SANDRA SATIE MIZOKAMI

EFEITO ANTI-INFLAMATÓRIO DO ÁCIDO PIMARADIENÓICO E EFEITOS ANALGÉSICO E ANTI-INFLAMATÓRIO DO ÁCIDO CAURENÓICO EM CAMUNDONGOS

Londrina
2016

SANDRA SATIE MIZOKAMI

EFEITO ANTI-INFLAMATÓRIO DO ÁCIDO PIMARADIENÓICO E EFEITOS ANALGÉSICO E ANTI-INFLAMATÓRIO DO ÁCIDO CAURENÓICO EM CAMUNDONGOS

Tese apresentada ao Programa de Pós-Graduação em Patologia Experimental da Universidade Estadual de Londrina, como requisito parcial à obtenção do título de Doutora em Patologia Experimental.

Orientador: Profº Dr. Waldiceu Aparecido Verri Jr.

Londrina
2016

Dados Internacionais de Catalogação-na-Publicação (CIP)

M685ef Mizokami, Sandra Satie.
Efeito anti-inflamatório do ácido pimaradienóico e efeitos analgésico e anti-inflamatório do ácido caurenóico em camundongos / Sandra Satie Mizokami. - Londrina, 2016.
144 f.: il.

Orientador: Waldiceu Aparecido Verri Junior.
Tese (Doutorado em Patologia Experimental) - Universidade Estadual de Londrina, Centro de Ciências Biológicas, Programa de Pós-Graduação em Patologia Experimental, 2016.
Inclui bibliografia.

1. Agentes antiinflamatórios - Teses. 2. Hiperagelsia - Teses. 3. Citocinas - Teses. I. Verri Júnior, Waldiceu Aparecido. II. Universidade Estadual de Londrina. Centro de Ciências Biológicas. Programa de Pós-Graduação em Patologia Experimental. III. Título.

CDU 616-092

SANDRA SATIE MIZOKAMI

EFEITO ANTI-INFLAMATÓRIO DO ÁCIDO PIMARADIENÓICO E EFEITOS ANALGÉSICO E ANTI-INFLAMATÓRIO DO ÁCIDO CAURENÓICO EM CAMUNDONGOS

Tese apresentada ao Programa de Pós-Graduação em Patologia Experimental da Universidade Estadual de Londrina, como requisito parcial à obtenção do título de Doutora em Patologia Experimental.

BANCA EXAMINADORA

_____
Orientador: Dr. Waldiceu Aparecido Verri Jr.
Universidade Estadual de Londrina - UEL

_____
Drª. Ana Carolina Rossaneis
Universidade Estadual de Londrina - UEL

_____
Drª. Andressa de Freitas Mendes Dionísio
Universidade Estadual de Londrina - UEL

_____
Drª. Glaura Scantarbulo Alves Fernandes
Universidade Estadual de Londrina - UEL

_____
Dr. Wander Rogério Pavanelli
Universidade Estadual de Londrina - UEL

Londrina, 04 de abril de 2016

Dedico este trabalho ao meu querido avô,
Que dizia que o estudo e o conhecimento são
os bens que jamais se perdem. Saudades!

AGRADECIMENTOS

Agradeço a Deus, pelas graças concedidas, me acompanhando e abençoando em todos os momentos de minha vida.

Agradeço aos meus queridos pais, Francisco e Eunice, que não mediram (e não medem) esforços para me educar e proporcionar o melhor para a minha formação, não só acadêmica, mas como pessoa. Em especial à minha mãe, fonte de minha admiração e exemplo de garra, determinação e sucesso, que me ensinou a nunca desanimar e desistir, mesmo frente às adversidades e tropeços da vida.

À minha irmã Miriam que é uma grande amiga e confidente. Presente em todos os momentos da minha vida, me aconselhando e me ajudando a seguir em frente.

Ao professor. Dr. Waldiceu Aparecido Verri Junior, por me aceitar em seu laboratório e proporcionar a satisfação de participar da sua linda equipe de pesquisa. Agradeço pelos ensinamentos, pela paciência e atenção. Mais que um orientador o senhor é um sinônimo de admirável dedicação e inteligência, um exemplo a seguir. Muito obrigada!

Aos colegas e amigos do Laboratório de Dor, Inflamação, Neuropatia e Câncer, que representam minha segunda família, sem vocês certamente essa jornada teria sido muito mais árdua e menos prazerosa. Obrigada!

Obrigada às 'Lindas da Dor': Camila, Cássia, Marília e Thacyana, pela paciência, pelos risos, pelos momentos de desabâfos, happys, conselhos e pela imensa ajuda. Adoro vocês!

Agradeço ao professor Dr. Nilton Syogo Arakawa, que disponibilizou os compostos utilizados neste trabalho, sem ele este trabalho não se realizaria.

Aos professores membros da banca (qualificação e defesa) por terem aceito o convite de participar da banca examinadora e disponibilizar seu tempo. Muito obrigada!

Aos professores e técnicos do Programa em Patologia Experimental da Universidade Estadual de Londrina e do Laboratório de Farmacologia da Faculdade de Medicina de Ribeirão Preto-USP. Obrigada.

Ao apoio financeiro do Conselho Nacional de Pesquisa (CNPq, Brasil), Coordenadoria de Aperfeiçoamento Pessoal de Nível Superior (CAPES, Brasil) e a Fundação Araucária.

Aos que não citei, mas que me ajudaram de forma direta ou indireta no percurso e realização deste trabalho. Muito Obrigada!

“Por vezes sentimos que aquilo que fazemos
não é senão uma gota de água no mar. Mas o
mar seria menor se lhe faltasse uma gota”
Madre Teresa de Calcutá

“Quando não compreendemos a dor, ela nos dilacera;
Quando entendemos seus fins, ela nos aperfeiçoa”
Provérbio Chinês

MIZOKAMI, Sandra Satie. **Efeito anti-inflamatório do ácido pimaradienóico e efeitos analgésico e anti-inflamatório.** 2016. 144 f. Tese (Doutorado em Patologia Experimental) – Universidade Estadual de Londrina. Londrina, 2016.

RESUMO

Estímulos inflamatórios ou lesões no tecido estimulam um intenso recrutamento de leucócitos, especialmente neutrófilos para o foco inflamado. Além disso, há a produção e liberação da cascata de citocinas, que levam à liberação de mediadores inflamatórios (prostanóides e aminas simpáticas), responsáveis pela dor inflamatória. Ácido Pimaradienóico (*ent*-pimara-8(14),15-dien-19-oic acid) e ácido Caurenóico (*ent*-kaur-16-en-19-oic acid) são diterpenos encontrados em plantas do cerrado brasileiro. Existem evidências do efeito analgésico e anti-inflamatório desses diterpenos. Nós investigamos estes efeitos em diferentes modelos de dor inflamatória em camundongos e seus mecanismos foram investigados. Em modelo de peritonite induzido por carragenina em camundongos, ácido Pimaradienóico inibiu o recrutamento de leucócitos totais e neutrófilos para a cavidade peritoneal de forma dose-dependente. Ácido Pimaradienóico também inibiu a formação de edema de pata e a atividade da mieloperoxidase (MPO) no tecido plantar induzido pela administração de carragenina. O mecanismo anti-inflamatório do ácido Pimaradienóico depende da manutenção da atividade anti-oxidante, produção de citocinas inflamatórias (TNF-α and IL-1β) e inibição da ativação do NFκB. Ácido caurenóico inibiu o recrutamento de leucócitos totais, neutrófilos e células mononucleares em modelo de peritonite induzida pela adminstração de LPS. Inibiu a produção de citocinas e ativação do NFκB no lavado peritoneal. Ácido Caurenóico também inibiu a hiperalgêsia mecânica e térmica, a atividade da MPO e o estresse oxidativo na pata indizido por LPS. Em modelo de constrição crônica do nervo ciático (CCI), tanto o pré como pós tratamento com ácido Caurenóico inibiu a hiperalgesia mecânica causada pela CCI. Ácido cauraenóico aumentou a expressão de RNAm de Nrf2 e inibiu a depleção de gluationa reduzida (GSH) na medula espinal. Além disso, o ácido Caurenóico inibiu a ativação de NFκB, ativação da microglia e de astrócitos, e a expressão de RNAm de citocinas (TNF-α e IL-33) na CCI. Nossos resultados demonstram que os diterpenos ácido Pimaradienóico e ácido Caurenóico apresentam efeito anti-inflamatório em diferentes modelos inflamatórios e representam importantes candidatos no controle da inflamação aguda e crônica.

Palavras-chave: Ácido Pimaradienóico. Ácido Caurenóico. Inflamação. Citocinas. NFκB.

MIZOKAMI, Sandra Satie. **Anti-inflammatory effect of pimaradienóic acid and analgésic and anti-inflammatory effects of kaurenóic acid in mice.** 2016. 144 p. Thesis (Doctoral Degree in Experimental Pathology) – Universidade Estadual de Londrina. Londrina, 2016.

ABSTRACT

Inflammatory stimuli or tissue injuries stimulate an intense recruitment of leukocytes, especially neutrophils to the inflammatory focus. Furthermore, there are production and release of cytokine cascades, which trigger the release of inflammatory mediators (prostanoids or sympathetic amines) responsible for inflammatory pain. Pimaradienoic acid (*ent*-pimara-8(14),15-dien-19-oic acid) and Kaurenoic acid (*ent*-kaur-16-en-19-oic acid) are diterpenes found in plants in the Brazilian savannas. Although there are evidences on the analgesic and anti-inflammatory effects theses diterpenes. We investigated this effect in different models of inflammatory pain and its mechanisms in mice were investigated further. In a model of carrageenan-induced peritonitis in mice, Pimaradienoic acid inhibited carrageenan-induced recruitment of total leukocytes and neutrophils to the peritoneal cavity in a dose-dependent manner. Pimaradienoic acid also inhibited carrageenan-induced paw edema and myeloperoxidase (MPO) activity in the paw skin. The anti-inflammatory mechanism of Pimaradienoic acid depended on maintaining paw skin antioxidant activity, production of inflammatory cytokines (TNF-α and IL-1β) and inhibits the NFκB activation. Kaurenoic acid inhibited the recruitment of total leukocytes, neutrophils and mononuclear cells in LPS-induced peritonitis in mice. Inhibited LPS-induced production of cytokines and NFκB activation in peritoneal lavage. Kaurenoic acid also inhibited LPS-induced mechanical and thermal hyperalgesia, MPO activity and oxidative stress in the paw. In a model of neuropathic pain induced by chronic constriction injury (CCI) of sciatic nerve, pre or post-treatment with Kaurenoic acid inhibited CCI-induced mechanical hyperalgesia. Kaurenoic acid enhanced Nrf2 mRNA expression and inhibited the depletion of the antioxidant reduced glutathione (GSH) in the spinal cord of CCI mice. Furthermore, Kaurenoic acid inhibited the NFκB activation, activation of microglia and astrocyte and mRNA expression of cytokines (TNF-α and IL-33) in CCI. Our results provide evidences that the diterpenes Pimaradienoic acid and Kaurenoic acid presents anti-inflammatory effect in different inflammatory models, and thus represent important candidates to control to acute and chronic inflammation.

Keywords: Pimaradienoic acid. Kaurenoic acid. Inflammation. Cytokines. NFκB.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

ALT	Alanina aminotransferase
AMPc	Adenosina monofosfato cíclico
AST	Aspartato aminotransferase
ATP	Adenosina trifosfato
BALF	Lavado bronco-alveolar
CCI	Injúria por constrição crônida
CD14	*Cluster* de diferenciação 14
$CDCl_3$	Clorofórmio deuterado
CFA	Adjuvante completo de Freund
DMSO	Dimetilsulfóxido
DRG	Gânglio da raiz dorsal
ELISA	Ensaio Imunoenzimático
eNOS	Óxido nítrico sintase endotelial
ERK	*Extracellular-signal-regulated Kinase*
EROS	Espécies reativas de oxigênio
GFAP	Proteína glial fibrilar ácida
GGT	Gama-glutamiltransferase
GMPc	Guanosina monofosfato ciclico
HeLa	(*Henrietta Lacks' cervical)* células de câncer cervical
HIV	Vírus da imunodeficiência humana
HPLC	Cromatográfia líquida de alta eficiência
IASP	*International Association for the Study of Pain*
Iba-1	Proteína de ligação do calcio inonizado
Ig E	Imunoglobulina E
IL	Interleucina
IL-1β	Interleucina 1-beta
iNOS	Óxido nítrico sintase induzível
IP3	Inositol fosfato
JNK	*c- Jun N-terminal Kinase*
KA	*Kaurenoic acid*
KC	queratinocitos
KCl	Cloreto de potássio

KT5823	inibidor das proteínas quinases dependente de GMPc/PKG
LTB_4	Leucotrieno B4
L-NAME	L-nitro-arginina metil ester; inibidor não seletivo da oxido nítrico sintase
LPS	Lipopolissacarídeo
MAPK	mitógeno ativado por proteina quinase
MDA	Malondialdeído
mg/Kg	miligrama por quilograma
mNOS	Óxido nítrico sintase mitocondrial
MPO	Mieloperoxidase
NFκB	Fator de transcrição nuclear *kappa* B
NO	Óxido nítrico
NOS	Óxido nítrico sintase
nNOS	Óxido nítrico neuronal
ODQ	(1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) inibidor da guanilato ciclase
PA	*Pimaradienóic acid*
pH	Potencial hidrogeniônico
PG	Prostaglandina
PGE_2	Prostaglandina E2
PKA	Proteína quinase A
PKC	Proteína quinase C
PKG	Proteína quinase G
PLA_2	Fosfolipase A_2
SNC	Sistema nervoso central
TNF-α	Fator de necrose tumoral
TGF-β	Fator de transformação de crescimento beta
TLR4	Receptor *toll* do tipo 4
TPA	12-O-tetradecanoilforbol acetato
δ	Deslocamento químico (ppm)

SUMÁRIO

1. Introdução

1.1. Processo Inflamatório

A inflamação é um importante componente da imunidade inata e seu desenvolvimento tem por função destruir ou inativar o agente agressor e reparar, quando possível, o tecido lesionado. Pode ser desencadeado por diferentes estímulos por exemplo: bactérias, vírus, isquemia, interação antígeno-anticorpo, lesões traumáticas ou provocado por agentes físicos ou químicos (TAKEUCHI; AKIRA, 2010). O processo inflamatório pode ser dividido em duas fases: aguda e crônica.

A inflamação aguda inicia-se rapidamente após o contato com o agente inflamatório e possui curta duração, cerca de horas a poucos dias. Envolvem alterações vasculares e celulares e apresenta o desenvolvimento de cinco sinais cardinais: rubor, calor, edema, dor e dependendo da intensidade e extensão do processo inflamatório pode ocorrer a perda da função (COTRAN; KUMAR; COLLINS, 2000). Dentre as alterações vasculares que ocorrem durante a inflamação temos a vasodilatação de arteríolas e vênulas e abertura de capilares induzida pela liberação de mediadores químicos, como histamina e óxido nítrico (NO). Este processo induz ao aumento do volume sanguíneo no local, resultando no rubor ou eritema, e aumento de temperatura local (calor). O aumento do volume local eleva a pressão hidrostática em conjunto com a vasodilação e abertura de lacunas entre as células endoteliais, contribuindo para o aumento da permeabilidade e extravassamento de líquido rico em proteínas (exsudado) e formação do edema (COTRAN; KUMAR; COLLINS, 2000).

Apesar do volume sanguíneo aumentar no foco inflamatório, há diminuição da sua velocidade durante o fluxo sanguíneo devido a abertura de capilares. Essa redução da velocidade permite a saída dos leucócitos da coluna central do fluxo sanguíneo ou sua marginalização. Essa marginalização aliada a ativação de moléculas de adesão ao endotélio, contribui para a posterior transmigração e migração dos leucócitos para o foco inflamatório. No foco inflamatório, essas células tentam combater e eliminar o agente agressor através de mecanismos como fagocitose, pinocitose, liberação de enzimas e grânulos microbicidas, produção de

espécies reativas de oxigênio (ROS) e ativação de mediadores inflamatórios (COTRAN; KUMAR; COLLINS, 2000; CUNHA et al., 2008). A liberação de mediadores inflamatórios (por exemplo histamina leucotrienos, aminas simpáticas, citocinas dentre outros) contribue para o desenvolvimento da dor inflamatória e lesão tecidual (CUNHA et al., 2008; VERRI et al., 2006).

Caso o organismo não consiga eliminar o patógeno ou agente agressor há o desenvolvimento da cronificação do processo (COTRAN; KUMAR; COLLINS, 2000). De duração prolongada (semanas, meses ou anos), a inflamação crônica ocorre na presença de inflamação ativa, com infiltrado de macrófagos, linfócitos e plamócitos, juntamente com a destruição tecidual e reparação envolvendo proliferação de novos vasos (angiogênese) e cicatrização por fibrose (COTRAN; KUMAR; COLLINS, 2000).

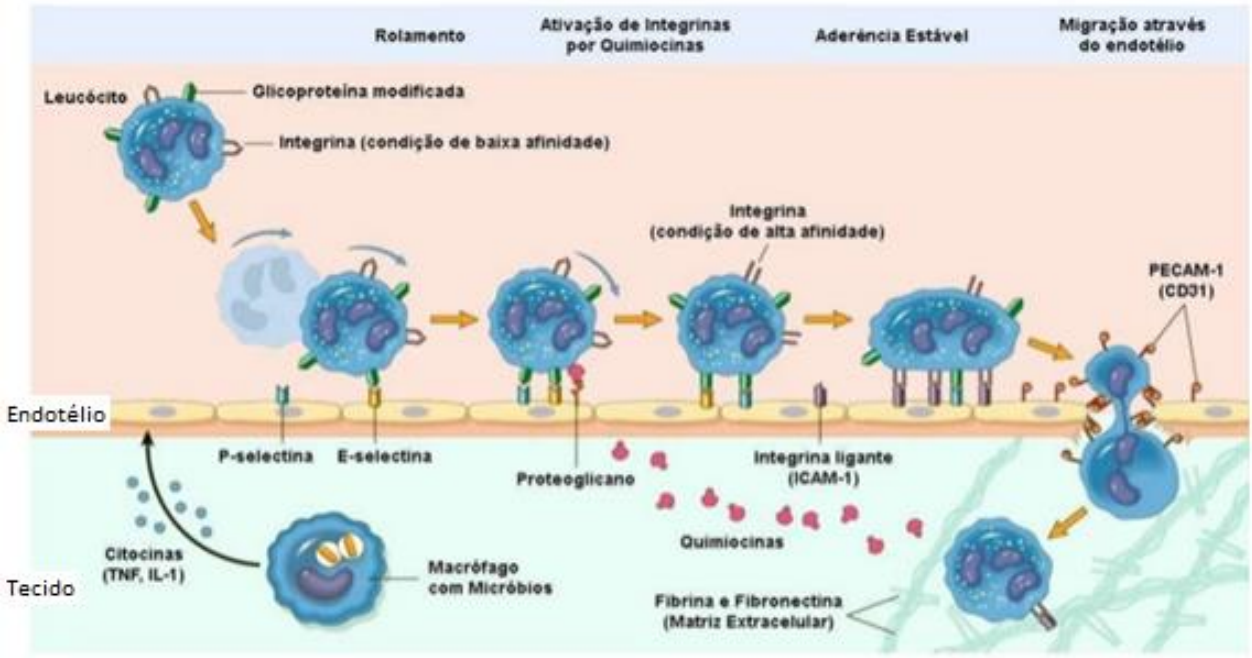
1.2. Migração Celular

Os neutrófilos são as primeiras células do sistema imunológico a chegarem ao local do dano e são responsáveis pela eliminação do agente infeccioso. Entretanto, em certos casos ocorre falha em controlar o recrutamento dessas células (FERREIRA et al., 2009), sendo responsáveis pelo dano tecidual e associados ao desenvolvimento e cronicidade de inúmeras doenças como artrite reumatóide, asma, esclerose múltipla, doença pulmonar obstrutiva crônica e colite ulcerativa (DAL-SECCO et al., 2003; GUERRERO et al., 2006; PAULA-NETO et al., 2011). Isso se dá ao fato de que os neutrófilos quando chegam ao local inflamado são ativados e passam a produzir substâncias tóxicas para o tecido (FERREIRA et al., 2009).

O processo de migração celular ocorre primeiramente pelo rolamento, seguido de adesão firme dos leucócitos ao endotelio e posterior transmigração pelo tecido (Figura 1). O rolamento é mediado por moléculas de adesão da família das selectinas, glicoproteínas que apresentam um domínio N-terminal extracelular que se ligam a açúcares. Essa ligação a carboidratos fornece uma interação fraca entre leucócitos e endotélio vascular resultando no rolamento e também ativação de integrinas. Posteriormente, os leucócitos interagem com moléculas de adesão da

superfamília das imunoglobulinas (VCAM, ICAM, PCAM) permitindo a adesão firme dos leucócitos e posterior migração pelo tecido (COTRAN; KUMAR; COLLINS, 2000; DAL-SECCO et al., 2003; LEITE et al., 2009; SADIK; LUSTER, 2012).

Figura 1 – Ativação e migração de leucócitos



Fonte: COTRAN; KUMAR; COLLINS, 2000)

A correta migração dos leucócitos ativados até o foco inflamatório ocorre mediante a liberação de substâncias quimioatrativas. Dentre elas podemos citar os componentes do sistema complemento C3a e C5a, leucotrienos (LTB_4), fator de agregação plaquetária, citocinas pró- inflamatórias e quimiocinas (DAL-SECCO et al., 2003; SADIK; LUSTER, 2012). Concomitante à liberação de fatores quimioatrativos, há a liberação de mediadores anti-inflamatórios, como as interleucinas (IL) 10 e 4 e lipoxina, que irão modular de forma negativa o recrutamento de leucócitos (DAL-SECCO et al., 2003).

Os leucócitos também estão relacionados ao desenvolvimento da dor, mas o real mecanismo de como ocorre essa sensibilização dos nociceptores pelos leucócitos ainda não foi totalmente esclarecido (CUNHA; VERRI, 2006). Em modelo de inflamação induzida pela adminstração de carragenina, os leucócitos recrutados, principalmente neutrófilos, contribuem para o desenvolvimento da hiperalgesia por

produzirem prostaglandina E_2 e o pré-tratamento com Fucoidin (inibidor da adesão de neutrófilos) reverteu a hiperalgesia induzida pela carragenina (CUNHA et al., 2008).

1.3. Dor Inflamatória ou Nociceptiva

A dor é um sentimento desagradável, porém essencial para o sistema de defesa do corpo. Se não sentíssemos dor não teríamos, por exemplo, a ação de retirada do pé quando pisamos em algo pontiagudo ou a retirada do braço de uma chama quente. Segundo a *International Association from the Study of Pain* (IASP), a dor é considerada uma experiência sensorial e emocional desagradável, associada ao dano tecidual potencial ou real (MERSKEY, 1980) e, subjetiva pois a intensidade de sua percepção pode variar de pessoa para pessoa.

A dor persistente pode ser subdividida segundo sua origem em dor nociceptiva e dor neuropática. A dor nociceptiva resulta da ativação direta de nociceptores em resposta a um agente agressor. A dor neuropática ou neurogênica origina-se devido a lesões de nervos periféricos ou do sistema nervoso central (KLAUMANN; WOUK; SILLAS, 2008).

Nocicepção (do latim *nocere*, ‘nocivo’) consiste nos processos de transdução, transmissão e modulação de sinais gerados em resposta a um estímulo nocivo (KLAUMANN; WOUK; SILLAS, 2008). Essa transmissão e modulação de sinais ocorre primariamente nos nociceptores ou neurônios nociceptivos, que são terminações nervosas livres de fibras aferentes primárias. Existem três tipos principais de fibras aferentes primárias que transmitem os estímulos periféricos ao sistema nervoso central (SNC): as fibras do tipo Aβ, Aδ e as do tipo C. As fibras Aδ e Aβ são mielinizadas e as do tipo C são amielinizadas. As fibras Aδ conduzem os estímulos mais rapidamente, em torno de 5-30 m/s e respondem a estímulos térmicos e mecânicos, enquanto as fibras do tipo C conduzem os estímulos a uma baixa velocidade (< 1m/s) porém com maior intensidade e respondem a estímulos de origem térmica, mecânica ou química (FERREIRA et al., 2009). Existem também uma outra classe de nociceptores chamados de “silenciosos” ou “adormecidos”

(*silent* ou *sleeping nociceptors*) descritos por Schaible e Schimidt em 1988 (revisado por FERREIRA et al., 2009). Estes nociceptores não respondem a estímulos térmicos ou mecânicos mas são ativados durante o processo inflamatório (FERREIRA et al., 2009). Cada fibra transmite um tipo de sinal doloroso, como por exemplo, a estimulação de fibras do tipo Aδ produzem uma dor na forma de picada ou ferroada, enquando fibras do tipo C produzem dor em forma de queimação (KLAUMANN; WOUK; SILLAS, 2008).

A liberação de mediadores inflamatórios como prostaglandina (PG), aminas simpática, substância P, histamina, serotonina, citocinas e quimiocinas agem sobre as fibras C e nociceptores silenciosos ativando segundos mensageiros como a via da adenosina monofosfato ciclico (AMPc), proteínas quinases A e C (PKA e PKC). Ocorre também a fosforilação de canais iônicos, como os de sódio, cálcio e potássio voltagem-dependente, presentes na membrana dos neurônios, levando a um aumento do influxo intracelular de sódio e cálcio. O resultado disto, são alterações nos potenciais de repouso, despolarização e consequente excitabilidade da membrana neuronal e transmissão do impulso nervoso da periferia até a medula espinal, tálamo e córtex somatossensorial, onde será interpretada como dor (FERREIRA et al., 2009; KLAUMANN; WOOUK; SILLAS, 2008; VERRI et al., 2006)

1.4. Dor Neuropática ou Neurogênica

A dor neuropática é uma síndrome complexa resultante de lesões ou disfunções de fibras nervosas (WOOLF; MANNION, 1999). A lesão das fibras condutoras causa distúrbios das funções motoras e sensoriais e pode vir associada à hiperalgesia (aumento da resposta a um estímulo doloroso), parestesia (sensação de formigamento ou dormência), alodínia (dor resultante de estímulo não doloroso, por exemplo o vento) e dor espontânea (AUSTIN; MOALEM-TAYLOR, 2010; CAMPBELL; MEYER, 2006; SCHOLZ; WOOLF, 2007; WOOLF; MANNION, 1999). A dor neuropática pode originar de vários tipos de lesões ou doenças que afetam o sistema somatossensitivo periférico ou central, como diabetes *mellitus*, infecção por HIV, herpes-zoster, induzida por drogas, processos compressivos, lesão neural

traumática, neuralgia do trigêmio e câncer (CAMPBELL; MEYER, 2006; GAO; JI, 2010b; WOOLF; MANNION, 1999).

Estudos têm revelado a participação de outras células além dos neurônios no desenvolvimento da dor neuropática. Dentre elas destacam-se macrófagos, células da glia (células de Schwann, astrócitos e microglia), linfócitos T e neutrófilos, que levam à produção de citocinas pró-inflamatórias que irão contribuir para o dano axonal, modulação da atividade espontânea dos nociceptores e a sensibilidade a estímulos (GAO; JI, 2010a, 2010b; SCHOLZ; WOOLF, 2007).

Em modelo animal de injúria por constrição crônica (CCI) do nervo ciático há aumento da hiperalgesia, característico da dor neuropática (ZARPELON et al., 2016). Após a lesão do nervo, ocorre a ativação de nociceptores periféricos e esta ativação promove o recrutamento de células e a liberação de mediadores inflamatórios. O recrutamento de neutrófilos e a ativação de macrófagos e células de Schwann são orquestradas por quimiocinas e citocinas que contribuem para a degeneração axonal, também conhecida como degeneração *Walleriana* (GAO; JI, 2010a, 2010b; SCHOLZ; WOOLF, 2007; ZARPELON et al., 2016). Mediadores vasoativos, substância P, bradicinina e NO são liberados nos axônios lesionados causando edema e inchaço, proporcionando maior recrutamento de células do sistema imune agindo diretamente no axônio, gânglio da raiz dorsal (GRD) e SNC, sensibilizando-os (GAO; JI, 2010a, 2010b; SCHOLZ; WOOLF, 2007).

Em resposta à lesão, além da ativação de células de Schwann ocorre também a sua proliferação, que culmina na liberação de sinais químicos promovendo o crescimento irregular axonal e remielinização do axônio (SCHOLZ; WOOLF, 2007). Este fenômeno contribui para o surgimento de dores espôntaneas. Células de Schwann também ativam macrófagos residentes, neutrófilos e mastócitos a liberarem PG, citocinas pró-inflamatórias, incluindo IL-1β, lL-6, IL-12, IL-18, interferon gama e fator de necrose tumoral alfa (TNF-α), e citocinas anti-inflamatórias ou reguladores de função, como IL-10 e fator de transformação de crescimento beta (TGF-β)(GAO; JI, 2010b; SCHOLZ; WOOLF, 2007).

TNF-α, principal citocina envolvida na transmissão da dor (CUNHA et al., 1992; VERRI et al., 2006), ao se ligar ao seu receptor (TNFR) pode levar a fosforila-

ção e ativação da via das MAPK (proteínas ativadas por mitógenos) – ERK, JNK, p38-MAPK e PI3K, regulando a ativação do fator nuclear de transcrição *kappa* B (NFκB). Além disso, TNF-α induz a liberação de moleculas de adesão ICAM, favorecendo o recrutamento de leucócitos (GAO; JI, 2010b; GAO; ZHANG; JI, 2010; SCHOLZ; WOOLF, 2007) e ativam canais de sódio voltagem-dependente nos GRD facilitando a transmissão dolorosa (SCHOLZ; WOOLF, 2007).

Paralelamente a esses eventos, microglia e astrócitos sofrem ativação e proliferação. A ativação da microglia vem acompanhada de aumento do recrutamento de linfócitos T (SCHOLZ; WOOLF, 2007). A ativação da microglia é caracterizada pela expressão de vários marcadores como receptores do complemento CR3 ou CD11b (OX42), proteína de ligação do cálcio inonizado (Iba-1), *cluster* de diferenciação 14 (CD14) e receptor *toll* do tipo 4 (TLR4), bem como ativação da via p38-MAPK (AUSTIN; MOALEM-TAYLOR, 2010). Em resposta à sua ativação, microglia promove a produção de citocinas, quimiocinas e fatores neurotróficos que alteram a excitabilidade neuronal (TRANG; BEGGS; SALTER, 2012).

Astrócitos, a maior população de células do SNC, são capazes de manter a homeostase pela regulação extracelular de íons, prótons e concentrações de neurotransmissores ao seu redor (AUSTIN; MOALEM-TAYLOR, 2010). A ativação de astrócitos é morfologicamente caracterizada por hipertrofia do corpo e seus prolongamentos e proliferação de filamentos intermediários, proteína glial fibrilar ácida (GFAP), vementina ou nestina, e funcionalmente por aumento na produção de vários mediadores inflamatórios (AUSTIN; MOALEM-TAYLOR, 2010).

Existem diferentes terapias para o controle da dor neuropática, em geral voltadas para a diminuição da atividade neuronal, mas os tratamentos atuais deixam a desejar quanto a eficácia (CAMPBELL; MEYER, 2006; WOOLF; MANNION, 1999). Pacientes não respondem bem às terapias com anti-inflamatórios não esteroidais e a resistência e tolerância aos opiácios é comum (CAMPBELL; MEYER, 2006). Pacientes são também tratados com antidepressivos tricíclicos ou anticonvulsivantes, porém todos apresentam eficácia limitada (CAMPBELL; MEYER, 2006; WOOLF; MANNION, 1999).

1.5. Óxido Nítrico e Ativação da Via NO/GMPc/PKG/ K^+ATP

O óxido nítrico (NO) é formado pela óxido nítrico sintase (NOS) a partir da L-arginina tendo como subproduto a L-citrulina. Existem três isoformas de NOS: NOS neuronal (nNOS), NOS endotelial (eNOS) e NOS induzível (iNOS). A nNOS e eNOS são vias constitutivas dependentes de cálcio e agem em baixas concentrações. A iNOS é independente de cálcio intracelular e é ativada por macrófagos estimulados, por bactérias, toxinas e citocinas, e requer algumas horas para ser expressa. Uma vez sintetizada, libera quantidades maiores de NO . Há relatos da existência de uma quarta isoforma a mNOS presente em mitocondrias (DUSSE; VIEIRA; CARVALHO, 2003; COELHO, 2009; MICLESCU; GORDH, 2009).

O NO esta envolvido em muitos processos biológicos como vasodilatação do endotélio, relaxamento do músculo liso, inibição da adesão e agregação plaquetária, potencialização e depressão de longo prazo no hipocampo e cerebelo, age como neurotransmisssor com capacidade potencializadora, atua na memória e no aprendizado e regulação e liberação de outros neurotransmissores (CERQUEIRA; YOSHIDA, 2002; CURY et al., 2011; FLORA FILHO; ZILBERSTEIN, 2000; MONCADA; HIGGS, 2006). Desempenha sua função também no sistema imune, no qual ele é o principal mediador citotóxico de células imunes efetoras, sendo uma importante molécula reguladora do sistema imune (DUSSE; VIEIRA; CARVALHO, 2003).

Inúmeros autores relatam a participação do NO na regulação do recrutamento celular, porém existem divergências em relação ao seu papel como indutor ou agente inibitório do recrutamento celular. Dal-Secco e colaboradores (2003; 2006) demonstraram que o NO induz a diminuição no recrutamento de neutrófilos e isso se deve à redução na expressão de moléculas de adesão (ICAM) no endotélio, diminuindo assim o rolamento e a adesão de neutrófilos. Além disso, esta ação seria mediada via guanilato monofosfato cíclico (GMPc) (DAL SECCO et al., 2006; DAL-SECCO et al., 2003).

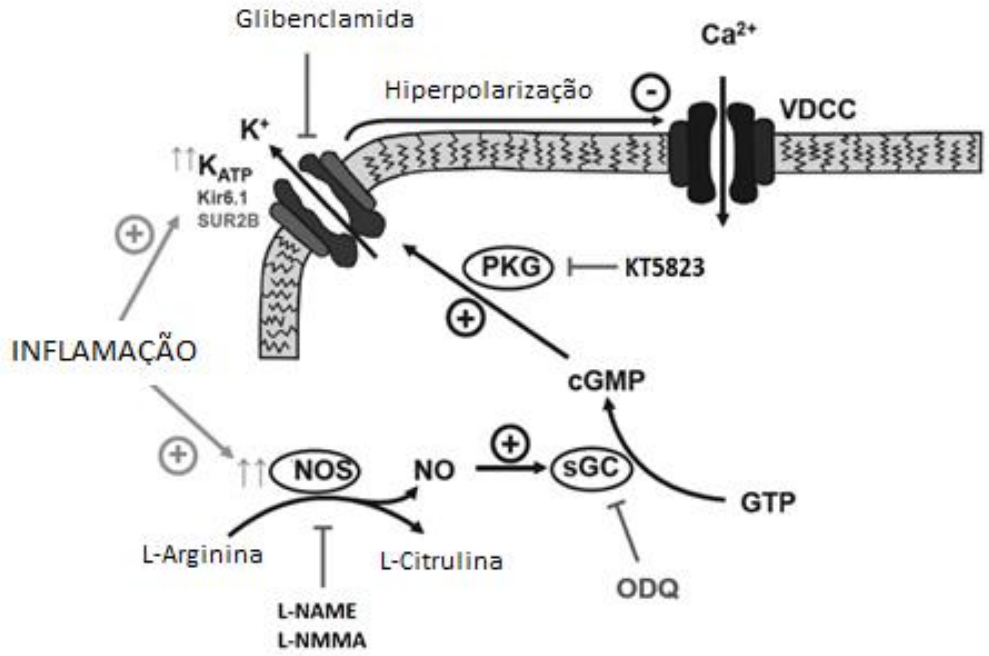
Estudos demonstram que o NO também participa na resposta inflamatória podendo ter efeitos tanto pró como anti-inflamatório, tais efeitos dependem da concentração, do local de atuação e do modelo utilizado para se avaliar a dor

(MICLESCU; GORDH, 2009). O NO formado pela NOS é rapidamente permeável através de membranas biológicas e ativa a enzima guanilato ciclase solúvel que produz um aumento nos níveis de guanosina monofosfato cíclico (GMPc). O GMPc estimula a proteína quinase dependente de GMPc, a PKG, que pode fosforilar diversas proteínas e ativar canais de potássio sensíveis ao ATP (K^+ATP), levando a hiperpolarização ou a saída de cálcio do citoplasma (FERREIRA et al., 2009; MISCLESCU; GORDH, 2009; MONCADA; HIGGS, 2006), contribuindo para a vasodilatação e modulação da transmissão sináptica (MICLESCU; GORDH, 2009; MONCADA; HIGGS, 2006).

A via de sinalização NO/GMPc/PKG/canais de K^+ATP contribui para a anti-hiperalgesia em modelos animais de dor e vem sendo alvo de diversos estudos de drogas potencialmente analgésicas (CUNHA et al., 2010; POSSEBON et al., 2014; SACHS; CUNHA; FERREIRA, 2004; STAURENGO-FERRARI et al., 2014). Drogas que bloqueiam de maneira direta a hiperalgesia agem bloqueando a sensibilização dos nociceptores por promover a abertura dos canais de potássio sensíveis a ATP, no qual a saída desse íon da célula contrabalançaria o limiar de ativação aumentado devido à modulação de canais de sódio e cálcio durante a sensibilização neuronal (SACHS; CUNHA; FERREIRA, 2004).

Para se avaliar o efeito de novas substâncias sobre a via de sinalização do óxido nítrico são utilizados diversos inibidores da via L-arginina/NO/GMPc/PKG/K^+ATP, uma vez que o uso de inibidores irá coibir a produção de NO e toda a cascata de eventos posteriores (Figura 2) (FERREIRA, et. al, 2008).

