest that the mechanism of action these anti-inflammatory activity is linked mainly to the blocking action of chemical mediators such as histamine and serotonin.

The model of pleurisy induced by carrageenan injection is also widely used in the evaluation of anti-inflammatory agents. The number of migrated leukocytes, volume of fluid and IL-1 levels has its peak at 4 h after carrageenan application [29]. Carrageenan is an irritant which triggers a cascade of reactions that result in the influx of cells and fluid into the pleural cavity [30-33]. These influx of cells and fluid is connected, at least in part, to the presence of the complement components, since inhibitors of complement are functional in pleurisy [32]. The study showed that there was a decrease in total leukocyte

migration and proteins dosage for the groups EEPA (100 mg/kg) and CI- 1 (1 and 3 mg/kg). We suggest that the mechanism action of these two substances is similar to the agents that block the complement system.

Von Frey filaments are widely used in pain research [34]. In murine, time measurement of hind-limb withdrawal in response to von Frey filaments [35] is used to evaluate mechanical nociception [36-38]. The filaments are allocated perpendicularly to the plantar surface of the hind paw, and then held in this same position using enough force to cause a slight bend in the filament: abrupt withdrawal of the hind paw from the stimulus, or flinching behaviour immediately following removal of the stimulus are included as positive responses [37].

Some *Piper* species also had been studied in experimental models of nociception. *P. tuberculatum* and its isolated compound 3,4,5-t rimethoxyhydroxycinnamic [39] showed antinociceptive effect. In another study [40], *Piper auritum* K. showed anxiolytic and antinociceptive effect. Other *Piper* species with anti-hypernociception effect include: conocarpan and orientin obtained from *Piper solmsianum* [41], Piper betle [42], *Piper sarmentosum* [43] and *Piper laetispicum* [44].

Our results showed that both EEPA (100 mg/kg) and IC-1 (3 mg/kg) have anti-alloydinic effect when administered orally to mice, with maximum inhibitions of 64 % and 32 %, respectively, at 4h.

Conclusion

We concluded that the ethanolic extract of *P. amalago* L. and IC-1 have anti-inflammatory effect in experimental models of paw edema and pleurisy induced by carrageenan both performed in mice and, therefore, the isolated compound is responsible, at least in part, for this anti-inflammatory activity.

Our study also demonstrates that both EEPA and the IC-1 have antihyperalgesic effect in the carrageenan induced pain.

These findings support the use of this plant as an anti-inflammatory agent in popular medicine and show that it has a potential for the development of a phytomedicine with systemic anti-inflammatory effect. However, studies aiming the investigation of possible toxic effects of its components are needed.

Conflict of interest

No conflict to disclose.

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Legends to Figures

Figure 1 – Chemical structure of compound N-[7-(3', 4'-Methylenedioxyphenyl)-2(Z), 4(Z)-heptadienoyl] pyrrolidine (IC-1).

Figure 2 (A, B, C and D) – Effect of EEPA on carrageenan-induced paw oedema in mice. Animals received vehicle (0.9% saline solution), EEPA (10, 30 or 300 mg/kg, p.o.) or dexamethasone (1 mg/kg, s.c.). After 1 h, carrageenan (300 μg/paw) was injected into the intraplantar surface. The bars express the mean ± SEM of 5-6 animals. Statistical analyze of one-way ANOVA followed by Student-Newman-Keuls post-test were performed to compare differences among the means. ****P*<0.001.

Figure 3 (A, B, C and D) – Effect of N-[7-(3',4'-methylenedioxyphenyl)-2(Z),4(Z)-heptadiene]pyrrolidine on carrageenan-induced paw oedema in mice. Animals received vehicle (0.9% saline solution), N-[7-(3',4'-methylenedioxyphenyl)-2(Z),4(Z)-heptadiene]pyrrolidine (0.3, 1 or 3 mg/kg, p.o.) or dexamethasone (1 mg/kg, s.c.). After 1 h, carrageenan (300 μg/paw) was injected into the intraplantar surface. The bars express the mean ± SEM of 5-6 animals. Statistical analyze of one-way ANOVA followed by Student-Newman-Keuls post-test were performed to compare differences among the means. ***P*<0.01, ****P*<0.001.

Figure 4 (A and B) – Effect of EEPA on carrageenan-induced leukocyte migration and protein levels in mice. Animals received vehicle (0.9% saline solution), EEPA (10, 30 or 100 mg/kg, p.o.) or dexamethasone (1 mg/kg, s.c.). After 1 h, carrageenan (300 μg in 100 μL of sterile saline solution) was injected into the pleural cavity. It was performed a pleural lavage with 1000 μL of saline solution after 4h of carrageenan injection. Graph (A) shows the total leucocyte counted in the four external quadrants of Neubauer chamber

($x10^7$) and graph (B) shows the proteins levels determined in ELISA. Statistical analyze of one-way ANOVA followed by Student-Newman-Keuls post-test were performed to compare differences among the means. ***P*<0.01, ****P*<0.001.

Figure 5 (A and B) – Effect of N-[7-(3',4'-methylenedioxyphenyl)-2(Z),4(Z)-heptadiene]pyrrolidine on carrageenan-induced leukocyte migration and protein levels in mice. Animals received vehicle (0.9% saline solution), IC-1 (0.3, 1 or 3 mg/kg, p.o.) or dexamethasone (1 mg/kg, s.c.). After 1 h, carrageenan (300 μg in 100 μL of sterile saline solution) was injected into the pleural cavity. It was performed a pleural lavage with 1000 μL of saline solution after 4h of carrageenan injection. Graph (A) shows the total leucocyte counted in the four external quadrants of Neubauer chamber ($x10^6$) and graph (B) shows the proteins levels determined in ELISA. Statistical analyze of one-way ANOVA followed by Student-Newman-Keuls post-test were performed to compare differences among the means. **P<0.05,* ***P*<0.01, ****P*<0.001.

Figure 6 (A, B, C and D) - Effect of EEPA and IC-1 on carrageenan-induced pain in mice. Animals received orally vehicle (0.9% saline solution), EEPA (100 mg/kg) or IC-1 (3 mg/kg). After 1 h, carrageenan (300 μg/paw) was injected into the intraplantar surface. Mechanical hyperalgesia was evaluated using a digital analgesymeter – von Frey filaments, at times 3 and 4h after the Cg injection. The bars express the mean ± SEM of 6 animals. Statistical analyze of unpaired student t test were performed to compare difference among the means. **P<0.05,* ***P*<0.01, ****P*<0.001.

Figures

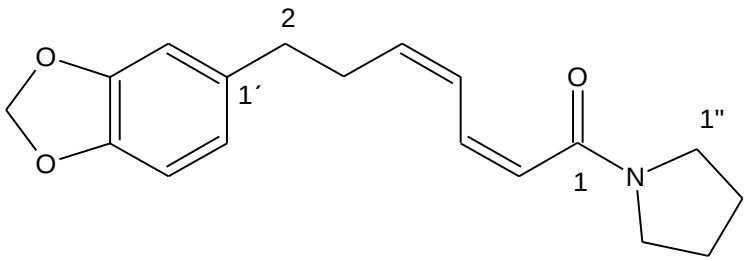
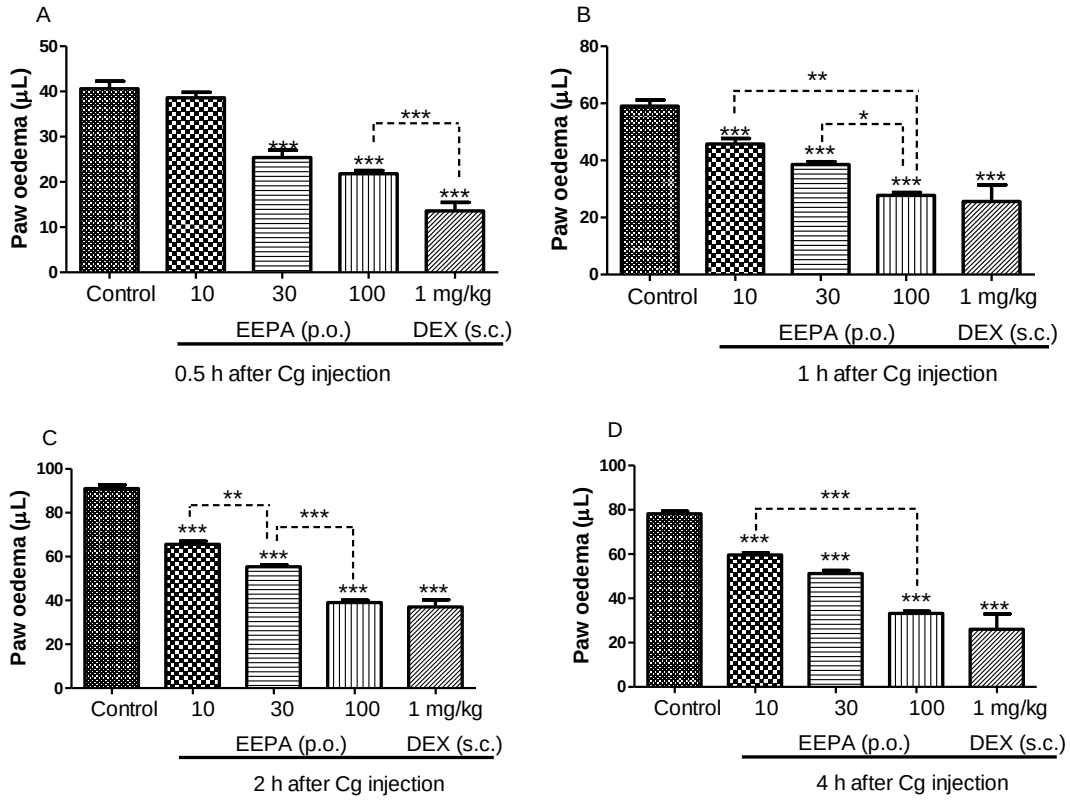


Figure 1.

