Universidade Estadual de Londrina

JULIANA APARECIDA MACRI

ATIVIDADE IMUNOMODULADORA, MICROBICIDA E MECANISMO DE AÇÃO DO ÁCIDO CAURENÓICO NA LEISHMANIOSE EXPERIMENTAL

Londrina
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Dissertação apresentada ao Programa de Pós-Graduação em Patologia Experimental da Universidade Estadual de Londrina, como requisito parcial para a obtenção do título de Mestre em Patologia Experimental.

Orientador: Prof. Dr. Wander Rogério Pavanelli

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BANCA EXAMINADORA

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

_____
Prof. Dr. Ivete Conchon Costa
Universidade Estadual de Londrina - UEL

_____
Prof. Dr. José Mauricio Sforcin
Universidade Estadual Paulista Júlio de Mesquita Filho- UNESP

Londrina, 27 de Fevereiro de 2014.

Dedico este trabalho aos meus pais e avós, porque são minhas raízes eternas; ao meu marido, fonte de paciência e amor e aos meus amigos, por tornarem esta jornada mais alegre.

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Que os vossos esforços desafiem as impossibilidades, lembrai-vos de que as grandes coisas do homem foram conquistadas do que parecia impossível.

Charles Chaplin

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RESUMO

A infecção por *Leishmania (Leishmania) amazonensis* pode causar lesões locais e graves nos seres humanos, sendo esta condição clínica conhecida como leishmaniose tegumentar americana (LTA). Atualmente, a LTA continua sendo uma doença negligenciada além de não possuir uma terapêutica eficaz. O ácido caurenóico (AC) é um diterpeno presente em diversas plantas, dentre elas a *Sphagneticola trilobata (L.) pruski*. Diversos efeitos biológicos deste composto já foi comprovado em diferentes modelos experimentais, dentre eles destaca-se a ação antiparasitaria, anti-inflamatória e analgésica. Neste trabalho propomos a utilização do ácido caurenóico no modelo de leishmaniose experimental *in vitro.* Para tanto, investigou-se a atividade imunomoduladora em macrófagos de camundongos BALB/c, assim como o seu efeito leishmanicida em promastigotas de *L. amazonensis* ou em macrófagos peritoniais de animais susceptiveis (BALB/c) infectados com estes protozoários. Nossos resultados evidenciam a propriedade imunomoduladora do AC, que apresentou perfil antioxidante em modelo não inflamatório. Além disso, nossos dados evidenciam a atividade leishmanicida direta em formas promastigotas de *L.amazonensis, assim como* em macrófagos após a infecção. O mecanismo envolvido nessa atividade pode ser relacionado a capacidade que este diterperno possui em reestabeler a produção de óxido nítrico (NO) dependente da atividade da cNOS em macrófagos infectados. Esta produção esteve relacionada ao aumento dos níveis de IL-1β ativo e aumento da expressão de inflamossomo NRLP-12, mecanismo este, dependente da concentração. Juntos, os resultados evidenciam as propriedades imunomoduladora e leishmanicida do AC *in vitro*.

Palavras-chave: Leishmaniose. Ácido caurenóico. Imunomodulação.

SILVA, Juliana Aparecida Macri Santana.**Activity immunomodulatory, microbicidal and mechanism of action of kaurenoic acid in experimental leishmaniasis.** 2014. 90p. Dissertation (Master's degree in Experimental Pathology) – Universidade Estadual de Londrina, Londrina, 2014.

ABSTRACT

Infection with *Leishmania (Leishmania) amazonensis* can cause local and serious injuries in humans, representing the clinical condition known as american cutaneous leishmaniasis (ACL). Currently, ACL remains a neglected disease besides having no effective therapy. The kaurenoic acid (KA) is a diterpene present in many plants, including the *Sphagneticola trilobata (L.) Pruski*. Several biological effects of this compound has been confirmed in different experimental models, among them stands the antiparasitic, anti - inflammatory and analgesic action. In this study, we propose the use of kaurenoic acid in *in vitro* experimental model. To this end, we investigated the immunomodulatory activity in macrophages from BALB/c mice, as well as its leishmanicidal effect on *L. amazonensis* promastigotes or peritoneal macrophages of susceptible animals (BALB/c) infected with these protozoa. Our results demonstrate the immunomodulatory property of KA, which showed antioxidant profile in non-inflammatory model. Moreover, our data show the direct antileishmanial activity in promastigote forms *L.amazonensis* as well as in macrophages after infection. The mechanism involved in this activity may be related to the capacity which it has diterperno reestabeler in producing activity-dependent cNOS in infected macrophages nitric oxide (NO). This production was related to increased levels of active IL-1β , and increased expression of inflamossomo NRLP-12, this mechanism-concentration dependent. Together, the results suggest the immunomodulatory properties and Leishmanicidal KA *in vitro*.