Figura 2 – Via L-Arginina/NO/GMPc/PKG/K^+ATP

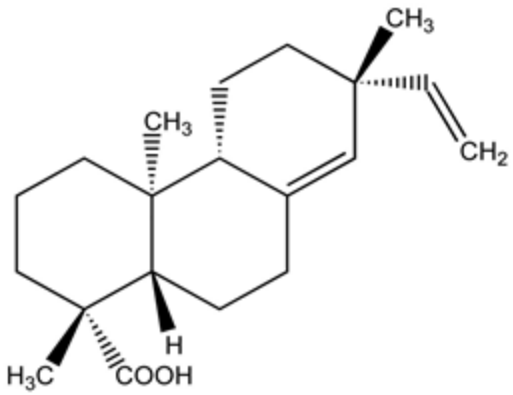


Fonte: MATHIAS; VON DER WEID, 2013 - modificado

1.6. Ácido Pimaradienóico

Ácido Pimaradienóico (ent-pimara-8(14),15-dien-19oic acid) (Figura 3) é um diterpeno pimarano pertencente à família das Asteraceas. No Brasil são encontrados cerca de 35 espécies dessa família, a maioria em regiões de cerrados brasileiro. Os diterpenos pimaranos apresentam diversas atividades, tais como: ação antiespasmódica e relaxante sobre o músculo liso (AMBROSIO;SCHORR; DA COSTA, 2004; AMBROSIO et al., 2002;), anti-inflamatória (KANG et al., 2008; LIM et al., 2009; MIZOKAMI et al., 2016; POSSEBON et al., 2014), antimicrobiana CHEUNG; FU; SMAL, 1994) e analgésica (OKUYAMA; NISHIMURA; YAMAZAKI, 1991; POSSEBON et al., 2014).

Figura 3 – Estrutura química do ácido Pimaradienóico



Fonte: AMBROSIO et al., 2002

Em relação ao seu efeito anti-inflamatório, o ácido Pimaradienóico na dose de 100 μM se mostrou eficaz ao inibir, *in vitro,* a produção de NO e PGE_2, ciclooxigenase-2 e IL-6, bem como induziu o aumento da expressão de RNA mensageiro para iNOS e ativou NFκB em culturas de macrófagos RAW 264.7 estimuladas com LPS (KANG et al., 2008). *In vivo,* o ácido Pimaradienóico inibiu a formação de edema de pata induzido pela administração de carragenina em camundongos (LIM et al, 2009; POSSEBON et al., 2014). Em modelo de dor manifesta, o tratamento com ácido Pimaradienóico nas doses de 1-100 mg/kg inibiu o número de contorções abdominais induzido pela adminstração intraperitoneal de ácido acético (OKUYAMA; NISHIMURA; YAMAZAKI, 1991; POSSEBON et al., 2014) e inibiu o número de sacudidas e o tempo de lambida na pata após adminstração de solução de formalina 1,5% ou Adjuvante completo de Freund (CFA) na dose de 10 mg/kg, administrado por via oral (POSSEBON et al., 2014). Estes experimentos estão relacionados com a liberação de histamina, serotonina, prostaglandinas e citocinas, no qual culminam na instalação e no desenvolvimento do processo inflamatório (BORGHI et al., 2013; PAVAO-DE-SOUZA et al., 2012).

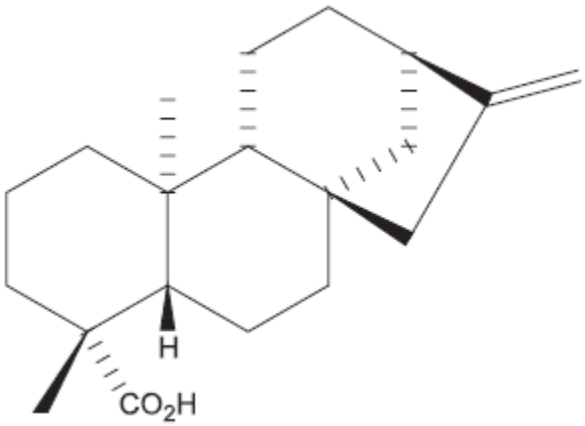
Ácido Pimaradienóico (10 mg/kg) também inibiu a hiperalgesia mecânica induzida pelos estímulos hiperalgésicos CFA e carragenina por inibir a ativação de NFκB, produção de citocinas (TNF-α, IL-1β, IL-33 e IL-10) e ativar a via NO-cGMP-PKG-canais de potássio sensíveis ao ATP (POSSEBON et al., 2014). Em relação à

sua toxicidade, o ácido Pimaradienóico não alterou os níveis plasmáticos de aspartato aninotrasferase (AST) e alanina aminotrasferase (ALT), não havendo dano hepático após sete dias de tratamento (POSSEBON et al., 2014).

1.7. Ácido Caurenóico

Ent-kaur-16-en-19-oic acid ou ácido Caurenóico (Figura 4) é um diterpeno presente em maior quantidade na *Sphagneticola trilobata* (L.) Pruski (sinônimos: *Wedelia Paludosa, Acmela brasiliensis*), também conhecida popularmente como pseudo-arnica, margaridão, mal-me-quer-do-brejo, vedelia ou picão da praia (FUCINA et al., 2012; MEOTTI et al., 2006). Participa como caurano intermediário na biogênese das giberelinas, hormônios que controla várias funções biológicas da planta como germinação, crescimento, floração e defesa contra microrganismos (CASTRILLO et al., 2001).

Figura 4 – Estrutura química do ácido Caurenóico



Fonte: TIRAPELLI et al., 2004

O ácido Caurenóico apresenta diversas atividades farmacológicas, dentre as quais destacam-se sua ação antibacteriana (SILVA et al., 2002; YATSUDA et al., 2005), anti-parasitária (MACRI et al., 2014; MIRANDA et al., 2015; VIEIRA et al., 2002), anti-cancerígeno (COSTA-LOTUFO et al., 2002; LIZARTE NETO et al., 2013), anti-asmático (CHO et al., 2010), antiespasmótico e vasorelaxante (AMBROSIO et al., 2004; TIRAPELLI et al., 2004), anti-glicêmico (BRESCIANI et al., 2004), analgé-

sico (BLOCK et al., 1998; MIZOKAMI et al., 2012) e anti-inflamatório (BOLLER et al., 2010; CHOI et al., 2011; DÍAZ-VICIEDO et al., 2008; LIM et al., 2009; LYU et al., 2011; MIZOKAMI et al., 2012; PAIVA et al., 2002).

Em relação à sua atividade antibacteriana, o ácido Caurenóico mostra ser mais eficaz contra bactérias do tipo gram-positivas. Apresenta atividade tanto bacteriostática quanto bactericida contra *Streptococcus mutans, Staphylococcus aureus, Staphylococcus epidermidis* e *Bacillus cereus* (OKOYE et al., 2012; SILVA et al., 2002; YATSUDA et al., 2005) e ineficaz contra o crescimento de bactérias gram-negativas como *Escherichia coli* (OKOYE et al., 2012; SILVA et al., 2002) e *Pseudomonas aeruginosa* (SILVA et al., 2002). Essa ação diferente em relação ao tipo de bactéria pode ser devido aos componentes existentes na parede das bactérias gram-negativas, que impediria o ácido Caurenóico de atravessar a membrana bacteriana e chegar até o citoplasma para desenvolver sua ação (WILKENS et al., 2002). Ácido Caurenóico também se mostrou eficaz ao inibir a adesão e formação de placa bacteriana causada por *Streptococcus mutans* (JEONG et al., 2013; YATSUDA et al., 2005), o maior causador de placa bacteriana e cáries dentais. *Streptococcus mutans* pode metabolizar açucares em ácido lático e fórmico, reduzindo o pH do dente, desmineralizando o esmalte do dente e levando ao surgimento de caries (JEONG et al., 2013).

Seu efeito anti-tumorogênico foi testado *in vitro* em diferentes culturas de células tumorais apresentando efeito citotóxico e antiproliferativo contra células tumorais do cólon e mama (COSTA-LOTUFO et al., 2002; HENRY et al., 2006), da próstata (HENRY et al., 2006), do rim, pancrêas e HeLa (OKOYE et al., 2014), e contra glioblastoma (tumor maligno cerebral) (LIZARTE NETO et al., 2013) e células leucêmicas (COSTA-LOTUFO et al., 2002). O mecanismo molecular envolvido nesta atividade inclui a inibição de fatores oncogênicos e a ativação da via apoptótica através da inibição da ativação de NFκB e ativação de caspase 8 (FERNANDES et al., 2013; LIZARTE NETO et al., 2013; OKOYE et al., 2014).

Cho e colaboradores (2010) avaliaram a atividade anti-espasmótica do diterpeno extraído da raiz da *Aralia cordata*. Em seus experimentos, foi avaliada a ação do ácido Caurenóico na resistência das vias aéreas na fase imediata e tardia da asma induzida por Ig-E em porcos sensibilizados com ovalbumina. Foram avaliados

também o recrutamento de neutrófilos e a presença de mediadores químicos no fluido lavado broncoalveolar (BALF). Ácido Caurenóico na dose de 50 mg/Kg diminuiu a resistência das vias aéreas na fase tardia da asma em mais de 40% e o recrutamento de eosinófilos e neutrófilos para o pulmão, principais células responsávés pelo quadro inflamatório da asma. Diminuiu a liberação de histamina, a atividade da fosfolipase A2 (PLA_2) e a peroxidase eosinofilica (EPO) analisadas no BALF. Além disso, revelou efeito semelhante ao encontrado com drogas anti-asmáticas disponíveis no mercado (CHO et al., 2010).

Tirapelli (2004) verificou *in vitro*, o efeito do ácido Caurenóico em modelo de contração de artérias isoladas de ratos. O endotélio da artéria aorta íntegra ou seus anéis foram conectados a um transdutor de força e as contrações do músculo induzidas por fenilefrina ou cloreto de potássio (KCl). O pré-tratamento com ácido Caurenóico (10, 50 e 100 μM) reduziu de maneira dose-dependente as contrações induzidas por fenilefrina e KCl tanto no endotélio da aorta íntegra quanto nos anéis aórticos e causou o relaxamento do vaso. Isto se deve ao fato do ácido caurenóico bloquear os canais de cálcio pela ativação da via NO/GMPc e abertura dos canais de potássio (TIRAPELLI et al., 2004).

A ação analgésica do ácido Caurenóico foi primeiramente demonstrada por Block e colaboradores em 1998. Extrato contendo ácido caurenóico extraído da *Wedelia paludosa* foi administrado via intraperitoneal nas doses de 1-60 mg/Kg e o número de contorções abdominais induzido pela administração de ácido acético 0,6 % foi avaliada. A dose de 3 mg/Kg do extrato inibiu 55% o número de contorções induzido por ácido acético (BLOCK et al., 1998). Corroborando com esses dados, ácido Caurenóico extraído da *Annona reticulada* L. Bark e *Sphagneticola trilobata* também inibiu o número de contorções abdomais induzida por ácido acético (CHAVAN et al., 2012; MIZOKAMI et al., 2012). Tratamento com ácido Caurenóico na dose de 10 mg/Kg, por via oral, inibiu o número de contorções abdominais induzido pela adiminstração de PBQ (Phenil-p-benzoquinona) e inibiu o número de sacudidas e o tempo de lambida na pata estimuladas com solução de formalina ou CFA (MIZOKAMI et al., 2012).

Em modelos de colite induzido pela administração de ácido acético, o diterpeno reduziu significativamente o dano tecidual e o edema do cólon em ratos na dose

de 100 mg/Kg. Diminuiu de maneira dose-dependente a atividade da mieloperoxidase (MPO) para o local inflamado (PAIVA et al., 2002). MPO é uma enzima presente dentro dos leucócitos e utilizada de forma indireta na análise de migração de leucócitos, especialmente neutrófilos (CASAGRANDE et al., 2006; MIZOKAMI et al., 2016), Ácido Caurenóico diminuiu também a concentração de malondialdeido (MDA), um indicador da lipoperoxidação lipídica. Em modelos de colite induzida por ácido acético o aumento da MPO e MDA podem gerar radicais livres e consequente instalação do processo inflamatório e a formação de ulcerações (PAIVA et al., 2002).

Ademais, a aplicação tópica do ácido Caurenóico inibiu a formação de edema de orelha induzido por óleo de cróton (dose de 1 mg/mL; DE CARLI et al., 2009) e 12-O-tetradecanoilforbol acetato (TPA; dose de 0,1-1 mg/orelha; BOLLER et al., 2010) em camundongos. O composto extraído da *Aralia continentalis* também inibiu a formação de edema de pata em camundongos induzido pela administração de carragenina, porém não inibiu de forma significativa a produção de PE_2 na concentração de 100 µM em culturas de células de macrófagos RAW 264.7 ativadas por LPS. Os autores sugerem que esta diferença na atividade anti-inflamatória em modelos *in vivo* e *in vitro* pode estar relacionada ao mecanismo de ação do ácido Caurenóico, como a inibição de citocinas pró-inflamatórias (LIM et al., 2009).

Nosso laboratório também confirmou o efeito anti-inflamatório do ácido Caurenóico em modelo de hiperalgesia induzido por carragenina, PGE_2 e CFA. Tratamento com ácido Caurenóico na dose de 10 mg/Kg por via oral, inibiu a hiperalgesia mecânica induzida por essas drogas e o mecanismo anti-inflamatório do ácido Caurenóico esta relacionado à diminuição na produção de citocinas pró-inflamatória (TNF-α e IL-1β), produção de NO e ativação da via anti-hiperalgésica GMPc/PKG/K^+ATP (MIZOKAMI et al., 2012). Outros autores também demonstram a participação do ácido Caurenóico em inibir a ativação de NFκB e consequentemente, a produção de mediadores inflamatórios (CASTRILLO et al., 2001; CHOI et al., 2011).

Em relação à toxicidade, o ácido Caurenóico demonstrou ser seguro não apresentando alterações em exames bioquímicos, histológicos e de avaliação comportamental (BÜRGER et al., 2005; MIZOKAMI et al., 2012). Bürger e colaboradores em 2005, avaliaram a toxicidade aguda e subaguda do diterpeno. Camundongos

receberam por via oral extrato hidroalcoólico contendo ácido Caurenóico na dose 100-4.000 mg/Kg em dose única (toxicidade aguda) ou durante 15 dias (toxicidade subaguda). O tratamento agudo e subagudo com o composto hidroalcoólico não apresentou nenhuma mudança em relação ao comportamento, ao sistema respiratório, nervoso, gastrointestinal ou metabolismo do animal durante o tratamento. Também não foi apresentado nenhum efeito cutâneo ou aumento da taxa de mortalidade. A análise dos níveis de AST, ALT e gama-glutamiltransferase (GGT) não demonstraram lesão hepatocelular e a análise macroscópica do fígado, pulmão, coração, baço e rim não apresentou nenhuma alteração em relação à cor e textura, indicando que o ácido Caurenóico não apresenta toxicidade quando administrado em altas doses ou por período mais prolongado (BÜRGER et al., 2005).

Baseado nos dados revisados nós propomos investigar o efeito analgésico e anti-inflamatório dos diterpenos ácido Pimaradienóico e ácido Caurenóico em diferentes modelos inflamatórios em camundongos.

2. Objetivos

2.1. Objetivo Geral

Investigar a ação analgésica e anti-inflamatória dos diterpenos ácido Pimaradienóico e ácido Caurenóico em diferentes modelos de inflamação em camundongos bem como seus possíveis mecanismos de ação.

2.2. Objetivos Específicos

- Verificar a ação anti-inflamatória do ácido Pimaradienóico em modelo de peritonite induzida pela adminstração de carragenina;

- Avaliar o efeito do pós tratamento com ácido Caurenóico no modelo de peritonite induzida por LPS;

- Verificar a ação analgésica e anti-inflamatória do ácido Caurenóico no modelo de peritonite induzido por LPS;

- Verificar a ação analgésica e anti-inflamatória do ácido Caurenóico no após a administração de LPS na pata do camundongo;

- Avaliar a ação anti-inflamatória do ácido Caurenóico em modelo de dor neuropática pela constrição do nervo ciático (CCI);

- Verificar se a ação anti-inflamatória do ácido Caurenóico permanece durante o tratamento por sete dias no modelo de CCI;

3. Artigos

Este trabalho foi realizado pelo Laboratório de Dor, Inflamação, Neuropatia e Câncer e é composto por 03 artigos.

Artigo 1: Pimaradienoic Acid Inhibits Carrageenan-Induced Inflammatory Leucocyte Recruitment and Edema in Mice: Inhibition of Oxidative Stress, Nitric Oxide and Cytokine Production.

O artigo 1 foi aceito e segue as normas da revista Plos One (ISSN: 1932-6203).

Artigo 2: Kaurenoic Acid Reduces Lipopolysaccharide-Induced Leukocyte Recruitment, Hyperalgesia and Oxidative Stress in Mice.

A formatação do artigo 2 segue as normas da Revista Journal Natural Products (ISSN:0163-3864)

Artigo 3: Kaurenoic Acid Inhibits Ongoing Chronic Constriction Injury-Induced Neuropathic Pain by Inhibing Spinal Cord Neuroinflammation.

A formatação do artigo 3 segue as normas da Revista Neuropharmacology (ISSN: 0028-3908).

ARTIGO 1

PLOS ONE

RESEARCH ARTICLE

Pimaradienoic Acid Inhibits Carrageenan-Induced Inflammatory Leukocyte Recruitment and Edema in Mice: Inhibition of Oxidative Stress, Nitric Oxide and Cytokine Production

Sandra S. Mizokami[1☯], Miriam S. N. Hohmann[1☯], Larissa Staurengo-Ferrari[1☯], Thacyana T. Carvalho[1☯], Ana C. Zarpelon[1☯], Maria I. Possebon[2☯], Anderson R. de Souza[3☯], Rodrigo C. S. Veneziani[3☯], Nilton S. Arakawa[2☯], Rubia Casagrande[2‡*], Waldiceu A. Verri, Jr.[1‡*]

1 Departamento de Ciências Patológicas - Centro de Ciências Biológicas, Universidade Estadual de Londrina, Londrina, Paraná, Brazil, 2 Departamento de Ciências Farmacêuticas - Centro de Ciências de Saúde, Universidade Estadual de Londrina, Londrina, Paraná, Brazil, 3 Núcleo de Pesquisa em Ciências Exatas e Tecnológicas, Universidade de Franca, Franca, São Paulo, Brazil

☯ These authors contributed equally to this work.

‡ These authors share senior authorship and contributed equally to this work.

* rubiacasa@uel.br (RC); waverri@uel.br (WAV)

Citation: Mizokami SS, Hohmann MSN, StaurengoFerrari L, Carvalho TT, Zarpelon AC, Possebon MI, et al. (2016) Pimaradienoic Acid Inhibits CarrageenanInduced Inflammatory Leukocyte Recruitment and Edema in Mice: Inhibition of Oxidative Stress, Nitric Oxide and Cytokine Production. PLoS ONE 11(2): e0149656. doi:10.1371/journal.pone.0149656

Editor: Bernhard Ryffel, French National Centre for Scientific Research, FRANCE

Received: November 18, 2015

Accepted: February 3, 2016

Published: February 19, 2016

Data Availability Statement: All relevant data are within the paper files.

Funding: This work was supported by Brazilian grants from Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil-SSM MSNH TTC ACZ ARS RC WAV), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil-LSF RCSV RC WAV), Ministério da Ciência, Tecnologia e Inovação (MCTI)/ Secretaria da Ciência, Tecnologia e Ensino Superior (SETI)/ Fundação Araucária, and Paraná State Government (Brazil- MSNH ACZ RC WAV). ACZ received a post-doc fellowship from CAPES/Fundação Araucária. LSF received a post-doc fellowship of CNPq. The specific roles of these authors are articulated in the 'author contributions' section. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Pimaradienoic acid (PA; *ent*-pimara-8(14),15-dien-19-oic acid) is a pimarane diterpene found in plants such as Vigueira arenaria Baker (Asteraceae) in the Brazilian savannas. Although there is evidence on the analgesic and *in vitro* inhibition of inflammatory signaling pathways, and paw edema by PA, its anti-inflammatory effect deserves further investigation. Thus, the objective of present study was to investigate the anti-inflammatory effect of PA in carrageenan-induced peritoneal and paw inflammation in mice. Firstly, we assessed the effect of PA in carrageenan-induced leukocyte recruitment in the peritoneal cavity and paw edema and myeloperoxidase activity. Next, we investigated the mechanisms involved in the anti-inflammatory effect of PA. The effect of PA on carrageenan-induced oxidative stress in the paw skin and peritoneal cavity was assessed. We also tested the effect of PA on nitric oxide, superoxide anion, and inflammatory cytokine production in the peritoneal cavity. PA inhibited carrageenan-induced recruitment of total leukocytes and neutrophils to the peritoneal cavity in a dose-dependent manner. PA also inhibited carrageenan-induced paw edema and myeloperoxidase activity in the paw skin. The anti-inflammatory mechanism of PA depended on maintaining paw skin antioxidant activity as observed by the levels of reduced glutathione, ability to scavenge the ABTS cation and reduce iron as well as by the inhibition of superoxide anion and nitric oxide production in the peritoneal cavity. Furthermore, PA inhibited carrageenan-induced peritoneal production of inflammatory cytokines TNF-α and IL-1β. PA presents prominent anti-inflammatory effect in carrageenan induced inflammation by reducing oxidative stress, nitric oxide, and cytokine production. Therefore, it seems to be a promising anti-inflammatory molecule that merits further investigation.

Introduction

Inflammation is a common mechanism of many diseases. Despite the importance of controlling inflammation, the current anti-inflammatory drugs present many side effects that limit their clinical use [1]. Therefore, it is still necessary to develop novel anti-inflammatories. Inflammatory cardinal signs include the development of pain, erythema, heat, edema, and loss of function. An important non-clinical sign of inflammation involves the recruitment of leukocytes to the inflammatory foci [2]. Upon an inflammatory stimulus, resident cells produce cytokines to communicate the threat to other cells and respond to it. Cytokines activate the endothelial cells to express adhesion molecules and chemoattract leukocytes to the inflammatory foci [3]. These leukocytes are mainly neutrophils in acute inflammation. At the inflammatory foci, neutrophils produce reactive oxygen species such as superoxide anion and nitric oxide [3], which induce tissue damage by oxidative stress and forming the highly reactive and deleterious peroxynitrite [4]. Due to the harmful effects of exacerbated inflammation, the use of anti-inflammatories is a useful clinical tool to control inflammation and reduce tissue damage [1].

Ent-pimara-8(14),15-dien-19-oic acid is a pimarane diterpene known as pimaradienoic acid (PA). Various plants produce PA, especially *Vigueira arenaria* Baker (Asteraceae), which presents PA in high concentrations. *V. arenaria* is a herbaceous plant native of the Brazilian savannas [5–7]. The pharmacological activities of PA include the antispasmodic and relaxant actions on vascular smooth muscle and inhibition of rat carotid contractions [7–9], and antimicrobial activity [10, 11].

Furthermore, evidence supports the anti-inflammatory action of PA as follows. *In vitro*, PA inhibits LPS-induced production of IL-6, nitric oxide (NO) and prostaglandin (PG) E_2 as well as cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) mRNA expression, and NF-κB activation in RAW 264.7 macrophages [12]. *In vivo*, PA inhibited carrageenan-induced inflammatory paw edema in mice [13]. Moreover, PA inhibited acetic acid-induced inflammatory abdominal writhing response [14, 15]. PA also inhibited carrageenan- and complete Freund´s adjuvant-induced mechanical hyperalgesia by inhibiting paw skin NFκB activation, cytokine production, and activating the nitric oxide (NO)/ cyclic guanosine monophosphate (cGMP)/ ATP-sensitive potassium channels signaling pathway [14]. Importantly, PA does not in-

duce gastric or liver damage within a seven days treatment protocol [14], which corroborates its potential applicability to control inflammation with reduced side effects compared to nonsteroidal anti-inflammatory drugs. It is also not expected that PA would present corticoid-like side effects since these occur as a result of binding to corticoid receptor and activation of corticoid responsive genes [16].

Despite the above-mentioned evidence, it remains to be determined whether PA inhibits other inflammatory parameters in addition to paw edema and its mechanisms *in vivo.* Therefore, the anti-inflammatory effect and mechanisms of PA were investigated in the present study.

Materials and Methods

Animals

Male Swiss mice (20–25 g), from Universidade Estadual de Londrina, Londrina, Paraná, Brazil, were used in this study. Mice housing was in standard clear plastic cages with free access to food and water, a light/dark cycle of 12:12 h, and kept at 21°C. In all behavioral testing, experiments were performed between 9 a.m. and 5 p.m. in a temperature-controlled room. Before sample collection, mice were terminally anesthetized with 3% isoflurane followed by decapitation. The Ethics Committee of the Universidade Estadual de Londrina (CEUA-UEL) specifically approved this study (process number 1531.2013.76). Every effort was made to minimize the number of animals used and their suffering.

Plant material

M. Magenta collected *Viguiera arenaria* Baker (Asteraceae) at Itirapina—SP (22°13 S, 47°54W, SP, Brazil), identified the plant material, and deposited a voucher specimen under the code SPF #61 in the herbarium of the University of São Paulo (SP, Brazil). Prof. F. B. Costa gently provided the plant material [7, 8, 14]. The Genetic Heritage Management Council (CNPq, Brazil, Process #010055/2012-6) authorized collecting *V. arenaria*. It is noteworthy to mention that *V. arenaria* is not endangered or protected specie.

Extraction and isolation

Extraction of air-dried tuberous roots (980 g) from *V. arenaria* was with CH_2Cl_2 for 30 minutes using a sonicator to yield 82 g of crude extract. After suspension in MeO-HyH_2O (9:1, v/v), the crude extract was exhaustively washed with hexane and CH_2Cl_2 to yield 39.5 g (hexane phase) and 25.0 g (CH_2Cl_2 phase). The hexane phase was chromatographed over Si gel using vacuum liquid chromatography to yield six fractions: F1 (0.5 g), F2 (13.3 g), F3 (14.2 g), F4 (5.3 g), F5 (2.4 g) and F6 (3.6 g). Fraction F2 furnished the diterpene PA. Isolation and purification steps were carried out by flash chromatography (hexane-EtOAc), PTLC (Si gel, hexane-EtOAc or hexane-$CHCl_3$) and recrystallization from MeOH. The structure of the diterpene was established by comparison of the 1H and 13C NMR spectral data with those reported in the literature [6, 7, 14].

Drugs

The compounds used in this study were carrageenan and dimethyl sulfoxide (DMSO) (Santa Cruz Biotechnology, Santa Cruz, CA, United States). Nitroblue Tetrazolium (NBT) (SigmaAldrich, St. Louis, MO, USA). Mouse IL-1β and TNF-α ELISA kits obtained from eBioscience (San Diego, CA, USA).

Experimental proticols

The optical rotation of PA was measured in $CHCl_3$ using a Perkin Elmer 241 polarimeter. Nuclear magnetic resonance (NMR) spectra were run on a Bruker DPX 400 spectrometer (400 MHz for 1H and 100 MHz for 13C). Samples were dissolved in $CDCl_3$, and the spectra were calibrated with the solvent signals at δ 7.26 (1H) and δ 77.0 (13C). Mass spectrometric analysis was performed at low resolution on a Micromass Quattro-LC instrument (Manchester, UK) provided with an ESI ion source and a triple quadrupole mass analyzer. Solutions were dissolved in MeOH-H_2O 8:2 (v/v) and infused into the ESI source at a flow-rate of 5 μL/minute, using a Harvard apparatus model 1746 (Holliston, MA) syringe pump. Vaccum-liquid chromatography (VLC) was carried out using silica gel 60H (Merck, art. 7736) in glass columns with 5–10 cm i.d.29 High-performance liquid chromatography (HPLC) analysis was accomplished using a Shimadzu CBM-20A liquid chromatography controller, operating

with LC solution software, equipped with a Shimadzu UV-DAD detector SPD-M20A and a Shimadzu ODS column (4.6 x 250 mm, 5 µm, 100 Å) [6, 7].

During the experiments, mice received per oral (p.o.) treatment with PA (0.1, 1 and 10 mg/ kg) or vehicle (saline) 30 minutes before intraperitoneal (i.p.; 500 µg) or intraplantar (i.pl.; 300 µg) carrageenan injection. The doses of the inflammatory stimulus were determined previously in our laboratory in pilot studies and previous work [17–19]. The number of total leukocytes, neutrophils, and mononuclear cells recruited to the peritoneal cavity was evaluated 6 hours after carrageenan (500 µg/cavity) injection. Paw edema was evaluated 0–5 hours after carrageenan (300 µg/paw) injection, and at 5 hours animals were euthanized and paw skin samples were collected for MPO activity. Oxidative stress in the paw, and superoxide anion levels, quantification of NBT positive cells, and nitric oxide production in the peritoneal cavity were assessed at 3 hours following carrageenan-stimuli. Further, and TNFα and IL-1β cytokines levels in the peritoneal cavity were determined at 6 hours. The inflammatory models and time points of sample collection were of pilot studies and previous work [18, 20–23].

Recruitment of leukocyte to peritoneal cavity in mice

The recruitment of leukocytes to peritoneal cavity was assessed 6 hours after carrageenan injection. After mice euthanasia, the peritoneal cavity cells were harvested by washing the cavity with 2 mL of phosphate-buffered saline (PBS) containing 1 mM of EDTA. The volumes recovered were similar in all experimental groups and equated to approximately 90% of the injected volume. The total leukocytes counts were performed with a Newbauer chamber, and differential cell counts (100 cells total) were carried out on cytocentrifuge slides (Cytospin 3; Shandon Southern Products, Astmoore, UK) stained with panotic solutions (Laborclin, Pinhais, Paraná, Brazil). The results are the number of total leukocytes, neutrophils or mononuclear cells x 10^6 [17, 18, 24].

Paw edema test

The volume of the mice paw was measured using an analog caliper (Digmatic Caliper, Mitutoyo Corporation, Kanagawa, Japan) before (basal) and at indicated time

points after carrageenan injection (VT). The amount of paw swelling for each mouse and the difference between VT and basal was the edema value (mm/paw)[18, 25].

Mieloperoxidase activity (MPO)

The neutrophil migration to paw was indirectly evaluated by the MPO activity kinetic-colorimetric assay [26]. Briefly, samples were collected in 50 mM K_2PO_4 buffer (pH 6.0) containing 0.5% HTAB and were homogenized using Ultra-Turrax[1] (IKA T10 Basic, CQA Química, Paulínea, SP). Then the homogenates were centrifuged at 16,100 g for 2 minutes at 4°C. Fifteen µL of the resulting supernatant was mixed with 200 µL of 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/mL o-dianisidine dihydrochloride and 0.05% hydrogen peroxide and was assayed spectrophotometrically for MPO activity determination at 450 nm (BEL SP2000UV, Photonics, São Paulo, SP, Brazil). The results of MPO activity were expressed as the number of neutrophils per mg of tissue by using a standard curve of neutrophils (100,000–1,562.5 cells). Neutrophils for the standard curve were from the peritoneal cavity of Swiss mice, 6 hours after i.p. stimulus with thioglycolate broth (1 mL, 5%, Becton Dickinson, MD, USA). The number of neutrophils was determined by total counts in Neubauer chamber and differential counts in slides stained by Rosenfelt method. We obtained 96% of neutrophils in a pool of 10 mice. Neutrophils were suspended in K_2HPO_4 buffer containing HTAB and stored at -80°C until use.

Reduced glutathione (GSH) levels measurement

Samples of paw skin were collected and maintained at −80°C for at least 48 h. Samples were homogenized in 200 µL of 0.02 M EDTA. The homogenate was mixed with 25 µL of trichloroacetic acid 50% and was homogenized three times over 15 minutes. The mixture was centrifuged (15 minutes x 1500 g at 4°C). The supernatant was added to 200 µL of 0.2 M TRIS buffer, pH 8.2, and 10 µL of 0.01M DTNB. After 5 minutes, the absorbance was measured at 412 nm (Multiskan GO, Thermo Scientific) against a blank reagent with no supernatant. A standard GSH curve was formed. The results are GSH per mg of protein.

ABTS and FRAP assays

The ability of samples to resist oxidative damage was determined by their free radical scavenging (ABTS [2,2'-Azinobis-3-ethylbenzothiazoline 6-sulfonic acid] assay) and ferric reducing ability (FRAP assay). The tests were adapted to a 96-well microplate format as previously described [21]. Plantar tissue samples were collected 3 hours after carrageenan i.pl. injection (300 µg, 25 µL) and homogenized immediately in ice-cold KCl buffer (500 µL, 1.15% w/v). The homogenates were centrifuged (200 g × 10 minutes at 4°C), and the supernatants were used in both assays. Diluted ABTS solution (200 µL) was mixed with 10 µL of sample in each well. After 6 minutes of incubation at 25°C, the absorbance was measured at 730 nm. For FRAP assay, the supernatants (10 µL) were mixed with the freshly prepared FRAP reagent (150 µL). The reaction mixture was incubated at 37°C for 30 minutes, and the absorbance was measured at 595 nm (Multiskan GO Thermo Scientific). The results of ABTS and FRAP assays were equated against a standard Trolox curve (0.02–20 nmol).

NBT assay

The quantification of superoxide anion production in tissue homogenates (10 mg/mL in 1.15% KCl) was performed using the NBT assay as previously described [27]. Briefly, 50 µL of homogenate were incubated with 100 µL of NBT (1 mg/mL) in 96-well plates at 37°C for 1 hour. The mixture was then carefully removed from wells and the reduced formazan solubilized by adding 120 µL of KOH 2 M and 140 µL of DMSO. The absorbance was measured at 600 nm (Multiskan GO ThermoScientific). The weight of samples was used for data normalization and results presented as NBT reduction (OD at 600 nm).

Nitric oxide production

Nitrite (NO_2^-) concentration in peritoneal exudate was determined by the Griess reaction as an indicator of nitric oxide production as previously reported [28]. Briefly, 100 µL of samples and 100 µL of Griess reagent (mix of 2% sulphanilamide in 5% phosphoric acid and 0.2% N(1-naphthyl) ethylenediamine hydrochloride—NEED) were mixed in 96-well ELISA plate. Absorbance was measured at 550 nm, and the levels

of NO_2^- were determined using a standard curve of $NaNO_2$. Results are µM of NO_2^- per cavity.

Cytokine measurement

Mice were treated with vehicle or PA (10 mg/kg, p.o.) 30 minutes before carrageenan (500 µg/ cavity) stimulus. Six hours after the carrageenan injection, animals were terminally anesthetized, and the peritoneal cavity cells were harvested and washing by introducing 1 mL PBS containing 1 mM of EDTA. The cytokines (TNF-α and IL-1β) levels were determined by an enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (eBioscience) and as described previously [23]. Results are pg of cytokine/cavity

Statistical analysis

Results are presented as means ± SEM of measurements made on 6 mice in each group per experiment and are representative of two independent experiments. Differences between groups were evaluated by analysis of variance (ANOVA) followed by Tukey's test. All statistical analyzes were performed using Graph Pad Prism (La Jolla, 5 CA). Statistical differences were considered to be significant at $P<0.05$.

Results

Pimaradienoic acid (PA) inhibits carrageenan-induced total leukocyte and neutrophil recruitment in the peritoneal cavity

Mice received per oral (p.o.) treatment with PA (Fig 1)[29] (0.1–10 mg/kg, 2% DMSO diluted in saline) 30 minutes before intraperitoneal (i.p.) injection of carrageenan (500 μg/cavity) and the recruitment of total leukocytes, neutrophils, and mononuclear cells was assessed at 6 hours after stimulus. Carrageenan injection induced a significant increase in the recruitment of total leukocytes (Fig 2A) and neutrophils (Fig 2B), but not mononuclear cells (Fig 2C). Only the dose of 10 mg/kg of PA inhibited carrageenan-induced total leukocyte (Fig 2A) and neutrophil (Fig 2B) recruitment. Therefore, 10 mg/kg was selected for the next experiments. No effect was observed in the number of mononuclear cells for any of the doses of PA tested (Fig 2C).

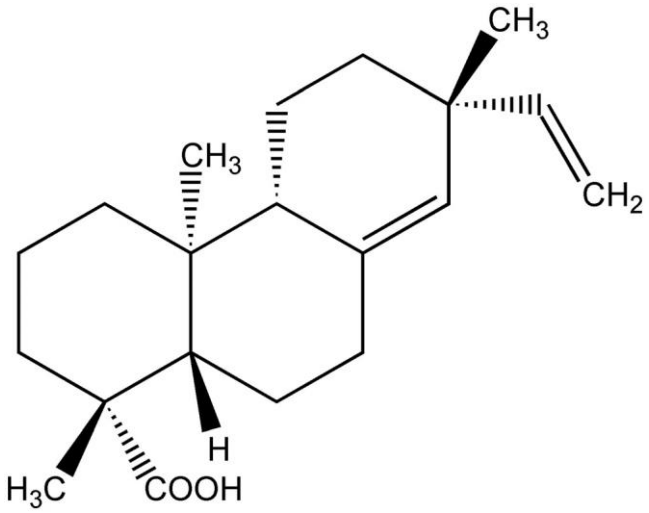


Fig 1. Pimaradienoic acid (PA) structure. Chemical structure of PA[29].