Iwamoto *et al.*, 2014.



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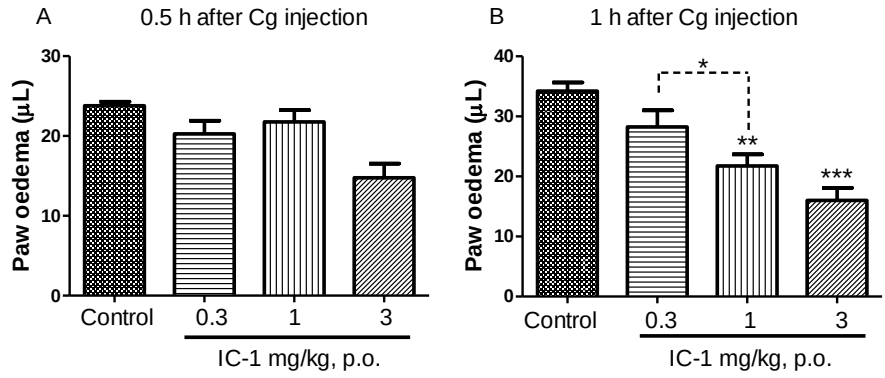

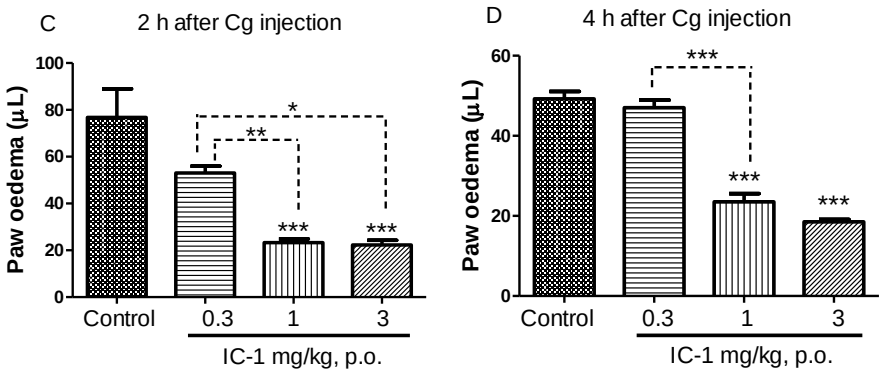

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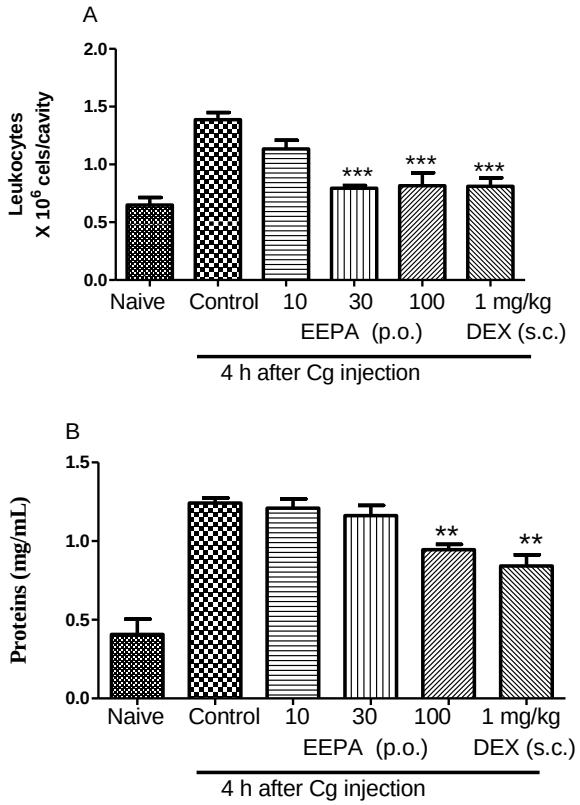

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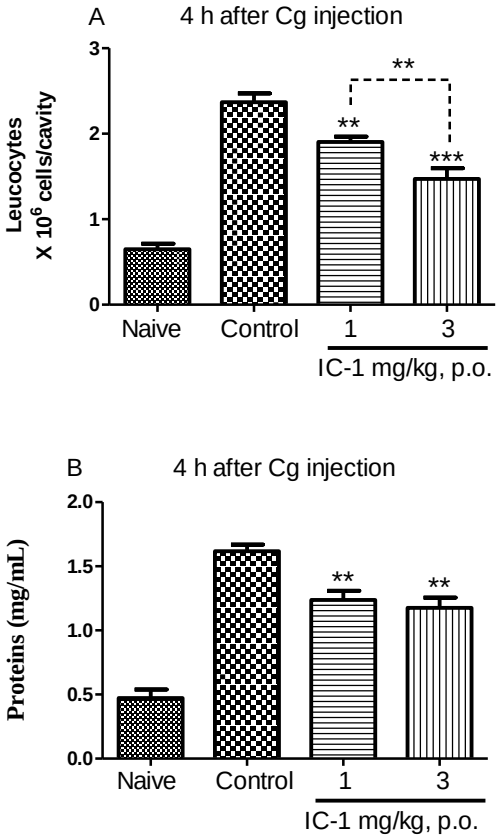

Figure 5.

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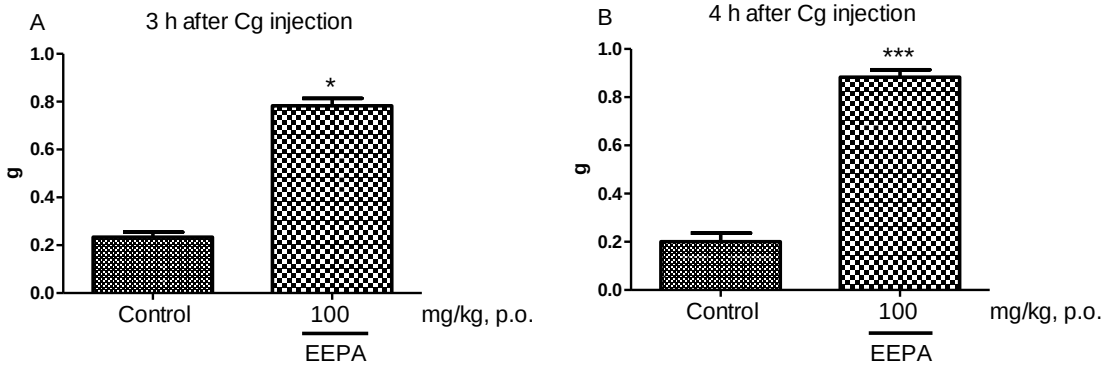

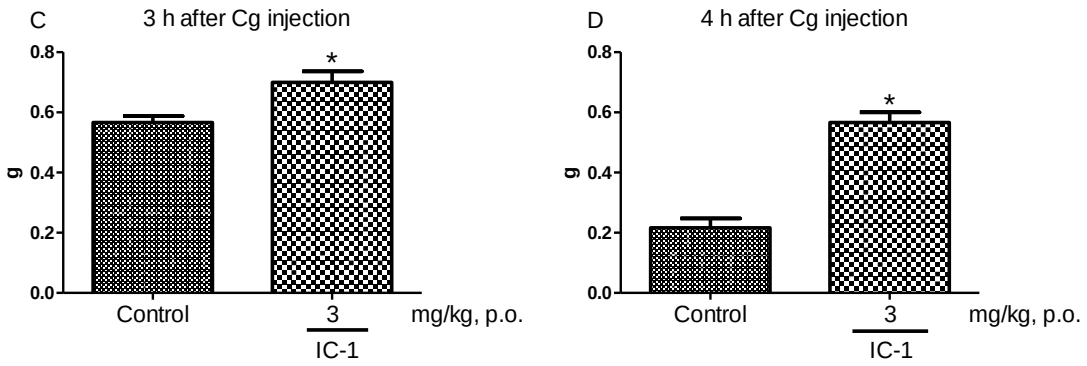

Figure 6.