Keywords: Leishmaniais. Kaurenoic acid. Immunomodulador effect.

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LISTA DE ABREVIATURAS

AC	Acido caurenóico
BALB/c	Linhagem de camundongos isogênicos
C57BL/6	Linhagem de camundongos isogênicos
Células Th1	Células T helper tipo 1
Células Th2	Células T helper tipo 2
cNOS	Oxido nítrico constitutiva
COX-1	Ciclooxigenase-1
COX-2	Ciclooxigenase-2
DMSO	Dimetilsulfóxide
ELISA	Ensaio imunoenzimático
EROS	Espécies Reativas de Oxigênio
GMP	Gaunosina monofosfato cíclico
Gp63	Glicoproteína de 63Kda expressa na superfície de promastigotas e amastigotas
HPLC	*High-permance liquid chromatography*
IDRM	Teste de intradermorreação de Montenegro
IL	Interleucina
IL- 4	Interleucina-04
IL-10	Interleucina-10
IL-12	Interleucina-12
IL-1β	Interleucina-1β
IL-8	Interleucina-8
INF-γ	Interferon-gama
KA	*Kaurenoic acid*
KCL	Cloreto de sódio
LC	Leishmaniose cutânea
LCD	Leishmaniose cutânea difusa
LCM	Leishmaniose cutâneo-mucosa
L-NAME	L-nitro arginina metil ester;
LPS	Lipopolissacarídeo
LT	Leishmaniose Tegumentar
LTA	Leishmaniose Tegumentar Americana

MDA	Malondialdeido
MDA	Malondialdeído
MPO	Mieloperoxidase
MTT	“3-[4,5-dimethylthiazol-2]-2,5-diphenyltetrazolium bromide”
NADPH	Nicotinamida adenina dinnucleotídeo fosfato
NFκB	Fator nuclear κB
NO	Óxido nítrico
NOS	Oxido nítrico sintase
NRf2	*Nuclear factor erythroid-related factor 2*
NRLP-12	Receptor do tipo Nod contendo domínio pirina do tipo 12
PGE2	Prostaglandina-2
PPG	Proteofosfoglicano
TEP	1,1,3,3-tetraetoxipropane
TGF-β	Fator de Trasformação do Crescimento – β
TNF-α	Fator de necrose tumoral- α
TRAP	*Trapping Antioxidant Parameter*
VLC	*Vaccum liquidchromatography*

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1. INTRODUÇÃO

1.1. As Leishmanioses

As leishmanioses são doenças parasitárias de caráter zoonóticas consideradas um grave problema de saúde pública, principalmente nos países em desenvolvimento, sendo classificada como doença negligenciada pela Organização Mundial de Saúde, juntamente com a doença de Chagas, tuberculose, hanseníase e malária (Morel, 2006).

Apresentam-se basicamente em duas formas clínicas, sendo elas: a leishmaniose visceral (LV), que acomete visceras, principalmente baço e fígado e a leishmaniose tegumentar (LT) com acometimento de pele, cartilagens e/ou mucosa (Neves, 2011). Estas manifestações são determinadas pela espécie do parasito e pelo estado imunológico do hospedeiro (Reithingher et al, 2007).

Nas Américas, a forma tegumentar é conhecida como Leishmaniose Tegumentar Americana (LTA), apresentando alta incidência, ampla distribuição e grande complexidade, podendo acarretar lesões destrutivas, desfigurantes e até mesmo incapacitantes (Schriefer et al., 2005; Lonardoni et al., 2006; Monteiro et al., 2008).