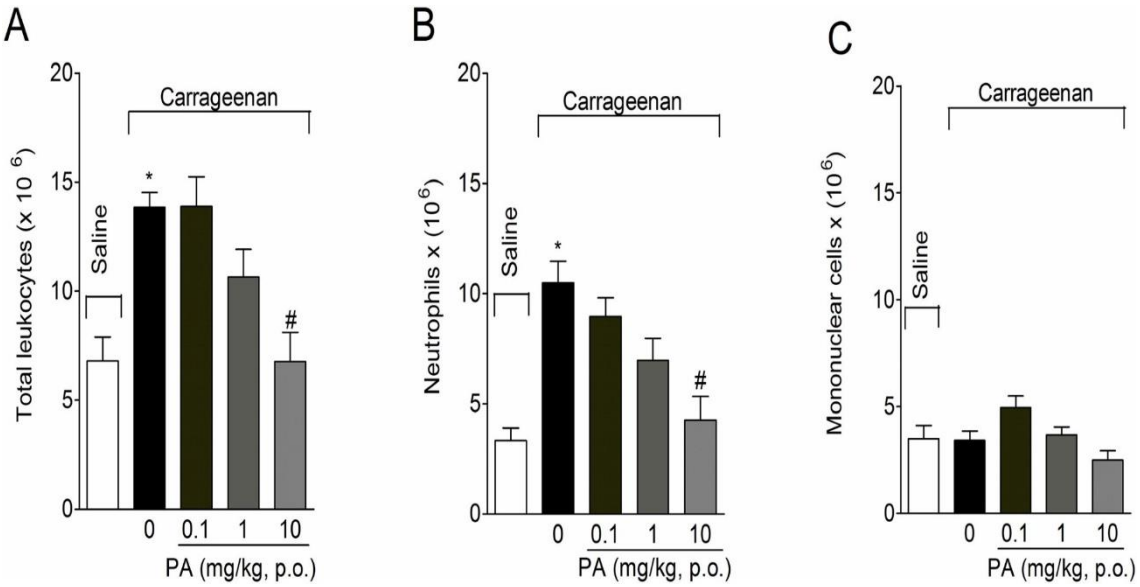


Fig 2. Pimaradienoic acid (PA) inhibits carrageenan-induced total leukocyte and neutrophil recruitment in the peritoneal cavity. Mice were treated per oral (p.o.) with PA (0.1–10 mg/kg) or vehicle (DMSO 2% diluted in saline) 30 minutes before carrageenan (500 µg/ cavity) intraperitoneal (i.p.) injection. The (A) total number of leukocytes, (B) neutrophils and (C) mononuclear cells was evaluated 6 hours after carrageenan injection. Results are means ± SEM of six mice per group per experiment and are representative of two separate experiments. [*p < 0.05 compared to the saline group; #p < 0.05 compared to the vehicle group (One-way ANOVA followed by Tukey's test)].

PA inhibits carrageenan-induced paw edema and myeloperoxidase activity

Mice received p.o. treatment with PA (10 mg/kg, 2% DMSO diluted in saline) 30 minutes before intraplantar (i.pl.) injection of carrageenan (300 µg/paw). PA significantly inhibited carrageenan-induced paw edema at 1, 3 and 5 hours (Fig 3A) and myeloperoxidase activity at 5 hours (Fig 3B) after stimulus injection.

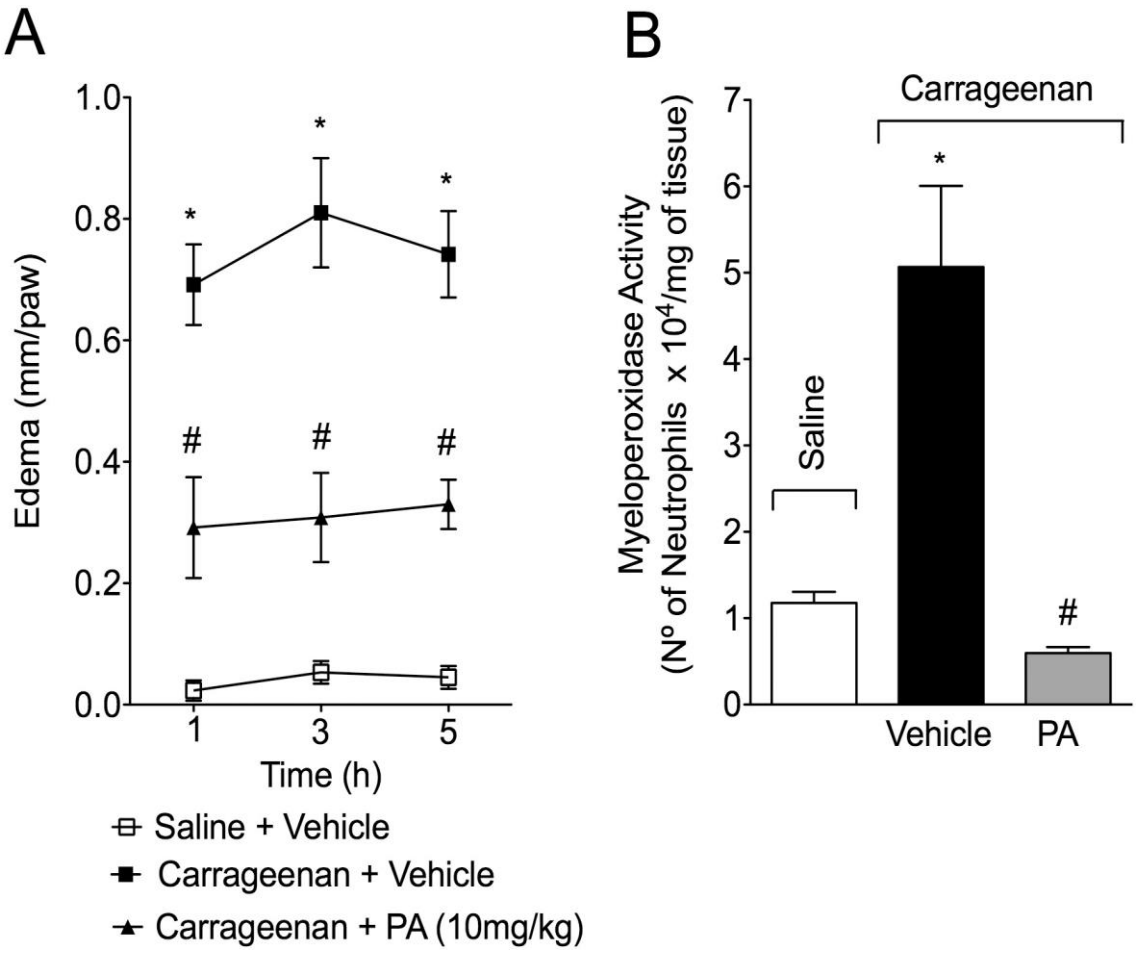


Fig 3. Pimaradienoic acid (PA) inhibits carrageenan-induced paw edema and myeloperoxidase (MPO) activity. Mice were treated per oral (p.o.) with PA (10 mg/kg) or vehicle (DMSO 2% diluted in saline) 30 minutes before the intraplantar (i.pl.) injection of carrageenan (300 µg/paw). The evaluation of (A) paw edema was at 1–5 hours and (B) MPO activity at 5 hours after carrageenan injection. Results are means ± SEM of six mice per group per experiment, and are representative of two separate experiments. [*p< 0.05 compared with the saline group, #p <0.05 compared to the vehicle group (One-way ANOVA followed by Tukey's test)].

PA inhibits carrageenan-induced oxidative stress in the paw skin

To assess the protective role of PA on carrageenan-induced oxidative stress; mice received p.o. treatment with PA (10 mg/kg, 2% DMSO diluted in saline) 30 minutes before i.pl. injection of carrageenan (300 µg/paw) and were terminally anesthetized 3 hours after the stimulus. Carrageenan-induced a reduction in GSH levels (Fig 4A), ABTS scavenging ability (Fig 4B) and ferric reducing ability (Fig 4C), which were prevented by PA treatment. In agreement, treatment with PA also inhibited the carrageenan induced increase of superoxide production (NBT assay; Fig 4D). Together, these data demonstrate that PA inhibits carrageenan-induced tissue oxidative stress.

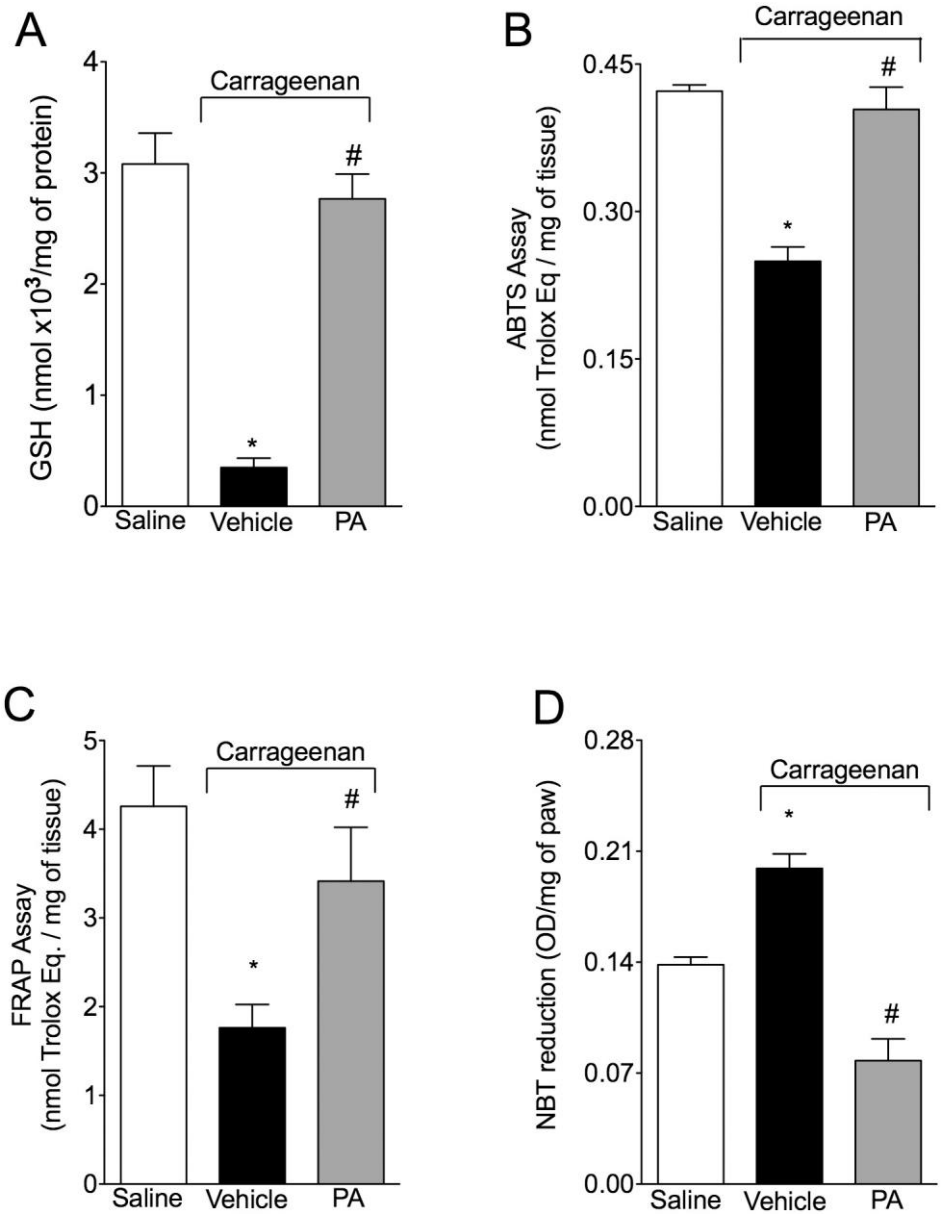


Fig 4. Pimaradienoic acid (PA) inhibits carrageenan-induced oxidative stress. Mice were treated per oral (p.o.) with PA (10 mg/kg) or vehicle (DMSO 2% diluted in saline) 30 minutes before the intraplantar (i.pl.) injection of carrageenan (300 µg/paw). Paw skin (A) GSH levels, (B) $ABTS^{+}$ scavenging activity, (C) the ability to reduce iron (FRAP), and (D) superoxide anion production were determined 3 hours after carrageenan injection. Results are means ± SEM of six mice per group per experiment, and are representative of two separate experiments. [*p<0.05 compared to the saline group; #p<0.05 compared to inflammatory stimulus group. (One-way ANOVA followed by Tukey's test)].

PA inhibits carrageenan-induced oxidative stress in the peritoneal cavity

Mice received p.o. treatment with PA (10 mg/kg, 2% DMSO diluted in saline) 30 minutes before i.p. injection of carrageenan (500 µg/paw). PA significantly inhibited carrageenan-induced NBT reduction in the peritoneal exudate (Fig 5A) at 3 hours, demonstrating that PA inhibits carrageenan-induced superoxide anion production. Furthermore, PA also reduced the number of NBT positive cells in the peritoneal cavity (Fig 5B) at this same time point. Therefore, PA not only reduces the total superoxide anion production (Figs 4D and 5A) but also reduces the number of cells producing superoxide anion (Fig 5B).

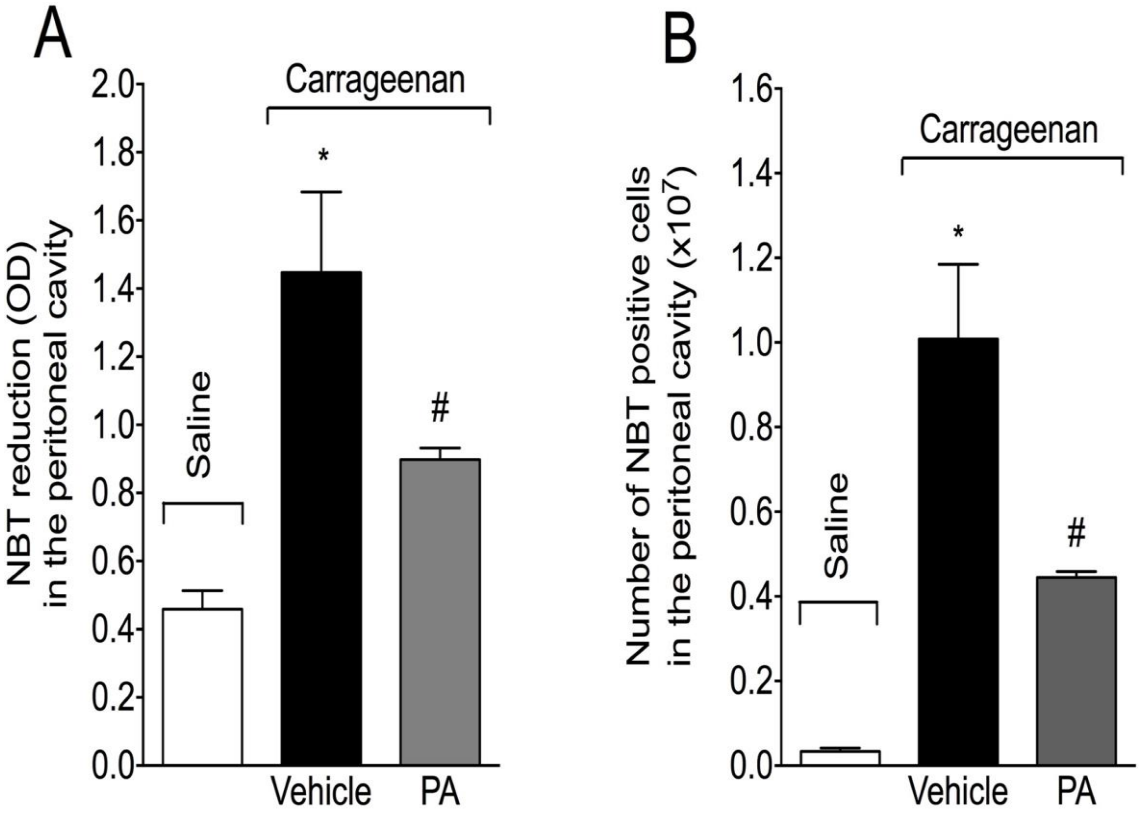


Fig 5. Pimaradienoic acid (PA) inhibited carrageenan-induced superoxide anion production. Mice were treated per oral (p.o.) with PA (10 mg/kg) or vehicle (DMSO 2% diluted in saline) 30 minutes before the intraperitoneal (i.p.) injection of carrageenan (500 µg/cavity). (A) Superoxide anion production and (B) NBT reaction positive cells (presence of formazan crystals) were determined in peritoneal cavity exudates 3 hours after carrageenan injection. Results are means ± SEM of six mice per group per experiment, and are representative of two

separate experiments. [*p< 0.05 compared with the saline group, and #p< 0.05 compared to the vehicle group (One-way ANOVA followed by Tukey's test)].

PA inhibits carrageenan-induced nitric oxide (NO) production in the peritoneal cavity

Mice received p.o. treatment with PA (10 mg/kg, 2% DMSO diluted in saline) 30 minutes before i.p. injection of carrageenan (500 µg/paw). PA significantly inhibited carrageenan-induced nitrite production in the peritoneal exudates (Fig 6) at 3 hours.

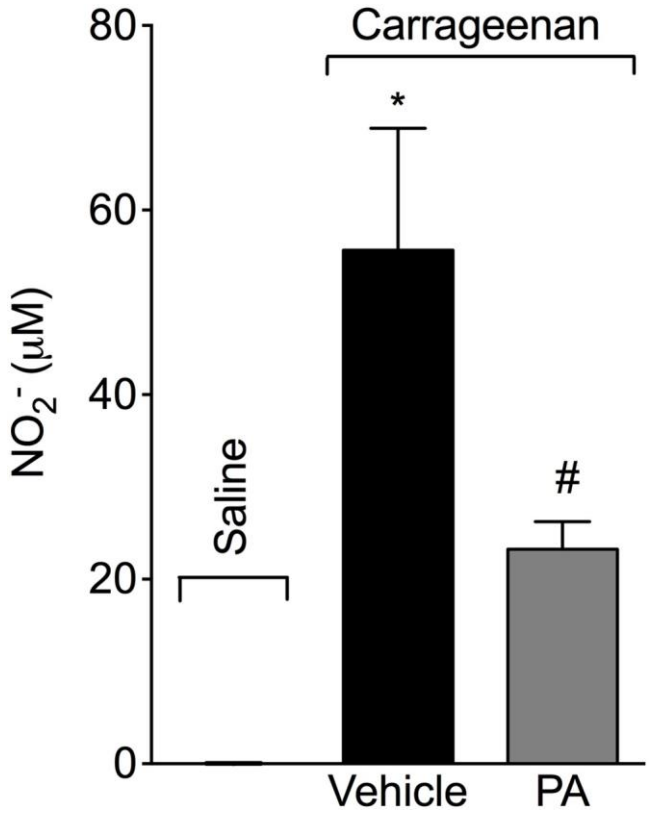


Fig 6. Pimaradienoic acid (PA) inhibits carrageenan-induced nitric oxide (NO) production. Mice were treated per oral (p.o.) with PA (10 mg/kg) or vehicle (DMSO 2% diluted in saline) 30 minutes before the intraperitoneal (i.p.) injection of carrageenan (300 µg/paw). Nitrite production in peritoneal exudates was determined 3 hours after carrageenan injection. Results are means ± SEM of six mice per group per experiment, and are representative of two separate experiments. [*p<0.05 compared with the saline group, and #p< 0.05 compared to the vehicle group (One-way ANOVA followed by Tukey's test)].

PA inhibits carrageenan-induced TNF-α and IL-1β production in the peritoneal cavity

Mice received p.o. treatment with PA (10 mg/kg, 2% DMSO diluted in saline) 30 minutes before i.p. injection of carrageenan (500 µg/paw). PA significantly inhibited carrageenan-induced TNF-α (Fig 7A) and IL-1β (Fig 7B) production in the peritoneal exudate at 6 hours. Therefore, in addition to inhibiting carrageenan-induced oxidative stress, PA also inhibits carrageenan-induced cytokine production.

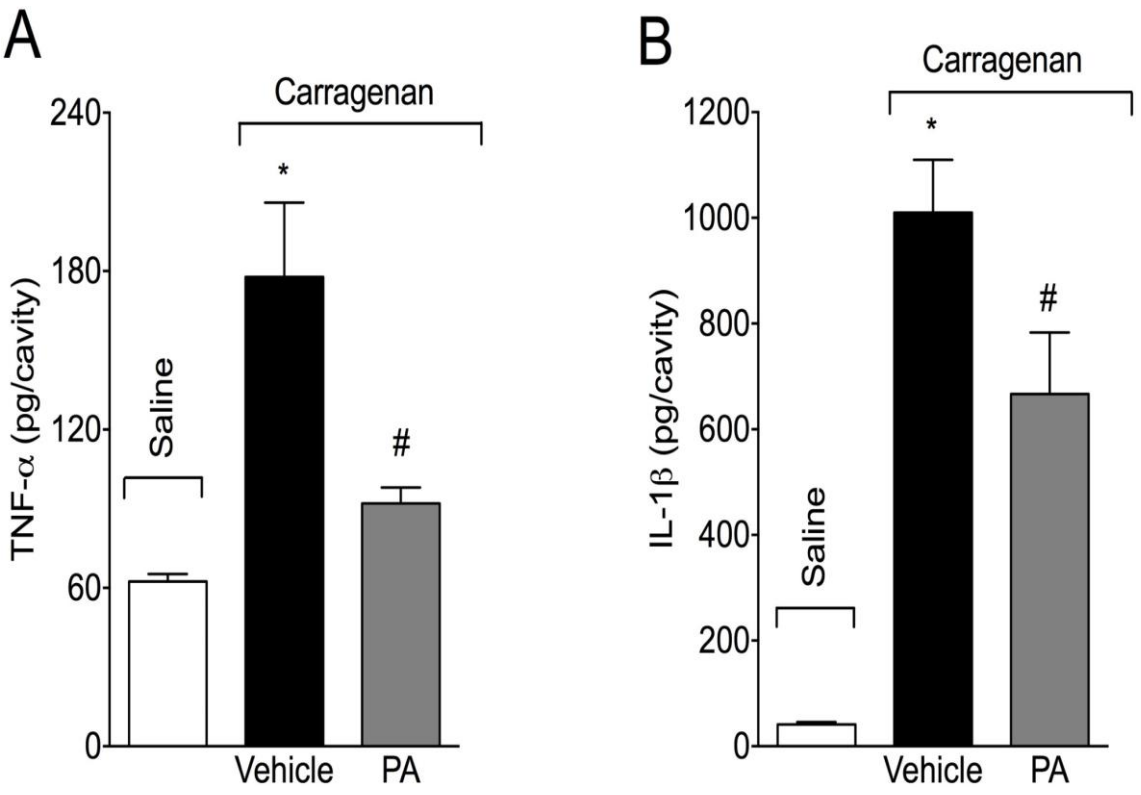


Fig 7. Pimaradienoic acid (PA) inhibits carrageenan-induced peritoneal cytokine production. Mice were treated per oral (p.o.) with PA (10 mg/kg) or vehicle (DMSO 2% diluted in saline) 30 minutes before the intraperitoneal (i.p.) injection of carrageenan (500 µg/cavity). Peritoneal exudate samples were harvested 6 hours after carrageenan injection for (A) TNF-α and (B) IL-1β determination by ELISA. Results are means ± SEM of six mice per group per experiment, and are representative of two separate experiments. [*$p<0.05$ compared with the saline group, #$p<0.05$ compared to vehicle group (One-way ANOVA followed by Tukey's test)].

Discussion

The present data demonstrate that pimaradienoic acid (PA) inhibits carrageenan-induced leukocyte recruitment and edema. The mechanisms involved in the anti-inflammatory effect of PA depended on inhibiting oxidative stress, nitric oxide and cytokine production in mice. Importantly, to our knowledge, this is the first study demonstrating that PA reduces oxidative stress *in vivo*.

We have demonstrated that PA inhibits acetic acid-induced writhing response irrespective of the route of administration, i.p., s.c. or p.o. route. Nevertheless, the p.o. route achieved only slightly lower effect than i.p. route, suggesting that the study with PA p.o. is suitable considering that this is a useful and easy route of administration to conscious patients [14]. PA inhibited carrageenan-induced total leukocyte and neutrophil recruitment to the peritoneal cavity in a dose-dependent manner. Neutrophils are the first cell type recruited in innate immune response as observed in the carrageenan model. Therefore, it is coherent that the increase of total leukocytes is predominantly reflecting the neutrophil counts [3]. The dose of PA of 10 mg/kg is the same used in a previous study on the analgesic effect of PA, which suggests that PA at this dose inhibits inflammation and pain. Nevertheless, studies of other groups observed analgesic and anti-inflammatory effects of PA with higher doses than the present study. For instance, PA inhibited carrageenan-induced paw edema by 22% at the dose of 100 mg/kg [13]. We observed up to 64% inhibition of carrageenan-induced paw edema by PA at 3 hours. Possible explanations for this divergence include that PA may present a U or bell shape effect, different doses of carrageenan (50 µg/paw versus 300 µg/paw), mouse strain (ICR versus Swiss mice), method of paw edema evaluation (plethysmometer versus caliper), and time point of evaluation (5 hours versus 1–5 hours) used in [13] and the present study, respectively.

Importantly, the carrageenan-induced paw inflammation was also useful to demonstrate that PA inhibits the increase of myeloperoxidase activity in the paw skin, which is an indirect measurement of neutrophil and macrophage counts. The result on myeloperoxidase in the paw skin corroborated the data on total leukocyte and neutrophil counts in the peritoneal cavity, demonstrating that PA inhibits carrageenan-induced leukocyte recruitment irrespectively of the tissue or cavity. Both neutrophils and macrophages contribute to MPO activity in inflamed

tissues, although neutrophils are the major source of MPO, accounting for approximately 5% of their dry mass [26]. Moreover, not all subpopulations of macrophages are MPO sources, and they depend on the type of inflammatory model (acute versus late model) [30]. Furthermore, the present assay did not detect MPO activity of up to 1 x 10^5 macrophages (data not shown). Taking into account that the carrageenan model is an acute inflammatory model, PA did not alter monocyte recruitment to the peritoneal cavity, and the employed method to assess MPO activity is selective to neutrophils [31–33], we suggest that PA reduces carrageenan-induced inflammation by inhibiting neutrophil recruitment.

Although other mechanisms, for example, apoptosis or macrophage efferocytosis, are also important in the reduction in the number of neutrophils at the site of inflammation [34], this does not seem to be the case for PA. Firstly, apoptotic leukocytes or apoptotic bodies were not observed during the examination of the recruitment of leukocytes to the peritoneal cavity (data not shown). Further, the only evidence of PA-induced apoptosis is DNA damage in liver cells of Swiss mice induced by doses 4–8 fold higher (40–80 mg/kg, p.o.) than used in the present study (0.1–10 mg/kg, p.o.) [35]. Secondly, previous studies have shown the importance of IL10 in maintaining phagocytosis/efferocytosis during inflammatory conditions [36]. However, PA does not increase IL-10 levels in the paw tissue following carrageenan stimulus but reduces its levels [14]. Collectively, these data suggest that the reduction in the carrageenan-induced increase in the number of neutrophils in the paw and peritoneal cavity of mice is due to the reduction in neutrophil recruitment rather than increasing the neutrophil apoptosis or efferocytosis.

Carrageenan-induced inflammation depends on tissue oxidative stress [18]. As PA inhibited carrageenan inflammation, it was conceivable to determine whether PA also inhibits oxidative stress. Carrageenan significantly reduced GSH levels, the tissue ability to scavenge ABTS cation radical and to reduce iron (FRAP) as well as increased superoxide anion production. PA inhibited all these parameters of oxidative stress in the paw skin. These data on paw skin oxidative stress suggests that the inhibition of carrageenan-induced oxidative stress may also explain the analgesic effect of PA [14]. Only high concentrations (IC_{50} > 100 µg/mL) of PA exhibit DPPH radical scavenging activity in vitro [37]. The fact that scavenging free radical is not the unique mechanism that an antioxidant molecule may present might explain this ap-

parent divergence. Inhibition of lipid peroxidation, iron chelation, and scavenging other free radicals are important antioxidant mechanisms [38]. It is noteworthy that the in vivo antioxidant effect of PA may be indirect by inhibiting the inflammatory process [39, 40]. Activated neutrophils are major sources of reactive oxygen species (ROS) at the site of inflammation, thus, the reduction in oxidative stress may be the result of reduced neutrophil recruitment by PA [41]. Further, the inhibition of TNF-α and IL-1β by PA may also have contributed to the reduction in oxidative stress since inflammatory cytokines can enhance ROS and reactive nitrogen species (RNS) production via NADPH oxidase and iNOS enzymes, respectively [40, 42].

PA also inhibited carrageenan-induced superoxide anion production in the peritoneal exudate and the counts of NBT positive peritoneal cells. Therefore, the PA inhibition of carrageenan-induced superoxide anion was also accompanied by a reduction of cellular activation of superoxide anion production. Although a pimaradiene diterpene with a similar structure to PA, acanthoic acid, had more prominent effect in reducing ROS production by monocytes/macrophages than by neutrophils [43], a noticeable difference between the amount of ROS induced by the stimuli used to activate monocytes/macrophages and neutrophils could be observed. In this sense, it was difficult to conclude that acanthoic acid acts similarly or differently on the production of ROS by these cells. Thus, it is possible that PA acts on the reduction of ROS production in both macrophages and neutrophils. However, future studies are necessary to elucidate the effects of PA on ROS/RNS production by each cell type.

PA also inhibited carrageenan-induced NO production in the peritoneal exudate. *In vitro* evidence showed that PA inhibits LPS-induced iNOS mRNA expression and NO production by RAW 264.7 macrophages [12]. Macrophages are important cells in the peritoneal cavity and a possible source of NO during inflammation, therefore, a potential target of PA effect [44, 45]. In addition to macrophages, neutrophils also produce NO [46]. Therefore, the reduction in neutrophil recruitment into the peritoneal cavity might have accounted for the PA reduction of carrageenan-induced NO production in the peritoneal cavity. NO has a significant role as a microbicidal molecule. However, NO reacts with superoxide anion forming peroxynitrite, which presents deleterious effects such as tissue lesion [4]. Therefore, the effect of PA of limiting superoxide anion and NO production may have as a consequence the decrease of tissue lesions together with the reduction of oxidative stress. Evidence demonstrates

that PA induces vasorelaxation in an eNOS-derived NO production manner [47]. However, in the present experimental condition, the NO production is dependent on iNOS and not eNOS, which is in agreement with *in vitro* data demonstrating that PA inhibits LPS-induced iNOS mRNA and protein expression by macrophages [12].

Cytokines are important mediators of inflammation. In fact, targeting cytokines reduces carrageenan-induced paw edema, leukocyte recruitment and myeloperoxidase activity [25, 48]. PA inhibited carrageenan-induced TNF-α and IL-1β production in the peritoneal exudates. The cytokines selection took into account their proven role in inflammatory diseases and as therapeutic targets [49]. PA has been shown to inhibit cytokine production via inhibition of NFκB. For instance, PA inhibits LPS-induced IL-6 production by RAW 264.7 macrophages by inhibiting the mitogen-activated protein kinases p38 and ERK (extracellular-regulated kinase) [12]. In vivo, PA inhibited carrageenan-induced hyperalgesia and NFκB activation in the paw skin [14]. The inhibition of NFκB activation might also explain the reduction of NO production since the expression of iNOS is NFκB dependent [50]. Therefore, it is reasonable that the PA anti-inflammatory effect observed in the present study might depend on inhibiting carrageenan-induced NFκB activation.

It is noteworthy to mention that despite PA inhibition of carrageenan-induced leukocyte recruitment, superoxide anion, and NO production, it is unlikely that PA treatment would facilitate infection since PA is an antimicrobial molecule [51]. PA inhibits the growth of endodontic bacteria such as *Porphyromonas gingivalis, Prevotella nigrescens, Prevotella intermedia, Prevotella buccae, Fusobacterium nucleatum, Bacteroides fragilis, Actinomyces naeslundii, A. viscosus, Peptostreptococcus micros, Enterococcus faecalis and Aggregatibacter actinomycetemcomitans* [51], bacteria involved in caries such as *Streptococcus salivarius, S. sobrinus, S. mutans, S. mitis, S. sanguinis and Lactobacillus casei* [52], and gram positive bacteria such as *Bacillus cereus, B. subtilis, Enterococcus faecalis, E. hirae, Kocuria rhizophila, Staphylococcus aureus strains, S. epidermidis, Streptococcus agalactiae, S. dysgalactiae, S. pneumonia, S. pyogenes* [53].

Concluding, the present study demonstrates the anti-inflammatory effect of PA on carrageenan-induce leukocyte recruitment and edema and suggests some possible mechanisms of action such as inhibition of oxidative stress, cellular activation, NO and cytokine production.

References

1. Peterson K, McDonagh M, Thakurta S, Dana T, Roberts C, Chou R, et al. Drug Class Review: Nonsteroidal Antiinflammatory Drugs (NSAIDs): Final Update 4 Report. Drug Class Reviews. Portland (OR) 2010.
2. Ashley NT, Weil ZM, Nelson RJ. Inflammation: Mechanisms, Costs, and Natural Variation. Annu Rev Ecol Evol Syst. 2012; 43:385–406.
3. Medzhitov R. Origin and physiological roles of inflammation. Nature. 2008; 454:428–35. doi: 10.1038/ nature07201 PMID: 18650913.
4. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev. 2007; 87:315–424. doi: 10.1152/physrev.00029.2006 PMID: 17237348; PubMed Central PMCID: PMC2248324.
5. Schilling EE, Da Costa FB, Lopes NP, Heise PJ. Brazilian species of Viguiera (Asteraceae) exhibit low levels of its sequence variation. Edinb J Bot. 2000; 57:323–32.
6. Ambrósio SR, Schorr K, Da Costa FB. Terpenoids of Viguiera arenaria (Asteraceae) Biochemical Systematics and Ecology. 2004; 32:221–4.
7. Tirapelli CR, Ambrosio SR, Coutinho ST, de Oliveira DC, da Costa FB, de Oliveira AM. Pharmacological comparison of the vasorelaxant action displayed by kaurenoic acid and pimaradienoic acid. J Pharm Pharmacol. 2005; 57:997–1004. doi: 10.1211/0022357056578 PMID: 16102255.
8. Tirapelli CR, Ambrosio SR, da Costa FB, Coutinho ST, de Oliveira DC, de Oliveira AM. Analysis of the mechanisms underlying the vasorelaxant action of kaurenoic acid in the isolated rat aorta. Eur J Pharmacol. 2004; 492:233–41. doi: 10.1016/j.ejphar.2004.04.003 PMID: 15178370.
9. Ambrosio SR, Tirapelli CR, da Costa FB, de Oliveira AM. Kaurane and pimarane-type diterpenes from the Viguiera species inhibit vascular smooth muscle contractility. Life Sci. 2006; 79:925–33. doi: 10. 1016/j.lfs.2006.05.015 PMID: 16780894.
10. Ambrosio SR, Furtado NA, de Oliveira DC, da Costa FB, Martins CH, de Carvalho TC, et al. Antimicrobial activity of kaurane diterpenes against oral pathogens. Z Naturforsch C. 2008; 63:326–30. PMID: 18669015.

11. Rubio J, Calderon JS, Flores A, Castroa C, Cespedes CL. Trypanocidal activity of oleoresin and terpenoids isolated from Pinus oocarpa. Z Naturforsch C. 2005; 60:711–6. PMID: 16320613.

12. Kang OH, Chae HS, Choi JG, Oh YC, Lee YS, Kim JH, et al. Ent-pimara-8(14), 15-dien-19-oic acid isolated from the roots of Aralia cordata inhibits induction of inflammatory mediators by blocking NF-kappaB activation and mitogen-activated protein kinase pathways. Eur J Pharmacol. 2008; 601:179–85. doi: 10.1016/j.ejphar.2008.10.012 PMID: 18938152.

13. Lim H, Jung HA, Choi JS, Kim YS, Kang SS, Kim HP. Anti-inflammatory activity of the constituents of the roots of Aralia continentalis. Arch Pharm Res. 2009; 32:1237–43. doi: 10.1007/s12272-009-1909-3 PMID: 19784580.

14. Possebon MI, Mizokami SS, Carvalho TT, Zarpelon AC, Hohmann MS, Staurengo-Ferrari L, et al. Pimaradienoic acid inhibits inflammatory pain: inhibition of NF-kappaB activation and cytokine production and activation of the NO-cyclic GMP-protein kinase G-ATP-sensitive potassium channel signaling pathway. J Nat Prod. 2014; 77:2488–96. doi: 10.1021/np500563b PMID: 25394199.

15. Okuyama E, Nishimura S, Yamazaki M. Analgesic principles from Aralia cordata Thunb. Chem Pharm Bull (Tokyo). 1991; 39:405–7. PMID: 2054865.

16. Schacke H, Docke WD, Asadullah K. Mechanisms involved in the side effects of glucocorticoids. Pharmacol Ther. 2002; 96:23–43. PMID: 12441176.

17. Martinez RM, Zarpelon AC, Zimermann VVM, Georgetti SR, Baracat MM, Fonseca MJV, et al. Tephrosia sinapou extract reduces inflammatory leukocyte recruitment in mice: effect on oxidative stress, nitric oxide and cytokine production. Rev Bras Farmacogn. 2012; 22:587–97.

18. Valerio DA, Cunha TM, Arakawa NS, Lemos HP, Da Costa FB, Parada CA, et al. Anti-inflammatory and analgesic effects of the sesquiterpene lactone budlein A in mice: inhibition of cytokine productiondependent mechanism. Eur J Pharmacol. 2007; 562:155–63. doi: 10.1016/j.ejphar.2007.01.029 PMID: 17320857.

19. Mizokami SS, Arakawa NS, Ambrosio SR, Zarpelon AC, Casagrande R, Cunha TM, et al. Kaurenoic acid from Sphagneticola trilobata Inhibits Inflammatory Pain: effect on cytokine production and activation of the NO-cyclic GMP-protein kinase G-ATP-sensitive potassium channel signaling pathway. J Nat Prod. 2012; 75:896–904. doi: 10.1021/np200989t PMID: 22574649.

20. Casagrande R, Georgetti SR, Verri WA Jr., Dorta DJ, dos Santos AC, Fonseca MJ. Protective effect of topical formulations containing quercetin against UVB-induced oxidative stress in hairless mice. J Photochem Photobiol B. 2006; 84:21–7. doi: 10.1016/j.jphotobiol.2006.01.006 PMID: 16495072.

21. Navarro SA, Serafim KG, Mizokami SS, Hohmann MS, Casagrande R, Verri WA Jr. Analgesic activity of piracetam: effect on cytokine production and oxidative stress. Pharmacol Biochem Behav. 2013; 105:183–92. doi: 10.1016/j.pbb.2013.02.018 PMID: 23474372.

22. Verri WA Jr., Cunha TM, Parada CA, Poole S, Cunha FQ, Ferreira SH. Hypernociceptive role of cytokines and chemokines: targets for analgesic drug development? Pharmacol Ther. 2006; 112:116–38. doi: 10.1016/j.pharmthera.2006.04.001 PMID: 16730375.