Iwamoto *et al.*, 2014

5.2 Artigo científico 2 - Archives of Pharmacal Research

THE HYPOTENSIVE AND VASORELAXANT EFFECT OF *Piper amalago* L. **(PIPERACEAE) LEAVES EXTRACT IN RATS**

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ABSTRACT

Piper species are used in Brazilian folk medicine to reduce blood pressure and this effect was demonstrated in some studies with different species. There is no study with *Piper amalago* involving blood pressure and therefore we investigated the effect of ethalonic extract of *Piper amalago* (EEPA) in aorta relaxation and in mean arterial pressure (MAP) of spontaneously hypertensive rats (SHR). Relaxation responses to EEPA were obtained in precontracted (phenylephrine 1 µM) thoracic aorta rings of *Wistar* rats. EEPA promoted aorta relaxation in a dose-dependent manner. Preincubation of tissues with NO inhibitor N(G)-nitro-L-arginine methyl ester (100 µM), soluble guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (10 µM) and the removal of the endothelium did not reduce the relaxation responses to the plant extract. Finally, EEPA decreased the MAP in a dose-dependent manner and killed all rats (n=3) by hypotension in the highest dose tested. In conclusion, EEPA has effect on lowering the blood pressure and relaxation of aorta smooth muscle by EEPA is not correlated with mechanisms involving activation of the NO-cGMP pathway.

Keywords: *Piper amalago;* aorta; hypotensive; medicinal plants.

Introduction

Hypertension (high blood pressure) is an important risk factor for cardiovascular mortality and morbidity. Worldwide this disease is estimated to cause 7.5 million deaths, which are about 12.8% of the total of all deaths (WHO, 2014). Lowering blood pressure largely reduces the chances of developing renal damage, heart failure, cerebral vascular disease and arterial coronary disease (Paulis and Unger, 2010).

Natural products are recognized as an important source of antihypertensive agents. A classical example is the reserpine extract from *Rauwolfia serpentina*. Several extracts and isolated compounds obtained from plants were tested with good results againt hypertension, such as: *Berberis integerrima* Bunge. (Berberidaceae), *Crataegus microphylla* C. Koch (Rosaceae), *Nymphaea alba* L. (Nymphaeaceae), *Onopordon acanthium* L. (Asteraceae), *Quercus infectoria* G. Olivier. (Fagaceae) and *Rubus sp*. (Rosaceae) (Sharifi *et al.*, 2013).

Some species of *Piper* (like *Piper nigrum* and *Piper truncatum*) are used in Brazilian folk medicine to reduce blood pressure (Raimundo *et al.*, 2009, Hlavackova *et al.*, 2010). But there are no studies with *Piper amalago*, therefore we performed an *in vitro* study of relaxation of thoracic aorta isolated from *Wistar* rats and an *in vivo* study with mean arterial pressure (MAP) on Spontaneously Hypertensive Rats (SHR). In this way, we investigated the hypotensive and vasorrelaxant effects of the ethanolic extract of *P. amalago* L. in rats.

Materials and Methods

Animals

Adult male Spontaneously Hipertensive Rats (SHR) and *Wistar* rats, weighing from 200 to 220 g, were obtained from CEMIB - State University of Campinas. All the animals were kept under standard condition, with rat chow and water *ad libitum*. All experimental procedures that involved animal studies were approved by the Ethics Committee on Animal Use CEUA (CEUA/UNICAMP – Protocol 3324-1).

Plant Material

P.amalago leaves were collected in Dourados – MS – Brazil, in August 2008, and identified by Elsie Franklin Guimarães (Instituto de Pesquisas Jardim Botânico, Rio de Janeiro, RJ, Brazil). A voucher specimen of the species *P. amalago* (DDMS 4410) is deposited in the herbarium of the Universidade Federal da Grande Dourados, MS, Brazil. The species was collected in the following geographical coordinates: *P. amalago* (S22012'42, 9 ", WO54054'55, 6").

In vivo studies

Rats were anaesthetized with thiopental (30 mg/kg i.p.) injection. A polyethylene catheter (PE 50) was inserted into the right femoral artery. This catheter was connected to

an infusion pump of saline and to a pressure transducer (PowerLab® system, Australia) to record changes in arterial pressure.

After 30 min of stabilization of the mean arterial pressure (MAP), a bolus of EEPA (5 – 60 mg/kg in 0.2 mL) were administered into the left femoral vein.

In vitro studies

The rats were euthanatized with CO_2 inhalation, then decapitated and exsanguinated. The thoracic aorta were rapidly isolated and put in Krebs-Henseleit nutritive solution (mM): NaCl (117), KCl (4.7), $CaCl_2$ (2.5), $MgSO_4$ (1.2), $NaHPO_4$ (1.2), $NaHCO_3$ (25) and $C_6H_{12}0_6$ (11), pH 7.4, 37 °C. Perivascular tissue was removed and aortic rings with approximately 5 mm in length were cut. These aortic rings were suspended in organ chambers containing 10 mL of Krebs-Henseleit solution at 36.5 ºC and pH 7.3 ~ 7.5, aerated with carbogen O_2:CO_2 (95:5%).

A tension of 10 mN was applied to the tissues. This tension was readjusted and Krebs-Henseleit solution was changed every 10 minutes until stabilization (~40 min).

Changes in tension were measured using isometric transducers (ADInstruments®, Australia) and recorded with PowerLab 4/30 system of data acquisition (Software version 7.3.7 PRO, ADInstruments®, Australia).

The vasorelaxant effect of EEPA (0.0005 to 0.5 mg) were evaluated in the pre-contracted aortic rings with phenylephrine (PE 3 µM). We tested EEPA on intact aortic rings, rings with denuded endothelium, rings pre-incubated with L-NAME (100 µM, 30 min prior) and on rings pre-incubated with ODQ ([1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one] - 10 µM, 30 min prior).

Statistical Analysis

Results are expressed as mean±SEM. One-way analysis of variance (ANOVA) followed by Student Newman-Keuls test was used for statistical evaluation. *P* values were considered significant when lower than 0.05.

Results

EEPA reduced the MAP of SHRs in doses of 5, 15 and 30 mg/kg. All animals died (n=4) by hypotension at 60 mg/kg. The mean of decreases in MAP were: 19.333 mmHg (for 5 mg/kg), 68 mmHg (for 15 mg/kg) and 77 mmHg (for 30 mg/kg) (Fig. 1). Vehicle used to prepare the EEPA solution (saline 0.9%) was administered to each animal prior to the administration of the EEPA and did not alter MAP.

In vitro, EEPA relaxed thoracic aorta rings in a dose-dependent manner. Removal of the endothelium did not alter this relaxation. When L-NAME (100 µM) or ODQ (10 µM) were incubated with tissues 30 minutes prior to EEPA incubation, results were the same (Fig. 2 and Tab. I).

Discussion

MAP experiments showed that after EEPA administration (5, 15 and 30 mg/kg) and MAP decrease, blood pressure returned to the normal (hypertensive - SHR) state after about 2-3 hours. Interestingly, it was also observed that EEPA caused sialorrhea in the animals of group 30 mg/kg but not in 5 and 15 mg/kg. Possibly this dose is acting in muscarinic receptors and further tests are needed.

Relaxation by EEPA did not alter with removal of the endothelium or 30 minutes prior incubation with L-NAME/ODQ. These data indicates that relaxation by EEPA is not correlated to the NO-cGMP pathway. The experiments with removal of the endothelium demonstrated that EEPA do not works due to endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadzki, 1980). L-NAME (L-NG-Nitroarginine Methyl Ester) is a nitric oxide synthase (NOS) inhibitor and requires hydrolysis of the methyl ester by cellular esterases to become a functional inhibitor (L-NNA / L-N^G-Nitroarginine). L-NNA is a competitive inhibitor nNOS (neuronal), eNOS (endothelium) and iNOS (inducible), leading to a decrease in nitric oxide (NO) bioavailability (Rees *et al.*, 1990, Kopincova *et al.*, 2012). ODQ is a guanylate cyclase irreversible inhibitor apparently by oxidation of the heme group of the enzyme (Schrammel *et al.*, 1996, Friebe and Koesling, 2003, Zhao *et al.*, 2000).

The removal of the heme group by the action of detergents (Foerster *et al.*, 1996) or its oxidation by ODQ (Zhao *et al.*, 2000) potentiated the action of BAY 58-2667 in isolated sGC (soluble guanylyl cyclase) (Stasch *et al.*, 2002). Intravenous administration of L-NAME (50 mg/kg) or ODQ (5 mg/kg) produce an increase in blood pressure and lung in

mice, whereas co-administration of BAY 60-2770 (10, 30 and 100 mg/kg) significantly reduce the values of the pressures more effectively compared to baseline levels (in the absence of L-NAME or ODQ) (Pankey *et al.*, 2011). Our functional findings showed that both L-NAME and ODQ potentiated the relaxation induced by EEPA, similar to what happens with the sGC activators (BAY 58-2667 and BAY 60-2770) in normotensive rats.