Distribuída mundialmente, as leishmanioses ocorrem principalmente em áreas tropicais e subtropicais, sendo endêmicas em 98 países. Na forma visceral, aproximadamente 90% dos casos ocorrem em Bangladesh, Brasil, Índia, Nepal e Sudão. A LT é mais amplamente distribuída com cerca de um terço dos casos ocorrendo nas Américas, Mediterrâneo ou Ásia ocidental do Oriente Médio para a Ásia Central. Os dez países com maior número de casos estimados são: Afeganistão, Argélia, Brasil, Colômbia, Costa Rica, Etiópia, Irã, Peru, Sudão e Síria, que juntos

representam 70 à 75% da incidência global estimada de LT (WHO, 2014).

O Brasil é considerado área endêmica destas parasitoses, apresentando no ano de 2013, 10.150 casos notificados de leishmanioses, sendo 9.735 referentes a LTA e 1.415 casos da forma visceral (Ministério da Saúde/SVS - Sistema de Notificação de Agravos de Notificação – SINAN NET -2014).

No estado do Paraná, a LTA já foi notificada em 276 dos 399 municipios, principalmente das regiões norte e oeste. Em 2013, foram notificados 169 casos de LTA e nenhum caso de LV neste estado (Ministério da Saúde/SVS – Sistema de Notificação de Agravos de Notificação – SINAN NET 2014).

Estas parasitoses são consideradas doenças emergentes e re-emergentes, com aumento de incidência nas últimas décadas devido a migração de pessoas da zona rural para a urbana em busca de oportunidades de trabalho, migração por causa de guerras, alterações climáticas, baixa condição sócio-economica e co-infecção de HIV/*Leishmania* (Goto e Lindoso, 2010). Além disso, mudanças no padrão de transmissão da doença tem sido sugeridas, uma vez que inicialmente as leishmanioses eram consideradas zoonoses, que ocasionalmente acometiam pessoas em contato com as florestas. Posteriormente a doença passou a ocorrer em zonas rurais e atualmente também acontece em regiões periurbanas (Brasil, 2007).

1.2. Formas Clínicas da Leishmaniose Tegumentar Americana

A LTA é considerada uma enfermidade polimórfica, que atinge a pele e as mucosas. Os parasitos ao serem inoculados no hospedeiro mamífero formam, geralmente uma única lesão limitada no local da picada, porém dependendo da espécie do protozoário e da resposta imune do hospedeiro, a doença pode apresentar

amplo espectro de severidade e uma série de formas clínicas, tais como: cutânea localizada; mucocutânea e cutanea-difusa (Garnier; Croft, 2002).

Nas Américas existem pelo menos 11 espécies dermotrópicas de *Leishmania*, das quais sete já foram identificadas no Brasil como causadoras de LTA. Destas espécies, seis pertencem ao subgênero *Viannia* – *Leishmania (Viannia) braziliensis, Leishmania (Viannia) guyanensis, Leishmania (Viannia) lainsoni, Leishmania (Viannia) naiffi, Leishmania (Viannia) lindenberg e Leishmania (Viannia) shawi* – e uma espécie do subgênero *Leishmania* – *Leishmania (Leishmania) amazonensis* (Ministério da Saúde, 2007).

A forma clínica cutânea localizada, apresenta-se com lesão única (mais comum), múltiplas ou disseminadas por via hematogênica. Inicialmente forma-se uma mácula, seguida por pápula, nódulo e progressivamente aumenta de tamanho até ulcerar, este processo pode levar semanas, meses ou anos após a infecção inicial. Na forma ulcerativa o parasito está confinado a pele e a lesão pode apresentar variações de tamanho e formato dependentes do tempo de evolução. Essas lesões ulcerativas são caracterizadas como indolores e apresentar fundo granuloso e bordas elevadas (Reveiz et al., 2013).

Algumas espécies do subgênero *Viannia*, como a *L. braziliensis*, pode disseminar-se à mucosas distantes da lesão primária, ocasionando lesões secundárias, especialmente na região nasofaríngea caracterizando a forma clínica mucocutânea (Reveiz et al., 2013). Esse tipo de lesão apresenta um processo lento, de curso crônico, ocorrendo por extensão direta de uma lesão primaria ou através da via hematogênica (Genaro, 2005). Nesta forma clínica não ocorre cura espontânea e caso não tratada corretamente ocorre mutilação ou destruição da área afetada com perda da qualidade de vida do paciente (Reveiz et al., 2013).