23. Verri WA Jr., Cunha TM, Ferreira SH, Wei X, Leung BP, Fraser A, et al. IL-15 mediates antigen-induced neutrophil migration by triggering IL-18 production. Eur J Immunol. 2007; 37:3373–80. doi: 10.1002/eji. 200737488 PMID: 17979156.

24. Dal Secco D, Paron JA, de Oliveira SH, Ferreira SH, Silva JS, Cunha FQ. Neutrophil migration in inflammation: nitric oxide inhibits rolling, adhesion and induces apoptosis. Nitric Oxide. 2004; 9:153–64. doi: 10.1016/j.niox.2003.11.001

25. Zarpelon AC, Cunha TM, Alves-Filho JC, Pinto LG, Ferreira SH, McInnes IB, et al. IL-33/ST2 signalling contributes to carrageenin-induced innate inflammation and inflammatory pain: role of cytokines, endothelin-1 and prostaglandin E2. Br J Pharmacol. 2013; 169:90–101. doi: 10.1111/bph.12110 PMID: 23347081; PubMed Central PMCID: PMC3632241.

26. Bradley PP, Priebat DA, Christensen RD, Rothstein G. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. J Invest Dermatol. 1982; 78:206–9. PMID: 6276474.

27. Campanini MZ, Pinho-Ribeiro FA, Ivan AL, Ferreira VS, Vilela FM, Vicentini FT, et al. Efficacy of topical formulations containing Pimenta pseudocaryophyllus extract against UVB-induced oxidative stress and inflammation in hairless mice. J Photochem Photobiol B. 2013; 127:153–60. doi: 10.1016/j. jphotobiol.2013.08.007 PMID: 24041853.

28. Prado WA, Schiavon VF, Cunha FQ. Dual effect of local application of nitric oxide donors in a model of incision pain in rats. Eur J Pharmacol. 2002; 441:57–65. PMID: 12007920.

29. Ambrosio SR, Tirapelli CR, Bonaventura D, De Oliveira AM, Da Costa FB. Pimarane diterpene from Viguiera arenaria (Asteraceae) inhibit rat carotid contraction. Fitoterapia. 2002; 73:484–9. PMID: 12385871.

30. Sugiyama S, Okada Y, Sukhova GK, Virmani R, Heinecke JW, Libby P. Macrophage myeloperoxidase regulation by granulocyte macrophage colony-stimulating factor in human atherosclerosis and
implications in acute coronary syndromes. Am J Pathol. 2001; 158:879–91. doi: 10.1016/S0002-9440 (10)64036-9 PMID: 11238037; PubMed Central PMCID: PMC1850342.

31. Pulli B, Ali M, Forghani R, Schob S, Hsieh KL, Wojtkiewicz G, et al. Measuring myeloperoxidase activity in biological samples. PLoS One. 2013; 8:e67976. doi: 10.1371/journal.pone.0067976 PMID: 23861842; PubMed Central PMCID: PMC3702519.

32. Klebanoff SJ. Myeloperoxidase: friend and foe. J Leukoc Biol. 2005; 77:598–625. doi: 10.1189/jlb. 1204697 PMID: 15689384.

33. Suzuki K, Ota H, Sasagawa S, Sakatani T, Fujikura T. Assay method for myeloperoxidase in human polymorphonuclear leukocytes. Anal Biochem. 1983; 132:345–52. PMID: 6312841.

34. Martin KR, Ohayon D, Witko-Sarsat V. Promoting apoptosis of neutrophils and phagocytosis by macrophages: novel strategies in the resolution of inflammation. Swiss Med Wkly. 2015; 145:w14056. doi: 10. 4414/smw.2015.14056 PMID: 25701669.

35. Kato FH, Viana NI, Santini CB, de Souza CG, Veneziani RC, Ambrosio SR, et al. Assessment of the in vitro and in vivo genotoxic and antigenotoxic effects of pimaradienoic acid in mammalian cells. Mutat Res. 2012; 749:87–92. doi: 10.1016/j.mrgentox.2012.09.001 PMID: 22985806.

36. Michlewska S, Dransfield I, Megson IL, Rossi AG. Macrophage phagocytosis of apoptotic neutrophils is critically regulated by the opposing actions of pro-inflammatory and anti-inflammatory agents: key role for TNF-alpha. FASEB J. 2009; 23:844–54. doi: 10.1096/fj.08-121228 PMID: 18971259.

37. Komala I, Ito T, Nagashima F, Yagi Y, Asakawa Y. Cytotoxic, radical scavenging and antimicrobial activities of sesquiterpenoids from the Tahitian liverwort Mastigophora diclados (Brid.) Nees (Mastigophoraceae). J Nat Med. 2010; 64:417–22. doi: 10.1007/s11418-010-0423-8 PMID: 20458547.

38. Lu JM, Lin PH, Yao Q, Chen C. Chemical and molecular mechanisms of antioxidants: experimental approaches and model systems. J Cell Mol Med. 2010; 14:840–60. doi: 10.1111/j.1582-4934.2009. 00897.x PMID: 19754673; PubMed Central PMCID: PMC2927345.

39. Maioli NA, Zarpelon AC, Mizokami SS, Calixto-Campos C, Guazelli CF, Hohmann MS, et al. The superoxide anion donor, potassium superoxide, induces pain and inflammation in mice through production of reactive oxygen species and cyclooxygenase-2. Braz J Med Biol Res. 2015; 48:321–31. doi: 10.1590/1414-431X20144187 PMID: 25714890; PubMed Central PMCID: PMC4418362.

40. Li JM, Fan LM, Christie MR, Shah AM. Acute tumor necrosis factor alpha signaling via NADPH oxidase in microvascular endothelial cells: role of p47phox phosphorylation and binding to TRAF4. Mol Cell Biol. 2005; 25:2320–30. doi: 10.1128/MCB.25.6.2320-2330.2005 PMID: 15743827; PubMed Central PMCID: PMC1061612.

41. Mittal M, Siddiqui MR, Tran K, Reddy SP, Malik AB. Reactive oxygen species in inflammation and tissue injury. Antioxid Redox Signal. 2014; 20:1126–67. doi: 10.1089/ars.2012.5149 PMID: 23991888; PubMed Central PMCID: PMC3929010.

42. Drapier JC, Hibbs JB Jr. Differentiation of murine macrophages to express nonspecific cytotoxicity for tumor cells results in L-arginine-dependent inhibition of mitochondrial iron-sulfur enzymes in the macrophage effector cells. J Immunol. 1988; 140:2829–38. PMID: 2451695.

43. Kang HS, Kim YH, Lee CS, Lee JJ, Choi I, Pyun KH. Suppression of interleukin-1 and tumor necrosis factor-alpha production by acanthoic acid, (-)-pimara-9(11),15-dien-19-oic acid, and it antifibrotic effects in vivo. Cell Immunol. 1996; 170:212–21. PMID: 8660820.

44. Saito S, Nakano M. Nitric oxide production by peritoneal macrophages of Mycobacterium bovis BCGinfected or non-infected mice: regulatory role of T lymphocytes and cytokines. J Leukoc Biol. 1996; 59:908–15. PMID: 8691077.

45. Biswas SK, Sodhi A, Paul S. Regulation of nitric oxide production by murine peritoneal macrophages treated in vitro with chemokine monocyte chemoattractant protein 1. Nitric Oxide. 2001; 5:566–79. doi: 10.1006/niox.2001.0370 PMID: 11730364.

46. Sanchez de Miguel L, Arriero MM, Farre J, Jimenez P, Garcia-Mendez A, de Frutos T, et al. Nitric oxide production by neutrophils obtained from patients during acute coronary syndromes: expression of the nitric oxide synthase isoforms. J Am Coll Cardiol. 2002; 39:818–25. PMID: 11869847.

47. Tirapelli CR, Ambrosio SR, da Costa FB, de Oliveira AM. Evidence for the mechanisms underlying the effects of pimaradienoic acid isolated from the roots of Viguiera arenaria on rat aorta. Pharmacology. 2004; 70:31–8. doi: 10.1159/000074240 PMID: 14646354.

48. Joosten LA, Netea MG, Kim SH, Yoon DY, Oppers-Walgreen B, Radstake TR, et al. IL-32, a proinflammatory cytokine in rheumatoid arthritis. Proc Natl Acad Sci U S A. 2006; 103:3298–303. doi: 10.1073/ pnas.0511233103 PMID: 16492735; PubMed Central PMCID: PMC1413916.

49. Verri WA Jr., Souto FO, Vieira SM, Almeida SC, Fukada SY, Xu D, et al. IL-33 induces neutrophil migration in rheumatoid arthritis and is a target of anti-TNF therapy. Ann Rheum Dis. 2010; 69:1697–703. doi: 10.1136/ard.2009.122655 PMID: 20472598.

50. Wu CH, Huang CM, Lin CH, Ho YS, Chen CM, Lee HM. Advanced glycosylation end products induce NF-kappaB dependent iNOS expression in RAW 264.7 cells. Mol Cell Endocrinol. 2002; 194:9–17. PMID: 12242023.

51. Carvalho TC, Simao MR, Ambrosio SR, Furtado NA, Veneziani RC, Heleno VC, et al. Antimicrobial activity of diterpenes from Viguiera arenaria against endodontic bacteria. Molecules. 2011; 16:543–51. doi: 10.3390/molecules160100543 PMID: 21233793.

52. Porto TS, Rangel R, Furtado NA, de Carvalho TC, Martins CH, Veneziani RC, et al. Pimarane-type diterpenes: antimicrobial activity against oral pathogens. Molecules. 2009; 14:191–9. doi: 10.3390/ molecules14010191 PMID: 19127247.

53. Porto TS, Furtado NA, Heleno VC, Martins CH, Da Costa FB, Severiano ME, et al. Antimicrobial entpimarane diterpenes from Viguiera arenaria against Gram-positive bacteria. Fitoterapia. 2009; 80:432– 6. doi: 10.1016/j.fitote.2009.06.003 PMID: 19524643.

ARTIGO 2

Kaurenoic Acid Reduces Lipopolysaccharide-Induced Leukocyte Recruitment, Hyperalgesia and Oxidative Stress in Mice

Sandra S. Mizokami,[†] Nilton S. Arakawa,[‡] Thiago H. Hayashida,[‡] Sergio R. Ambrosio,[§] Thiago M. Cunha,[⊥] José C. Alves-Filho,[⊥] Fernando Q. Cunha,[⊥] Rubia Casagrande,[‡] Waldiceu A. Verri, Jr[†,*]

[†] Departamento de Ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina, 86057-970 Londrina, Paraná, Brazil.

[‡] Departamento de Ciências Farmacêuticas, Centro de Ciências da Saúde, Universidade Estadual de Londrina, 86038-350 Londrina, Paraná, Brazil.

[§] Núcleo de Pesquisa em Ciências Exatas e Tecnológicas, Universidade de Franca, 14404-600 Franca, São Paulo, Brazil.

[⊥] Departamento de Farmacologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, 14049-900 Ribeirão Preto, São Paulo, Brazil.

*Corresponding Author:

Waldiceu A. Verri Jr. Tel: + 55 43 3371-4979. Fax: + 55 43 3371-4387. E-mail: waldiceujr@yahoo.com.br; waverri@uel.br

ABSTRACT

Kaurenoic acid (KA) presents anti-inflammatory and antibacterial effects. LPS-triggered inflammatory response, pain and tissue lesion are important clinical features of gram-negative bacterial infection. The effect of KA on lipopolysaccharide (LPS)-induced leukocyte recruitment, hyperalgesia and oxidative stress in mice were investigated in the present study. Pre and post per oral treatment with KA inhibited the recruitment of total leukocytes, neutrophils and mononuclear cells. KA also inhibited LPS-induced mechanical and thermal hyperalgesia, myeloperoxidase (MPO) activity and oxidative stress in the paw. The KA inhibition of LPS-induced leukocyte recruitment was prevented by L-NAME, ODQ, KT5823 and glibenclamide treatments, demonstrating that KA mechanism depends on inducing NO production and activating the cyclic GMP–protein kinase G–ATP-sensitive potassium channel pathway. Furthermore, KA also inhibited LPS-induced production of TNF-α, IL-1β and IL-33, and enhanced IL-10 production. In agreement with the effect of KA over pro-inflammatory cytokines, KA inhibited the NFκB activation. Thus, KA inhibits inflammation triggered by the bacterial component LPS rendering it as a suitable candidate to control excessive inflammation during gram-negative infection.

Keywords: Kaurenoic acid; lipopolysaccharide-LPS; leukocyte recruitment; cytokines; hyperalgesia.

There is intense leukocyte recruitment during the inflammatory response against gram-negative bacteria infection. Neutrophils are the major leukocytes recruited to the inflammatory foci in acute response having an essential role in protecting the host in bacterial infection. Although apoptotic recruited neutrophils have an important role in the resolution of inflammation, neutrophils are also associated with hyperalgesia and tissue damage.[1] Furthermore, recruited neutrophils contribute to the pathogenicity of inflammatory and autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, psoriasis, asthma and ulcerative colitis.[2–5]

Upon the treatment with antibiotics such as ceftazidime and release of neutrophil products, there is bacterial lysis and release of lipopolysaccharide (LPS) present in gram-negative bacterial wall.[6] In turn, LPS activates Toll-like receptor 4 (TLR4) inducing further inflammation, pain and tissue destruction, reinforcing the importance of inhibiting the pro-inflammatory effects of LPS. The LPS/TLR4 signaling-induced inflammation and pain depends on the production cytokines such as tumor necrosis factor alpha (TNF-α), interleukin 1-beta (IL-1β) and interleukin 33 (IL-33).[7–11] These cytokines induce the rolling, adhesion, transmigration and guide recruited neutrophils in the extravascular space.[2,9,12] Cytokines also induce the production of other chemoattractant factors that further increase the leukocyte recruitment,[10] and mediate LPS-induced inflammatory hyperalgesia.[7,13]

Kaurenoic acid [*ent*-kaur-16-en-19-oic acid (KA)] is a diterpene extracted from *Sphagneticola trilobata* (L.) Pruski, and exhibits analgesic, anti-inflammatory, antiprotozoal, antimicrobial, anticancer, hypoglycemic, anti-asthmatic, antispasmodic and vasorelaxant activities.[14–24] Other plant sources of KA include *Annona glabra, Aralia continentalis, Aralia cordata, Copaifera langsdorffii, Mikania glomerata, Mikania laev-*

igata and Vigueira robusta.[17,25–29] We have demonstrated that KA presents analgesic and anti-inflammatory activity by diminishing cytokines production (TNF-α and IL-1β) and activating the nitric oxide (NO)–cyclic GMP–protein kinase G–ATP-sensitive potassium channel signaling pathway in the carrageenan model of paw inflammation.[20] KA also inhibits complete Freund´s adjuvant-induced mechanical hyperalgesia, and the overt pain-like behavior induced by acetic acid, phenyl-p-benzoquinone and formalin.[20] Evidence shows that KA inhibits the leukocyte recruitment in acetic acid-induced colitis and asthma-induced neutrophil recruitment.[17,23] Furthermore, KA inhibits LPS-induced activation of NFκB[16,30] and induces Nrf2 activation[31] in RAW 264.7 macrophages *in vitro*. However, the effects of KA over LPS-induced inflammation and pain *in vivo* as well as its mechanisms at therapeutic doses have not been addressed.

Based on the information presented above, we investigated whether KA inhibits LPS-induced inflammation and pain, and if these effects depend on inhibiting cytokines production, oxidative stress, activating the NO–cyclic GMP–protein kinase G–ATP-sensitive potassium channel and nuclear factor kappa B (NFκB) activation signaling pathway.

RESULTS AND DISCUSSION

Kaurenoic Acid (KA) Reduces LPS-Induced Leukocyte Recruitment to the Peritoneal Cavity. In the first series of experiments, the effect of KA in LPS-induced leukocyte recruitment to the peritoneal cavity was evaluated. Mice were treated per orally (po) with KA (1, 3 and 10 mg/kg) or vehicle (2% DMSO diluted in

saline, 10 mL/kg) 30 min before intraperitoneal (ip) LPS injection (200 ng/cavity). LPS induced significant recruitment of total leukocytes (Figure 1A), neutrophils (Figure 1B) and mononuclear cells (Figure 1C) to the peritoneal cavity 6 h after injection. All doses of KA significantly reduced the LPS-induced total leukocytes recruitment (Figure 1A) in the peritoneal cavity. The dose of 3 mg/kg of KA reduced significantly the LPS-induced recruitment of neutrophils compared with dose of 1 mg/kg (Figure 1B), and 10 mg/kg inhibited the LPS-induced recruitment of neutrophils and mononuclear cells compared to vehicle group and all other doses of KA (Figure 1B-C). Therefore, the dose of 10 mg/kg of KA was selected for the following experiments. Corroborating our results, KA inhibited the recruitment of inflammatory leukocytes and prevented tissue damage in acetic acid-induced colitis.[23] In a model of IgE-mediated asthma in guinea pigs, KA inhibited the recruitment of leukocytes, mainly eosinophils and neutrophils as well as histamine, phospholipase A_2 and eosinophil peroxidase activity in bronchoalveolar lavage fluid.[17]

Post-Treatment with KA Reduces LPS-Induced Leukocyte Recruitment to Peritoneal Cavity. The effect of post-treatment with KA was also tested in LPS-induced peritonitis. Mice received LPS ip injection (200 ng/cavity) and 30 min after were treated with KA (10 mg/kg, po) or vehicle (2% DMSO in saline, po). After six hours, the number total leukocytes and differential cell counting were performed (Figure 2). The post-treatment with KA also reduced significantly the LPS-induced recruitment of total leukocytes (Figure 2A), neutrophils (Figure 2B) and mononuclear cells (Figure 2C) to the peritoneal cavity. These results show that KA can be effective in the treatment of diseases in which there is intense recruitment of cells even when

administered after the inflammatory process has begun. Of note, this is the first study to demonstrate that KA is active in post-treatment regimen.

Treatment with KA Reduces LPS-Induced Hyperalgesia and Myeloperoxidase (MPO) Activity. The antinociceptive effect of KA was tested in LPS-induced mechanical hyperalgesia, thermal hyperalgesia and MPO activity (an indirect indicator of neutrophil recruitment) in the paw. Mice were treatment with KA (1, 3 and 10 mg/kg, po) or vehicle (2% DMSO in saline, po), and after 30 min received an intraplantar (ipl) injection of LPS (200 ng/paw). The mechanical (Figure 3A) and thermal (Figure 3B) hyperalgesia were evaluated 0-5 h after LPS injection. Immediately after the last measurement, mice were terminally anesthetized and plantar paw skin samples were collected for MPO activity measurement (Figure 3C). The dose of 1 mg/kg of KA showed no effect on LPS-induced mechanical hyperalgesia, thermal hyperalgesia and MPO activity, while the dose of 3 mg/kg inhibited the mechanical hyperalgesia at 3 h, (Figure 3A) and thermal hyperalgesia only in the first h (Figure 3B), and no effect in MPO activity (Figure 3C). Treatment with 10 mg/kg of KA inhibited the LPS-induced mechanical (Figure 3A) and thermal (Figure 3B) hyperalgesia between 1-5 h and MPO activity (Figure 3C). The LPS injection into hindpaw induces mechanical[7] and thermal[8] hyperalgesia. The recruited neutrophils contribute to the development of hyperalgesia by producing prostaglandin E_2 and leukotriene B_4.[1,32] The MPO is an enzyme expressed by neutrophils and macrophages.[33] The inhibition of LPS-induced tissue MPO activity corroborates the data of Figures 1 and 2 of inhibition of LPS-induced leukocyte recruitment in the peritoneal cavity.

Treatment with KA Reduces LPS-Induced Oxidative Stress in the Paw Skin. Oxidative imbalance plays an important role in inflammatory hyperalgesia and leukocyte recruitment as well as the inhibition of leukocyte recruitment to inflamed tissue contributes to the inhibition of oxidative stress.[34,35] Mice were treatment with KA (10 mg/kg, po) or vehicle (2% DMSO in saline, po) 30 min after received LPS (200 ng/paw) injection. After 3 h, mice were terminally anesthetized, the plantar paw skin samples collected, and the antioxidant capacity was assessed by endogenous reduced glutathione (GSH) levels (Figure 4A), ability to scavenge 2,2′-Azinobis-3-ethylbenzothiazoline 6-sulfonic acid (ABTS) assay (Figure 4B), quantification of superoxide anion production by nitroblue tetrazolium (NBT) reduction (Figure 4C) and lipid peroxidation by determining thiobarbituric acid reactive substance (TBARS) (Figure 4D). LPS significantly reduced GSH levels (Figure 4A), the tissue ability to scavenge ABTS (Figure 4B), increased superoxide anion production (Figure 4C) and the lipid peroxidation (Figure 4D), which were prevented by KA treatment. These data demonstrate that KA inhibits LPS-induced oxidative stress, which is a contributing mechanism to the inhibition of LPS-induced hyperalgesia and inflammatory leukocyte recruitment.

L-NAME Prevents in a Dose-Dependent Manner the KA Inhibition of LPS-Induced Leukocyte Recruitment to the Peritoneal Cavity. KA activates the NO-dependent cyclic GMP–protein kinase G–ATP-sensitive potassium channel pathway to induce analgesia[20] and vasodilatation.[22] Despite the importance of activating this pathway to reduce leukocyte recruitment,[2,36–39] it has not been determined whether inhibition of LPS-induced leukocyte recruitment by KA depends on activating the NO

- cyclic GMP–protein kinase G–ATP-sensitive potassium channel pathway. Mice were pre-treated with L-NAME (L-nitro-arginine methyl ester, NOS inhibitor; 10, 30 and 100 mg/kg, ip) 30 min before treatment with KA (10 mg/kg, po) or vehicle (2% DMSO in saline, po). After an additional 30 min, mice received the LPS ip injection (200 ng/cavity) and the number of total leukocytes and differential cell counting were evaluated after 6 h (Figure 5). L-NAME prevented in a dose-dependent manner the KA inhibition of LPS-induced leukocyte recruitment to the peritoneal cavity (Figure 5A-C). The dose of 10 mg/kg of L-NAME did not affect the inhibitory effect of KA over LPS-induced leukocyte recruitment (total leukocytes, neutrophils and mononuclear cells). On the other hand, 30 and 100 mg/kg of L-NAME significantly inhibited the effect of KA (Figure 5A-C). The inhibitory effect of 100 mg/kg of L-NAME was also statistically different compared to the dose 10 mg/kg of L-NAME. There was no difference between the doses of 30 and 100 mg/kg of L-NAME (Figure 5A-C).

These results reinforce the role of NO in the control of recruitment of leukocytes.[2,37] NO is produced by nitric oxide synthase (NOS) through the conversion of L-arginine to L-citrulline and NO. There are three NOS isoforms: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS). The nNOS and eNOS are constitutively expressed and dependent on intracellular Ca^{+2} for their activity whereas the iNOS is an inducible form that is active independently of intracellular Ca^{+2}. Inducers of iNOS include LPS and cytokines.[2,40] Further supporting the importance of NO to controlling leukocyte recruitment, pharmacological inhibitors and knocking down NOS increase neutrophil adhesion to endothelium and transmigration of leukocytes.[2,37,38,41,42] There are evidences that this effect is mediated via cyclic guanosine monophosphate – protein kinase G – ATP sensitive potassium channel (cGMP-PKG-ATP-sensitive potassium channel).[2,36–39] Therefore, we also evaluated whether the effect of KA in inhibit-

ing LPS-induced leukocyte recruitment to the peritoneal cavity depends on activating the cGMP-PKG-ATP-sensitive potassium channel signaling pathway.

ODQ Prevents the KA Inhibition of LPS-Induced Leukocyte Recruitment to the Peritoneal Cavity. Mice were pre-treated with ODQ (soluble cGMP inhibitor; 1 mg/kg, ip) 30 min before treatment with KA (10 mg/kg, po) or vehicle (2% DMSO in saline, po). After an additional 30 min, mice received the LPS ip injection (200 ng/cavity), and the number total leukocytes and differential cell counting were determined (Figure 6). The treatment with ODQ reversed the KA inhibition of LPS-induced total leukocyte (Figure 6A), neutrophil (Figure 6B) and mononuclear cell (Figure 6C) to the peritoneal cavity. Therefore, KA inhibition of LPS-induced leukocyte recruitment depends on activating NO and cGMP. In agreement with this result, NO down-regulates the endothelial cells expression of adhesion molecules such as P-selectin, ICAM-1 and V-CAM-1 in a cGMP-dependent manner.[36]

KT5823 Prevents the KA Inhibition of LPS-Induced Leukocyte Recruitment to the Peritoneal Cavity. PKG deficient mice present enhanced C5a-induced chemotaxis reinforcing that this kinase down-regulates leukocyte recruitment. PKG also inactivates the transcription factor NFκB, contributing to the reduction of adhesion molecules expression on vessels.[43] cGMP activates PKG, therefore, the effect of inhibiting PKG over KA anti-inflammatory mechanism was tested. Mice were treated with KT5823 (PKG inhibitor; 0.5 μg/mice, ip) 10 min before treatment with KA (10 mg/kg, po) or vehicle (2% DMSO in saline, po). After an additional 30 min, mice received the LPS injection (200 ng/cavity), and the number of total leukocytes and dif-

ferential cell counting were determined (Figure 7). The treatment with KT5823 prevented the KA inhibition of LPS-induced total leukocyte (Figure 7A), neutrophil (Figure 7B) and mononuclear cell (Figure 7C) recruitment.

Pre-Treatment with Glibenclamide Prevents KA Inhibition of LPS-Induced Leukocyte Recruitment to the Peritoneal Cavity. Next, we investigated whether activation of ATP sensitive potassium channel was involved in the KA inhibition of LPS-induced leukocyte recruitment.[44] Mice were pre-treated with Glibenclamide (ATP-sensitive potassium channel blocker; Gli, 1 mg/kg, ip) 30 min before treatment with KA (10 mg/kg, po) or vehicle (2% DMSO in saline, po). After an additional 30 min, mice received the LPS injection (200 ng/cavity), and the number of total leukocytes and differential cell counting were determined (Figure 8). The treatment with glibenclamide prevented the KA inhibition of LPS-induced leukocyte (Figure 8A), neutrophil (Figure 8B) and mononuclear cell (Figure 8C) recruitment. The results of Figures 5-8 demonstrate that KA inhibits the LPS-induced leukocyte recruitment by activating the NO-cGMP-PKG-ATP-sensitive potassium channel pathway. It is noteworthy to mention that none of the inhibitors presented an effect per se in the doses used in this study as observed in Figures 5 (L-NAME), 6 (ODQ), 7 (KT5823) and 8 (Glibenclamide).

KA Reduces LPS-Induced Pro-Inflammatory Cytokine Production and Enhances Anti-Inflammatory Cytokine Production. Intraperitoneal injection of LPS induces expressive increase of rolling and adhesion of leukocytes on endothelial

cells. This interaction of endothelial cells and leukocytes depends on endothelial cells activation by cytokines, which start expression adhesion molecules.[45,46] In fact, cytokines are crucial mediators involved in leukocyte recruitment during LPS inflammation and pain.[7,46,47] Therefore, the possible effect of KA on LPS-induced pro-inflammatory (TNF-α, IL-1β and IL-33) and anti-inflammatory (IL-10) cytokine production was evaluated. Mice were treatment with KA (10 mg/kg, po) or vehicle (2% DMSO in saline, po), and after 30 min, received LPS ip injection (200 ng/cavity). Cytokine production in the peritoneal cavity of mice was determined at indicated time points (Figure 9). LPS increased the concentration of TNF-α, IL-1β and IL-33 in the peritoneal exudates at all evaluated time points, except by IL-33 at 30 min. These data is in accordance with the pronounced effect of LPS of inducing cytokine production at varied tissues.[7,8,11] The treatment with KA reduced the LPS-induced production of TNF-α (Figure 9A), IL-1β (Figure 9B) and IL-33 (Figure 9C) in the peritoneal cavity. The KA inhibition of LPS-induced TNF-α, IL-1β and IL-33 production was consistent over time. IL-10 is an anti-inflammatory cytokine that inhibits leukocyte recruitment and hyperalgesia.[10,48] IL-10 is co-released with pro-inflammatory cytokines during inflammation with the role of limiting the inflammatory response and hyperalgesia.[49] KA treatment consistently enhanced LPS-induced IL-10 production at 1, 2, 4 and 6 h after stimulus injection (Figure 9D). Therefore, it is conceivable that the effect of KA of inhibiting LPS-induced leukocyte recruitment depends on inhibiting the production of the pro-inflammatory cytokines TNF-α, IL-1β and IL-33 as well as enhancing the production of the anti-inflammatory cytokine IL-10.

Treatment with KA Inhibits the NFκB Activation. Mice were treated with KA (10 mg/kg, po) or vehicle (2% DMSO in saline, po), and after 30 min, received LPS ip injection (200 ng/cavity). The peritoneal exudates were collected 2 h after LPS stimulus. Treatment with KA inhibited the LPS-induced NFκB activation as observed by an inhibition of LPS-induced decrease of the total NFκB p65/phosphorylated NFκB p65 ratio (Figure 10). The LPS-induced mechanical hyperalgesia and MPO activity in the mice paw mice depends on activation of TLR4/MyD88 signaling in a TLR4/TRIF-independent manner.[7] TLR4/MyD88 signaling leads to NFκB activation and consequent production of pro-hyperalgesic cytokines and other nociceptor sensitizing molecules such as prostaglandins and sympathetic amines that contributes to the inflammatory process and hyperalgesia.[7] Moreover, oxidative stress also induces the NFκB activation.[50] For instance, superoxide anion activates NFκB resulting in pro-inflammatory/hyperalgesic cytokine production.[50] Treatment with KA prevented the depletion of GSH levels and reduction of ABTS scavenging activity as well as decreased the production of superoxide anion and TBARS (Figure 4), and these effects might account to reducing NFκB activation, and TNF-α, IL-1β and IL-33 production. Corroborating our *in vivo* results, KA inhibited the LPS-induced NFκB activation and the TNF-α secretion in RAW 264.7 macrophages.[30]

In conclusion, we demonstrated that KA exerts anti-inflammatory and analgesic effects by inhibiting LPS-induced peritonitis and paw inflammation. KA reduced the total leukocyte, neutrophil and mononuclear cell recruitment as well as mechanical hyperalgesia, thermal hyperalgesia, and MPO activity. Regarding the mechanism of action of KA, we observed that KA inhibited oxidative stress and pro-inflammatory cytokine production (TNF-α, IL-1β and IL-33). The modulation of these mediators of

the inflammatory process depended on inhibition of NFκB activation. Nevertheless, KA not only presents inhibitory mechanisms, but also active mechanisms to control inflammation and pain since KA enhanced the production of the anti-inflammatory cytokine IL-10 and triggered the NO-cGMP-PKG-ATP-sensitive potassium channel signaling pathway. Therefore, the present data further support KA as a potential anti-inflammatory and analgesic molecule.

EXPERIMENTAL SECTION

Experimental protocols. Mice received per oral (po) pre-treatment with Kaurenoic acid (KA, 1, 3 and 10 mg/kg, diluted in 2% DMSO in saline) or vehicle (2% DMSO diluted in saline, 10 mL/kg of body weight) 30 min before intraperitoneal (ip) injection of LPS (200 ng/200 μL/cavity) or post-treated with KA (10 mg/kg, diluted in 2% DMSO in saline) 30 min after LPS (200 ng/200 μL/cavity) injection. The effect of KA (1, 3 and 10 mg/kg, po) on LPS-induced mechanical and thermal hyperalgesia was determined between 0-5 h after stimulus LPS (200 ng/25 μL/paw) and MPO activity 5 h after LPS injection. To investigate the role of the NO–cyclic GMP–protein kinase G–ATP-sensitive potassium channel signaling pathway, the following drugs were used: L-NAME (L-nitro-arginine methyl ester, NOS inhibitor, 10, 30 and 100 mg/kg, ip, diluted in saline, 30 min), ODQ (soluble cGMP inhibitor; 1 mg/kg, ip, diluted in 2% DMSO in saline 30 min), KT5823 (PKG inhibitor; 0.5 μg/mice, ip, diluted in 2% DMSO in saline, 10 min) and glibenclamide (ATP-sensitive potassium channel blocker; 1 mg/kg, ip, diluted in 5% of Tween 80 in saline, 30 min). Oxidative stress in the paw was assessed at 3 h following LPS-stimuli. Further, TNFα, IL-1β, IL-33 and IL-10 levels in the peritoneal cavity were determined between 0.5-6 h and NFκB activity at 2 h

after LPS injection. The selected doses of drugs were chosen based on pilot studies and previous standardization in our laboratory.[2,8,20,36]

Animals. Male Swiss mice (20-25 g), from the Universidade Estadual de Londrina, Londrina, Paraná, Brazil, were used in this study. Mice were housed in standard clear plastic cages with free access to food and water, a light/dark cycle of 12:12 h, and kept at 21 ^{o}C. All behavioral testing was performed between 9 a.m. and 5 p.m. in a temperature-controlled room. Animal care and handling procedures were in accordance with the International Association for Study of Pain (IASP) guidelines and approved by the Ethics Committee of the Universidade Estadual de Londrina (process number 12105.2012.67). Every effort was made to minimize the number of animals used and their suffering.

Extraction and isolation of Kaurenoic acid. The air-dried roots were pulverized and then extracted exhaustively with dichloromethane (900 mL) at room temperature, to give 1.2 g of crude extract, which was suspended in 300 mL of methanol−H2O (9:1) and filtered. The soluble fraction was partitioned using *n*-hexane (300 mL, four times), which resulted in a 0.7 g *n*-hexane-soluble fraction after solvent evaporation under reduced pressure. The *n*-hexane-soluble fraction was chromatographed over silica gel 60 (0.063−0.200 mm) using vacuum-liquid chromatography with *n*-hexane and increasing amounts of ethyl acetate as eluents (250 mL each fraction). The second fraction (0.41 g) was washed with cold methanol, to afford Kaurenoic acid (KA, *ent*-kaur-16-en-19-oic acid; 800 mg, purity 96%, as determined by HPLC), exhibiting $[\alpha]^{20}_{D}$ – 110, similar to a previous report.[51] EIMS *m/z* 325 $[M + Na]^{+}$ and compared by ^{1}H (CDCl3, 400 MHz) and ^{13}C (CDCl3, 100 MHz) spectroscopy with an authentic standard and literature data.[52]

Drugs and stimuli. The following materials were obtained from the sources indicated: saline (NaCl 0.9%; Fresenius Kabi Brasil Ltda. Aquiraz, CE, Brazil), dimethyl sulfoxide (DMSO), *Esquerichia coli* lipopolysaccharide (LPS), N^G-nitro-L-arginine methyl ester (L-NAME) and glybenclamide were obtained from Sigma-Aldrich (St. Louis, MO, USA). 2,3,9,10,11,12-hexahydro-10R-methoxy-2,9-dimethyl-1-oxo-9S,12R-epoxy-1diindolo [1,2,3-fg:3',2', 1'-kl] pyrrol [3,4-i][1,6]benzodiazocine-10-carboxylic acid, methyl ester (KT5823) from **Cayman Chemical Company** (Ann Arbor, Michigan, USA), and 1H-(1,2,4)-oxadiazolol-(4,3-a)quinoxalin-1-one (ODQ) was obtained Santa Cruz Biotechnology (CA, USA). Mouse TNF-α, IL-1β, IL-33 and IL-10 ELISA kits were obtained from eBioscience (San Diego, CA, USA).

Recruitment of leukocytes to peritoneal cavity of mice. Leukocyte recruitment to the peritoneal cavity of mice was induced by ip injection of *Esquerichia coli* lipopolysaccharide (LPS; 200 ng/200 μL/cavity). Control group received only saline ip (200 μL/cavity). Six h after LPS injection, mice were terminally anesthetized, 2 mL of phosphate-buffered saline (PBS) containing 1 mM of EDTA was injected ip, and peritoneal exudates were collected to determine the cell counts. The total leukocyte counts were performed with a Newbauer chamber, and differential cell counts (100 cells per slide) were carried out on microscope slides stained with **Panotic kit** (Laborclin, Pinhais, Paraná, Brazil). The results were expressed as the number of total leukocytes, neutrophils or mononuclear cells x 10^6 per cavity.[2,8]

Electronic pressure-meter test. Mechanical hyperalgesia was assessed as previously reported.[53] In a quiet room, mice were placed in acrylic cages (12×10×17 cm) with wire grid floors, 30 min before testing. The test consisted of evoking a hind paw reflex with a hand-held force transducer (electronic anesthesiometer; Insight,

Ribeirão Preto, SP, Brazil) adapted with a 0.5 mm^2 polypropylene tip. The investigator was trained to apply the tip perpendicularly to the central area of the hind paw with a gradual increase in pressure. The end point was characterized by the removal of the paw followed by clear flinching movements, and the intensity of pressure was recorded automatically. The animals were tested before (basal) and after treatment and stimulus, and the value for each interval was an average of three measurements. The results are expressed by delta (Δ) withdrawal threshold (in g), calculated by subtracting the mean measurements obtained at 1, 3 or 5 h after ipl stimulus from the basal (zero h) mean measurements.

Hot plate test. Thermal hyperalgesia was evaluated before and after stimulus. The test was performed as previously reported.[54] In brief, mice were placed in a 10 cm wide glass cylinder on a hot plate (Hot Plate HP-2002, Insight Equipamentos, Ribeirao Preto, SP, Brazil) maintained at 50 °C. The reaction time was scored when the animal jumped, flinched or licked its paws. The normal latency (reaction time) were: saline 19.80±0.20 s; LPS 19.60±0.40 s; KA 1 mg/kg 19.74± 0.26 s; KA 3 mg/kg 19.40±0.40 s; KA 10 mg/kg 19.75±0.25 s. A maximum latency (cutoff) was set at 20 s to avoid tissue damage.