Studies with other species of *Piper* genus also demonstrated hypotensive effect. Eudesmin extracted from *Piper truncatum* induced a vascular relaxation in rat aorta mediated by the release of nitric oxide (NO) and prostanoid through the involvement of histamine receptor present in the endothelial cells (Raimundo *et al.*, 2009). Piperine from *Piper nigrum* partially prevented the raise of blood pressure caused by chronic L-NAME administration and the authors concluded that the effect is probably caused by the blockage of voltage-dependent calcium channels (Hlavackova *et al.*, 2010).

Conclusion

Results showed that EEPA has a hypotensive effect in spontaneously hypertensive rats. The vasorelaxation effect on thoracic aorta from *Wistar* rats is endothelium-independent and seems to be not correlated to NO-cGMP pathway.

Conflict of interest

No conflict to disclose

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Legend to table and figures

Tab. 1 – Efficacy (Emax) and the negative logarithm of concentration that produces 50% of the maximum response (pEC50) of each incubated drug. Control/Vehicle, EEPA alone, EEPA tested in aortic ring with denuded endothelium (E-), L-NAME 100 µM incubated 30 min prior to the EEPA (L-NAME) and ODQ 10 µM incubated 30 min prior to the EEPA (ODQ). ***$p<0.001$

Fig. 1 – Reduction (mmHg) of the Mean Arterial Pressure (MAP) of SHRs. EEPA were tested in doses of 5, 15, 30 and 60 mg/kg/0.2 mL (data of 60 mg/kg not shown because all animals from this group died). Pressure was recorded with LabChart® software installed on a computer connected to a PowerLab® system of pressure transducer (ADInstruments™).

Fig. 2 – Effect of EEPA (0.0005 mg to 0.2 mg) on aorta relaxation *in vitro.* Aortas were isolated from *Wistar* rats. Changes were recorded with LabChart® software installed on a computer connected to a PowerLab® system of force transducer (ADInstruments™). L-NAME (N5-[imino(nitroamino)methyl]-L-ornithine) 100 µM and ODQ (1H-[1,2, 4]oxadiazolo[4,3-a]quinoxalin-1-one) 10 µM were incubated with aortas during 30 minutes prior to the contraction of tissues. E- means that endothelia from aortas were removed in this group. Acetylcholine 1 µM was used to confirm the absence of endothelium.

Table and Figures

	E_{max}	pEC_{50}
EEPA	109.51 ± 2.67	0.043 ± 0.003
E-	107.08 ± 0.01	0.008 ± 0.0004***
L-NAME	112.51 ± 3.22	0.006 ± 0.0006***
ODQ	114.93 ± 2.07	0.0259 ± 0.002***

*** p<0.001

Table I.

Iwamoto *et al.*, 2014.

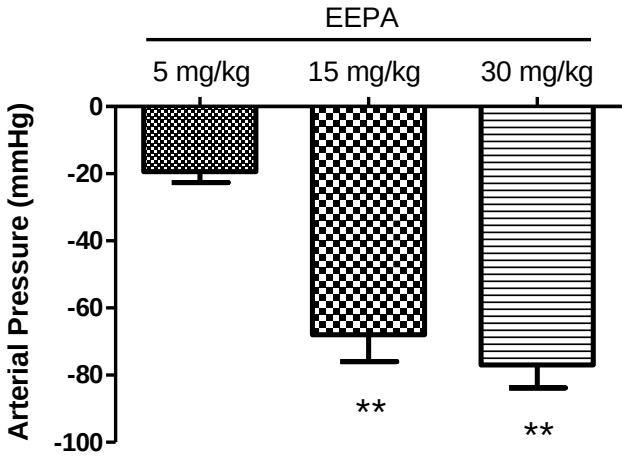

Figure 1.

Iwamoto *et al.*, 2014.

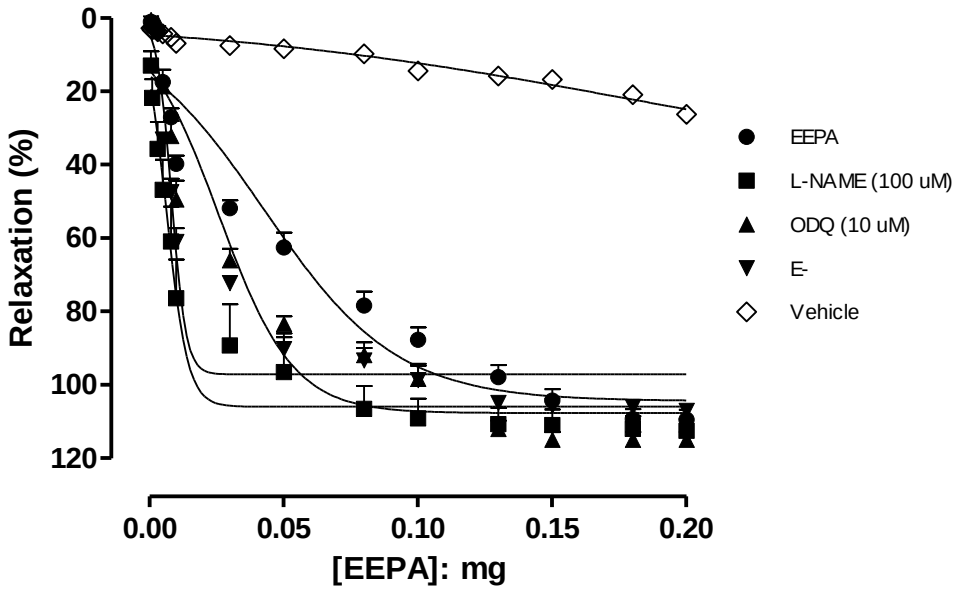

Figure 2.

Iwamoto *et al.*, 2014.

6 APÊNDICES

6.1 Normas da Journal of Inflammation para Submissão de Trabalhos

JOURNAL OF INFLAMMATION

Instructions for authors

Research Articles

Submission process

Manuscripts must be submitted by one of the authors of the manuscript, and should not be submitted by anyone on their behalf. The submitting author takes responsibility for the article during submission and peer review.

Please note that *Journal of Inflammation* levies an article-processing charge on all accepted Research Articles; if the submitting author's institution is a BioMed Central member the cost of the article-processing charge may be covered by the membership (see About page for detail). Please note that the membership is only automatically recognised on submission if the submitting author is based at the member institution.

To facilitate rapid publication and to minimize administrative costs, *Journal of Inflammation* prefers online submission.

Files can be submitted as a batch, or one by one. The submission process can be interrupted at any time; when users return to the site, they can carry on where they left off.

See below for examples of word processor and graphics file formats that can be accepted for the main manuscript document by the online submission system. Additional files of any type, such asmovies, animations, or original data files, can also be submitted as part of the manuscript.

During submission you will be asked to provide a cover letter. Use this to explain why your manuscript should be published in the journal, to elaborate on any issues relating to our editorial policies in the 'About *Journal of Inflammation*' page, and to declare any potential competing interests. You will be also asked to provide the contact details (including email addresses) of potential peer reviewers for your manuscript. These should be experts in their field, who will be able to provide an objective assessment of the manuscript. Any suggested peer reviewers should not have published with any of the authors of the manuscript within the past five years, should not be current collaborators, and should not be members of the same research institution. Suggested reviewers will be considered alongside potential reviewers recommended by the Editor-in-Chief and/or Editorial Board members.

Assistance with the process of manuscript preparation and submission is available from BioMed Central customer support team.

We also provide a collection of links to useful tools and resources for scientific authors on our Useful Tools page.

File formats

The following word processor file formats are acceptable for the main manuscript document:

- Microsoft word (DOC, DOCX)

- Rich text format (RTF)
- Portable document format (PDF)
- TeX/LaTeX (use BioMed Central's TeX template)
- DeVice Independent format (DVI)

TeX/LaTeX users: Please use BioMed Central's TeX template and BibTeX stylefile if you use TeX format. During the TeX submission process, please submit your TeX file as the main manuscript file and your bib/bbl file as a dependent file. Please also convert your TeX file into a PDF and submit this PDF as an additional file with the name 'Reference PDF'. This PDF will be used by internal staff as a reference point to check the layout of the article as the author intended. Please also note that all figures must be coded at the end of the TeX file and not inline.

If you have used another template for your manuscript, or if you do not wish to use BibTeX, then please submit your manuscript as a DVI file. We do not recommend converting to RTF.

For all TeX submissions, all relevant editable source must be submitted during the submission process. Failing to submit these source files will cause unnecessary delays in the publication procedures.

Preparing main manuscript text

General guidelines of the journal's style and language are given below.