A forma clínica cutânea-difusa é causada pela *L. amazonensis* no Brasil, esta forma clínica de leishmaniose é considerada de ocorrência rara, que acomete pacientes com anergia e deficiência na resposta imune celular à antígenos de *Leishmania*. Caracteriza-se por lesões difusas não ulceradas e distribuídas amplamente na pele, particularmente nas extremidades. Este tipo de lesão assume caráter crônico e progressivo que persiste por toda a vida do paciente (Ashford, 2000). O paciente tem resultado negativo no teste de Montenegro e não possui resposta terapêutica satisfatória (Genaro, 2005; Brasil, 2007).

1.3. Agente etiológico e ciclo biológico

As leishmanioses são causadas por protozoários da família dos Trypanosomatidae, da ordem Kinetoplastida, do gênero *Leishmania*. Estes protozoários são transmitidos pela picada de insetos vetores, classificados como hospedeiros intermediários, pertencentes à ordem Diptera, família Psychodidae, subfamília Phlebotominae, gênero *Lutzomyia* no novo mundo ou *Phlebotomus* no velho mundo (Killick-Kendrick, 1999; Bates, 2007). No Brasil são conhecidos como mosquito palha, birigui e tatuquira (Brasil, 2007).

Aproximadamente 700 espécies de insetos vetores foram descritas e cerca de 30 estão provavelmente envolvidos na transmissão da leishmaniose (Bates, 2007). Algumas dessas espécies possuem estreita relação com espécies de *Leishmania* e seus hospedeiros vertebrados, sendo portanto vetores específicos de algumas das formas clínicas das leishmanioses conforme a região, por exemplo, o parasito *L. braziliensis* pode ter como vetor no Brasil o *Lutzomia wellcomei*, *Lu. complexus*, *Lu. intermedia, Lu. amazonensis, Lu. paraensis, Lu. whitmani, Lu. pessoai*. Por outro lado, o parasito *L. amazonensis* tem como vetores o *Lu. flaviscutellata*, *Lu.*

olmeca nociva e *Lu. reducta* (Killick-Kendrick, 1999).

O ciclo de vida do parasito *Leishmania* compreende dois estágios, a forma promastigota medindo entre 14 e 20 µm (forma flagelada) encontrada no trato digestivo dos insetos vetores e a amastigota (sem flagelo externo) medindo entre 2,1 e 3,2 µm encontrada no citoplasma dos fagócitos dos hospedeiros vertebrados, sendo estes os mamíferos, canídeos, roedores, marsupiais (gambá), e primatas, incluindo o homem (Van Assche et al., 2011). Muitas leishmanioses possuem caráter de zoonose, embora também ocorra a forma antropozoonótica (Reithinger et al., 2007; Van Assche et al., 2011).

Ao picar o individuo ou animal parasitado, os flebotomineos ingerem juntamente com o sangue ou linfa, macrófagos infectados com formas amastigotas de *Leishmania*. Durante a digestão do sangue, as formas amastigotas diferenciam-se em promastigotas procíclicas no tudo digestório do inseto vetor, formas delgadas com cerca de 1,5 µm de largura e 20 µm de comprimento, capazes de se dividir e não infectantes. Essas formas se multiplicam por divisão binária e colonizam o tubo digestório do inseto vetor. De acordo com a posição ocupada pelo parasito no inseto, foram descritos dois subgeneros: *Leishmania (Leishmania)*, para os parasitos que se aderem ao epitélio do intestino anterior, e *Leishmania (Viannia)*, para os que aderem ao epitélio posterior. Em seguida as promastigotas prócicliclas, passam por um processo chamado metaciclogênese, diferenciando-se em formas promastigotas metacíclicas (forma infectante). Nesta fase os parasitos deslocam-se do intestino do inseto vetor e migram para a probóscide e na próxima picada o parasita é inoculado no hospedeiro mamífero (Awasthi, 2004; Gupta et al 2001; Rey, 2001; Kaye e Scott, 2011).