Myeloperoxidase (MPO) activity. The neutrophil migration to the paw skin was evaluated by the MPO kinetic-colorimetric assay.[55,56] Samples were collected in 200 μL of 50 mM K_2PO_4 buffer (pH 6.0) containing 0.5% of HTAB, and homogenized in ice-cold using a Tissue-Tearor (Biospec®). After the homogenates were centrifuged at 16100 g for 2 min, the resulting supernatants (15 μL) were mixed with with 200 μL 50 mM K_2PO_4 buffer, pH 6.0, containing 0.167 mg/mL *o*-dianisidine dihydrochloride and 0.015% hydrogen peroxide. The MPO activity absorbance was determi-

nation at 450 nm (Multiskan GO ThermoScientific). The results were presented as the MPO activity (number of neutrophils x 10^4/ mg of skin paw tissue).

Cytokine measurement. The production of cytokines (TNF-α, IL-1β, IL-33 and IL-10) was determined by an enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (eBioscience).[8] The results are expresses as pg of cytokine/cavity.

Reduced glutathione (GSH) levels measurement. Samples of paw skin were collected and maintained at −80 °C for at least 48 h. Samples were homogenized in 200 μL of 0.02 M EDTA. The homogenate was mixed with 25 μL of trichloroacetic acid 50% and was homogenized three times over 15 min. The mixture was centrifuged (15 min x 1500 g at 4 °C). The supernatant was added to 200 μL of 0.2 M TRIS buffer, pH 8.2, and 10 μL of 0.01M DTNB. After 5 min, the absorbance was measured at 412 nm (Multiskan GO, Thermo Scientific) against a blank reagent with no supernatant. A standard GSH curve was formed. The results were expressed as nmol of GSH x 10^3 per mg of protein.

ABTS assays. The ability of samples to resist oxidative damage was determined by their free radical scavenging (ABTS [2,2'-Azinobis-3-ethylbenzothiazoline 6-sulfonic acid] assay). The test was adapted to a 96-well microplate format as previously described.[50] Plantar tissue samples were homogenized in ice-cold KCl buffer (500 μL, 1.15% w/v). The homogenates were centrifuged (200 g × 10 min at 4 °C), and the supernatants were used. Diluted ABTS solution (200 μL) was mixed with 10 μL of sample in each well. After 6 min of incubation at 25 °C, the absorbance was measured at 730 nm. (Multiskan GO Thermo Scientific). The results of ABTS assays were equated against a standard Trolox curve (0.02–20 nmol).

NBT assay. The quantification of superoxide anion production in tissue homogenates (10 mg/mL in 1.15% KCl) was performed using the NBT assay as previously described.[50] Briefly, 50 μL of homogenate were incubated with 100 μL of NBT (1 mg/mL) in 96-well plates at 37 °C for 1 h. The mixture was then carefully removed from wells and the reduced formazan solubilized by adding 120 μL of KOH 2 M and 140 μL of DMSO. The absorbance was measured at 600 nm (Multiskan GO Thermo-Scientific). The weight of samples was used for data normalization and results presented as NBT reduction (OD/ mg of tissue).

Lipid Peroxidation. The lipid peroxidation was evaluated by TBARS levels assay as previously described.[34] Briefly, 10% TCA was added to the homogenate, and the mixture was centrifuged (1000 g, 3 min, 4 °C) to precipitateprot eins. The protein-free supernatant was then separated and mixed with TBA (0.67%). The mixture was kept in a water bath (15 min, 100 °C). Malondialdehyde (MDA), an intermediate product of lipid peroxidation, was determined by the difference between absorbances at 535 and 572 nm (Multiskan GO ThermoScientific).

NFκB activity. Samples of the peritoneal exudate were collected in ice-cold PBS buffer and centrifuged (200 g × 10 min × 4 °C), and resuspended in lysis buffer (Cell Signaling). The supernatants were used to assess the levels of total and phosphorylated NFκB p65 subunit by ELISA (PathScan, Cell Signaling) according to the manufacturer's directions. The results were expressed as OD of samples (total p65/phospho-p65) at 450 nm (Multiskan GO Thermo Scientific).

Statistical analyses. Results are representative of two independent experiments and are presented as the means ± S.E.M. (n = 6 per group in each experiment). Two-way analysis of variance (ANOVA) was used to compare the groups and

doses at all times when the parameters were measured at different times after the stimulus injection. The analyzed factors were treatments, time and time *versus* treatment interaction. When there was a significant time *versus* treatment interaction, one-way ANOVA followed by Tukey's *t* test was performed for each time. Alternatively, when the treatment was evaluated once after the stimulus injection, the differences between responses were evaluated by one-way ANOVA followed by Tukey's *t* test. Statistical differences were considered to be significant at $P<0.05$.

ACKNOWLEDGMENTS

This work was supported by grants from São Paulo Research Foundation (FAPESP) under grant agreements number 2011/19670-0 (Thematic project) and 2013/08216-2 (Center for Research in Inflammatory Disease-CRID), Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Ministério da Ciência, Tecnologia e Inovação (MCTI), Secretaria da Ciência, Tecnologia e Ensino Superior (SETI)/Fundação Araucária and Governo do Estado do Paraná (Brazil).

REFERENCES

(1) Cunha, T. M.; Verri, W. A.; Schivo, I. R.; Napimoga, M. H.; Parada, C. A; Poole, S.; Teixeira, M. M.; Ferreira, S. H.; Cunha, F. Q. *Journal of leukocyte biology* **2008**, *83* (4), 824–832.

(2) Dal-Secco, D.; Paron, J. A.; De Oliveira, S. H. P.; Ferreira, S. H.; Silva, J. S.;

Cunha, F. D. Q. *Nitric Oxide - Biology and Chemistry* **2003**, *9* (3), 153–164.

(3) Guazelli, C. F. S.; Fattori, V.; Colombo, B. B.; Georgetti, S. R.; Vicentini, F. T. M. C.; Casagrande, R.; Baracat, M. M.; Verri, W. A. *Journal of Natural Products* **2013**, *76*, 200–208.

(4) Guerrero, A. T. G.; Verri, W. A.; Cunha, T. M.; Silva, T. A.; Rocha, F. A C.; Ferreira, S. H.; Cunha, F. Q.; Parada, C. A. *Pharmacology Biochemistry and Behavior* **2006**, *84* (2), 244–251.

(5) Malech, H. L.; Gallin, J. I. *The New England journal of medicine* **1987**, *317* (11), 687–694.

(6) Ginsburg, I.; Koren, E. *Expert review of anti-infective therapy* **2008**, *6* (4), 453–462.

(7) Calil, I. L.; Zarpelon, A. C.; Guerrero, A. T. G.; Alves-Filho, J. C.; Ferreira, S. H.; Cunha, F. Q.; Cunha, T. M.; Verri, W. A. *PLoS ONE* **2014**, *9* (3), 2–9.

(8) Ruiz-Miyazawa, K. W.; Pinho-Ribeiro, F. A.; Zarpelon, A. C.; Staurengo-Ferrari, L.; Silva, R. L.; Alves-Filho, J. C.; Cunha, T. M.; Cunha, F. Q.; Casagrande, R.; Verri, W. A. *Chemico-Biological Interactions* **2015**, *237*, 9–17.

(9) Sadik, C. D.; Kim, N. D.; Luster, A. D. *Trends in immunology* **2011**, *32* (10), 452–460.

(10) Verri, W. A.; Cunha, T. M.; Parada, C. A.; Poole, S.; Cunha, F. Q.; Ferreira, S. H. *Pharmacology & therapeutics* **2006**, *112*, 116–138.

(11) Verri, W. A.; Souto, F. O.; Vieira, S. M.; Almeida, S. C. L.; Fukada, S. Y.; Xu,

D.; Alves-Filho, J. C.; Cunha, T. M.; Guerrero, A. T. G.; Mattos-Guimaraes, R. B.; Oliveira, F. R.; Teixeira, M. M.; Silva, J. S.; McInnes, I. B.; Ferreira, S. H.; Louzada-Junior, P.; Liew, F. Y.; Cunha, F. Q. *Annals of the rheumatic diseases* **2010**, *69* (9), 1697–1703.

(12) Sadik, C. D.; Luster, A. D. *Journal of Leukocyte Biology* **2012**, *91* (2), 207–215.

(13) Ferreira, S. H.; Lorenzetti, B. B.; Bristow, A. F.; Poole, S. *Nature* **1988**, *334* (6184), 698–700.

(14) Block, L. C.; Santos, A. R.; de Souza, M. M.; Scheidt, C.; Yunes, R. A.; Santos, M. A.; Monache, F. D.; Filho, V. C. *Journal of ethnopharmacology* **1998**, *61* (1), 85–89.

(15) Boller, S.; Soldi, C.; Marques, M. C. a; Santos, E. P.; Cabrini, D. A.; Pizzolatti, M. G.; Zampronio, A. R.; Otuki, M. F. *Journal of Ethnopharmacology* **2010**, *130* (2), 262–266.

(16) Choi, R. J.; Shin, E. M.; Jung, H. A.; Choi, J. S.; Kim, Y. S. *Phytomedicine* **2011**, *18* (8-9), 677–682.

(17) Cho, J. H.; Lee, J. Y.; Sim, S. S.; Whang, W. K.; Kim, C. J. *Pulmonary Pharmacology and Therapeutics* **2010**, *23* (3), 190–199.

(18) Silva, E. A.; Takahashi, J. A.; Boaventura, M. A. D.; Oliveira, A. B. *Phytochemistry* **1999**, *52* (3), 397–400.

(19) Bresciani, L. F. V; Yunes, R. A.; Bürger, C.; De Oliveira, L. E.; Bóf, K. L.; Cechinel-Filho, V. *Zeitschrift fur Naturforschung - Section C Journal of Biosciences* **2004**, *59* (3-4), 229–232.

(20) Mizokami, S. S.; Arakawa, N. S.; Ambrosio, S. R.; Zarpelon, A. C.; Casagrande, R.; Cunha, T. M.; Ferreira, S. H.; Cunha, F. Q.; Verri, W. A. *Journal of Natural Products* **2012**, *75* (5), 896–904.

(21) Fernandes, V. C.; Coppede, J.; Martins, J. S.; Rizo, W. F.; Beleboni, R. O.; Marins, M.; Pereira, P. S.; Fachin, A. L.; Biotecnologia, U. De. *Genetics and Molecular Research* **2013**, *12* (2), 1005–1011.

(22) Tirapelli, C. R.; Ambrosio, S. R.; Da Costa, F. B.; Coutinho, S. T.; De Oliveira, D. C. R.; De Oliveira, A. M. *European Journal of Pharmacology* **2004**, *492* (2-3), 233–241.

(23) Paiva, L. A. F.; Gurgel, L. A.; Silva, R. M.; Tomé, A. R.; Gramosa, N. V.; Silveira, E. R.; Santos, F. A.; Rao, V. S. N. *Vascular Pharmacology* **2002**, *39* (6), 303–307.

(24) Miranda, M. M.; Panis, C.; da Silva, S. S.; Macri, J. A.; Kawakami, N. Y.; Hayashida, T. H.; Madeira, T. B.; Acquaro, V. R.; Nixdorf, S. L.; Pizzatti, L.; Ambrósio, S. R.; Cecchini, R.; Arakawa, N. S.; Verri, W. A.; Conchon Costa, I.; Pavanelli, W. R. *Mediators of inflammation* **2015**, *2015*, 392918.

(25) Costa-Lotufo, L. V.; Cunha, G. M. A; Farias, P. A. M.; Viana, G. S. B.; Cunha, K. M. A.; Pessoa, C.; Moraes, M. O.; Silveira, E. R.; Gramosa, N. V.; Rao, V. S. N. *Toxicon* **2002**, *40* (8), 1231–1234.

(26) Lim, H.; Jung, H. A.; Choi, J. S.; Kim, Y. S.; Kang, S. S.; Kim, H. P. *Archives of Pharmacal Research* **2009**, *32* (9), 1237–1243.

(27) Silva, R. Z. da; Rios, E. de M.; Silva, M. Z. da; Leal, L. de F.; Yunes, R. A.;

Miguel, O. G.; Cechinel-Filho, V. *Visão Acadêmica. Curitiba-PR* **2002**, *3*, 59–64.

(28) Cavalcanti, B. C.; Costa-Lotufo, L. V.; Moraes, M. O.; Burbano, R. R.; Silveira, E. R.; Cunha, K. M. A.; Rao, V. S. N.; Moura, D. J.; Rosa, R. M.; Henriques, J. A. P.; Pessoa, C. *Food and Chemical Toxicology* **2006**, *44* (3), 388–392.

(29) Ambrosio, S. R.; Tirapelli, C. R.; da Costa, F. B.; de Oliveira, A. M. *Life Sciences* **2006**, *79* (10), 925–933.

(30) Castrillo, A.; de Las Heras, B.; Hortelano, S.; Rodriguez, B.; Villar, A.; Bosca, L. *The Journal of biological chemistry* **2001**, *276* (19), 15854–15860.

(31) Lyu, J. H.; Lee, G. S.; Kim, K. H.; Kim, H. W.; Cho, S. I.; Jeong, S. Il; Kim, H. J.; Ju, Y. S.; Kim, H. K.; Sadikot, R. T.; Christman, J. W.; Oh, S. R.; Lee, H. K.; Ahn, K. S.; Joo, M. *Journal of Ethnopharmacology* **2011**, *137* (3), 1442–1449.

(32) Guerrero, A. T. G.; Verri, W. A.; Cunha, T. M.; Silva, T. A.; Schivo, I. R. S.; Dal-Secco, D.; Canetti, C.; Rocha, F. A. C.; Parada, C. A.; Cunha, F. Q.; Ferreira, S. H. *Journal of leukocyte biology* **2008**, *83* (1), 122–130.

(33) Mizokami, S. S.; Hohmann, M. S. N.; Staurengo-Ferrari, L.; Carvalho, T. T.; Zarpelon, A. C.; Possebon, M. I.; de Souza, A. R.; Veneziani, R. C. S.; Arakawa, N. S.; Casagrande, R.; Verri, W. A. *Plos One* **2016**, *11* (2), e0149656.

(34) Calixto-Campos, C.; Carvalho, T. T.; Hohmann, M. S. N.; Pinho-Ribeiro, F. A.; Fattori, V.; Manchope, M. F.; Zarpelon, A. C.; Baracat, M. M.; Georgetti, S. R.; Casagrande, R.; Verri, W. A. *Journal of Natural Products* **2015**, *78* (8), 1799–

1808.

(35) Hattori, H.; Subramanian, K. K.; Sakai, J.; Jia, Y.; Li, Y.; Porter, T. F.; Loison, F.; Sarraj, B.; Kasorn, A.; Jo, H.; Blanchard, C.; Zirkle, D.; McDonald, D.; Pai, S.-Y.; Serhan, C. N.; Luo, H. R. *Proceedings of the National Academy of Sciences of the United States of America* **2010**, *107* (8), 3546–3551.

(36) Dal-Secco, D.; Moreira, A. P.; Freitas, A.; Silva, J. S.; Rossi, M. A.; Ferreira, S. H.; Cunha, F. Q. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* **2006**, *15* (1), 77–86.

(37) Leite, A. C. R. M.; Cunha, F. Q.; Dal-Secco, D.; Fukada, S. Y.; Girão, V. C. C.; Rocha, F. A. C. *British journal of pharmacology* **2009**, *156* (5), 818–825.

(38) Ahluwalia, A.; Foster, P.; Scotland, R. S.; McLean, P. G.; Mathur, A.; Perretti, M.; Moncada, S.; Hobbs, A. J. *Proceedings of the National Academy of Sciences of the United States of America* **2004**, *101* (5), 1386–1391.

(39) Da Silva-Santos, J. E.; Santos-Silva, M. C.; Cunha, F. de Q.; Assreuy, J. *The Journal of pharmacology and experimental therapeutics* **2002**, *300* (3), 946–951.

(40) Staurengo-Ferrari, L.; Mizokami, S. S.; Fattori, V.; Silva, J. J.; Zanichelli, P. G. P. G.; Georgetti, S. R.; Baracat, M. M.; Da França, L. G.; Pavanelli, W. R.; Casagrande, R.; Verri, W. A.; da França, L. G.; Pavanelli, W. R.; Casagrande, R.; Verri, W. A. *Naunyn-Schmiedeberg's Archives of Pharmacology* **2014**, *387* (11), 1053–1068.

(41) Hickey, M. J.; Granger, D. N.; Kubes, P. *Acta physiologica Scandinavica* **2001**,

173 (1), 119–126.

(42) Hossain, M.; Qadri, S. M.; Liu, L. *Journal of Inflammation* **2012**, *9* (1), 28.

(43) Dal-Secco, D.; Freitas, A.; Abreu, M. A.; Garlet, T. P.; Rossi, M. A.; Ferreira, S. H.; Silva, J. S.; Alves-Filho, J. C.; Cunha, F. Q. *Naunyn-Schmiedeberg's archives of pharmacology* **2010**, *381* (6), 483–493.

(44) Dal-Secco, D.; Cunha, T. M.; Freitas, A.; Alves-Filho, J. C.; Souto, F. O.; Fukada, S. Y.; Grespan, R.; Alencar, N. M. N.; Neto, A. F.; Rossi, M. A.; Ferreira, S. H.; Hothersall, J. S.; Cunha, F. Q. *Journal of immunology (Baltimore, Md. : 1950)* **2008**, *181* (6), 4287–4298.

(45) Verri, W. A.; Cunha, T. M.; Magro, D. A.; Guerrero, A. T. G.; Vieira, S. M.; Carregaro, V.; Souza, G. R.; Henriques, M. D. G. M. O.; Ferreira, S. H.; Cunha, F. Q. *Naunyn-Schmiedeberg's Archives of Pharmacology* **2009**, *379* (3), 271–279.

(46) Santos, D. R.; Calixto, J. B.; Souza, G. E. P. *British journal of pharmacology* **2003**, *139* (2), 271–278.

(47) Medvedev, A. E.; Kopydlowski, K. M.; Vogel, S. N. *Journal of immunology (Baltimore, Md. : 1950)* **2000**, *164* (11), 5564–5574.

(48) Martinez, R. M.; Longhi-Balbinot, D. T.; Zarpelon, A. C.; Staurengo-Ferrari, L.; Baracat, M. M.; Georgetti, S. R.; Sassonia, R. C.; Verri, W. A.; Casagrande, R. *Archives of Pharmacal Research* **2014**.

(49) Borghi, S. M.; Pinho-Ribeiro, F. A.; Zarpelon, A. C.; Cunha, T. M.; Alves-Filho, J. C.; Ferreira, S. H.; Cunha, F. Q.; Casagrande, R.; Verri, W. A. *Experimental*

physiology **2015**, *100* (5), 531–544.

(50) Pinho-Ribeiro, F. A.; Hohmann, M. S. N.; Borghi, S. M.; Zarpelon, A. C.; Guazelli, C. F. S.; Manchope, M. F.; Casagrande, R.; Verri, W. A. *Chemico-Biological Interactions* **2015**, *228*, 88–99.

(51) Okuyama, E.; Nishimura, S.; Yamazaki, M. *Chemical & pharmaceutical bulletin* **1991**, *39* (2), 405–407.

(52) de Andrade, B. B.; Moreira, M. R.; Ambrosio, S. R.; Furtado, N. A. J. C.; Cunha, W. R.; Heleno, V. C. G.; Silva, A. N.; Simão, M. R.; da Rocha, E. M. P.; Martins, C. H. G.; Veneziani, R. C. S. *Natural product communications* **2011**, *6* (6), 777–780.

(53) Cunha, T. M.; Verri, W. A.; Vivancos, G. G.; Moreira, I. F.; Reis, S.; Parada, C. A.; Cunha, F. Q.; Ferreira, S. H. *Brazilian Journal of Medical and Biological Research* **2004**, *37* (3), 391–399.

(54) Verri, W. A.; Molina, R. O.; Schivo, I. R. S.; Cunha, T. M.; Parada, C. A.; Poole, S.; Ferreira, H.; Cunha, F. Q. *The Journal of pharmacology and experimental therapeutics* **2005**, *315* (2), 609–615.

(55) Casagrande, R.; Georgetti, S. R.; Verri, W. A.; Dorta, D. J.; dos Santos, A. C.; Fonseca, M. J. V. *Journal of Photochemistry and Photobiology B: Biology* **2006**, *84* (1), 21–27.

(56) Bradley, P. P.; Priebat, D. A.; Christensen, R. D.; Rothstein, G. *The Journal of investigative dermatology* **1982**, *78* (3), 206–209

LEGENDS TO FIGURES

Figure 1. KA reduces LPS-induced leukocyte recruitment to the peritoneal cavity. Mice were treated with KA in three different doses (1, 3 and 10 mg/kg) 30 min before injection of LPS-induced leukocytes recruitment to peritoneal cavity. The total number of leukocytes (Panel A), neutrophils (Panel B) and mononuclear cells (Panel C) were evaluated six hour after LPS stimulus. Results are presented as the means ± S.E.M. of six mice per group per experiment, and are representative of two separate experiments. $^{*}P<0.05$ compared with the saline groups; $^{\#}P<0.05$ compared with the vehicle groups; $^{**}P<0.05$ compared with the vehicle groups and the dose of 1 mg/kg of KA; $^{f}P<0.05$ compared with the vehicle groups and the doses of 1 and 3 mg/kg of KA (One-way ANOVA followed by Tukey's *t* test).

Figure 2. Post-treatment with KA reduces LPS-induced leukocyte recruitment to peritoneal cavity. Mice were treated with KA (10 mg/kg) 30 min after injection of LPS-induced leukocytes recruitment to peritoneal cavity. The number of total leukocytes (Panel A), neutrophils (Panel B) and mononuclear cells (Panel C) were evaluated six hour after LPS stimulus. Results are presented as the means ± S.E.M. of six mice per group per experiment, and are representative of two separate experiments. $^{*}P<0.05$ compared with the saline groups; $^{\#}P<0.05$ compared with the vehicle groups (One-way ANOVA followed by Tukey's *t* test).

Figure 3. Treatment with KA reduces LPS-induced hyperalgesia and MPO activity. Mice were treated with KA (1, 3 and 10 mg/kg) 30 min after injection of LPS (200

ng/paw). The mechanical (Pane A) and thermal (Panel B) hyperalgesia were evaluated 0-5 h after LPS injection. MPO activity (Panel C) was determined in plantar skin tissue collected 5 h after LPS injection. Results are presented as the means ± S.E.M. of six mice per group per experiment, and are representative of two separate experiments. $^{*}P<0.05$ compared with the saline groups; $^{\#}P<0.05$ compared with the vehicle groups; $^{**}P<0.05$ compared with the vehicle groups and the dose of 1 mg/kg of KA (Two-way repeated measures ANOVA followed by Tukey's *t* test).

Figure 4. Treatment with KA reduces LPS-induced oxidative stress in the paw skin. Mice were treated with KA (10 mg/kg) 30 min after injection of LPS (200 ng/paw). The GSH levels (Panel A), free radical scavenging ability (ABTS assay, panel B), superoxide anion production (NBT assay, panel C) and lipid peroxidation (TBARS assay, panel D) was determined in plantar skin tissue collected 3 h after LPS injection. Results are presented as the means ± S.E.M. of six mice per group per experiment, and are representative of two separate experiments. $^{*}P<0.05$ compared with the saline groups; $^{\#}P<0.05$ compared with the vehicle groups; (One-way ANOVA followed by Tukey's *t* test).

Figure 5. L-Name prevents in a dose-dependent manner the KA inhibition of LPS-induced leukocyte recruitment to the peritoneal cavity. Mice were pre-treated with L-NAME (10-100 mg/kg; NOS inhibitor) and 30 min after treated with KA (10 mg/kg). Added 30 min after treatment mice received LPS injection. The total number of leukocytes (Panel A), neutrophils (Panel B) and mononuclear cells (Panel

C) were evaluated six hour after LPS stimulus. Results are presented as the means ± S.E.M. of six mice per group per experiment, and are representative of two separate experiments. $^{*}P<0.05$ compared with the saline groups; $^{\#}P<0.05$ compared with the vehicle groups; $^{**}P<0.05$ compared with the KA group; $^{ƒ}P<0.05$ compared with the KA groups and the L-NAME 10 mg/kg plus KA group (One-way ANOVA followed by Tukey's *t* test).

Figure 6. ODQ prevents the KA inhibition of LPS-induced leukocytes recruitment to the peritoneal cavity. Mice were pre-treated with ODQ (1 mg/kg; soluble cGMP inhibitor) 30 min before treatment with KA (10 mg/kg). Added 30 min after treatment mice received LPS injection. The number of total leukocytes. The number of total leukocytes (Panel A), neutrophils (Panel B) and mononuclear cells (Panel C) were evaluated six hour after LPS stimulus. Results are presented as the means ± S.E.M. of six mice per group per experiment, and are representative of two separate experiments. $^{*}P<0.05$ compared with the saline groups; $^{\#}P<0.05$ compared with the vehicle groups; $^{**}P<0.05$ compared with the KA group (One-way ANOVA followed by Tukey's *t* test).

Figure 7. KT5823 prevents the KA inhibition of LPS-induced leukocyte recruitment to the peritoneal cavity. Mice were pre-treated with KT5823 (0.5 µg/mice; PKG inhibitor) 10 min before treatment with KA (10 mg/kg). Added 30 min after treatment mice received LPS injection. The number of total leukocytes. The number of total leukocytes (Panel A), neutrophils (Panel B) and mononuclear cells (Panel C)

were evaluated six hour after LPS stimulus. Results are presented as the means ± S.E.M. of six mice per group per experiment, and are representative of two separate experiments. $^{*}P<0.05$ compared with the saline groups; $^{\#}P<0.05$ compared with the vehicle groups; $^{**}P<0.05$ compared with the KA group (One-way ANOVA followed by Tukey's *t* test).

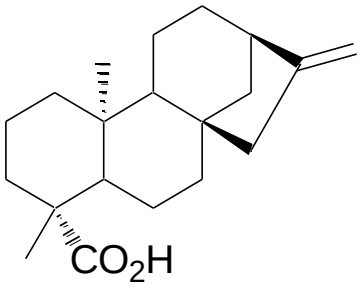
Figure 8. Glibenclamide prevents the KA inhibition of LPS-induced leukocyte recruitment to the peritoneal cavity. Mice were pre-treated with Glibenclamide (1 mg/kg; Gli; ATP-sensitive potassium channel blocker) 30 min before treatment with KA (10 mg/kg). Added 30 min after treatment mice received LPS injection. The number of total leukocytes. The number of total leukocytes (Panel A), neutrophils (Panel B) and mononuclear cells (Panel C) were evaluated six hour after LPS stimulus. Results are presented as the means ± S.E.M. of six mice per group per experiment, and are representative of two separate experiments. $^{*}P<0.05$ compared with the saline groups; $^{\#}P<0.05$ compared with the vehicle groups; $^{**}P<0.05$ compared with the KA group (One-way ANOVA followed by Tukey's *t* test).

Figure 9. KA inhibits LPS-induced pro-inflammatory cytokine and enhances anti-inflammatory cytokine production. Mice were treated with KA (10 mg/kg) 30 min before injection of LPS. At indicated times after the injection stimuli (0.5 – 6 h) the cytokines TNF-α (Panel A), IL-1β (Panel B), IL-33 (Panel C) and IL-10 (Panel D) levels were determined. Results are presented as the means ± S.E.M. of six mice per group per experiment, and are representative of two separate experiments. $^{*}P<0.05$

compared with the saline groups; $^{\#}P<0.05$ compared with the vehicle groups (One-way ANOVA followed by Tukey's *t* test).

Figure 10. KA inhibits LPS-induced NFκB activation. Mice were treated with KA (10 mg/kg) 30 min after injection of LPS. The NFκB activation was determined 2 h after LPS injection. Results are presented as the means ± S.E.M. of six mice per group per experiment, and are representative of two separate experiments. $^{*}P<0.05$ compared with the saline groups; $^{\#}P<0.05$ compared with the vehicle groups; (One-way ANOVA followed by Tukey's *t* test).

Structure Sheet



Kaurenoic acid (KA)

Figure 1

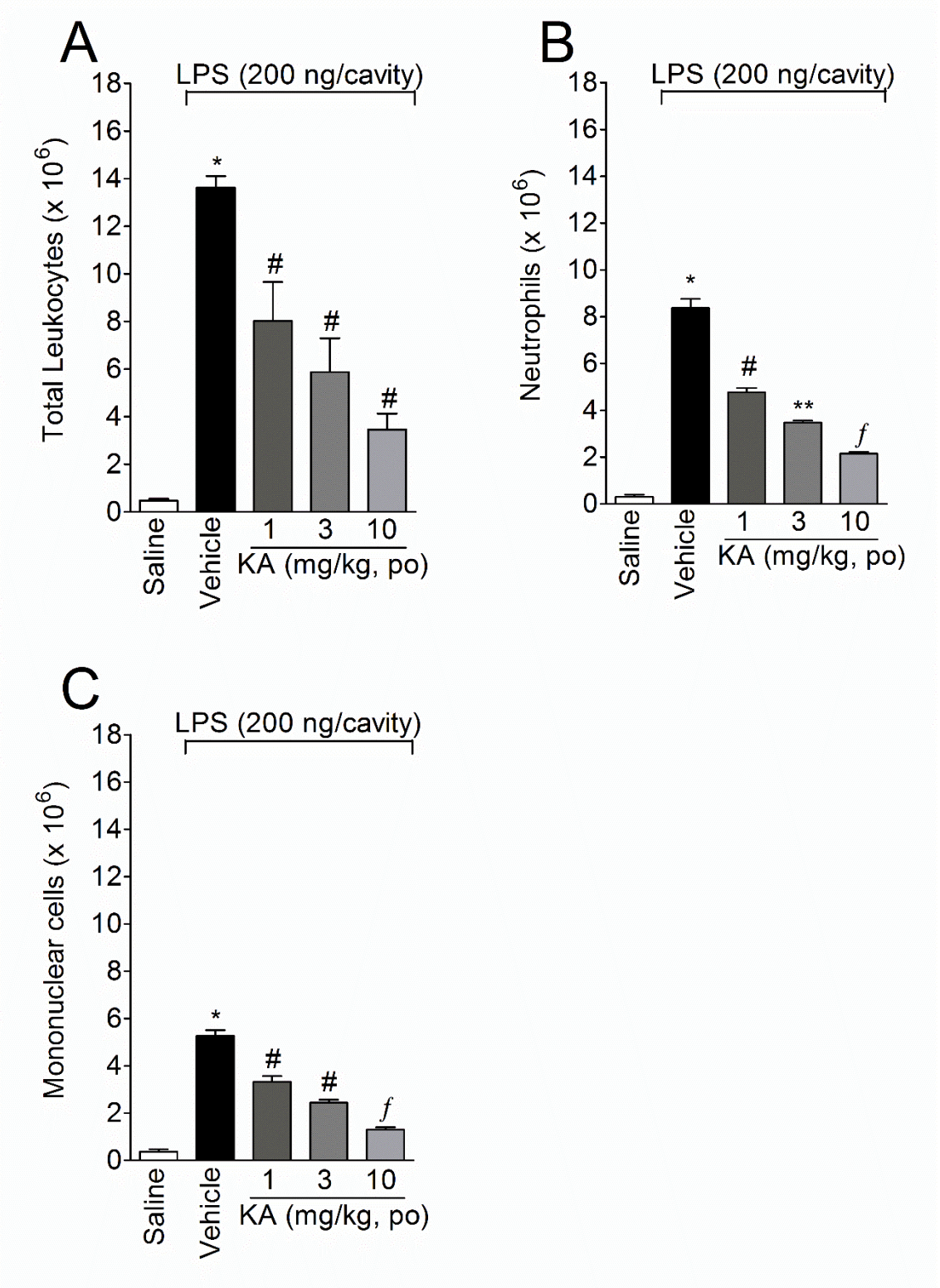

A
LPS (200 ng/cavity)
Total Leukocytes (x 10^6)
18
16
14
12
10
8
6
4
2
0
*
#
#
#
Saline
Vehicle
1
3
10
KA (mg/kg, po)
B
LPS (200 ng/cavity)
Neutrophils (x 10^6)
*
#
**
f
Saline
Vehicle
1
3
10
KA (mg/kg, po)
C
LPS (200 ng/cavity)
Mononuclear cells (x 10^6)
*
#
#
f
Saline
Vehicle
1
3
10
KA (mg/kg, po)

Figure 2

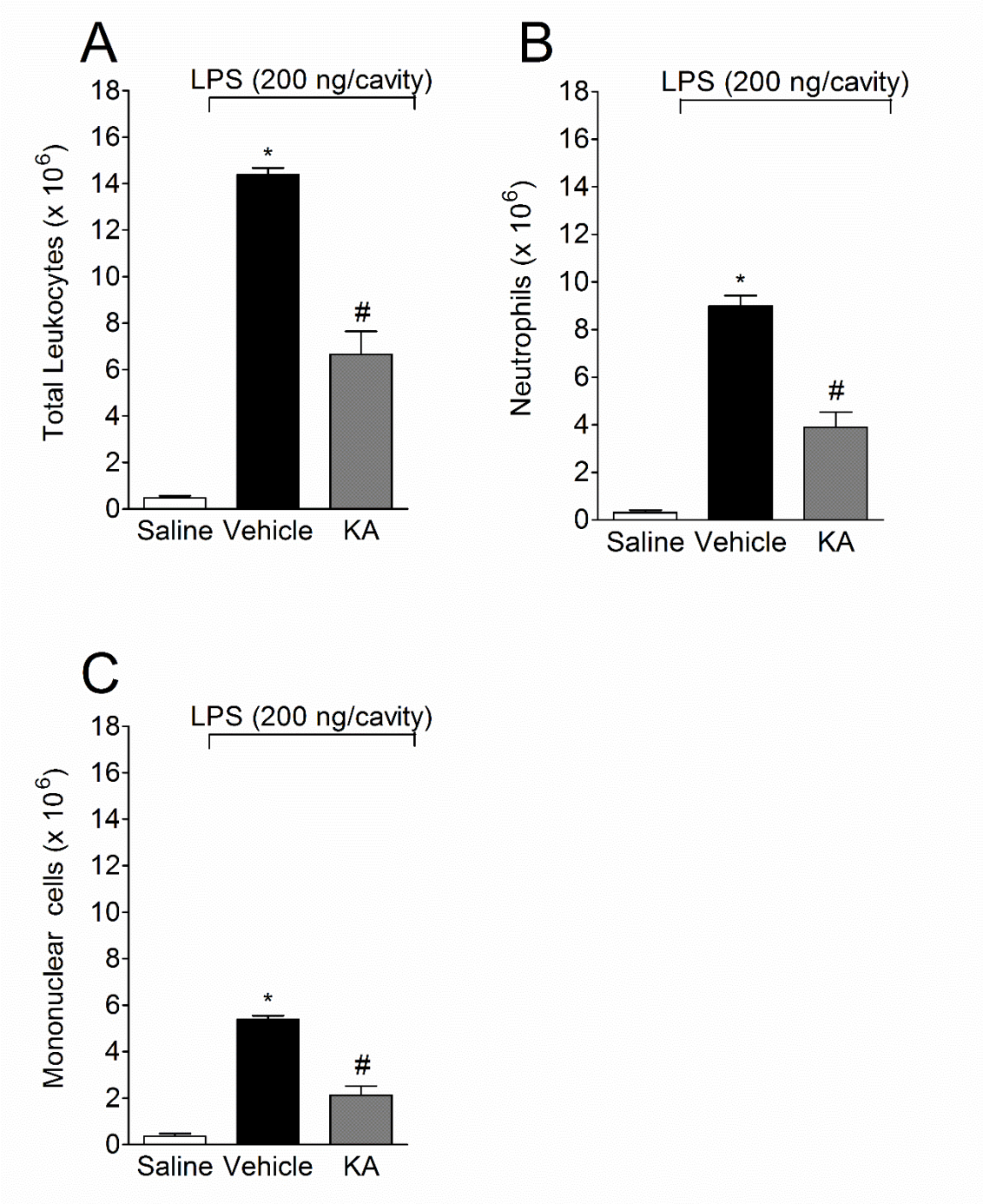

A
LPS (200 ng/cavity)
Total Leukocytes (x 10^6)
18
16
14
12
10
8
6
4
2
0
*
#
Saline
Vehicle
KA
B
LPS (200 ng/cavity)
Neutrophils (x 10^6)
18
16
14
12
10
8
6
4
2
0
*
#
Saline
Vehicle
KA
C
LPS (200 ng/cavity)
Mononuclear cells (x 10^6)
18
16
14
12
10
8
6
4
2
0
*
#
Saline
Vehicle
KA