Overview of manuscript sections for Research Articles

Manuscripts for Research Articles submitted to *Journal of Inflammation* should be divided into the following sections (in this order):

- Title page
- Abstract
- Keywords
- Background
- Methods
- Results and discussion
- Conclusions
- List of abbreviations used (if any)

- Competing interests
- Authors' contributions
- Authors' information
- Acknowledgements
- Endnotes
- References
- Illustrations and figures (if any)
- Tables and captions
- Preparing additional files

The **Accession Numbers** of any nucleic acid sequences, protein sequences or atomic coordinates cited in the manuscript should be provided, in square brackets and include the corresponding database name; for example, [EMBL:AB026295, EMBL:AC137000, DDBJ:AE000812, GenBank:U49845, PDB:1BFM, Swiss-Prot:Q96KQ7, PIR:S66116].

The databases for which we can provide direct links are: EMBL Nucleotide Sequence Database (EMBL), DNA Data Bank of Japan (DDBJ), GenBank at the NCBI (GenBank), Protein Data Bank (PDB), Protein Information Resource (PIR) and the Swiss-Prot Protein Database (Swiss-Prot).

You can download a template (Mac and Windows compatible; Microsoft Word 98/2000) for your article.

For reporting standards please see the information in the About section.

Title page

The title page should:

- provide the title of the article
- list the full names, institutional addresses and email addresses for all authors
- indicate the corresponding author

Please note:

- the title should include the study design, for example "A versus B in the treatment of C: a randomized controlled trial X is a risk factor for Y: a case control study"
- abbreviations within the title should be avoided

Abstract

The Abstract of the manuscript should not exceed 350 words and must be structured into separate sections: **Background,** the context and purpose of the study; **Methods**, how the study was performed and statistical tests used; **Results**, the main findings; **Conclusions**, brief summary and potential implications. Please minimize the use of abbreviations and do not cite references in the abstract. **Trial registration**, if your research reports the results of a controlled health care intervention, please list your trial registry, along with the unique identifying number (e.g. **Trial registration**: Current Controlled Trials ISRCTN73824458). Please note that there should be no space between the letters and numbers of your trial registration number. We recommend manuscripts that report randomized controlled trials follow the CONSORT extension for abstracts.

Keywords

Three to ten keywords representing the main content of the article.

Background

The Background section should be written in a way that is accessible to researchers without specialist knowledge in that area and must clearly state - and, if helpful, illustrate - the background to the research and its aims. Reports of clinical research should, where appropriate, include a summary of a search of the literature to indicate why this study was necessary and what it aimed to contribute to the field. The section should end with a brief statement of what is being reported in the article.

Methods

The methods section should include the design of the study, the setting, the type of participants or materials involved, a clear description of all interventions and comparisons, and the type of analysis used, including a power calculation if appropriate. Generic drug names should generally be used. When proprietary brands are used in research, include the brand names in parentheses in the Methods section.

For studies involving human participants a statement detailing ethical approval and consent should be included in the methods section. For further details of the journal's editorial policies and ethical guidelines see 'About this journal'.

For further details of the journal's data-release policy, see the policy section in 'About this journal'.

Results and discussion

The Results and discussion may be combined into a single section or presented separately. Results of statistical analysis should include, where appropriate, relative and absolute risks or risk reductions, and confidence intervals. The Results and discussion sections may also be broken into subsections with short, informative headings.

Conclusions

This should state clearly the main conclusions of the research and give a clear explanation of their importance and relevance. Summary illustrations may be included.

List of abbreviations

If abbreviations are used in the text they should be defined in the text at first use, and a list of abbreviations can be provided, which should precede the competing interests and authors' contributions.

Competing interests

A competing interest exists when your interpretation of data or presentation of information may be influenced by your personal or financial relationship with other people or organizations. Authors must disclose any financial competing interests; they should also reveal any non-financial competing interests that may cause them embarrassment were they to become public after the publication of the manuscript.

Authors are required to complete a declaration of competing interests. All competing interests that are declared will be listed at the end of published articles. Where an author gives no competing interests, the listing will read 'The author(s) declare that they have no competing interests'.

When completing your declaration, please consider the following questions:

Financial competing interests

- In the past five years have you received reimbursements, fees, funding, or salary from an organization that may in any way gain or lose financially from the publication of

this manuscript, either now or in the future? Is such an organization financing this manuscript (including the article-processing charge)? If so, please specify.

- Do you hold any stocks or shares in an organization that may in any way gain or lose financially from the publication of this manuscript, either now or in the future? If so, please specify.
- Do you hold or are you currently applying for any patents relating to the content of the manuscript? Have you received reimbursements, fees, funding, or salary from an organization that holds or has applied for patents relating to the content of the manuscript? If so, please specify.
- Do you have any other financial competing interests? If so, please specify.

Non-financial competing interests

Are there any non-financial competing interests (political, personal, religious, ideological, academic, intellectual, commercial or any other) to declare in relation to this manuscript? If so, please specify.

If you are unsure as to whether you, or one your co-authors, has a competing interest please discuss it with the editorial office.

Authors' contributions

In order to give appropriate credit to each author of a paper, the individual contributions of authors to the manuscript should be specified in this section.

According to ICMJE guidelines, An 'author' is generally considered to be someone who has made substantive intellectual contributions to a published study. To qualify as an author one should 1) have made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; 2) have been involved in drafting the manuscript or revising it critically for important intellectual content; 3) have given final approval of the version to be published; and 4) agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Each author should have participated sufficiently in the work to take public responsibility for appropriate portions of the content. Acquisition of funding, collection of data, or general supervision of the research group, alone, does not justify authorship.

We suggest the following kind of format (please use initials to refer to each author's contribution): AB carried out the molecular genetic studies, participated in the sequence

alignment and drafted the manuscript. JY carried out the immunoassays. MT participated in the sequence alignment. ES participated in the design of the study and performed the statistical analysis. FG conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

All contributors who do not meet the criteria for authorship should be listed in an acknowledgements section. Examples of those who might be acknowledged include a person who provided purely technical help, writing assistance, or a department chair who provided only general support.

Authors' information

You may choose to use this section to include any relevant information about the author(s) that may aid the reader's interpretation of the article, and understand the standpoint of the author(s). This may include details about the authors' qualifications, current positions they hold at institutions or societies, or any other relevant background information. Please refer to authors using their initials. Note this section should not be used to describe any competing interests.

Acknowledgements

Please acknowledge anyone who contributed towards the article by making substantial contributions to conception, design, acquisition of data, or analysis and interpretation of data, or who was involved in drafting the manuscript or revising it critically for important intellectual content, but who does not meet the criteria for authorship. Please also include the source(s) of funding for each author, and for the manuscript preparation. Authors must describe the role of the funding body, if any, in design, in the collection, analysis, and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication. Please also acknowledge anyone who contributed materials essential for the study. If a language editor has made significant revision of the manuscript, we recommend that you acknowledge the editor by name, where possible.

The role of a scientific (medical) writer must be included in the acknowledgements section, including their source(s) of funding. We suggest wording such as 'We thank Jane Doe who provided medical writing services on behalf of XYZ Pharmaceuticals Ltd.'

Authors should obtain permission to acknowledge from all those mentioned in the Acknowledgements section.

Endnotes

Endnotes should be designated within the text using a superscript lowercase letter and all notes (along with their corresponding letter) should be included in the Endnotes section. Please format this section in a paragraph rather than a list.

References

All references, including URLs, must be numbered consecutively, in square brackets, in the order in which they are cited in the text, followed by any in tables or legends. Each reference must have an individual reference number. Please avoid excessive referencing. If automatic numbering systems are used, the reference numbers must be finalized and the bibliography must be fully formatted before submission.

Only articles, datasets, clinical trial registration records and abstracts that have been published or are in press, or are available through public e-print/preprint servers, may be cited; unpublished abstracts, unpublished data and personal communications should not be included in the reference list, but may be included in the text and referred to as "unpublished observations" or "personal communications" giving the names of the involved researchers. Obtaining permission to quote personal communications and unpublished data from the cited colleagues is the responsibility of the author. Footnotes are not allowed, but endnotes are permitted. Journal abbreviations follow Index Medicus/MEDLINE. Citations in the reference list should include all named authors, up to the first 30 before adding '*et al.*'..

Any *in press* articles cited within the references and necessary for the reviewers' assessment of the manuscript should be made available if requested by the editorial office.

Style files are available for use with popular bibliographic management software:

- BibTeX
- EndNote style file
- Reference Manager
- Zotero

Examples of the *Journal of Inflammation* reference style are shown below. Please ensure that the reference style is followed precisely; if the references are not in the correct style they may have to be retyped and carefully proofread.

All web links and URLs, including links to the authors' own websites, should be given a reference number and included in the reference list rather than within the text of the manuscript. They should be provided in full, including both the title of the site and the URL, in the following format: **The Mouse Tumor Biology Database** [http://tumor.informatics.jax.org/mtbwi/index.do]. If an author or group of authors can clearly be associated with a web link, such as for weblogs, then they should be included in the reference.