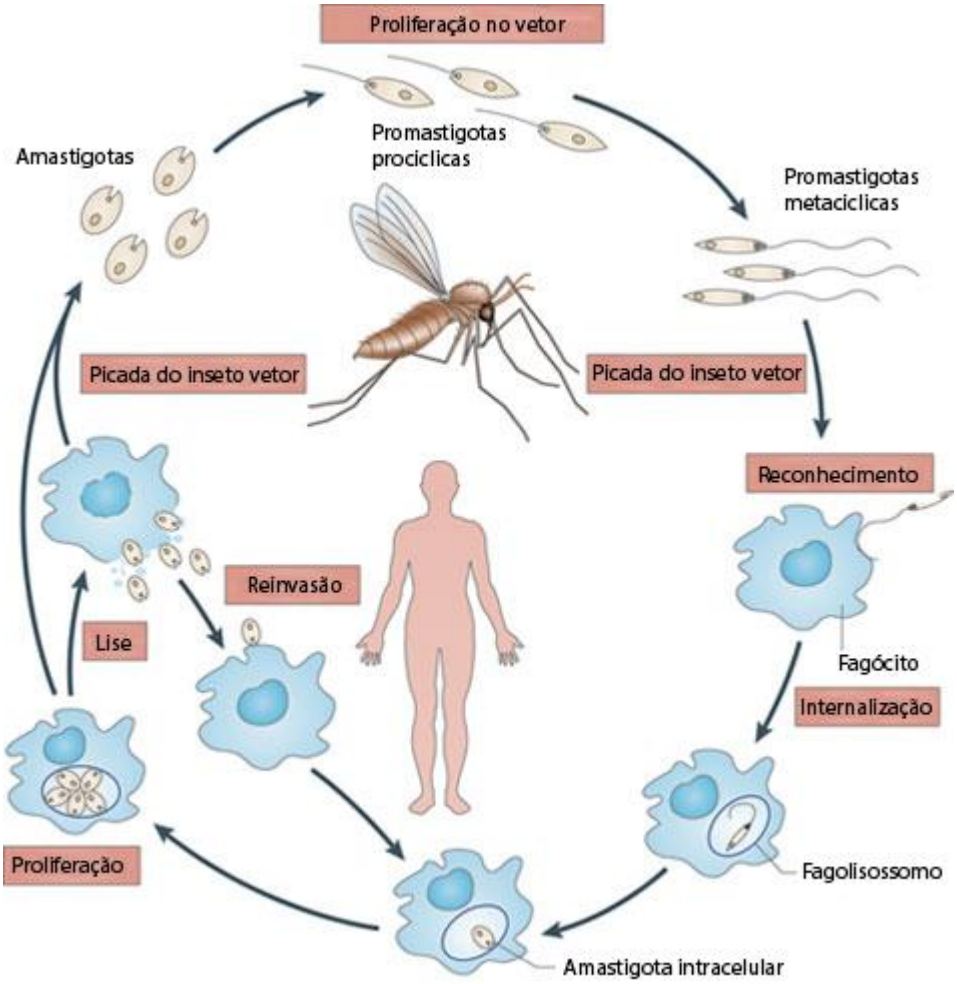

Figura 1: ciclo biológico da Leishmaniose. Adaptado de Kaye e Scott, 2011.

Ao serem inoculadas no hospedeiro vertebrados, estas formas infectantes são fagocitadas principalmente por macrófagos, formando vacúolos parasitóforos onde se diferenciam para a forma amastigota e caso consigam evadir-se dos mecanismos microbicidas, dá-se início à multiplicação destas formas, recomeçando o ciclo (Figura 01) (Kaye e Scott, 2011).

1.4. Interação Parasita Hospedeiro e resposta imune

A instalação da LTA no hospedeiro é determinada por uma complexa associação entre os fatores relacionados a virulência do parasito e a resposta imunológica do hospedeiro. Ao ser inoculada na derme do hospedeiro, as promastigotas metacíclicas interagem com as proteínas do soro, sistema complemento e fluidos digestivo do inseto, assim como os receptores das células hospedeiras. Nesta fase inicial, um dos grandes desafios do parasito é estabelecer residência no interior dos fagócitos, que compõem a principal linha de defesa na leishmaniose (Genaro, 2005).