Figure 3

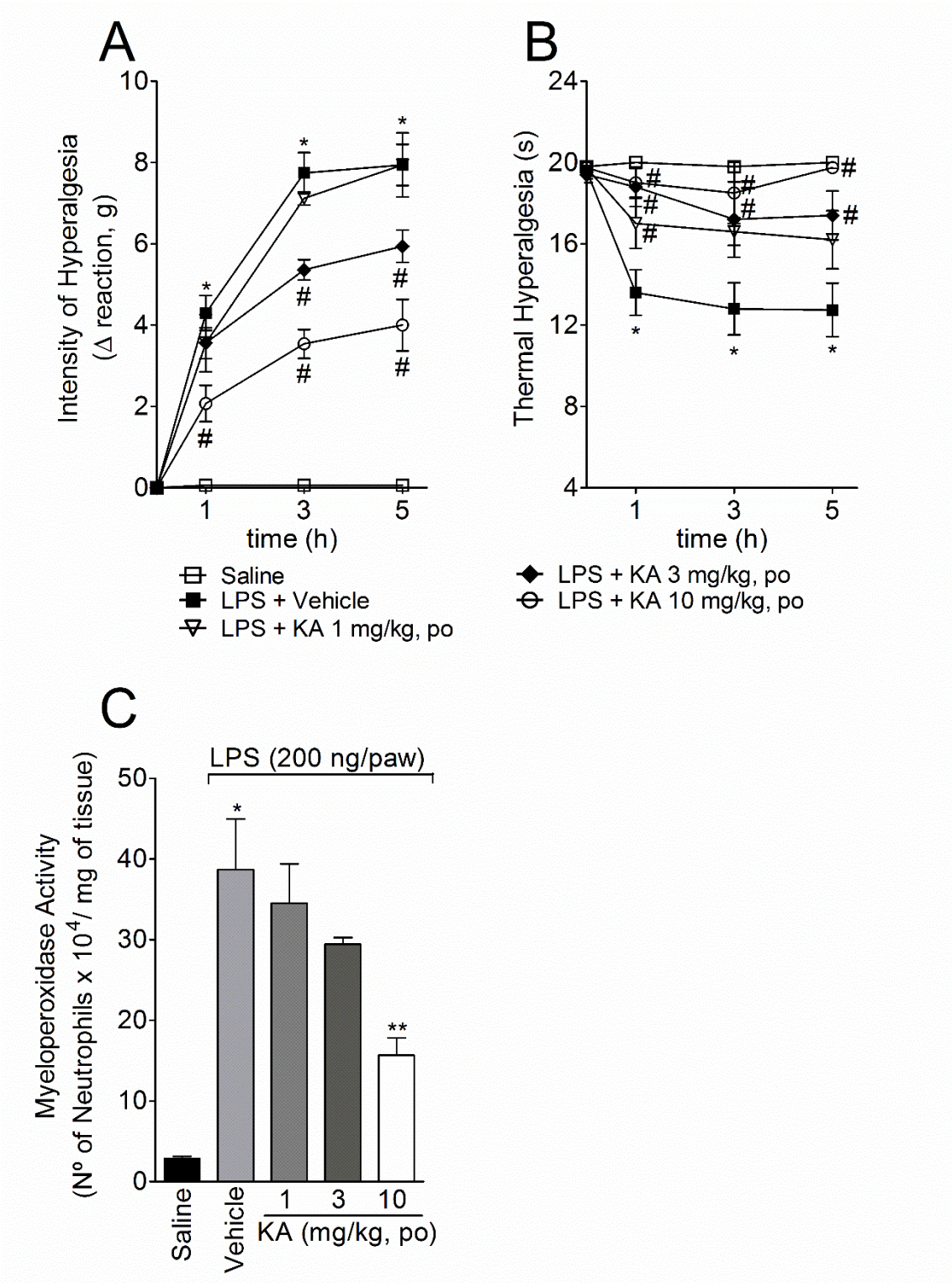

A
Intensity of Hyperalgesia
(Δ reaction, g)
time (h)
Saline
LPS + Vehicle
LPS + KA 1 mg/kg, po
B
Thermal Hyperalgesia (s)
time (h)
LPS + KA 3 mg/kg, po
LPS + KA 10 mg/kg, po
C
LPS (200 ng/paw)
Myeloperoxidase Activity
(Nº of Neutrophils x 10⁴/ mg of tissue)
Saline
Vehicle
1
3
10
KA (mg/kg, po)

Figure 4

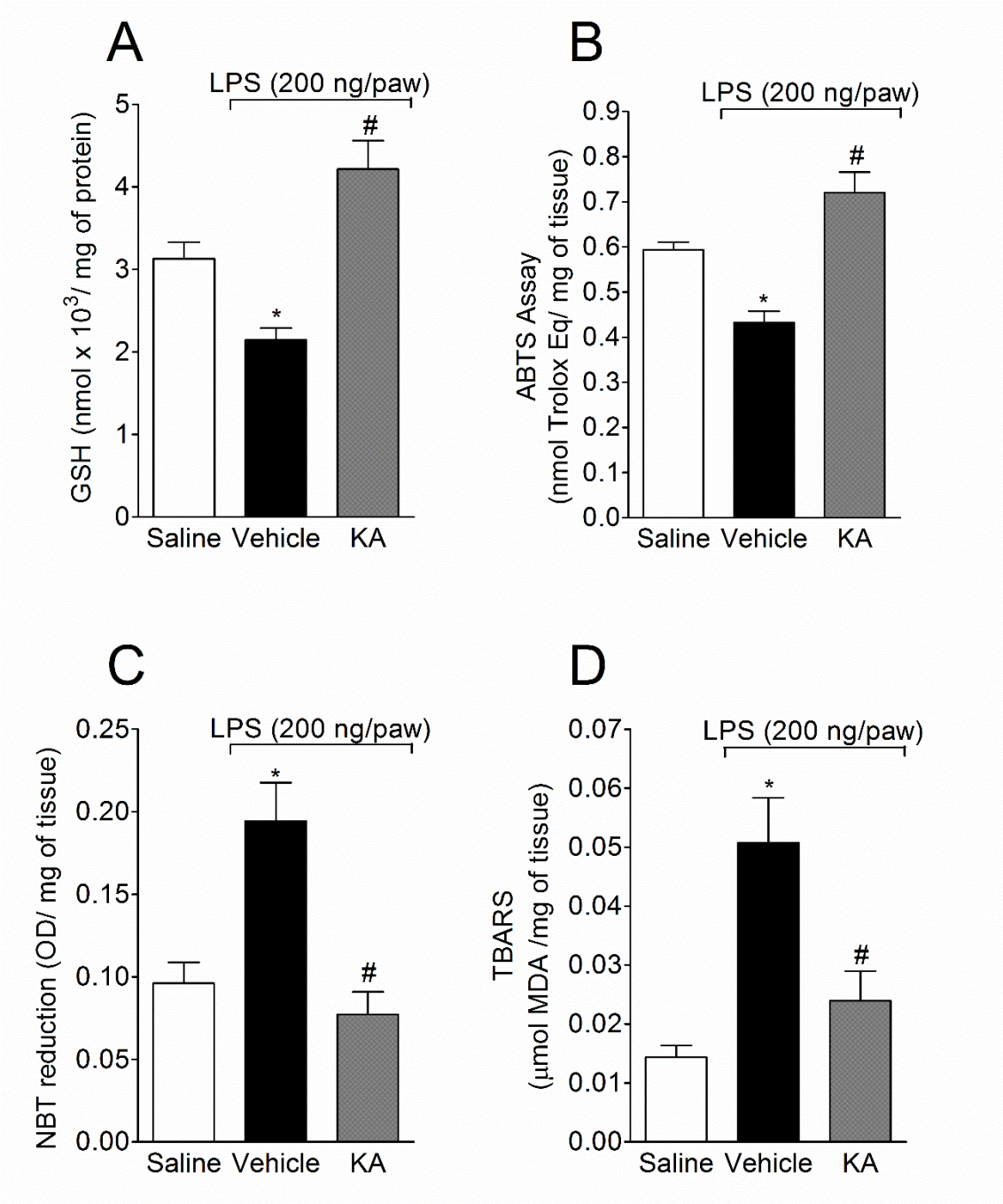

A
LPS (200 ng/paw)
GSH (nmol x 10³/ mg of protein)
Saline
Vehicle
KA
B
LPS (200 ng/paw)
ABTS Assay
(nmol Trolox Eq/ mg of tissue)
Saline
Vehicle
KA
C
LPS (200 ng/paw)
NBT reduction (OD/ mg of tissue)
Saline
Vehicle
KA
D
LPS (200 ng/paw)
TBARS
(µmol MDA /mg of tissue)
Saline
Vehicle
KA

Figure 5

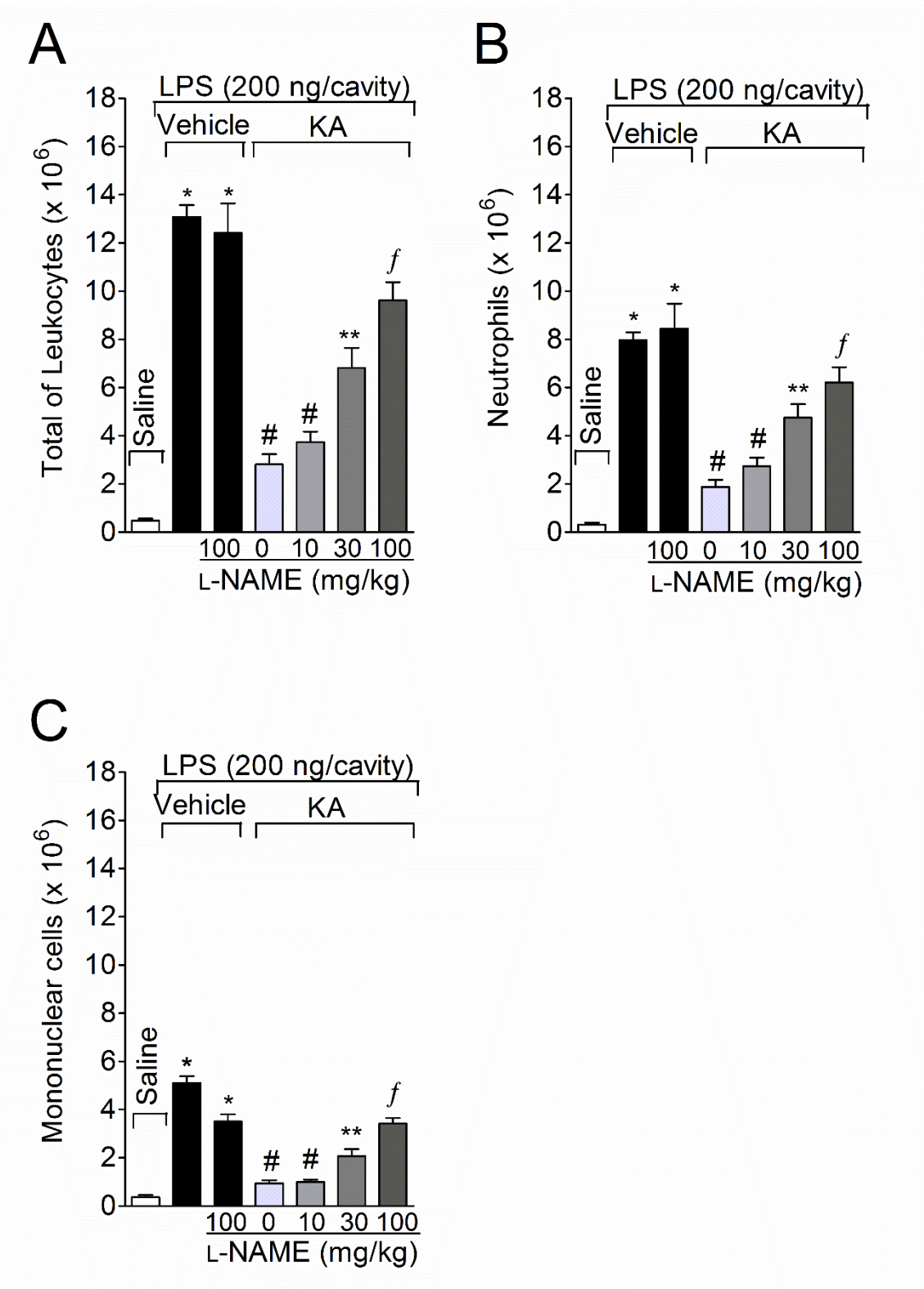

A
LPS (200 ng/cavity)
Vehicle
KA
Total of Leukocytes (x 10^6)
Saline
100 0 10 30 100
L-NAME (mg/kg)
B
LPS (200 ng/cavity)
Vehicle
KA
Neutrophils (x 10^6)
Saline
100 0 10 30 100
L-NAME (mg/kg)
C
LPS (200 ng/cavity)
Vehicle
KA
Mononuclear cells (x 10^6)
Saline
100 0 10 30 100
L-NAME (mg/kg)

Figure 6

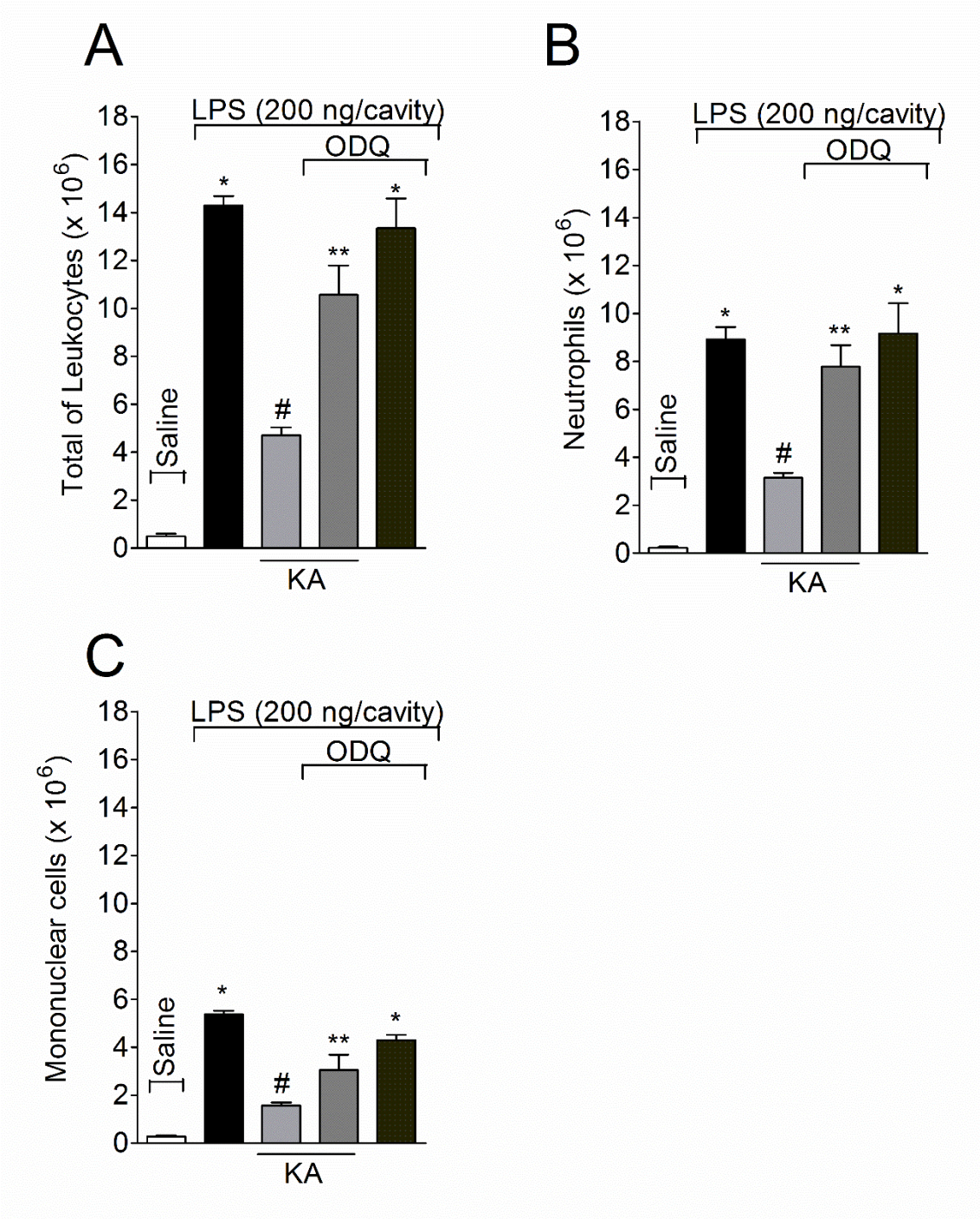

A
LPS (200 ng/cavity)
ODQ
Total of Leukocytes (x 10^6)
Saline
KA
B
LPS (200 ng/cavity)
ODQ
Neutrophils (x 10^6)
Saline
KA
C
LPS (200 ng/cavity)
ODQ
Mononuclear cells (x 10^6)
Saline
KA

Figure 7

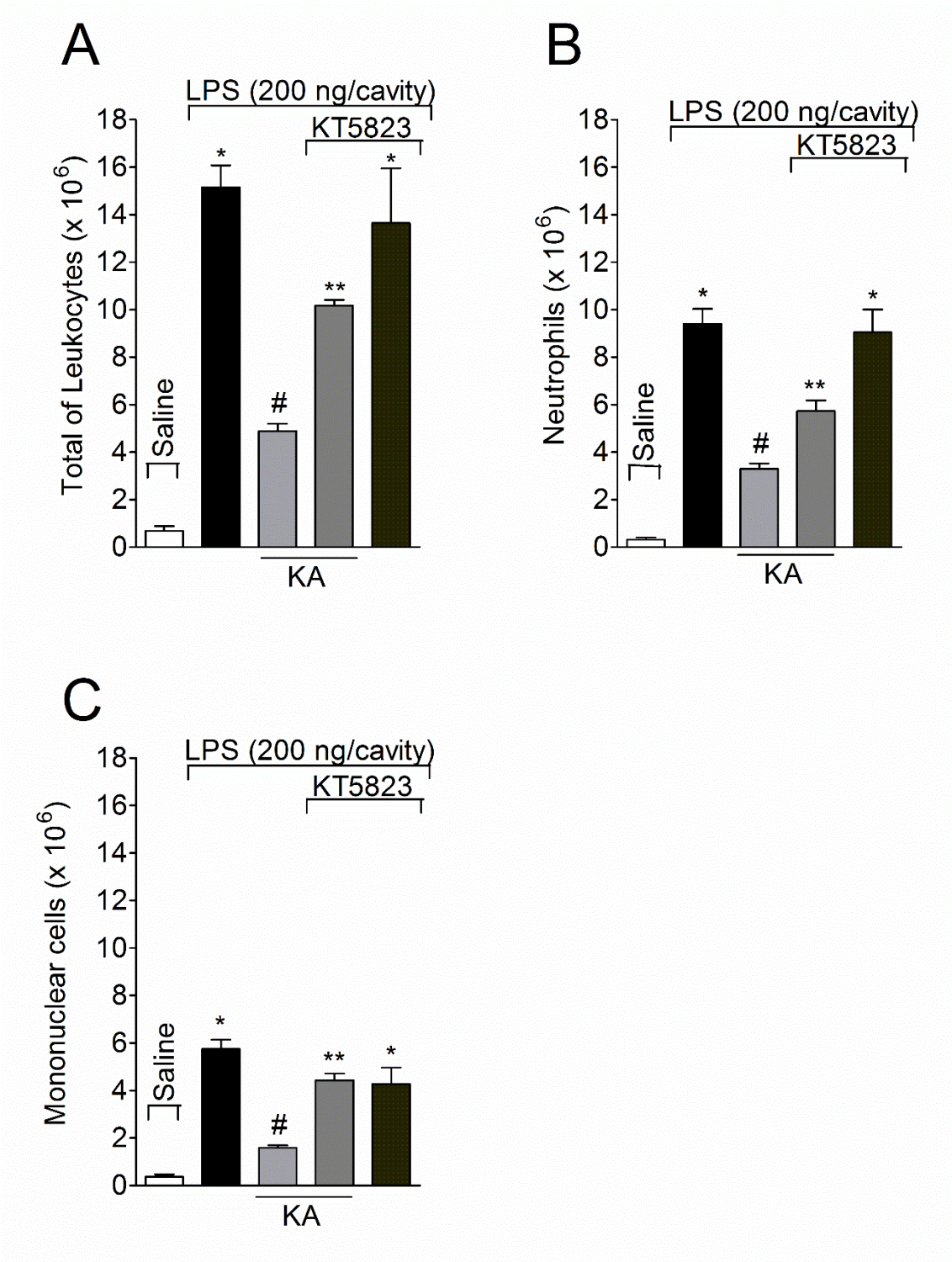

A
LPS (200 ng/cavity)
KT5823
Total of Leukocytes (x 10^6)
Saline
KA
B
LPS (200 ng/cavity)
KT5823
Neutrophils (x 10^6)
Saline
KA
C
LPS (200 ng/cavity)
KT5823
Mononuclear cells (x 10^6)
Saline
KA

Figure 8

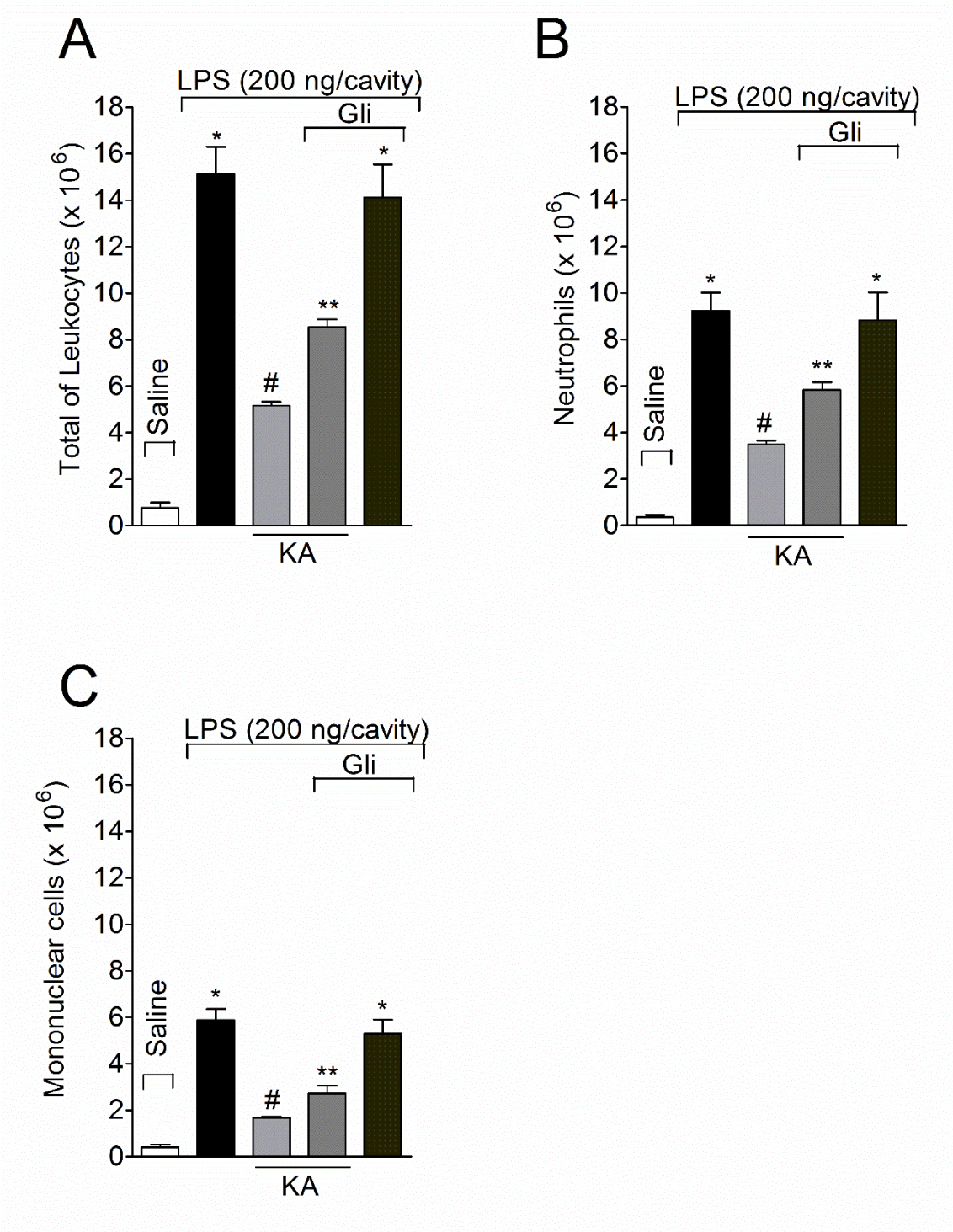

A
LPS (200 ng/cavity)
Gli
Total of Leukocytes (x 10^6)
Saline
KA
B
LPS (200 ng/cavity)
Gli
Neutrophils (x 10^6)
Saline
KA
C
LPS (200 ng/cavity)
Gli
Mononuclear cells (x 10^6)
Saline
KA

Figure 9

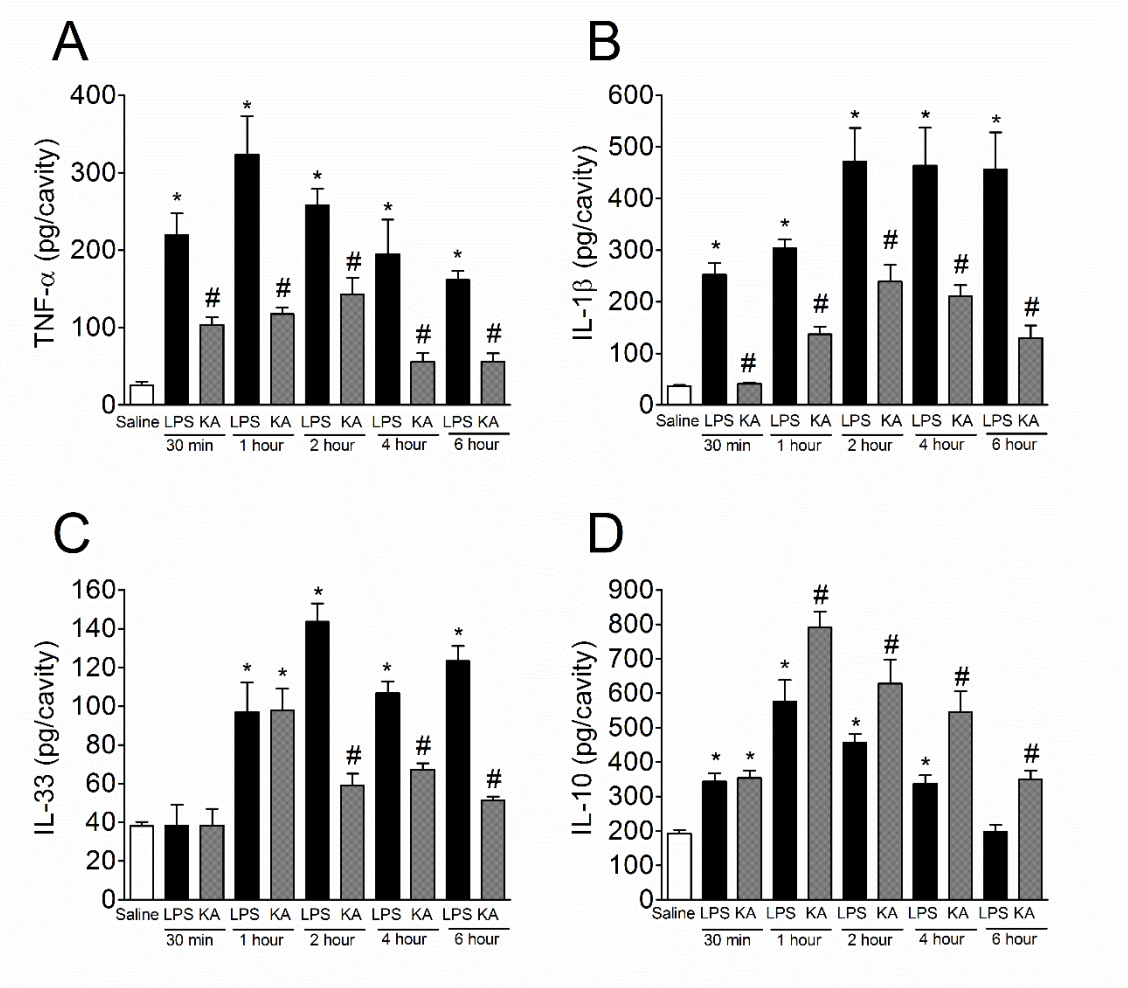
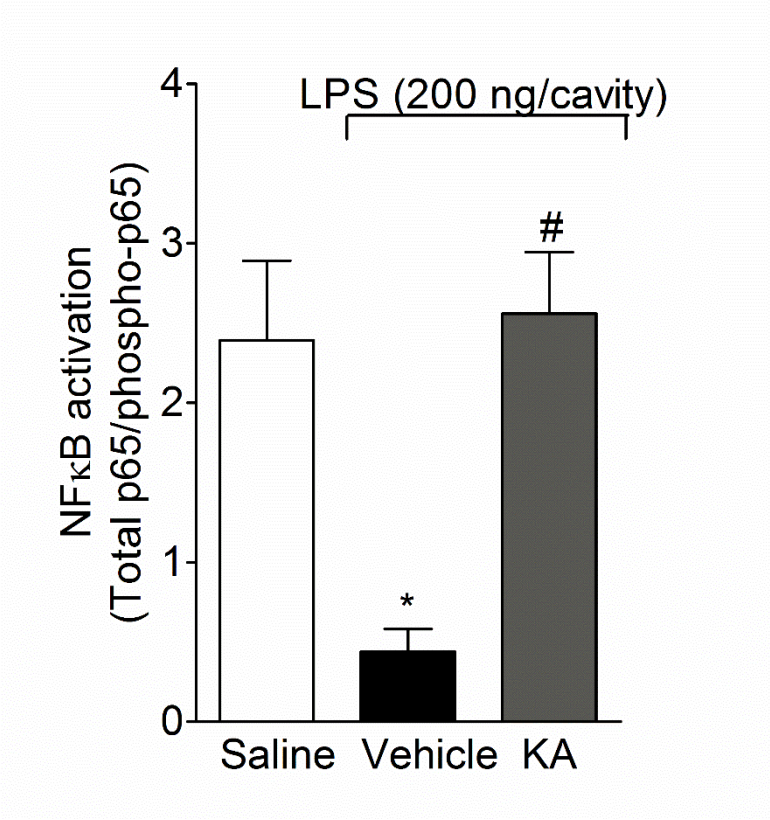

A
TNF-α (pg/cavity)
400
300
200
100
0
B
IL-1β (pg/cavity)
600
500
400
300
200
100
0
C
IL-33 (pg/cavity)
160
140
120
100
80
60
40
20
0
D
IL-10 (pg/cavity)
900
800
700
600
500
400
300
200
100
0
Saline LPS KA LPS KA LPS KA LPS KA LPS KA
30 min 1 hour 2 hour 4 hour 6 hour
*
#

Figure 10


LPS (200 ng/cavity)
NFκB activation
(Total p65/phospho-p65)
4
3
2
1
0
*
#
Saline
Vehicle
KA

ARTIGO 3

KAURENOIC ACID INHIBITS ONGOING CHRONIC CONSTRICTION INJURY-INDUCED NEUROPATHIC PAIN BY INHIBING SPINAL CORD NEUROINFLAMMATION.

Sandra S. Mizokami[a], Felipe A. Pinho-Ribeiro[a], Thiago H. Hayashida[b,] Nilton S. Arakawa[b], Sergio R. Ambrosio[c], Rubia Casagrande[b], Waldiceu A. Verri, Jr[a*]

[a]Departamento de Ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina, 86057-970 Londrina, Paraná, Brazil.

[b]Departamento de Ciências Farmacêuticas, Centro de Ciências da Saúde, Universidade Estadual de Londrina, 86038-350 Londrina, Paraná, Brazil.

[c]Núcleo de Pesquisa em Ciências Exatas e Tecnológicas, Universidade de Franca, 14404-600 Franca, São Paulo, Brazil.

***Author for correspondence:** Waldiceu A. Verri Jr (waldiceujr@yahoo.com.br or waverri@uel.br). Present address: Departamento de Ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina. Rod. Celso Garcia Cid, Km 380, PR 445, Cx. Postal 10.011, 86057-970, Londrina, Paraná, Brazil. Fax: + 55 43 3371-4387, Tel: + 55 43 3371-4979.

Abstract

Neuropathic pain induced by nerve injury is associated with a variety of neuroinflammatory changes, including peripheral and central sensitization, that lead to hyperalgesia, paresthesia, allodynia and spontaneous pain. Kaurenoic acid (KA) is a diterpene with anti-inflammatory properties in pre-clinical models of diseases. The aim this study was to evaluate the effects of KA in a mouse model of neuropathic pain induced by chronic constriction injury (CCI) of the sciatic nerve. Seven days after CCI surgery, mice were treated with KA and the mechanical hyperalgesia evaluated. Samples from the spinal cord (L4-L6) were collected to evaluate the effects of KA in spinal cord neuroinflammation. Acute (at the 7th day post-CCI surgery) and prolonged (7-14th days post-CCI surgery) KA treatment inhibited CCI-induced mechanical hyperalgesia in all evaluated time points. Pre-treatment with inhibitors of the NO-cGMP-PKG-ATP-sensitive potassium channel signaling pathway impaired the analgesic effect of KA. Importantly, KA enhanced Nrf2 mRNA expression and inhibited the depletion of its downstream target, the antioxidant reduced glutathione (GSH) in the spinal cord of CCI mice. KA also inhibited the NFκB activation, and the mRNA expression of its downstream targets, the hyperalgesic cytokines TNF-α and IL-33. In agreement with a reduction of CCI-induced spinal cord inflammation, KA also inhibited CCI-induced activation of microglia and astrocyte as observed by reduced Iba-1 and GFAP mRNA expression., respectively. Our results provide evidences that KA inhibits spinal cord neuroinflammation, and thus represent an important candidate in controlling neuropathic pain.

Key-words: Kaurenoic acid; neuropathic pain; chronic constriction injury; sciatic nerve; spinal cord.

1

1. Introduction

Neuropathic pain is a complex syndrome that results from a nerve fiber injury or dysfunction such as occur in diabetes, autoimmune diseases, infections, trauma, and trigeminal neuralgia (Campbell and Meyer, 2006; Gao and Ji, 2010b; Woolf and Mannion, 1999). Damage of conductive fibers causes disturbances of sensory and motor functions, which is characterized by nociceptive pain and inflammation (Austin and Moalem-Taylor, 2010). Hyperalgesia (an increase of response to painful stimuli), paresthesia (tingling sensation), allodynia (pain resulting from a normally non-painful stimulus) and spontaneous pain are symptoms related to neuropathic pain (Austin and Moalem-Taylor, 2010; Campbell and Meyer, 2006; Scholz and Woolf, 2007; Woolf and Mannion, 1999). Furthermore, studies show that in addition to these symptoms, the patients eventually develop depression, sleep disorders, and physical function impairments (Gilron et al., 2015).

Studies have shown the participation of macrophages, glial cells (Schwann cells, astrocytes and microglia), T lymphocytes and neutrophils as well as oxidative stress, cytokines, chemokines and growth factors in neuropathic pain, which contribute to modulate axonal damage and spontaneous activity of nociceptors and sensitivity to stimuli (Austin and Moalem-Taylor, 2010; Scholz and Woolf, 2007; Zarpelon et al., 2016). Furthermore, in peripheral nerve injury, immune cells release pro-inflammatory mediators that can directly act on dorsal root ganglia (DRG) neurons and their axons leading to peripheral sensitization and axonal damage. DRG neurons also release mediators that activate glial cells in the DRG and spinal cord and vice-versa (Gao and Ji, 2010a; Scholz and Woolf, 2007; Zarpelon et al., 2016).

Several therapeutic drugs are available to controlling neuropathic pain, in general aiming to decrease neuronal activity, but without adequate effectiveness (Dworkin et al., 2003). Patients with neuropathic pain do not respond well to therapy with non-steroidal anti-inflammatory drugs and the resistance and tolerance to opiates is commonly observed. Nowadays, tricyclic antidepressants and anticonvulsants are extensively used to treat neuropathic pain, however all of them have limited effectiveness (Dworkin et al., 2003; Scholz and Woolf, 2007; Woolf and Mannion, 1999). Importantly, these drugs cause side effects such as nausea, sedation, drowsiness and dizziness (Gao and Ji, 2010a).

Diterpenes are natural compounds frequently used in the treatment of various diseases (Aquila et al., 2009; Tirapelli et al., 2008; Zhang et al., 2014). One of these compounds, the Kaurenoic acid (KA), possesses a wide variety of activities. For instance, KA presents antitumor activity by activating apoptotic pathways (Fernandes et al., 2013; Henry et al., 2006; Lizarte Neto et al., 2013; Okoye, 2014). KA affects the cardiovascular system by promoting antispasmodic and vasorelaxant actions, in part, by activating the NO-cGMP pathway (Tirapelli et al., 2008, 2004). KA also present anti-inflammatory potential since inhibited *in vitro* LPS-induced production of nitric oxide (NO) and NO synthase expression, prostaglandin E_2 and cyclooxygenase 2 expression, cytokine production and NFκB activation as well as induced Nrf2 activation in RAW 264.7 macrophages (Castrillo et al., 2001; Choi et al., 2011; Díaz-Viciedo et al., 2008; Lyu et al., 2011). Finally, KA is an analgesic molecule since inhibits inflammatory nociception in the models of acetic acid-induced writhings, phenyl-p-benzoquinone-induced writhings, formalin- and complete Freund´s adjuvant (CFA)-induced paw flinching and licking, and carrageenan- and CFA-induced mechanical hyperalgesia (Block et al., 1998; Mizokami et al., 2012). The analgesic

mechanism of KA depends, at least in part, by inhibiting pro-inflammatory cytokine production and activating the NO-cGMP (cyclic guanosine monophosphate)-PKG (protein kinase G)-ATP-sensitive potassium channel signaling pathway (Mizokami et al., 2012). These mechanisms of KA seem to be of importance since cytokines are targets to reduce pain in inflammatory and neuropathic pain conditions (Verri et al., 2006) and activating the NO-cGMP-PKG-ATP-sensitive potassium channel is an analgesic mechanism of morphine, dipyrone and other molecules with analgesic activity activate this signaling pathway (Cunha et al., 2010; Sachs et al., 2004; Zarpelon et al., 2013; Staurengo-Ferrari et al., 2014; Pinho-Ribeiro et al., 2016). However, the effect and mechanisms of KA in neuropathic pain remained to be investigated, which were the aims of the present study.

2. Material and methods

2.1. Animals

Male Swiss mice (20-25 g), from the Universidade Estadual de Londrina, Londrina, Paraná, Brazil, were used in this study. Mice were housed in standard clear plastic cages with free access to food and water, a light/dark cycle of 12:12 h. All behavioral testing was performed between 9 a.m. and 5 p.m. in a temperature-controlled room (21 ºC). Animal care and handling procedures were in accordance with the International Association for Study of Pain (IASP) guidelines and approved by the Ethics Committee of the Universidade Estadual de Londrina (process number 12105.2012.67).