Examples of the* Journal of Inflammation *reference style

Article within a journal

Koonin EV, Altschul SF, Bork P: **BRCA1 protein products: functional motifs.** *Nat Genet* 1996,**13:**266-267.

Article within a journal supplement

Orengo CA, Bray JE, Hubbard T, LoConte L, Sillitoe I: **Analysis and assessment of ab initio three-dimensional prediction, secondary structure, and contacts prediction.** *Proteins* 1999,**43**(Suppl 3):149-170.

In press article

Kharitonov SA, Barnes PJ: **Clinical aspects of exhaled nitric oxide.** *Eur Respir J*, in press.

Published abstract

Zvaifler NJ, Burger JA, Marinova-Mutafchieva L, Taylor P, Maini RN: **Mesenchymal cells, stromal derived factor-1 and rheumatoid arthritis [abstract].** *Arthritis Rheum* 1999, **42:**s250.

Article within conference proceedings

Jones X: **Zeolites and synthetic mechanisms.** In *Proceedings of the First National*

Conference on Porous Sieves: 27-30 June 1996; Baltimore. Edited by Smith Y. Stoneham: Butterworth-Heinemann; 1996:16-27.

Book chapter, or article within a book

Schnepf E: **From prey via endosymbiont to plastids: comparative studies in dinoflagellates.** In *Origins of Plastids. Volume 2.* 2nd edition. Edited by Lewin RA. New York: Chapman and Hall; 1993:53-76.

Whole issue of journal

Ponder B, Johnston S, Chodosh L (Eds): **Innovative oncology.** In *Breast Cancer Res* 1998, **10:**1-72.

Whole conference proceedings

Smith Y (Ed): *Proceedings of the First National Conference on Porous Sieves: 27-30 June 1996; Baltimore.* Stoneham: Butterworth-Heinemann; 1996.

Complete book

Margulis L: *Origin of Eukaryotic Cells.* New Haven: Yale University Press; 1970.

Monograph or book in a series

Hunninghake GW, Gadek JE: **The alveolar macrophage.** In *Cultured Human Cells and Tissues.*Edited by Harris TJR. New York: Academic Press; 1995:54-56. [Stoner G (Series Editor): *Methods and Perspectives in Cell Biology,* vol 1.]

Book with institutional author

Advisory Committee on Genetic Modification: *Annual Report.* London; 1999.

PhD thesis

Kohavi R: **Wrappers for performance enhancement and oblivious decision graphs.** *PhD thesis.* Stanford University, Computer Science Department; 1995.

Link / URL

The Mouse Tumor Biology Database [http://tumor.informatics.jax.org/mtbwi/index.do]

Link / URL with author(s)

Corpas M: **The Crowdfunding Genome Project: a personal genomics community with open source values** [http://blogs.biomedcentral.com/bmcblog/2012/07/16/the-crowdfunding-genome-project-a-personal-genomics-community-with-open-source-values/]

Dataset with persistent identifier

Zheng, L-Y; Guo, X-S; He, B; Sun, L-J; Peng, Y; Dong, S-S; Liu, T-F; Jiang, S; Ramachandran, S; Liu, C-M; Jing, H-C (2011): **Genome data from sweet and grain sorghum (Sorghum bicolor)**.*GigaScience*. http://dx.doi.org/10.5524/100012.

Clinical trial registration record with persistent identifier

Mendelow, AD (2006): **Surgical Trial in Lobar Intracerebral Haemorrhage**. Current Controlled Trials. http://dx.doi.org/10.1186/ISRCTN22153967

Preparing illustrations and figures

Illustrations should be provided as separate files, not embedded in the text file. Each figure should include a single illustration and should fit on a single page in portrait format. If a figure consists of separate parts, it is important that a single composite illustration file be submitted which contains all parts of the figure. There is no charge for the use of color figures.

Please read our figure preparation guidelines for detailed instructions on maximising the quality of your figures.

Formats

The following file formats can be accepted:

- PDF (preferred format for diagrams)
- DOCX/DOC (single page only)
- PPTX/PPT (single slide only)
- EPS
- PNG (preferred format for photos or images)
- TIFF
- JPEG
- BMP

Figure legends

The legends should be included in the main manuscript text file at the end of the document, rather than being a part of the figure file. For each figure, the following information should be provided: Figure number (in sequence, using Arabic numerals - i.e. Figure 1, 2, 3 etc); short title of figure (maximum 15 words); detailed legend, up to 300 words.

Please note that it is the responsibility of the author(s) to obtain permission from the copyright holder to reproduce figures or tables that have previously been published elsewhere.

Preparing tables

Each table should be numbered and cited in sequence using Arabic numerals (i.e. Table 1, 2, 3 etc.). Tables should also have a title (above the table) that summarizes the whole table; it should be no longer than 15 words. Detailed legends may then follow, but they should be concise. Tables should always be cited in text in consecutive numerical order.

Smaller tables considered to be integral to the manuscript can be pasted into the end of the document text file, in A4 portrait or landscape format. These will be typeset and displayed in the final published form of the article. Such tables should be formatted using the 'Table object' in a word processing program to ensure that columns of data are kept aligned when the file is sent electronically for review; this will not always be the case if columns are generated by simply using tabs to separate text. Columns and rows of data should be made visibly distinct by ensuring that the borders of each cell display as black lines. Commas should not be used to indicate numerical values. Color and shading may not be used; parts of the table can be highlighted using symbols or bold text, the meaning of which should be explained in a table legend. Tables should not be embedded as figures or spreadsheet files.

Larger datasets or tables too wide for a landscape page can be uploaded separately as additional files. Additional files will not be displayed in the final, laid-out PDF of the article, but a link will be provided to the files as supplied by the author.

Tabular data provided as additional files can be uploaded as an Excel spreadsheet (.xls) or comma separated values (.csv). As with all files, please use the standard file extensions.

Preparing additional files

Although *Journal of Inflammation* does not restrict the length and quantity of data included in an article, we encourage authors to provide datasets, tables, movies, or other information as additional files.

Please note: All Additional files **will be published** along with the article. Do not include files such as patient consent forms, certificates of language editing, or revised versions of the main manuscript document with tracked changes. Such files should be sent by email to editorial@journal-inflammation.com, quoting the Manuscript ID number.

Results that would otherwise be indicated as "data not shown" can and should be included as additional files. Since many weblinks and URLs rapidly become broken, *Journal of Inflammation*requires that supporting data are included as additional files, or deposited in a recognized repository. Please do not link to data on a personal/departmental website. The maximum file size for additional files is 20 MB each, and files will be virus-scanned on submission.

Additional files can be in any format, and will be downloadable from the final published article as supplied by the author. We recommend CSV rather than PDF for tabular data.

Certain supported files formats are recognized and can be displayed to the user in the browser. These include most movie formats (for users with the Quicktime plugin), mini-websites prepared according to our guidelines, chemical structure files (MOL, PDB), geographic data files (KML).

If additional material is provided, please list the following information in a separate section of the manuscript text:

- File name (e.g. Additional file 1)
- File format including the correct file extension for example .pdf, .xls, .txt, .pptx (including name and a URL of an appropriate viewer if format is unusual)
- Title of data
- Description of data

Additional files should be named "Additional file 1" and so on and should be referenced explicitly by file name within the body of the article, e.g. 'An additional movie file shows this in more detail [see Additional file 1]'.

Additional file formats

Ideally, file formats for additional files should not be platform-specific, and should be viewable using free or widely available tools. The following are examples of suitable formats.

- Additional documentation
  - PDF (Adode Acrobat)
- Animations
  - SWF (Shockwave Flash)
- Movies
  - MP4 (MPEG 4)
  - MOV (Quicktime)
- Tabular data
  - XLS, XLSX (Excel Spreadsheet)
  - CSV (Comma separated values)

As with figure files, files should be given the standard file extensions.

Mini-websites

Small self-contained websites can be submitted as additional files, in such a way that they will be browsable from within the full text HTML version of the article. In order to do this, please follow these instructions:

1. Create a folder containing a starting file called index.html (or index.htm) in the root.
2. Put all files necessary for viewing the mini-website within the folder, or sub-folders.
3. Ensure that all links are relative (ie "images/picture.jpg" rather than "/images/picture.jpg" or "http://yourdomain.net/images/picture.jpg" or "C:\Documents and Settings\username\My Documents\mini-website\images\picture.jpg") and no link is longer than 255 characters.
4. Access the index.html file and browse around the mini-website, to ensure that the most commonly used browsers (Internet Explorer and Firefox) are able to view all parts of the mini-website without problems, it is ideal to check this on a different machine.

5. Compress the folder into a ZIP, check the file size is under 20 MB, ensure that index.html is in the root of the ZIP, and that the file has .zip extension, then submit as an additional file with your article.