As infecções por *Leishmania* levam a uma ativação especfica da resposta imunológica por parte do hospedeiro. No local da picada ocorre intensa migração de neutrófilos e macrófagos. Os neutrófilos tem como principal função fagocitar a maioria dos parasitos presentes e produzir citocinas e quimiocinas que influenciem a resposta imune durante a infecção por *Leishmania (*Scapini et al., 2000; Peters et al.,2008).

No entanto, apenas neutrófilos e macrófagos ativados são capazes de eliminar os parasitos intracelulares. Assim, interação entre resposta imune inata e adquirida norteia a expressão da doença e o resultado da infecção (Launois et al. 1996). Desta forma, para se compreender os fatores envolvidos na resposta imune parasita-hospedeiro, Sacks e Noben Trauth (2002) estabeleceram um modelo de infecção experimental com *Leishmania major em* camundongos, no qual camundongos C57BL/6 apresentaram resistência à infecção enquanto que camundongos da linhagem BALB/c foram suscetíveis.

A resistência à doença ocorre quando prevalece a ativação preferencial da subpopulação de linfócitos T helper 1 (Th1), que por sua vez induzem

a síntese de diversas citocinas, principalmente Interferon-γ (IFN-γ) e Fator de Necrose Tumoral-α (TNF-α) e Interleucina- 1 β (IL-1β) que em conjunto levam a ativação dos macrófagos desencadeando o “burst oxidativo”, durante o processo de endocitose do parasito (Lima junior, 2013; Reis et al., 2006; Reithinger et al., 2007).

Este fenômeno se caracteriza pelo aumento da atividade respiratória da célula ativando enzimas como a nicotinamida adenina dinuceotídeo fosfato oxidase (NADPH oxidase) que transferem prótons para moléculas de oxigênio formando moléculas altamente reativas denominadas espécies reativas de oxigênio (EROS), como superóxidos, peróxido de hidrogênio e radicais hidroxila; e aumento na atividade da enzima óxido nítrico sintase induzível (iNOS), com consequente aumento na produção de NO, a principal molécula microbicida envolvida na eliminação do parasito (Mukbel et al., 2007; Van Assche et al., 2011; Lima-Junior et al., 2013)

Contudo, um predomínio de resposta Th2, com síntese das citocinas IL-4, IL-13, resulta no desenvolvimento da infecção intracelular, favorecendo a suscetibilidade a doença. O parasito apresenta estratégias para subverter e direcionar a resposta imune para esse tipo de resposta Th2 (Ji et al., 2003; Reis et al., 2006; Reithinger et al., 2007).

1.5. Diagnóstico

O diagnóstico da LTA apresenta dificuldades pelo fato de suas manifestações clínicas se assemelharem a patologias, como a hanseníase, canceres de pele, micoses cutâneas, paracoccidioidomicose e sífilis, patologias essas que também são comuns em áreas endêmicas a leishmaniose (Gontijo e Carvalho, 2003; Reithinger et al., 2007). Portanto o diagnóstico deve ser realizado pela associação da história clínica e epidemiológica, exame físico e testes laboratoriais.

O diagnóstico imunológico é altamente especifico e consiste de uma análise da biópsia ou aspirado de lesão em análise histopatológica; identificação de promastigotas em cultura *in vitro*, métodos sorológicos como ELISA (Enzyme-linked immunosorbent assay) e detecção de DNA através da reação de cadeia de polimerase (PCR), porém estes testes diagnósticos necessitam de infra-estrutura laboratorial, habilidade técnica, recursos humanos e material que são escassos principalmente nos países onde a leishmaniose é prevalente (Reithinger et al., 2007).

O teste de intradermorreação de Montenegro (IDRM) é um teste de hipersensibilidade tardia que possui alta sensibilidade e especificidade, muito utilizado devido a sua facilidade de aplicação, baixo, porém não distingue entre uma infecção recente ou tardia, sendo esta a única ferramenta disponível para diagnostico de LTA em muitos países carentes (Brasil, 2007; Reithinger et al., 2007).