2.2. *Extraction and isolation of Kaurenoic acid*

The air-dried roots of *Sphagneticola trilobata* were pulverized and then extracted exhaustively with dichloromethane (900 mL) at room temperature, to give 1.2 g of crude extract, which was suspended in 300 mL of methanol−H_2O (9:1) and filtered. The soluble fraction was partitioned using *n*-hexane (300 mL, four times), which resulted in a 0.7 g *n*-hexane-soluble fraction after solvent evaporation under reduced pressure. The *n*-hexane-soluble fraction was chromatographed over silica gel 60 (0.063−0.200 mm) using vacuum-liquid chromatography with *n*-hexane and increasing amounts of ethyl acetate as eluents (250 mL each fraction). The second fraction (0.41 g) was washed with cold methanol, to afford Kaurenoic acid (*ent*-kaur-16-en-19-oic acid; 800 mg, purity 96%, as determined by HPLC), exhibiting $[\alpha]^{20}_{D}$ – 110, similar to a previous report (Okuyama et al., 1991), EIMS *m/z* 325 $[M + Na]^+$ and compared by ^{1}H (CDCl3, 400 MHz) and ^{13}C (CDCl3, 100 MHz) spectroscopy with an authentic standard and literature data (de Andrade et al., 2011).

2.3. Drugs and stimuli

The following materials were obtained from the sources indicated: saline was obtained from Fresenius Kabi Brasil Ltda. (NaCl 0.9%; Aquiraz, CE, Brazil). Dimethyl sulfoxide (DMSO), N^G-nitro- L-arginine methyl ester **(**L-NAME) and glibenclamide were obtained from Sigma-Aldrich (St. Louis, MO, USA). 2,3,9,10,11,12-hexahydro-10R-methoxy-2,9-dimethyl-1-oxo-9S,12R-epoxy-1diindolo [1,2,3-fg:3',2', 1'-kl] pyrrol [3,4-i][1,6]benzodiazocine-10-carboxylic acid, methyl ester (KT 5823) from **Cayman Chemical Company** (Ann Arbor, MI, USA), 1H-(1,2,4)-oxadiazolol-(4,3-a)quinoxalin-

1-one (ODQ) was obtained Santa Cruz Biotechnology (Santa Cruz, CA, USA), and ketamine and xylazine from Sespo Industria e Comércio Ltda (Paulinia, SP, Brazil).

2.4. Model of chronic constriction injury (CCI)

Mice were anesthetized with ketamine and xylazine (1:1; 10 µL/10 g, of body weight, intramuscular) followed by trichotomy and asepsis of the surgery area. Surgical procedure was performed as previously described by Bennett & Xie (Bennett and Xie, 1988) with some modifications (Zarpelon et al., 2016). Briefly, an incision was performed in the rear leg and the distal portion of the sciatic nerve was tied with surgical thread (catgut 4-0). For the sham controls, mice underwent the same procedure without the constriction of the nerve.

2.5. Experimental protocols

The pain threshold to mechanical stimulus was measured before the surgery (basal response) and at the following intervals starting from the 7th day after CCI (peak of mechanical hyperalgesia) (Zarpelon et al., 2016): 0.5–24 h or daily 3 h after per oral (p.o.) treatment with KA (1, 10 and 30 mg/kg or 10 mg/kg, diluted in 2% DMSO in saline) or vehicle (2% DMSO in saline, 10 mL/kg of body weight). To investigate the role of the NO-cGMP-PKG-ATP-sensitive potassium channel signaling pathway in the analgesic effect of KA, the following drugs were administered before KA (10 mg/kg, p.o.) treatment: L-NAME [L-nitro-arginine methyl ester, NOS inhibitor, 100 mg/kg, intraperitoneal (i.p.), diluted in saline, 45 min), ODQ (soluble cGMP inhibitor; 1 mg/kg, i.p., diluted in 2% DMSO in saline, 30 min), KT5823 (PKG inhibitor; 0.5

μg/mice, i.p., diluted in 2% DMSO in saline, 5 min) and glibenclamide (ATP-sensitive potassium channel blocker; 1 mg/kg, p.o., diluted in 5% of Tween 80 in saline, 30 min). For GSH, NFκB and qPCR experiments, samples of spinal cord (L4-L6) were collected 3 h after treatment with KA (10 mg/kg, p.o.). For spinal cord samples, pools of 2 spinal cord were prepared, thus, a *n* = 1 represents two mice for the evaluation of GSH level or NFκB activation analyses. Pools of 4 spinal cords were also prepared, and a *n* = 1 represents four mice for qPCR analysis. The selected doses of drugs and time of sample collection were chosen based on pilot studies and previous data of our laboratory (Fattori et al., 2015; Hohmann et al., 2013; Mizokami et al., 2012; Pinho-Ribeiro et al., 2015; Zarpelon et al., 2016).

2.6. Mechanical hyperalgesia

The mechanical hyperalgesia test (Cunha et al., 2004) consisted of evoking a hindpaw flexion reflex with a hand-held force transducer (electronic anaesthesiometer; IITC Life Science) adapted with a 0.5 mm^2 polypropylene tip. The end point was the characterized by the removal of the paw followed by clear flinching movements. After the paw withdrawal, the intensity of the pressure was automatically recorded, and the value for the response was obtained by averaging three measurements. The animals were tested before and after the surgery and after of the treatments. The results are expressed by delta (Δ) withdrawal threshold (in g), calculated by subtracting from basal (before of the surgery) the mean measurements seven day after surgery (zero-time mean measurements), and the mean measurements obtained at 0.5 – 24 h and 1 – 7 days after treatment with KA.

2.7. Reduced glutathione (GSH) levels assay

Samples from spinal cord (L4-L6) were collected and maintained at –80 ºC for at least 48 h, and then homogenized with 200 µL of 0.02 M EDTA. Homogenates were mixed with 25 µL of 50% trichloroacetic acid, vortexed three times for 15 min, and centrifuged (15 min × 1500 *g* × 4 ºC), and the resulting supernatant added to 200 µL of 0.2 M Tris buffer, pH 8.2, and 10 µL of 0.01 M DTNB. After 5 min of incubation at room temperature, the absorbance was measured at 412 nm against a blank reagent with no supernatant (Hohmann et al., 2013; Sedlak and Lindsay, 1968). A standard curve of GSH was used and the results are reported as nmol of GSH per mg of tissue.

2.8. NFκB activity

Spinal cord samples (L4–L6) were collected and homogenized in ice-cold lysis buffer (Cell Signaling). Homogenates were centrifuged (200 *g* × 10 min × 4 °C) and the supernatants used to assess the levels of phosphorylated and total NFκB p65 subunit by ELISA according to the manufacturer's directions (PathScan, Cell Signaling). Absorbance was measured at 450 nm (Multiskan GO Thermo Scientific) and the results are presented as total p65/phospho-p65 ratio per 100 mg of tissue.

2.9. Reverse transcriptase (RT) and quantitative polymerase chain reaction (qPCR)

RT-qPCR was performed as previously described (Fattori et al., 2015). Spinal cord samples (L4–L6) were homogenized in TRIzol® reagent (Life Technologies), and total RNA was isolated according to manufacturer's directions. RNA purity was

confirmed by the 260/280 ratio. RT-PCR and qPCR were performed using GoTaq® 2-Step RT-qPCR System (Promega) on a StepOnePlusTM Real-Time PCR System (Applied Biosystems®). The relative gene expression was measured using the comparative $2^{-(\Delta\Delta Cq)}$ method and β-actin as the housekeeping gene. The primer sequences used were Nrf2, sense: 5´-TCACACGAGATGACGTTAGGGCAA-3´, antisense: 5´-TACAGTTCTGGGCGGCGGACTTTAT-3´; TNF-α, sense: 5´-TCTCATCAGTTCTATGGCCC-3´, antisense: 5´-GGGAGTAGACAAGGTACAAC-3´; IL-33, sense: 5´-TCC TTG CTT GGC AGT ATC CA-3´, antisense: 5´-TGC TCA ATG TGT CAA CAG ACG-3´; Iba-1: sense 5´-ATGGAGTTTGATCTGAATGGAAAT-3´, antisense: 5´-TCAGGGCAGCTCGGAGATAGCTTT-3´; GFAP: sense: 5´-GGCGCTCAATGCTGGCTTCA-3´, antisense: 5´-TCTGCCTCCAGCCTCAGGTT-3´; *β*-actin, sense: 5´-ACGTGCGTTTTACACCCTTT-3´, antisense: 5´-AAGCCATGCCAATGTTGTCT-3´.

2.10. Statistical analyses

Results are representative of two independent experiments and are presented as the means ± S.E.M. (*n* = 6 per group per experiment, representative of two separate experiments). Two-way analysis of variance (ANOVA) followed by Tukey's *t* test was used to compare the groups and doses at all times when the parameters were measured at different times after the CCI of sciatic nerve. The analyzed factors were treatments, time and time versus treatment interaction. When there was a significant time versus treatment interaction, one-way ANOVA followed by Tukey's *t*-test was performed for each time. On the other hand, when the nociceptive responses were presented as total values at indicated time period, the differences between respons-

es were evaluated by one-way ANOVA followed by Tukey's *t*-test. Statistical differences were considered to be significant at $P<0.05$.

3. Results

3.1. KA reduces CCI-induced mechanical hyperalgesia in mice

Seven day after CCI, mice were treated p.o. with KA (1, 10 and 30 mg/kg.) and the mechanical hyperalgesia was evaluated 0.5, 1, 3, 5, 7 and 24 h after treatment (Figure 1A). KA dose-dependently reduced CCI-induced mechanical hyperalgesia (Figure 1A). No significant effect was observed at the dose of KA of 1 mg/kg. On the other hand, the dose of 3 mg/kg of KA reduced the mechanical hyperalgesia between 1-7 h, and dose of 10 and 30 mg/kg of KA reduced the mechanical hyperalgesia already at 0.5 h after treatment and up to 7 h (Figure 1A). The dose of 10 mg/kg of KA was selected for the next experiments since was the lower dose of KA to achieve maximal antinociception within the shortest time. For the chronic treatment, mice were treated daily with KA (10 mg/kg, p.o.) for seven days and the mechanical hyperalgesia evaluated. KA inhibited CCI-induced mechanical hyperalgesia at all time points evaluated in the chronic treatment protocol (Figure 1B).

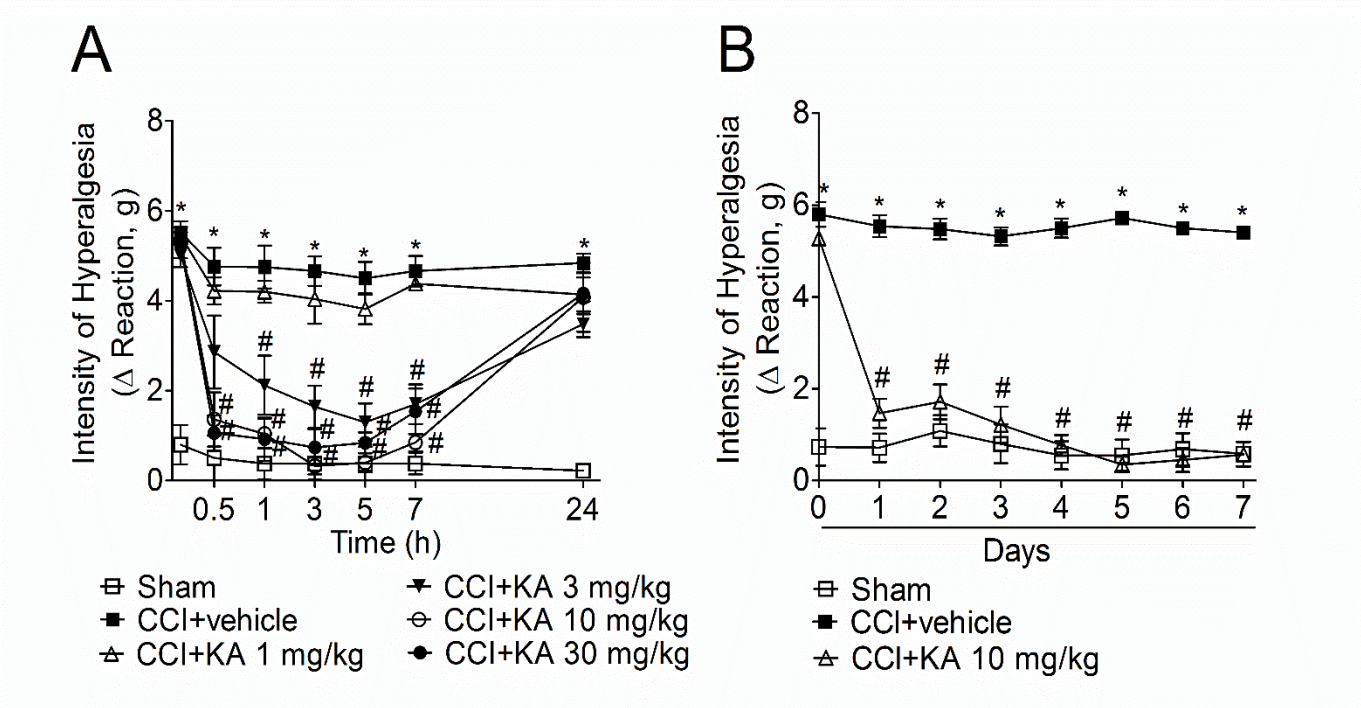


Figure 1. KA reduces CCI-induced mechanical hyperalgesia in mice. Seven days after surgery, mice were treated with KA (1, 3, 10 and 30 mg/kg, p.o.) and the mechanical hyperalgesia evaluated 0.5-24 h (Panel A). In panel B, mice were treated with KA 10 mg/kg, daily for 7 days and mechanical hyperalgesia evaluated daily after treatment. Results are presented as the means ± SEM (n = 6 per group per experiment, representative of two separate experiments). $^{*}P<0.05$ compared with the sham groups; $^{\#}P<0.05$ compared with the vehicle groups (Two-way ANOVA followed by Tukey's t test).

3.2. The analgesic effect of KA in CCI-induced mechanical hyperalgesia depends on activating the NO-cGMP-PKG-ATP-Sensitive Potassium Channel Signaling Pathway.

Seven days after CCI, mice were pre-treated with L-NAME (100 mg/kg, i.p.; NOS inhibitor), ODQ (1 mg/kg, i.p.; soluble guanilate cyclase inhibitor), KT5823 (0.5 μg/mice, i.p.; PKG inhibitor) or glibenclamide (1 mg/kg, p.o.; ATP-sensitive potassium channel blocker) before treatment with KA (10 mg/kg, p.o.). The mechanical hyperalgesia was evaluated 1-7 h after treatment with KA. Likewise, KA significantly reduced CCI-induced mechanical hyperalgesia at 1, 3, 5 and 7 h, which was reversed by the inhibitors L-NAME (Figure 2A), ODQ (Figure 2B), KT5823 (Figure 2C) and glibenclamide (Figure 2D). These results suggest that the anti-hyperalgesic effect of KA depends on triggering the triggering the NO-cGMP-PKG-ATP-sensitive potassium channel signaling analgesic pathway.

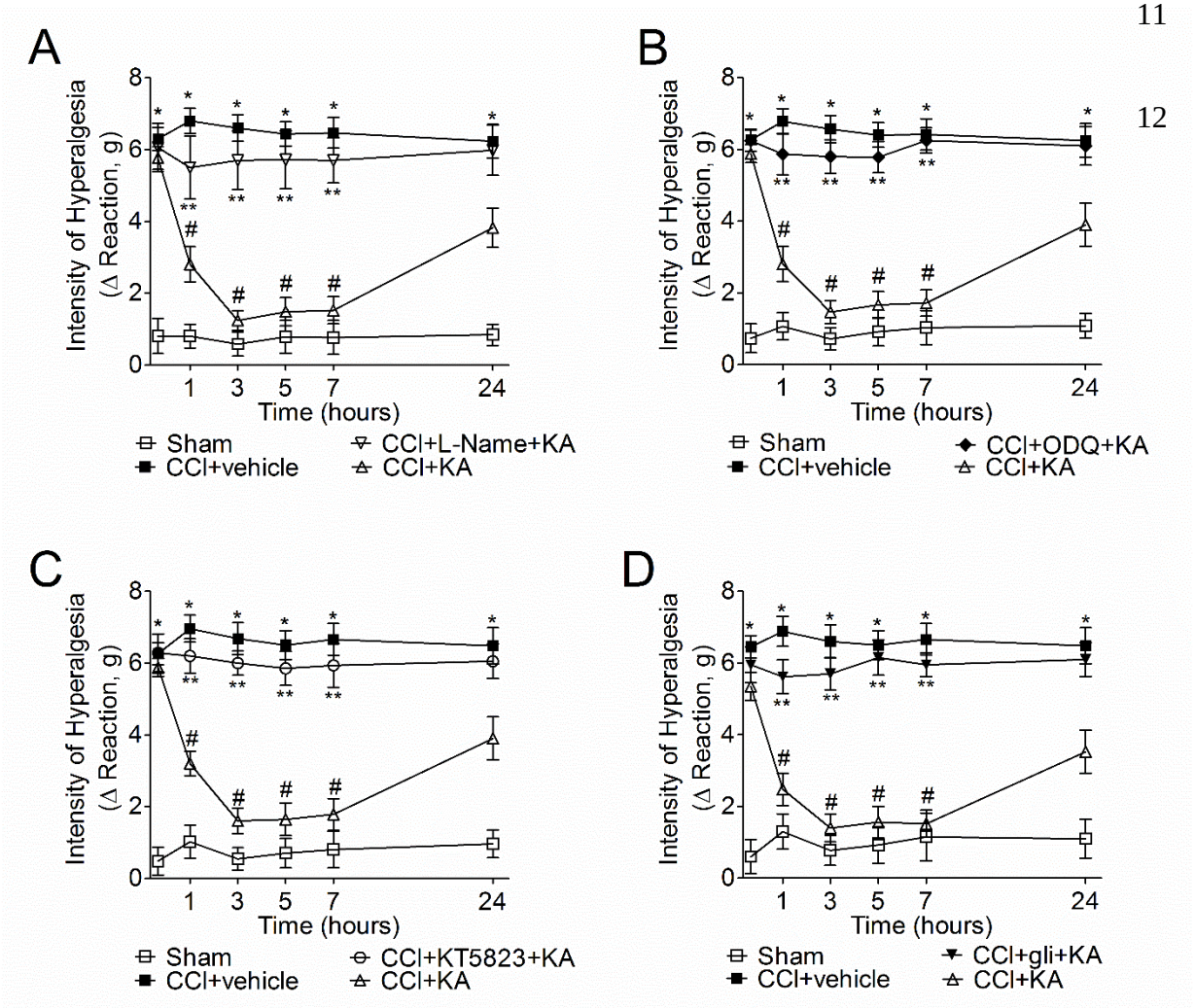


Figure 2. The analgesic effect of KA in CCI-induced mechanical hyperalgesia depends on activating the NO-cGMP-PKG-ATP-Sensitive Potassium Channel Signaling Pathway. Mice were pre-treated with L-NAME (Panel A), ODQ (Panel B), KT5823 (Panel C) or glibenclamide (Panel D) before administration KA (10 mg/kg, p.o.). Results are presented as the means ± SEM (n = 6 per group per experiment, representative of two separate experiments). $^{*}P<0.05$ compared with the sham group; $^{\#}P<0.05$ compared with the vehicle group; $^{**}P<0.05$ compared with the KA group (Two-way ANOVA followed by Tukey's t test).

3.3. Treatment with KA inhibits CCI-induced oxidative stress in the spinal cord.

Mice were treated with KA (10 mg/kg, p.o.), and after 3 h samples of spinal cord (L4-L6) were collected to determine GSH levels (Figure 3A) and Nuclear Factor Erythroid-2-Related Factor 2 (Nrf2) mRNA expression (Figure 3B). CCI-induced a decrease of GSH levels in the spinal cord, which was inhibited by KA (Figure 3A). CCI group presented a tendency of increasing Nrf2 mRNA expression in the spinal cord, which was significantly enhanced by KA treatment (Figure 3B).

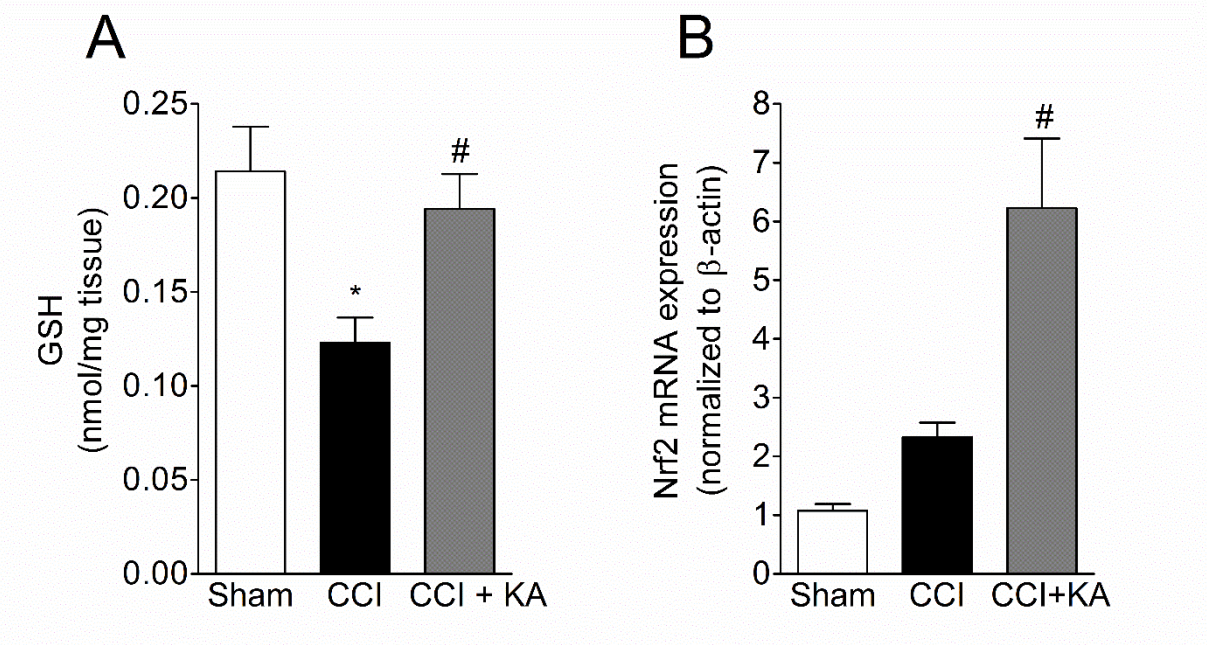


Figure 3. Treatment with KA inhibits CCI-induced oxidative stress in the spinal cord**.** Mice were treated with KA (10 mg/kg, p.o.) and after 3 h the samples of spinal cord were collected for measurement of GSH level (Panel A) and Nrf2 mRNA expression. Results are presented as the means ± SEM (*n* = 6 per group per experiment, representative of two separate experiments). $^{*}P<0.05$ compared with the sham groups; $^{\#}P<0.05$ compared with the vehicle groups (One-way ANOVA followed by Tukey's *t* test).

3.4. Treatment with KA inhibits CCI-induced spinal cord TNF-α and IL-33 mRNA expression.

Mice were treated with KA (10 mg/kg, p.o.) seven days after surgery. After 3 h, samples of spinal cord (L4-L6) were collected to determine TNF-α and IL-33 mRNA expression. CCI induced a significant increased in TNF-α (Figure 4A) and IL-33 (Fig-

ure 4B) mRNA expression. Treatment with KA inhibited the CCI-induced TNF-α (Figure 4A) and IL-33 (Figure 4B) mRNA expression.

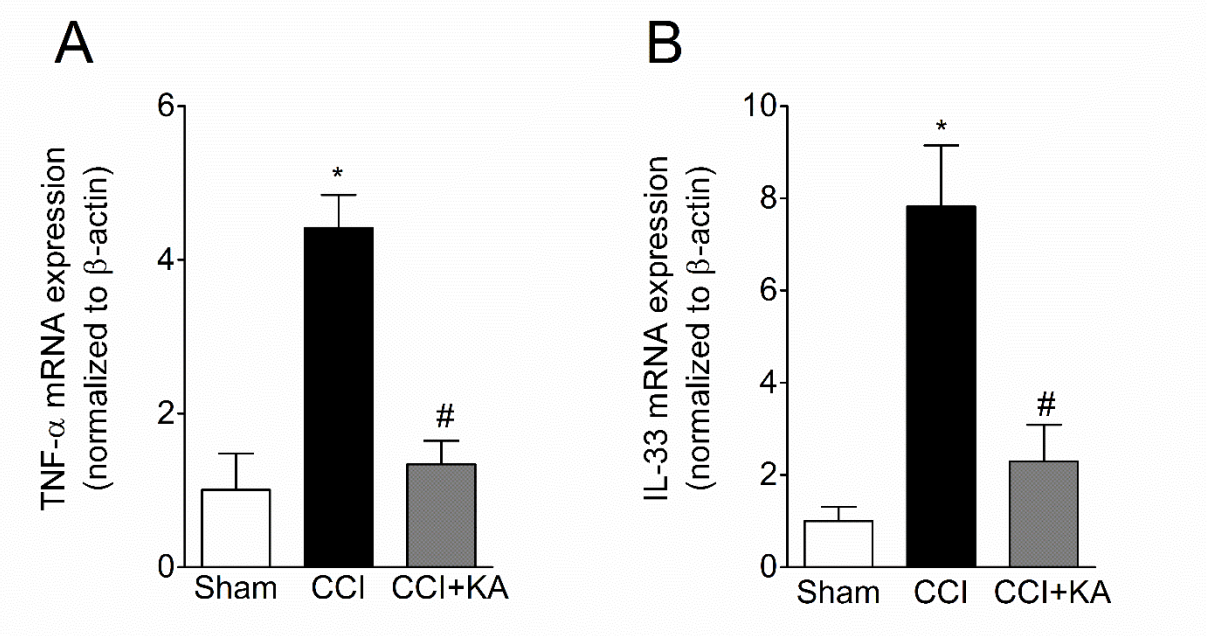


Figure 4. KA inhibits CCI-induced TNF-α and IL-33 mRNA expression. Mice were treated with KA (10 mg/kg, p.o.) and after 3 h samples of spinal cord were collected to analyze TNF-α (Panel A) and IL-33 (Panel B) mRNA expression by qPCR. Results are presented as the means ± SEM (n = 6 per group per experiment, representative of two separate experiments). $^{*}P<0.05$ compared with the sham groups; $^{\#}P<0.05$ compared with the vehicle groups (One-way ANOVA followed by Tukey's t test).

3.5. KA inhibits CCI-induced microglia and astrocytes activation in the spinal cord.

In order to evaluate whether KA treatment affected CCI-induced spinal cord glial cells activation the mRNA expression of microglia (Iba-1) and astrocytes (GFAP) were determined by qPCR (Figure 5). Mice were treated with KA (10 mg/kg, p.o.),

and after 3 h, samples of spinal cord (L4-L6) were collected to determine Iba-1 and GFAP mRNA expression. CCI significantly increase Iba-1 (Figure 5A) and GFAP (Figure 5B) mRNA expression. On the other hand, KA inhibited CCI-induced Iba-1 (Figure 5A) and GFAP (Figure 5B) mRNA expression in the spinal cord.

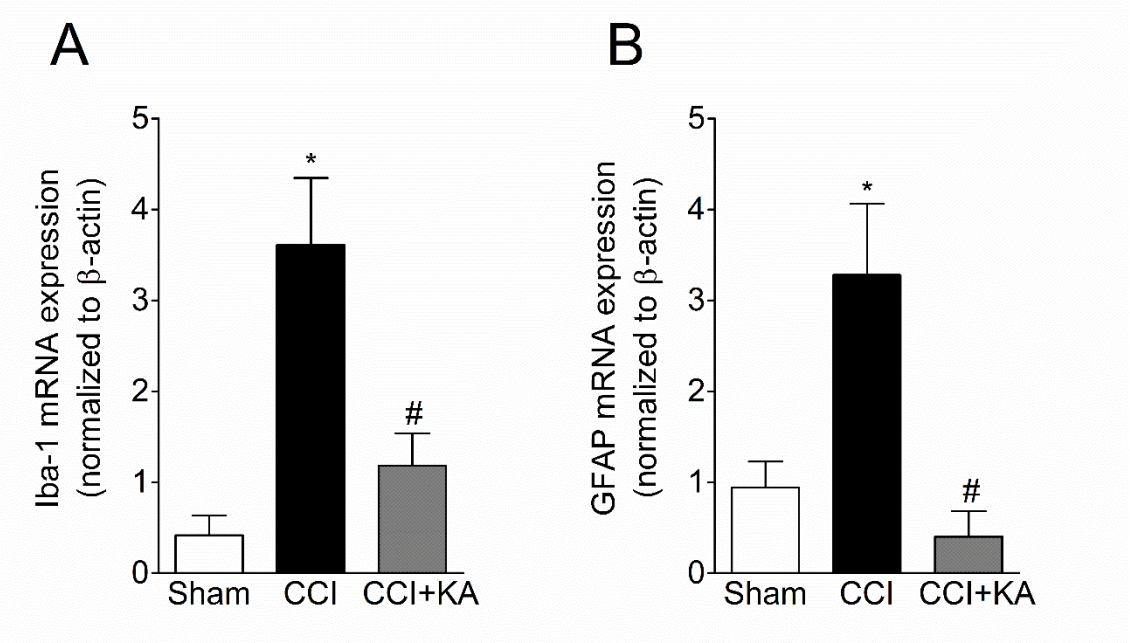


Figure 5. KA inhibits CCI-induced microglia and astrocyte activation in the spinal cord. Mice were treated with KA (10 mg/kg, p.o.) and after 3 h samples of spinal cord were collected to analyze Iba-1 (Panel A) and GFAP (Panel B) mRNA expression by qPCR. Results are presented as the means ± SEM (*n* = 6 per group per experiment, representative of two separate experiments). $^{*}P<0.05$ compared with the sham groups; $^{\#}P<0.05$ compared with the vehicle groups (One-way ANOVA followed by Tukey's *t* test).

3.6. KA inhibits CCI-induced NFκB activation in the spinal cord

Seven day after surgery, mice were treated with KA (10 mg/Kg, p.o.) and samples of spinal cord (L4-L6) were collected 3 h after treatment. Samples were processed and ELISA was used to evaluate the levels of total and phosphorylated NFκB p65 subunits. KA inhibited CCI-induced NFκB activation in the spinal cord (Figure 6).

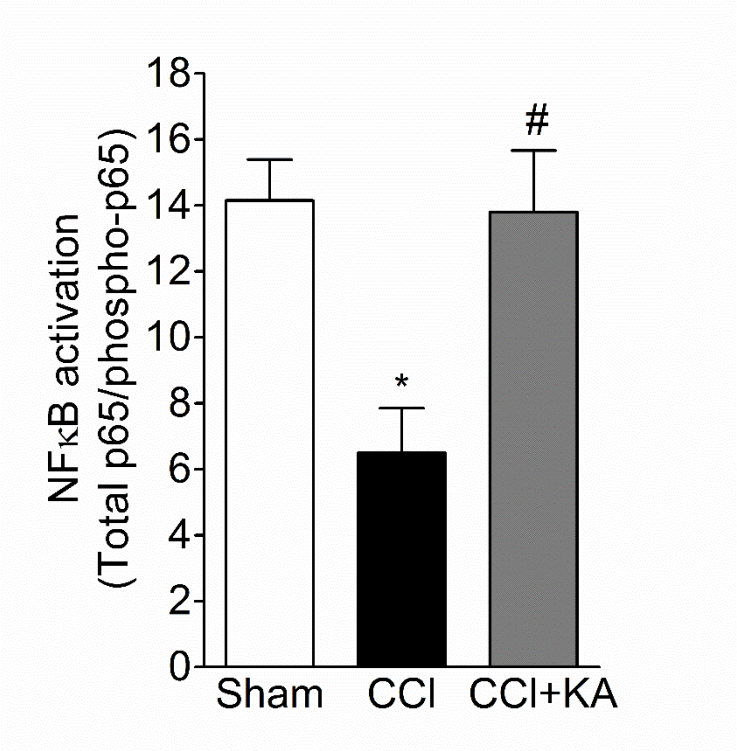


Figure 6. KA inhibited CCI-induced NFκB activation in the spinal cord. Mice were treated with KA (10 mg/kg, p.o.) and after 3 h samples of spinal cord were collected to analyze NFκB activation. Results are presented as the means ± SEM (*n* = 6 per group per experiment, representative of two separate experiments). $^{*}P<0.05$ compared with the sham groups; $^{\#}P<0.05$ compared with the vehicle groups (One-way ANOVA followed by Tukey's *t* test).

4. Discussion and Conclusion

Neuropathic pain caused by chronic constriction in sciatic nerve involves several mechanisms in peripheral and central systems. In the peripheral nerve neuropathy, Schwann cells activate resident cells, which promote the Wallerian degeneration of axonal nerve fibers. Additionally, these cells promote the release of several pro-inflammatory mediators such as cytokines, prostaglandin and reactive oxygen species (ROS) that contribute to peripheral sensitization (Gao and Ji, 2010a, 2010b; Salvemini et al., 2011; Scholz and Woolf, 2007). Nerve injury induces substantial changes in glial cells in the spinal cord leading to activation and proliferation of these cells, and promote the further release of pro-hyperalgesic cytokines and the central sensitization leading to the development and maintenance of neuropathic pain (Gao and Ji, 2010a, 2010b; Scholz and Woolf, 2007; Zarpelon et al., 2016).

Here we demonstrate that Kaurenoic acid (KA) treatment dose-dependently inhibited ongoing chronic constriction injury of sciatic nerve (CCI)-induced neuropathic pain in mice. Furthermore, the analgesic effect of KA was maintained with daily treatment during seven days. Subsequently, the mechanisms underlying the anti-hyperalgesic effect of KA were investigated. The analgesic effect of KA depends on activating the NO-cGMP-PKG-ATP-sensitive potassium channel signaling pathway as observed using the inhibitors L-NAME, ODQ, KT5823 and glibenclamide, respectively. This same pathway is also activated by some AINES such as diclofenac and dipyrone (Tonussi and Ferreira, 1994; Sachs et al., 2004), and opioids such as morphine (Cunha et al., 2010). NO and nitroxyl donors or substances that increase neuronal concentration of NO also cause analgesia by activating the cGMP-PKG-ATP-sensitive potassium channel signaling pathway (Cury et al., 2011; Staurengo-Ferrari

et al., 2014; Zarpelon et al., 2013). In fact, NO is able to stimulate cGMP production, leading to activation of PKG, which phosphorylates ATP-sensitive potassium channel. And the opening of these channels induces the hyperpolarization of nociceptive neurons reducing the depolarization and action potential transmission, thus, promoting analgesia (Cunha et al., 2010; Cury et al., 2011; Zarpelon et al., 2013).

Oxidative stress and ROS have been implicated in the development of persistent pain that results from nerve injury or inflammation (Pathak et al., 2014). Reduced glutathione (GSH) plays a critical role in protecting cells from oxidative stress as well as maintaining the thiol redox state. GSH is a tripeptide composed of glutamate, cysteine, and glycine residues. The depletion of GSH enhances oxidative stress, leading to neuronal degeneration (Salvemini et al., 2011). Hence, antioxidant compounds inhibits this process and have been the target of many studies. In the present data, KA inhibited the depletion of GSH levels in CCI and enhanced the expression of Nuclear Factor Erythroid-2-Related Factor 2 (Nrf2). Nrf2 is responsible for the expression of various detoxifying phase II enzymes, and inducible expression of antioxidant response elements (ARE), including GSH. The antioxidant activity and induction of Nrf2 can explain the analgesic effect of KA in CCI since in neuropathic pain models, inhibition of oxidative stress and inducing Nrf2 are analgesic mechanisms (Cederbaum, 2009; Lyu et al., 2011; Salvemini et al., 2011). Corroborating our data, KA induced Nrf2 activation in RAW 264.7 macrophages (Lyu et al., 2011).

Furthermore, pro-inflammatory cytokines also contribute to axonal damage and increased pain sensitivity in neuropathic pain (Austin and Moalem-Taylor, 2010; Scholz and Woolf, 2007: Zarpelon et al., 2016). Injury of sciatic nerve leads to up-regulation of TNF-α and its receptors in the nerve, DRG, Schwann cells, astrocytes

and microglia (Austin and Moalem-Taylor, 2010; Moalem and Tracey, 2006). TNFα/TNFR1 signaling activates p38 that phosphorylates tetrodotoxin-resistant sodium channels resulting DRG neuronal firing (Jin and Gereau, 2006). IL-33 and its receptor ST2 also participate in CCI-induced neuropathic pain. Upon CCI surgery, oligodendrocytes release IL-33 in the spinal cord, and IL-33 potently induces TNF-α and IL-1 production by glial cells (Zarpelon et al., 2016). TNF-α (Austin and Moalem-Taylor, 2010) and IL-33 (Zarpelon et al., 2016) activate PI3K, MAP kinases (ERK, JNK and p38), and NFκB enhancing cellular activation and cytokines production, therefore, mediating the spinal cord neuroinflammation in neuropathic pain. The intrathecal injection of TNF-α (Gao et al., 2010) and IL-33 (Zarpelon et al., 2016) also actives microglia and astrocytes in the spinal cord during the CCI-induced neuropathy. We found that KA reduces the TNF-α and IL-33 mRNA expression in spinal cord and inhibits the NFκB activation. These data corroborate that KA inhibits CCI-induced spinal cord activation of microglia and astrocytes by inhibiting NFκB activation and consequent cytokine production.

In the context of neuropathic pain, microglia are one of first spinal cord cell types to be activated and persists for at least several months. Microglia activation leads to increase in the synthesis of cytokines IL-1β, IL-6, IL-10, TNF-α and TGF-β that modulated the dorsal horn neuron and contributed to the development of neuropathic pain. On the other hand, cytokines also provide important autocrine feedback signals to microglial cells themselves (Scholz and Woolf, 2007). Astrocytes are the largest cell population in the CNS and its activation is morphologically characterized by hypertrophy and increased production of intermediate filaments, GFAP, vimentin and/or nestin, and functionally by increased production of a variety of pro-inflammatory substances (Gao and Ji, 2010a; Scholz and Woolf, 2007). Considering

the involvement of immune and glia cells in the development of neuropathic pain, pre-clinical studies with immunosuppressive drugs or drugs that interferes with glial functions for neuropathic pain have been studied. Examples of drugs that inhibit glial cells activation include fluorocitrate, propentofylline, minocycline and teriflunomide, which reduce cytokine release and attenuate pain-responsive behavior in several animal models of neuropathic pain (Gao and Ji, 2010a; Scholz and Woolf, 2007). We confirmed that CCI-induced activation of microglia and astrocytes by increasing Iba-1 and GFAP mRNA expression. KA treatment was effective to inhibit this activation in the spinal cord, demonstrating an important role in controlling CCI-induced glial cell activation in the spinal cord.