Style and language

General

Currently, *Journal of Inflammation* can only accept manuscripts written in English. Spelling should be US English or British English, but not a mixture.

There is no explicit limit on the length of articles submitted, but authors are encouraged to be concise.

Journal of Inflammation will not edit submitted manuscripts for style or language; reviewers may advise rejection of a manuscript if it is compromised by grammatical errors. Authors are advised to write clearly and simply, and to have their article checked by colleagues before submission. In-house copyediting will be minimal. Non-native speakers of English may choose to make use of a copyediting service.

Help and advice on scientific writing

The abstract is one of the most important parts of a manuscript. For guidance, please visit our page on Writing titles and abstracts for scientific articles.

Tim Albert has produced for BioMed Central a list of tips for writing a scientific manuscript. American Scientist also provides a list of resources for science writing. For more detailed guidance on preparing a manuscript and writing in English, please visit the BioMed Central author academy.

Abbreviations

Abbreviations should be used as sparingly as possible. They should be defined when first used and a list of abbreviations can be provided following the main manuscript text.

Typography

- Please use double line spacing.
- Type the text unjustified, without hyphenating words at line breaks.
- Use hard returns only to end headings and paragraphs, not to rearrange lines.

- Capitalize only the first word, and proper nouns, in the title.
- All pages should be numbered.
- Use the *Journal of Inflammation* reference format.
- Footnotes are not allowed, but endnotes are permitted.
- Please do not format the text in multiple columns.
- Greek and other special characters may be included. If you are unable to reproduce a particular special character, please type out the name of the symbol in full. **Please ensure that all special characters used are embedded in the text, otherwise they will be lost during conversion to PDF.**

Units

SI units should be used throughout (liter and molar are permitted, however).

6.2 Normas da Archives of Pharmacal Research para Submissão de Trabalhos

Archives of Pharmacal Research

Instruction for Authors

1. Aims and Scope

Archives of Pharmacal Research is an interdisciplinary journal devoted to the publication of original scientific research papers and reviews in the fields of drug discovery, drug development, and drug actions with a view to providing fundamental and novel information on drugs and drug candidates.

Manuscripts will be considered for publication on the condition that the results reported are based on original research that has not been published elsewhere in any journal.

Upon acceptance for publication of an article in *Archives of Pharmacal Research*, the author tacitly agrees to make available any materials used in the published experiments, or novel or natural products disclosed in the article that are not commercially available, so that other researchers may confirm the observations.

For the studies using natural extract, the journal will determine the acceptability of such papers on an individual basis. Natural product contribution must meet the following specific criteria: a) any natural extract must be defined, and appropriate information provided regarding the origin; b) the author must be able to state that the material under study is endotoxin free.

2. Types of Papers

Archives of Pharmacal Research considers manuscripts for publication in the following types of papers:

Research Articles. These are full-length descriptions of research that describe original and important pieces of work in detail from the fields covered by the journal.

Maximum length of manuscripts should not exceed 6,000 words (24 typescript pages) excluding figures and tables. At least, 4 figures or tables (in total) should be included. These manuscripts will undergo standard review and normally are not expedited.

Reviews. Invited or author-initiated review articles within the scope of *Archives of Pharmacal Research* will be considered for publication. Authors may submit a short synopsis to editors (pskor@korea.com) regarding content and length prior to submission. At least, 3 figures or tables (in total) should be included (for Review). Please note that reviews will be subjected to appropriate evaluation process.

Report on Investigational Drugs. We have created a new section called 'Report on Investigational Drugs', to cover recent updates in the field of drug development and discovery. The report should describe recent trends in new drug development among pharmaceutical and bio-venture companies, research institutes and universities. A focused report on one particular drug would be recommended. The report should contain a brief background, a description of drug candidate (e.g., effects and relevant experimental data) and its prospective view. The total length should be about 1000 words excluding references.

3. How to Submit Manuscripts

All submissions should be made online at the *Archives of Pharmacal Research* Editorial Manager site at www.editorialmanager.com/arpr. New users will first need to register. Once logged on to the site as an author, follow the instructions to submit your manuscript. Authors should submit the text, tables and artworks in electronic form via this web-based manuscript submission system.

Authors must include a cover letter that contains the title, authors, a brief outline of the work's originality, desired section of publication, corresponding author's name, address, telephone and fax numbers (including country and city codes), and e-mail address.

4. Review of Manuscripts

All manuscripts are first evaluated for their scientific content and significance by the editors and will be subjected to at least two independent reviewers. However, editors reserve the right to reject a manuscript without conducting an in-depth review if they feel that the manuscript is out of the scope or does not meet the minimal acceptance criteria for publication. The manuscript with incorrect format may be declined without further review.

5. Proofs

Authors are basically responsible for the factual accuracy of their papers. One set of proofs will be supplied for the author to check for typesetting accuracy, to be returned to the publisher within 3 days of receipt. No changes to the original manuscript will be allowed at this stage. In addition, the editors reserve the right to make any necessary correction to a paper prior to publication.

6. Transfer of Copyright

All authors must sign the 'Transfer of Copyright' agreement before the article can be published. This transfer agreement enables the Pharmaceutical Society of Korea to protect the copyrighted material for the authors, but does not relinquish the author's proprietary rights.

7. Publication Charges

The *Archives of Pharmacal Research* charges a publication fee of US $20 per printed page upon the acceptance of the manuscript on the form accompanying the proofs.

8. Preparation of Manuscripts

Manuscripts should be concisely written in English and typed double-spaced throughout on A-4 paper with margins of at least 3.0 cm. All pages should be numbered in succession, the title page being page 1.

Title Page. Each manuscript must have a title page, which includes only the title, the authors' names with their affiliation, a running title of not more than 50 characters including spaces, and mailing address, which includes telephone and fax numbers and e-mail address of the corresponding author. Place an asterisk after the name of the corresponding author. Author affiliations must be footnoted using superscript numbers. The title should be a brief phrase, not a complete sentence, describing the contents of the manuscript. Symbols, formulas, or arbitrary abbreviations should not be included in the title, except chemical symbols to indicate the structure of isotopically labeled compounds.

Abbreviation. The excessive use of abbreviations in the text is strongly discouraged. Authors should only use abbreviations sparingly and should always define an abbreviation when first used by placing it in parentheses after the full term, e.g.

Acetylcholinesterase (AChE). The metric system for all measurements should be expressed in lowercase letters without periods (ml, nm, min, etc.).

Drug Names. Drug names should be the official or approved names; trade names or common names may be given in brackets where the drug is first mentioned. The name of the manufacturer, not the address, should be given. The doses of the drugs should be given as unit weight/unit body weight, e.g. mmol/kg or mg/kg. Concentration should be given in terms of molarity (e.g., nM or mM), or as unit weight/unit volume solution (e.g., mg/ l) stating whether the weight refers to the salt or the active component of the drug.

9. Organization of Manuscripts

Each manuscript must begin with an **ABSTRACT** that summarizes the results obtained and the conclusion drawn. It should not exceed 200 words. A short list of six keywords or phrases should be supplied following the abstract.

Introduction. An introduction should first begin with general aspect for a non-specialist and then continue with the specific reason for undertaking the investigation. No attempt should be made to indicate the results obtained.

Materials and Methods. Procedures used in the work should be given in sufficient detail to permit the repetition by other researchers. Nevertheless, published procedures should be briefly summarized by mentioning the reference(s) and only described in detail if the procedures have been modified. The name of manufacturer should be specified without address (e.g., city and country).

[Ethics] All human and animal studies must have been approved by the author's institutional review board and the name of the review board should be stated. All clinical investigation must have been conducted according to Declaration of Helsinki principles. For the policies on the research and publication ethics not stated, 'Good Publication Practice Guidelines for Medical Journals (http://kamje.or.kr/publishing_ethics.html)' or 'Guidelines on good publication(http://www.publicationethics.org.uk/ guidelines)' can be applied.

Results. In this section, only observations should be described without discussion of their significance.

Discussion. The authors' interpretations of their observations (or findings) should be accompanied by an assessment of their significance in relation to previous work.

Conflict of Interest. All authors are requested to disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work.

Acknowledgements. The Acknowledgment section should include credits [initial(s) and last name] for technical assistance, financial support, and other appropriate recognition.

References. EndNote is a software product that we recommend to our journal authors to help simplify and streamline the research process. Using EndNote's bibliographic management tools, you can search bibliographic databases, build and organize your reference collection, and then instantly output your bibliography in any journal style.