1.6. Tratamento Leishmaniose

O tratamento atual da leishmaniose baseia-se na eliminação das formas amastigotas de *Leishmania*. Porém a localização intracelular, dificulta uma atuação mais eficaz dos fármacos atuais (Rodrigues et al, 2006)

As formas de tratamento para leishmaniose é baseada em quimioterapia, com uso de antimoniais pentavalentes como o antimoniato de meglumine ou antimoniato de N-metilglucamina (Glucantime®), e o estibogluconato de sódio (Pentostan®) (Brasil, 2007).

O Glucantime® é utilizado desde a década de 1940 no Brasil como principal droga de escolha no tratamento da leishmaniose, com esquema terapêutico composto por doses de 20mg/Kg/dia através de injeções intramusculares em ciclos de 20 a 30 dias (Brasil, 2007).

Seu mecanismo de ação é a inibição das enzimas da via glicolítica e da β-oxidação em amastigotas, porém é um metal pesado, e que possivelmente interfira em outras vias metabólicas da *Leishmania*, bem como a do hospedeiro (Silva-López, 2010), além disso possui efeitos tóxicos (Pelissari et al., 2011) e podem apresentar efeitos colaterais como artralgia, mialgia, disfunção gastrointestinal, cefaleia, insônia, aumento sérico das enzimas hepáticas, cardiotoxicidade e insuficiência renal (Lima et al., 2007; Silva-López, 2010), além das dificuldades de administração, altos custos, e muitas vezes a cura clínica não é acompanhada de cura parasitológica, pois têm sido observados parasitos na cicatriz de individuos clinicamente curados após o tratamento (Silva-López, 2010).

Outros medicamentos também são utilizados como a anfotericina B, a pentamidina o miltefosine e a paramomicina, classificadas como drogas alternativas no caso de resistência aos antimoniais, mas também não possuem um índice terapêutico favorável e apresentam variadas reações adversas assim como os antimoniais (Goto e Lindoso, 2010; Silva-López, 2010).

Portanto, os fármacos utilizados para tratamento das leishmanioses, apresentam uma série de problemas, dentre eles a resistência do parasito, os efeitos colaterais, que limitam sua utilização e forma de administração parenteral, que exigem a colaboração do paciente que muitas vezes abandonam o tratamento contribuindo assim para o aparecimento de cepas resistências. Assim, a busca de terapias alternativas e imunoprofiláticas têm sido recomendadas como prioridade estrategica de controle da doença (Armijos et al., 2004; Sen e Chatterjee, 2011). Faz-se necessário portanto, novas pesquisas para desenvolvimento de drogas eficazes, de baixo custo e com reações adversas menos agressivas (Silva-López, 2010; Sen e Chatterjee, 2011).

1.7. Ácido Caurenóico

O uso de produtos naturais com propriedades terapêuticas é tão antigo quanto a própria humanidade e por um longo tempo o uso de plantas em forma de chás, tinturas, pós e cataplasmas foram utilizados como drogas terapêuticas (Rates, 2001; Maciel et al., 2002; Balunas e Kinghorn, 2005).

É crescente o interesse em terapias de produtos naturais derivados de plantas, pois muitas vezes a medicina convencional pode ser ineficiente com muitos efeitos colaterais assim como no uso abusivo e incorreto de drogas sintéticas, além do alto custo ao consumidor, sendo as plantas de fácil acesso a população (Rates, 2001).

O estudo dos componentes vegetais constitui um campo imenso de novos conhecimentos científicos, podendo contribuir para o aprimoramento da medicina tradicional. As vantagens para desenvolvimento de pesquisas farmacológicas com plantas são de grande alcance social, permitindo que as informações sejam repassadas à população (Maciel et al., 2002).

As florestas da América do Sul possuem grande potencial para fornecer novos compostos leishmanicidas (Carvalho e Ferreira, 2001). Dentro dessa biodiversidade, está a planta *Sphagneticola trilobata (L.) Pruski* ou *Wedelia paludosa*, presente em várias regiões do Brasil, encontrada principalmente nos estados de Pernambuco, Bahia, Minas Gerais, São Paulo e Santa Catarina, conhecida popularmente como pseudo-arnica, margaridão, pingo-de-ouro, mal-me-quer-do-brejo, picão da praia ou apenas vedélia (Baccarin et al., 2009; Batista et al., 2009).