In addition to the suppressing CCI-induced spinal cord neuroinflammation, KA has low toxicity (Bürger et al., 2005; Mizokami et al., 2012). For instance, KA does not alter alanine aminotransferase, aspartate aminotransferase and gama-glutammyl transferase levels, which indicates no liver damage (Bürger et al., 2005; Mizokami et al., 2012), does not induce changes in hematological parameters (Bürger et al., 2005) and gastric mucosal lesion assessed by MPO activity in stomach samples (Mizokami et al., 2012).

In conclusion, KA inhibits CCI-induced neuropathic pain by mechanisms related to (i) activation of the NO-cGMP-PKG-ATP-sensitive potassium channel signaling pathway; (ii) inhibition of GSH levels depletion and enhancement of the Nrf2 expression; (iii) inhibition of pro-inflammatory cytokines expression; (iv) decrease of glial cells activation; and (v) inhibition of NFκB activation. All these activities account to decrease the CCI-induced spinal cord neuroinflammation and the consequent neuropathic pain.

Acknowledgments

This work was supported by grants from Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Ministério da Ciência, Tecnologia e Inovação (MCTI), Secretaria da Ciência, Tecnologia e Ensino Superior (SETI)/Fundação Araucária and Governo do Estado do Paraná (Brazil).

Conflicts of Interest

The authors declare no conflicts of interest.

References

Aquila, S., Weng, Z., Zeng, Y., Sun, H., Rı, L., 2009. Inhibition of NF- KB Activation and iNOS Induction by ent -Kaurane Diterpenoids in. J Nat Prod 72, 1269–1272.

Austin, P.J., Moalem-Taylor, G., 2010. The neuro-immune balance in neuropathic pain: Involvement of inflammatory immune cells, immune-like glial cells and cytokines. Journal of Neuroimmunology 229, 26–50. doi:10.1016/j.jneuroim.2010.08.013

Bennett, G.J., Xie, Y.K., 1988. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 33, 87–107.

Block, L.C., Santos, A.R., de Souza, M.M., Scheidt, C., Yunes, R.A., Santos, M.A., Monache, F.D., Filho, V.C., 1998. Chemical and pharmacological examination of

antinociceptive constituents of Wedelia paludosa. Journal of ethnopharmacology 61, 85–9.

Bürger, C., Fischer, D.R., Cordenunzzi, D.A., de Borba Batschauer, A.P., Filho, V.C., dos Santos Soares, A.R., 2005. Acute and subacute toxicity of the hydroalcoholic extract from Wedelia paludosa (Acmela brasiliensis) (Asteracea) in mice. Journal of Pharmacy and Pharmaceutical Sciences 8, 370–373.

Campbell, J.N., Meyer, R.A., 2006. Mechanisms of neuropathic pain. Neuron 52, 77–92. doi:10.1016/j.neuron.2006.09.021

Castrillo, A, de Las Heras, B., Hortelano, S., Rodriguez, B., Villar, A, Bosca, L., 2001. Inhibition of the nuclear factor kappa B (NF-kappa B) pathway by tetracyclic kaurene diterpenes in macrophages. Specific effects on NF-kappa B-inducing kinase activity and on the coordinate activation of ERK and p38 MAPK. The Journal of biological chemistry 276, 15854–15860. doi:10.1074/jbc.M100010200

Cederbaum, A., 2009. Nrf2 and antioxidant defense against CYP2E1 toxicity. Expert opinion on drug metabolism & toxicology 5, 1223–44. doi:10.1517/17425250903143769

Choi, R.J., Shin, E.M., Jung, H.A., Choi, J.S., Kim, Y.S., 2011. Inhibitory effects of kaurenoic acid from Aralia continentalis on LPS-induced inflammatory response in RAW264.7 macrophages. Phytomedicine 18, 677–682. doi:10.1016/j.phymed.2010.11.010

Cunha, T.M., Roman-Campos, D., Lotufo, C.M., Duarte, H.L., Souza, G.R., Verri, W. a, Funez, M.I., Dias, Q.M., Schivo, I.R., Domingues, A.C., Sachs, D., Chiavegatto, S., Teixeira, M.M., Hothersall, J.S., Cruz, J.S., Cunha, F.Q., Ferreira, S.H.,

2010. Morphine peripheral analgesia depends on activation of the PI3Kgamma/AKT/nNOS/NO/KATP signaling pathway. Proceedings of the National Academy of Sciences of the United States of America 107, 4442–4447. doi:10.1073/pnas.0914733107

Cunha, T.M., Verri, W.A., Vivancos, G.G., Moreira, I.F., Reis, S., Parada, C.A., Cunha, F.Q., Ferreira, S.H., 2004. An electronic pressure-meter nociception paw test for rats. Brazilian Journal of Medical and Biological Research 37, 391–399. doi:10.1590/S0100-879X2004000300018

Cunha, T.M., Verri, W.A., Silva, J.S., Poole, S., Cunha, F.Q., Ferreira, S.H., 2005. A cascade of cytokines mediates mechanical inflammatory hypernociception in mice. Proceedings of the National Academy of Sciences of the United States of America 102, 1755–1760.

Cury, Y., Picolo, G., Pacciari, V., Henrique, S., 2011. Nitric Oxide Pain and analgesia : The dual effect of nitric oxide in the nociceptive system. Nitric Oxide 25, 243–254. doi:10.1016/j.niox.2011.06.004

de Andrade, B.B., Moreira, M.R., Ambrosio, S.R., Furtado, N.A.J.C., Cunha, W.R., Heleno, V.C.G., Silva, A.N., Simão, M.R., da Rocha, E.M.P., Martins, C.H.G., Veneziani, R.C.S., 2011. Evaluation of ent-kaurenoic acid derivatives for their anticariogenic activity. Natural product communications 6, 777–80.

Díaz-Viciedo, R., Hortelano, S., Girón, N., Massó, J.M., Rodriguez, B., Villar, A., de las Heras, B., 2008. Modulation of inflammatory responses by diterpene acids from Helianthus annuus L. Biochemical and Biophysical Research Communications 369, 761–766. doi:10.1016/j.bbrc.2008.02.104

Dworkin, R.H., Backonja, M., Rowbotham, M.C., Allen, R.R., Argoff, C.R., Bennett, G.J., Bushnell, M.C., Farrar, J.T., Galer, B.S., Haythornthwaite, J.A, Hewitt, D.J., Loeser, J.D., Max, M.B., Saltarelli, M., Schmader, K.E., Stein, C., Thompson, D., Turk, D.C., Wallace, M.S., Watkins, L.R., Weinstein, S.M., 2003. Advances in neuropathic pain: diagnosis, mechanisms, and treatment recommendations. Archives of neurology 60, 1524–1534. doi:10.1001/archneur.60.11.1524

Fattori, V., Pinho-Ribeiro, F.A., Borghi, S.M., Alves-Filho, J.C., Cunha, T.M., Cunha, F.Q., Casagrande, R., Verri, W.A., 2015. Curcumin inhibits superoxide anion-induced pain-like behavior and leukocyte recruitment by increasing Nrf2 expression and reducing NF-kB activation. Inflammation Research 64, 993–1003. doi:10.1007/s00011-015-0885-y

Fernandes, V.C., Coppede, J., Martins, J.S., Rizo, W.F., Beleboni, R.O., Marins, M., Pereira, P.S., Fachin, A.L., Biotecnologia, U. De, 2013. The epimer of kaurenoic acid from Croton antisyphiliticus is cytotoxic toward B-16 and HeLa tumor cells through apoptosis induction. Genetics and Molecular Research 12, 1005–1011.

Gao, Y.-J., Ji, R.R., 2010a. Targeting Astrocyte Signaling for Chronic Pain. Neurotherapeutics 7, 482–493. doi:10.1016/j.nurt.2010.05.016

Gao, Y.-J., Ji, R.-R., 2010b. Chemokines, neuronal-glial interactions, and central processing of neuropathic pain. Pharmacology & therapeutics 126, 56–68. doi:10.1016/j.pharmthera.2010.01.002

Gao, Y.-J., Zhang, L., Ji, R.-R., 2010. Spinal injection of TNF-α-activated astrocytes produces persistent pain symptom mechanical allodynia by releasing monocyte chemoattractant protein-1. Glia 58, 1871–1880.

doi:10.1016/j.str.2010.08.012.Structure

Gilron, I., Baron, R., Jensen, T., 2015. Neuropathic Pain: Principles of Diagnosis and Treatment. Mayo Clinic Proceedings 90, 532–545. doi:10.1016/j.mayocp.2015.01.018

Jin, X., Gereau, R.W., 2006. Acute p38-mediated modulation of tetrodotoxin-resistant sodium channels in mouse sensory neurons by tumor necrosis factor-alpha. The Journal of neuroscience : the official journal of the Society for Neuroscience 26, 246–55. doi:10.1523/JNEUROSCI.3858-05.2006

Henry, G.E., Adams, L.S., Rosales, J.C., Jacobs, H., Heber, D., Seeram, N.P., 2006. Kaurene diterpenes from Laetia thamnia inhibit the growth of human cancer cells in vitro. Cancer Letters 244, 190–194. doi:10.1016/j.canlet.2005.12.022

Hohmann, M.S.N., Cardoso, R.D.R., Pinho-Ribeiro, F.A., Crespigio, J., Cunha, T.M., Alves-Filho, J.C., Da Silva, R.V., Pinge-Filho, P., Ferreira, S.H., Cunha, F.Q., Casagrande, R., Verri, W.A., 2013. 5-Lipoxygenase Deficiency Reduces Acetaminophen-Induced Hepatotoxicity and Lethality. BioMed Research International 1–13. doi:10.1155/2013/627046

Lizarte Neto, F.S., Tirapelli, D.P.C., Ambrosio, S.R., Tirapelli, C.R., Oliveira, F.M., Novais, P.C., Peria, F.M., Oliveira, H.F., Carlotti Junior, C.G., Tirapelli, L.F., 2013. Kaurene diterpene induces apoptosis in U87 human malignant glioblastoma cells by suppression of anti-apoptotic signals and activation of cysteine proteases. Brazilian Journal of Medical and Biological Research 46, 71–78. doi:10.1590/1414-431X20121423

Lyu, J.H., Lee, G.S., Kim, K.H., Kim, H.W., Cho, S.I., Jeong, S. Il, Kim, H.J., Ju, Y.S.,

Kim, H.K., Sadikot, R.T., Christman, J.W., Oh, S.R., Lee, H.K., Ahn, K.S., Joo, M., 2011. Ent-kaur-16-en-19-oic acid, isolated from the roots of Aralia continentalis, induces activation of Nrf2. Journal of Ethnopharmacology 137, 1442–1449. doi:10.1016/j.jep.2011.08.024

Mizokami, S.S., Arakawa, N.S., Ambrosio, S.R., Zarpelon, A.C., Casagrande, R., Cunha, T.M., Ferreira, S.H., Cunha, F.Q., Verri, W.A., 2012. Kaurenoic acid from Sphagneticola trilobata inhibits inflammatory pain: Effect on cytokine production and activation of the NO-cyclic GMP-protein kinase G-ATP-sensitive potassium channel signaling pathway. Journal of Natural Products 75, 896–904.

Moalem, G., Tracey, D.J., 2006. Immune and inflammatory mechanisms in neuropathic pain. Brain Research Reviews 51, 240–264. doi:10.1016/j.brainresrev.2005.11.004

Okoye, T.C., Akah, P.A., Nworu, C.S., Ezike, A.C., 2014. Kaurenoic Acid Isolated from the Root Bark of Annona senegalensis Induces Cytotoxic and Antiproliferative Effects against PANC-1 and HeLa cells. European Journal of Medicinal Plants 4, 579–589. doi:10.9734/EJMP/2014/6926

Okuyama, E., Nishimura, S., Yamazaki, M., 1991. Analgesic principles from Aralia cordata Thunb. Chemical & pharmaceutical bulletin 39, 405–7.

Pathak, N.N., Balaganur, V., Lingaraju, M.C., Kant, V., Latief, N., More, A.S., Kumar, D., Kumar, D., Tandan, S.K., 2014. Atorvastatin attenuates neuropathic pain in rat neuropathy model by down-regulating oxidative damage at peripheral, spinal and supraspinal levels. Neurochemistry International 68, 1–9. doi:10.1016/j.neuint.2014.01.014

Pinho-Ribeiro, F.A., Hohmann, M.S.N., Borghi, S.M., Zarpelon, A.C., Guazelli, C.F.S., Manchope, M.F., Casagrande, R., Verri, W.A., 2015. Protective effects of the flavonoid hesperidin methyl chalcone in inflammation and pain in mice: Role of TRPV1, oxidative stress, cytokines and NF-κB. Chemico-Biological Interactions 228, 88–99. doi:10.1016/j.cbi.2015.01.011

Pinho-ribeiro, F.A., Zarpelon, A.C., Fattori, V., Manchope, M.F., Mizokami, S.S., Casagrande, R., Verri, W.A., 2016. Neuropharmacology Naringenin reduces in fl ammatory pain in mice. Neurophar 105, 508–519.

Sachs, D., Cunha, F.Q., Ferreira, S.H. 2004. Peripheral analgesic blockade of hypernociception : Activation of arginine ¯NO ¯cGMP ¯protein kinase G ¯ATP-sensitive K ⁒ channel pathway. Proceedings of the National Academy of Sciences of the United States of America 101, 3680–3685.

Salvemini, D., Little, J.W., Doyle, T., Neumann, W.L., 2011. Roles of reactive oxygen and nitrogen species in pain. Free Radical Biology and Medicine 51, 951–966. doi:10.1016/j.freeradbiomed.2011.01.026

Scholz, J., Woolf, C.J., 2007. The neuropathic pain triad: neurons, immune cells and glia. Nature neuroscience 10, 1361–1368. doi:10.1038/nn1992

Sedlak, J., Lindsay, R.H., 1968. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman’s reagent. Analytical biochemistry 25, 192–205.

Staurengo-Ferrari, L., Mizokami, S.S., Fattori, V., Silva, J.J., Zanichelli, P.G., Georgetti, S.R., Baracat, M.M., da França, L.G., Pavanelli, W.R., Casagrande, R., Verri, W.A., 2014. The ruthenium nitric oxide donor, [Ru(HEDTA)NO], inhibits

acute nociception in mice by modulating oxidative stress, cytokine production and activating the cGMP/PKG/ATP-sensitive potassium channel signaling pathway. Naunyn-Schmiedeberg's Archives of Pharmacology 387, 1053–1068. doi:10.1007/s00210-014-1030-0

Tirapelli, C.R., Ambrosio, S.R., Da Costa, F.B., Coutinho, S.T., De Oliveira, D.C.R., De Oliveira, A.M., 2004. Analysis of the mechanisms underlying the vasorelaxant action of kaurenoic acid in the isolated rat aorta. European Journal of Pharmacology 492, 233–241. doi:10.1016/j.ejphar.2004.04.003

Tirapelli, C.R., Ambrosio, S.R., da Costa, F.B., de Oliveira, A.M., 2008. Diterpenes: a therapeutic promise for cardiovascular diseases. Recent patents on cardiovascular drug discovery 3, 1–8. doi:10.2174/157489008783331689

Tonussi, C.R., Ferreira, S.H., 1994. Mechanism of diclofenac analgesia: direct blockade of inflammatory sensitization. European journal of pharmacology 251, 173–9.

Verri, W.A., Cunha, T.M., Parada, C.A., Poole, S., Cunha, F.Q., Ferreira, S.H., 2006. Hypernociceptive role of cytokines and chemokines : Targets for analgesic drug development? Pharmacology & therapeutics 112, 116–138. doi:10.1016/j.pharmthera.2006.04.001

Verri, W.A., Souto, F.O., Vieira, S.M., Almeida, S.C.L., Fukada, S.Y., Xu, D., Alves-Filho, J.C., Cunha, T.M., Guerrero, A.T.G., Mattos-Guimaraes, R.B., Oliveira, F.R., Teixeira, M.M., Silva, J.S., McInnes, I.B., Ferreira, S.H., Louzada-Junior, P., Liew, F.Y., Cunha, F.Q., 2010. IL-33 induces neutrophil migration in rheumatoid arthritis and is a target of anti-TNF therapy. Annals of the rheumatic diseas-

es 69, 1697–703. doi:10.1136/ard.2009.122655

Woolf, C.J., Mannion, R.J., 1999. Neuropathic pain : aetiology , symptoms, mechanisms, and management. The Lancet 353, 1959–1964.

Zarpelon, A.C., Rodrigues, F.C., Lopes, A.H., Souza, G.R., Carvalho, T.T., Pinto, L.G., Xu, D., Ferreira, S.H., Alves-Filho, J.C., McInnes, I.B., Ryffel, B., Quesniaux, V.F.J., Reverchon, F., Mortaud, S., Menuet, A., Liew, F.Y., Cunha, F.Q., Cunha, T.M., Verri, W.A., 2016. Spinal cord oligodendrocyte-derived alarmin IL-33 mediates neuropathic pain. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 30, 54–65. doi:10.1096/fj.14-267146

Zarpelon, A.C., Souza, G.R., Cunha, T.M., Schivo, I.R.S., Marchesi, M., Casagrande, R., Pinge-Filho, P., Cunha, F.Q., Ferreira, S.H., Miranda, K.M., Verri, W.A., 2013. The nitroxyl donor, Angeli’s salt, inhibits inflammatory hyperalgesia in rats. Neuropharmacology 71, 1–9. doi:10.1016/j.neuropharm.2013.03.009

Zhang, D., Yang, R., Wang, S., Dong, Z., 2014. Paclitaxel: new uses for an old drug. Drug design, development and therapy 8, 279–84. doi:10.2147/DDDT.S56801

4. Considerações Gerais

Tendo em vista que o processo inflamatório envolve o recrutamento de leucócitos, produção de citocinas, formação de espécies reativas de oxigênio e ativação de fatores de transcrição que culminam na liberação de mais mediadores inflamatórios e que as drogas disponíveis hoje no mercado causam, em sua maioria, algum tipo de reação adversa, a busca de novos compostos que auxiliam no combate do processo inflamatório instalado vem crescendo.

Neste trabalho, nós demonstramos o efeito de dois compostos naturais (ácido Pimaradienóico e ácido Caurenóico) em diferentes modelos de hiperalgesia em camundongos. O ácido pimaradienóico possui atividade analgésica e anti-inflamatória, porém a maioria dos experimentos mostram somente sua ação *in* vitro. Neste trabalho demonstramos sua ação anti-inflamatória *in* vivo, em modelo de peritonite induzida pela administração de carragenina em camundongos. O ácido Pimaradienóico-se mostrou eficaz ao inibir o recrutamento de leucócitos para a cavidade peritoneal além de diminuir a hiperalgesia mecânica e a formação de edema de pata, inibindo a formação de estresse oxidativo e a produção de citocinas pró-hiperalgésicas.

O ácido Caurenóico demonstrou seu efeito analgésico e anti-inflamatório ao inibir o recrutamento de leucócitos para a cavidade peritoneal induzido pela adminstração de LPS em modelo de peritonite, bem como a hiperalgesia mecânica, tanto no modelo de inflamação induzido pela administração de LPS na pata quanto após a constrição crônica do nervo ciático (CCI). Seus efeitos analgésico e anti-inflamatório envolvem a diminuição do estresse oxidativo, a diminuição de citocinas pró-hiperalgésicas e aumento de citocina anti-hiperalgésica, e ativação da via NO-cGMP-PKG-canais de potássio sensivéis a ATP. No modelo de CCI, o ácido caurenóico inibiu a ativação de células da glia (astrócitos e microglia) e a ativação do fator de transcrição NFκB.

Com isso, podemos concluir que os diterpenos ácido Pimaradienóico e ácido Caurenóico apresentam efeito anti-inflamatório, podendo ser utilizados como novos compostos terapêuticos.

Referências Bibliográficas

AMBROSIO, S. R. et al. Role of the carboxylic group in the antispasmodic and vasorelaxant action displayed by kaurenoic acid. **The Journal of pharmacy and pharmacology**, v. 56, n. 11, p. 1407–1413, 2004.

AMBROSIO, S. R.; SCHORR, K.; DA COSTA, F. B. Terpenoids of *Viguiera arenaria* (Asteraceae). **Biochememical Systematics and Ecology**. v.32, p 221-224, 2004.

AMBROSIO, S. R. et al. Pimarane diterpene from Viguiera arenaria (Asteraceae) inhibit rat carotid contraction. **Fitoterapia**, v. 73, p. 484-489, 2002.

AUSTIN, P. J.; MOALEM-TAYLOR, G. The neuro-immune balance in neuropathic pain: Involvement of inflammatory immune cells, immune-like glial cells and cytokines. **Journal of Neuroimmunology**, v. 229, n. 1-2, p. 26–50, 2010.

BLOCK, L. C. et al. Chemical and pharmacological examination of antinociceptive constituents of Wedelia paludosa. **Journal of ethnopharmacology**, v. 61, n. 1, p. 85–9, maio 1998.

BOLLER, S. et al. Anti-inflammatory effect of crude extract and isolated compounds from Baccharis illinita DC in acute skin inflammation. **Journal of Ethnopharmacology**, v. 130, n. 2, p. 262–266, 2010.

BORGHI, S. M. et al. Vitexin inhibits inflammatory pain in mice by targeting TRPV1, oxidative stress, and cytokines. **Journal of Natural Products**, v. 76, n. 6, p. 1141–1146, 2013.

BRESCIANI, L. F. V et al. Seasonal variation of kaurenoic acid, a hypoglycemic diterpene present in Wedelia paludosa (Acmela brasiliensis) (Asteraceae). **Zeitschrift fur Naturforschung - Section C Journal of Biosciences**, v. 59, n. 3-4, p. 229–232, 2004.

BÜRGER, C. et al. Acute and subacute toxicity of the hydroalcoholic extract from Wedelia paludosa (Acmela brasiliensis) (Asteracea) in mice. **Journal of Pharmacy and Pharmaceutical Sciences**, v. 8, n. 2, p. 370–373, 2005.

CAMPBELL, J. N.; MEYER, R. A. Mechanisms of neuropathic pain. **Neuron**, v. 52, n. 1, p. 77–92, 5 out. 2006.

CASAGRANDE, R. et al. Protective effect of topical formulations containing quercetin against UVB-induced oxidative stress in hairless mice. **Journal of Photochemistry and Photobiology B: Biology**, v. 84, n. 1, p. 21–27, 2006.

CASTRILLO, A et al. Inhibition of the nuclear factor kappa B (NF-kappa B) pathway by tetracyclic kaurene diterpenes in macrophages. Specific effects on NF-kappa B-inducing kinase activity and on the coordinate activation of ERK and p38 MAPK. **The Journal of biological chemistry**, v. 276, n. 19, p. 15854–15860, 2001.

CERQUEIRA, N. F.; YOSHIDA, W. B. Óxido Nítrico: Revisão. **Acta Cirurgica Brasileira**, v. 17, n. 6, p. 417–423, 2002.

CHAVAN, M. J. et al. Analgesic and Antiinflammatory Activity of Kaur - 16 - en - 19 - oic acid from Annona reticulata L . Bark. v. 276, n. May 2010, p. 273–276, 2012.

CHEUNG, H. T.; FU, S. L.; SMAL, M. A. Inhibition of platelet agregation by diterpenes acids from *Pinus massoniana* resin. **arzneimittelforschung**, v.44, n.1, p. 17-25, 1994.

CHO, J. H. et al. Inhibitory effects of diterpene acids from root of Aralia cordata on IgE-mediated asthma in guinea pigs. **Pulmonary Pharmacology and Therapeutics**, v. 23, n. 3, p. 190–199, 2010.

CHOI, R. J. et al. Inhibitory effects of kaurenoic acid from Aralia continentalis on LPS-induced inflammatory response in RAW264.7 macrophages. **Phytomedicine**, v. 18, n. 8-9, p. 677–682, 2011.

COELHO, C. F. Avaliação do efeito anti-inflamatório do óxido nítrico administrado por via inalatória no modelo experimental de edema de pata induzido por carragenina em camundongos. 2009. Tese (doutorado). Universidade de São Paulo, Instituto de Ciências Biomédicas. São Paulo, 2009.

COSTA-LOTUFO, L. V. et al. The cytotoxic and embryotoxic effects of kaurenoic

acid, a diterpene isolated from Copaifera langsdorffii oleo-resin. **Toxicon**, v. 40, n. 8, p. 1231–1234, 2002.

COTRAN, R. S.; KUMAR, V. K.; COLLINS, T. Robbins - Patologia Estrutural e Funcional. In: 6. ed. Rio de Janeiro: Editora Guanabara Koogan S. A., 2000.

CUNHA, F. Q. et al. The pivotal role of tumor necrosis factor α in the development of inflammatory hyperalgesia. **Br. J. Pharmacol. 107,** 660–664, 1992.

CUNHA, T. M. et al. Crucial role of neutrophils in the development of mechanical inflammatory hypernociception. **Journal of leukocyte biology**, v. 83, n. 4, p. 824–832, 2008.

CUNHA, T. M. et al. Morphine peripheral analgesia depends on activation of the PI3Kgamma/AKT/nNOS/NO/KATP signaling pathway. **Proceedings of the National Academy of Sciences of the United States of America**, v. 107, n. 9, p. 4442–4447, 2010.

CUNHA, T. M.; VERRI, W. A. Neutrophils: are they hyperalgesic or anti-hyperalgesic? **Journal of leukocyte biology**, v. 80, n. 4, p. 727–728; author reply 729–730, 2006.

CURY, Y. et al. Nitric Oxide Pain and analgesia : The dual effect of nitric oxide in the nociceptive system. **Nitric Oxide**, v. 25, p. 243–254, 2011.

DAL SECCO, D. et al. Nitric oxide inhibits neutrophil migration by a mechanism dependent on ICAM-1: role of soluble guanylate cyclase. **Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society**, v. 15, n. 1, p. 77–86, ago. 2006.

DAL-SECCO, D. et al. Neutrophil migration in inflammation: Nitric oxide inhibits rolling, adhesion and induces apoptosis. **Nitric Oxide - Biology and Chemistry**, v. 9, n. 3, p. 153–164, 2003.

DE CARLI, R. B. G. et al. Topical anti-inflammatory effect of creams containing Kaurenoic acid isolated from Wedelia paludosa in mice. **Latin American Journal of Pharmacy**, v. 28, n. 4, p. 594–598, 2009.

DÍAZ-VICIEDO, R. et al. Modulation of inflammatory responses by diterpene acids from Helianthus annuus L. **Biochemical and Biophysical Research Communications**, v. 369, n. 2, p. 761–766, 2008.

DUSSE, L. M. S.; VIEIRA, L. M.; CARVALHO, M. DAS G. Nitric oxide revision. **Jornal Brasileiro de Patologia e Medicina Laboratórial**, v. 39, n. 4, p. 343–350, 2003.

FERREIRA, S. H. et al. Dor Inflamatória. In: Alves Neto O. **Dor - principio e prática.** 1ª Ed. São Paulo: Editora Artmed, 2009.

FERNANDES, V. C. et al. The epimer of kaurenoic acid from Croton antisyphiliticus is cytotoxic toward B-16 and HeLa tumor cells through apoptosis induction. **Genetics and Molecular Research**, v. 12, n. 2, p. 1005–1011, 2013.

FLORA FILHO, R.; ZILBERSTEIN, B. Óxido nítrico: o simples mensageiro percorrendo a complexidade. Metabolismo, síntese e funções. **Revista da Associação Médica Brasileira**, v. 46, n. 3, p. 265–271, 2000.

FUCINA, G. et al. Development and validation of a stability indicative HPLC-PDA method for kaurenoic acid in spray dried extracts of Sphagneticola trilobata (L.) Pruski, Asteraceae. **Talanta**, v. 101, p. 530–536, 2012.

GAO, Y.-J.; JI, R. R. Targeting Astrocyte Signaling for Chronic Pain. **Neurotherapeutics**, v. 7, n. 4, p. 482–493, 2010a.

GAO, Y.-J.; JI, R.-R. Chemokines, neuronal-glial interactions, and central processing of neuropathic pain. **Pharmacology & therapeutics**, v. 126, n. 1, p. 56–68, abr. 2010b.

GAO, Y.-J.; ZHANG, L.; JI, R.-R. Spinal injection of TNF-α-activated astrocytes produces persistent pain symptom mechanical allodynia by releasing monocyte chemoattractant protein-1. **Glia**, v. 58, n. 15, p. 1871–1880, 2010.

GUERRERO, A. T. G. et al. Hypernociception elicited by tibio-tarsal joint flexion in mice: A novel experimental arthritis model for pharmacological screening. **Pharmacology Biochemistry and Behavior**, v. 84, n. 2, p. 244–251, 2006.

HENRY, G. E. et al. Kaurene diterpenes from Laetia thamnia inhibit the growth of human cancer cells in vitro. **Cancer Letters**, v. 244, n. 2, p. 190–194, 2006.

JEONG, S. IL et al. Kaurenoic acid from Aralia continentalis inhibits biofilm formation of streptococcus mutans. **Evidence-based Complementary and Alternative Medicine**, v. 2013, p. 1-10, 2013.

KANG, O. H. et al. Ent-pimara-8(14), 15-dien-19-oic acid isolated from the roots of Aralia cordata inhibits induction of inflammatory mediators by blocking NF-kappaB activation and mitogen-activated protein kinase pathways. **Eur J Pharmacol,** v. 601, p.179-185, 2008.

KLAUMANN, P. R.; WOUK, A. F. P. F.; SILLAS, T. Pathophysiology of pain. **Archives of Veterinary Science**, v. 13, n. 1, p. 1–12, 2008.

LEITE, A. C. R. M. et al. Effects of nitric oxide on neutrophil influx depends on the tissue: role of leukotriene B4 and adhesion molecules. **British journal of pharmacology**, v. 156, n. 5, p. 818–25, mar. 2009.

LIM, H. et al. Anti-inflammatory activity of the constituents of the roots of Aralia continentalis. **Archives of Pharmacal Research**, v. 32, n. 9, p. 1237–1243, 2009.

LIZARTE NETO, F. S. et al. Kaurene diterpene induces apoptosis in U87 human malignant glioblastoma cells by suppression of anti-apoptotic signals and activation of cysteine proteases. **Brazilian Journal of Medical and Biological Research**, v. 46, n. 1, p. 71–78, 2013.

LYU, J. H. et al. Ent-kaur-16-en-19-oic acid, isolated from the roots of Aralia continentalis, induces activation of Nrf2. **Journal of Ethnopharmacology**, v. 137, n. 3, p. 1442–1449, 2011.

MACRI, J. A. et al. Immunomodulatory and Antioxidant Properties of Kaurenoic Acid on Macrophages of BALB/c in vitro. **American Journal of Immunology**, v. 10, n. 4, p. 183–188, 2014.

MATHIAS, R., VON DER WEID, P. Y. Involvement of the NO-cGMP-K(ATP) channel pathway in the mesenteric lymphatic pump dysfunction observed in the guinea pig model of TNBS-induced ileitis. American journal of **physiology**. Gastrointestinal and liver physiology 304, G623–34, 2013.

MEOTTI, F. C. et al. Protective effect of crude extract from Wedelia paludosa (Asteraceae) on the hepatotoxicity induced by paracetamol in mice. **The Journal of pharmacy and pharmacology**, v. 58, n. 1, p. 137–42, jan. 2006.

MERSKEY, H. Some features of the history of the idea of pain. **Pain**, v. 9, n. 1, p. 3–8, ago. 1980.

MICLESCU, A.; GORDH, T. Nitric oxide and pain: “Something old, something new”. **Acta Anaesthesiologica Scandinavica**, v. 53, n. 9, p. 1107–1120, 2009.

MIRANDA, M. M. et al. Kaurenoic Acid Possesses Leishmanicidal Activity by Triggering a NLRP12/IL-1β/cNOS/NO Pathway. **Mediators of inflammation**, v. 2015, p. 392918, jan. 2015.

MIZOKAMI, S. S. et al. Kaurenoic acid from Sphagneticola trilobata inhibits inflammatory pain: Effect on cytokine production and activation of the NO-cyclic GMP-protein kinase G-ATP-sensitive potassium channel signaling pathway. **Journal of Natural Products**, v. 75, n. 5, p. 896–904, 2012.

MIZOKAMI, S. S. et al. Pimaradienoic Acid Inhibits Carrageenan-Induced Inflammatory Leukocyte Recruitment and Edema in Mice: Inhibition of Oxidative Stress, Nitric Oxide and Cytokine Production. **Plos One**, v. 11, n. 2, p. e0149656, 2016.

MONCADA, S.; HIGGS, E. A. The discovery of nitric oxide and its role in vascular biology. **British journal of pharmacology**, v. 147 Suppl , n. S1, p. S193–201, 2006.

OKOYE, T. C. et al. Antimicrobial Effects of a Lipophilic Fraction and Kaurenoic Acid Isolated from the Root Bark Extracts of Annona senegalensis. **Evidence-based Complementary and Alternative Medicine**, v. 2012, 2012.

OKOYE, T. C. et al. Kaurenoic Acid Isolated from the Root Bark of Annona senegalensis Induces Cytotoxic and Antiproliferative Effects against PANC-1 and HeLa cells. **European Journal of Medicinal Plants**, v. 4, n. 5, p. 579–589, 10 jan. 2014.

OKUYAMA, E.; NISHIMURA, S.; YAMAZAKI, M. Analgesic principles from Aralia

cordata Thunb. **Chem Pharm Bull** (Tokyo). v. 39, p. 405-407, 1991.

PAIVA, L. A F. et al. Anti-inflammatory effect of kaurenoic acid, a diterpene from Copaifera langsdorffii on acetic acid-induced colitis in rats. **Vascular Pharmacology**, v. 39, n. 6, p. 303–307, 2002.

PAULA-NETO, H. A et al. Inhibition of guanylyl cyclase restores neutrophil migration and maintains bactericidal activity increasing survival in sepsis. **Shock**, v. 35, n. 1, p. 17–27, 2011.

PAVAO-DE-SOUZA, G. F. et al. Acetic acid- and phenyl-p-benzoquinone-induced overt pain-like behavior depends on spinal activation of MAP kinases, PI3K and microglia in mice. **Pharmacology Biochemistry and Behavior**, v. 101, n. 3, p. 320–328, 2012.

POSSEBON, M. I. et al. Activation and Cytokine Production and Activation of the NO − Cyclic. **Journal of Natural Products**, v. 77, n. 11, p. 2488–2496, 2014.

SACHS, D.; CUNHA, F. Q.; FERREIRA, S. H. Peripheral analgesic blockade of hypernociception : Activation of arginine ¯NO ¯cGMP ¯protein kinase G ¯ATP-sensitive K ⁒ channel pathway. **Proceedings of the National Academy of Sciences of the United States of America**, v. 101, n. 10, p. 3680–3685, 2004.

SADIK, C. D.; LUSTER, A. D. Lipid-cytokine-chemokine cascades orchestrate leukocyte recruitment in inflammation. **Journal of Leukocyte Biology**, v. 91, n. 2, p. 207–215, fev. 2012.

SCHOLZ, J.; WOOLF, C. J. The neuropathic pain triad: neurons, immune cells and glia. **Nature neuroscience**, v. 10, n. 11, p. 1361–1368, 2007.

SILVA, R. Z. DA et al. Investigação Fitoquímica e Avaliação da Atividade Antibacteriana da Mikania lanuginosa DC (ASTERACEAE). **Visão Acadêmica. Curitiba-PR**, v. 3, p. 59–64, 2002.

STAURENGO-FERRARI, L. et al. The ruthenium nitric oxide donor, [Ru(HEDTA)NO], inhibits acute nociception in mice by modulating oxidative stress, cytokine production and activating the cGMP/PKG/ATP-sensitive potassium channel signaling pathway.

Naunyn-Schmiedeberg's Archives of Pharmacology, v. 387, n. 11, p. 1053–1068, 2014.

TAKEUCHI, O.; AKIRA, S. Pattern recognition receptors and inflammation. **Cell**, v. 140, n. 6, p. 805–20, 19 mar. 2010.

TIRAPELLI, C. R. et al. Analysis of the mechanisms underlying the vasorelaxant action of kaurenoic acid in the isolated rat aorta. **European Journal of Pharmacology**, v. 492, n. 2-3, p. 233–241, 2004.

TRANG, T.; BEGGS, S.; SALTER, M. W. ATP receptors gate microglia signaling in neuropathic pain. **Experimental Neurology**, v. 234, n. 2, p. 354–361, 2012.

VERRI, W. A. et al. Hypernociceptive role of cytokines and chemokines : Targets for analgesic drug development? **Pharmacology & therapeutics**, v. 112, p. 116–138, 2006.

VIEIRA, H. S. et al. Novel derivatives of kaurenoic acid: Preparation and evaluation of their trypanocidal activity. **Journal of the Brazilian Chemical Society**, v. 13, n. 2, p. 151–157, 2002.

WILKENS, M. et al. Characterization of the bactericidal activity of the natural diterpene kaurenoic acid. **Planta Medica**, v. 68, n. 5, p. 452–454, 2002.

WOOLF, C. J.; MANNION, R. J. Neuropathic pain : aetiology , symptoms, mechanisms, and management. **The Lancet**, v. 353, p. 1959–1964, 1999.

YATSUDA, R. et al. Effects of Mikania genus plants on growth and cell adherence of mutans streptococci. **Journal of Ethnopharmacology**, v. 97, n. 2, p. 183–189, 2005.

ZARPELON, A. C. et al. Spinal cord oligodendrocyte-derived alarmin IL-33 mediates neuropathic pain. **FASEB journal : official publication of the Federation of American Societies for Experimental Biology**, v. 30, n. 1, p. 54–65, jan. 2016.