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Only papers closely related to the author's work should be cited. References should be assembled alphabetically. In the text, they should be referred by name and year (Harvard system). When referring to more than one paper from a same author from a same year, the alphabets a, b, c, etc. should be placed next to the year of publication to distinguish the articles. In the text, when referring to a work by sole author, the name of author should be given like (Robinson, 1998) and (Robinson, 1999; Jeong, 2000). When referring to a work by two authors, the name of authors should be given like (Robinson and Jeong, 2001). When referring to a work by more than three authors, the name of the first author should be given followed by et al. such as (Robinson et al., 2002). Literature references must consist of names and initials of all authors, title of the paper referred to, abbreviated title of the journal and the volume, year, and first and last page numbers of the paper. The style and punctuation of the references should confirm with the following examples:

Journals:

Lai,Y.-L.,Mehta,R.C.,Thacker,A.A.,Yoo,S.-D.,MacNamara,P.J., and DeLuca, P.P., Sustained bronchodilation with isoproterenol poly(glycolidecolactide) microspheres.*Arch. Pharm. Res.*, 10, 119–125 (1993).

Books:

Azria, M., *The Calctionins: Physiology and Pharmacology*.

Karger, London, (1989).

Borchartdt, R. T., Hidalgo, I. J., Hillgren, K. M. and Hu., M., Pharmaceutical applications of cell culture: An Overview, In

Wilson, G., Davis, S.S., Illum, L. and Zweibaum, A. (Eds.).

Pharmaceutical Application of Cell and Tissue Culture to Drug Transport. Plenum Press, New York, pp. 1–14, (1991).

Journal names should be abbreviated in accordance with Chemical Abstracts or Biological Abstracts List of Serials.

Experimental Data. If possible, statistical significance of the experimental data should be provided. Statistical probability (p) in tables, figures, figure legends and text should be expressed as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. For multiple comparisons within a table, footnotes italicized in lower case, superscript letters should be used and defined in the table legend. References to statistical methods of calculation should be provided. If statistical limits cannot be provided, the number of determinations and some indication of the variability and reliability of the results should be provided. For animal experimental data, doses and concentrations should be expressed as molar quantities (e.g., mmol/kg, mM) when comparisons are made between compounds having large differences in molecular weights. The routes of administration of test compounds and vehicles used should be indicated.

Figures. All figures (drawings, schemes, charts and photographs) should be numbered in one consecutive series of Arabic numerals in the order cited in the text.

1) Graphics: To ensure the highest print quality, your figures must be submitted in either TIF or EPS format according to the following minimum resolutions:

Black and white line art	1200dpi
Grayscale art	600dpi
Color art	300dpi

2) Colors: Remove all color from graphics, except for those graphics that you would like to have considered for publication in color. Color photographs will be printed at the Editors discretion, on the understanding that the authors will bear the cost.

3) Layout: Figures should be submitted in the actual size at which they should appear in the Journal. They may be printed in either single column (80 mm width) or double column (165 mm width) format. The single column format is preferred. The size of text in figures should be 8-10 points, except for single letter markers which may be 12 points. The use of sans serif font such as Helvetica is preferred. Numbers, letters, and symbols used inmulti-paneled figures must be consistent. Complex textures and shading to achieve a three- dimensional effect should be avoided. To show a pattern, a simple cross-hatch design should be used. Lines should be no thinner than 0.5 point. For a line graph, use standard symbols in the following order of preference: ʊ, ŏ, Ō, ʃ, D, ʃ; x and + should be avoided.

4) Legends: All legends should be typed consecutively in a separate section of the manuscript. Each legend must give a concise description of the figure and scheme concerned, together with any essential experimental details not described in the text. In particular, the key to any
symbols or distinctive line formats used on the figure must be given.

5) Contents: Abbreviations such as Me for CH3, Et for C2H5, and Ph (but not j) for C6H5 are acceptable. Make liberal use of "R and X groups" in equations, schemes, and structure blocks to avoid the repetition of similar structures. Do not repeat a structure; the number alone of an earlier structure can be used if a compound occurs several times. Schemes are numbered with Arabic numerals. Within schemes, structures should be numbered with boldface Arabic numerals, consecutively from left to right, top to bottom, regardless of the order in which the compounds are discussed in the text. Schemes should be footnoted in the manner described below for Tables. It is not necessary to give reagents and conditions in complete detail, since this detail is contained in the Experimental Section. Where needed, numbers such as NMR chemical shifts may be included directly on structural formulas.

Chemical Structures. Drawing preferences (preset in the ACS Stylesheet in ChemDraw) are as follows:

1. As drawing settings select:

chain angle	120º
bond spacing	18% of width
fixed length	14.4 pt (0.508 cm, 0.2 in.)

bold width	2.0 pt (0.071 cm, 0.0278 in.)
line width	0.6 pt (0.021 cm, 0.0084 in.)
margin width	1.6 pt (0.056 cm, 0.0222 in.)
hash spacing	2.5 pt (0.088 cm, 0.0347 in.)

2. As text settings

select:

font	Arial/Helvetica
size	10 pt

3. Under the preferences choose:

units	points
tolerances	5 pixels

4. Under page setup choose:

paper	US Letter
scale	100%

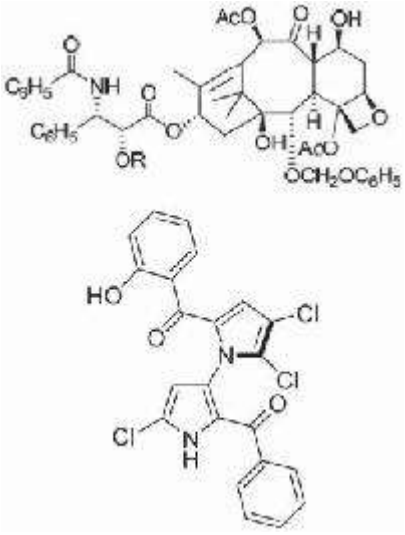
5. Using the ChemDraw ruler or appropriate margin settings, create structure blocks, schemes, and equations having maximum widths of 11.3 cm (onecolumn format) or 23.6 cm (two-column format). Note: if the foregoing preferences are selected as cm values, the ChemDraw ruler is calibrated in cm. ChemDraw graphics will be reduced to 75% during production.

6.Embolden compound numbers, but not atom labels or captions.

7. Authors are urged to use only a single configurational descriptor (heavy line or dashed line, but not both) when defining a stereocenter in a chemical structure. Atoms should be kept outside of rings wherever possible.

Rather than rectangular solid and dashed lines, authors should use solid and dashed wedges to indicate configurations, as shown below. Dots at ring junctions intended to represent hydrogen atoms should not be used. Structures should be drawn in a neat manner

ready for direct reproduction, and should not be cluttered or overlapping. Any arrows and numbering used for atoms in figures should not come into contact with bonds or ring systems. See an example of a prepared structure using ChemDraw with the specified preferences below. In molecules containing a chiral biphenyl axis, it is recommended that one of the aromatic rings be drawn in the plane of the paper and the second one be rotated out of the plane of the paper, to reflect the *P* or *M* conformation about the biphenyl bond (see below for example).



Tables. These should be numbered consecutively with Roman numerals in the order cited in the text. Tables should be formatted with horizontal lines only: vertical ruled lines are not required. Footnotes in tables should be given lowercase letter designations and be cited in the table by italic superscript letters. Each table should be provided with a descriptive heading, which, together with the individual column headings, should make the table, as nearly as possible, self-explanatory. In setting up tabulations, tables need to fit the type area of the journal page (17.8 × 25.4 cm) and the column width (8.5 cm). Arrangements that leave many columns partially filled or that contain much blank space should be avoided

6.3 Carta de aprovação do projeto pelo Comitê de Ética no Uso de Animais – CEUA/UFGD.

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DA GRANDE DOURADOS
PRÓ-REITORIA DE ENSINO DE PÓS-GRADUAÇÃO E PESQUISA

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

Dourados-MS, 4 de julho de 2013

Senhor Pesquisador:
Renan Donomae Iwamoto

O Projeto de sua responsabilidade – Protocolo nº. **025/2013 – CEUA/UFGD** - intitulado **"Avaliação da atividade anti-inflamatória do extrato bruto e compostos isolados de *Piper amalago* em camundongos"** foi integralmente **APROVADO** e poderá ser conduzido.

Ressaltamos que é de responsabilidade do (a) pesquisador (a) envio de notificação à CEUA sobre o término do projeto.

Prof. Dr. Fernando Miranda de Vargas Junior

Coordenador/CEUA

6.4 Carta de aprovação do projeto pelo Comitê de Ética no Uso de Animais – CEUA/UNICAMP.

UNICAMP

CEUA/Unicamp

Comissão de Ética no Uso de Animais
CEUA/Unicamp

CERTIFICADO

Certificamos que o projeto "**Avaliação da Atividade Vasorelaxante e Hipotensora de Piper amalago L. em Ratos**" (protocolo nº **3324-1**), sob a responsabilidade de **Prof. Dr. Gilberto de Nucci / Renan Donomae Iwamoto**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA**, **SISBIO** ou **CIBio**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **17 de março de 2014**.

Campinas, 17 de março de 2014.

Prof. Dr. Alexandre Leite Rodrigues de Oliveira
Presidente

Fátima Alonso
Secretária Executiva

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