Estudos fitoquímicos realizados com a planta *Sphagneticola trilobata (L.) Pruski*, indica que um dos seus principais componentes é o ácido caurenóico (AC)

ou ent-kaur ou ácido -16-em-19-oic (Figura 02), sendo este também presente em diversas plantas, entre as quais destacam-se *Aralia continentalis, Copaifera langsdorffii, Mikania glomerata* e *Mikania laevigata* (Paiva et al., 2002; Santos, 2005; Cavalcanti et al., 2006; Baccarin et al., 2009; Batista et al., 2009; Lim et al., 2009).

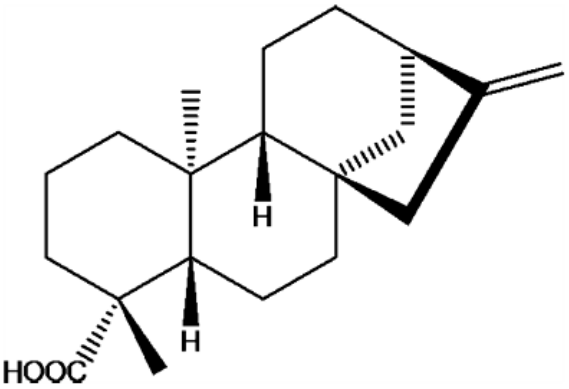

Figura 2: Estrutura química do ácido caurenóico.

O ácido caurenóico (AC) é um diterpeno com diversas atividades biológicas já comprovadas, dentre elas ação antiparasitária (Batista, Chiari e De Oliveira, 1999; Izumi et al., 2012; Santos et al., 2013), antimicrobiana (Yatsuda et al., 2005; Ambrosio et al., 2008), antifúngica (Santos, 2005; Baccarin et al., 2009), antinociceptiva (Baccarin et al., 2009), vasorrelaxante e hipotensiva (Tirapelli et al., 2004; Tirapelli et al., 2010) e hipoglicemiante (Bresciani et al., 2004).

Santos, et al., (2013), verificou *in vitro* o efeito direto do AC extraído do óleo de copaíba nas formas promastigotas e amastigotas de *L. amazonensis*. Através de microscopia eletrônica de varredura e citometria de fluxo confirmou alterações morfológicas e estruturais no parasito, principalmente aumento da permeabilidade da membrana plasmática e despolarização da membrama mitocondrial. Em estudo semelhante, porém utilizando *Trypanosoma cruzi* em seu

modelo, Izumi et al., (2012), verificou que o AC teve moderada atividade contra esses parasitos, não sendo capaz de causar lipoperoxidação na membrana de *T. cruzi.*

Ao utilizar óleo de copaíba (*Copaifera martii*) para o tratamento de leishmaniose experimental, Santos et al., (2011) obteve resultados positivos no tratamento oral com melhora significativa das lesões na pata. Os principais compostos químicos do óleo de copaíba utilizados neste trabalho eram representados por diterpenos (62,3%) e sesquiterpenos (37,7%), sendo o AC um dos principais diterpenos presentes. Fernandes e Freitas, 2007, atribuíram todas as propriedades biológicas da *Copaifera* spp. ao grupo de sesquiterpenos e diterpenos.

No modelo de edema de pata induzido por carregenina, Lim et al., (2009) e Choi et al., (2011) verificaram que o AC extraído da *Aralia continentalis* foi capaz de inibir o processo inflamatório, inibindo a ação da ciclooxigenase-1 (COX-1), ciclooxigenase-2 (COX-2) - precursores da síntese de prostaglandinas-2 (PGE2), importantes mediadores da inflamação - e diminuição dos níveis de IL-8, a qual é um potente quimioatraente para neutrófilos - células que apresentam papel importante no agravamento do processo inflamatório (AKDIS, et al, 2011). Choi et al. (2011), demonstrou ainda que a atividade anti-inflamatória do ácido caurenóico a nível celular se dá através da inibição do fator nuclear κB (NFκB), da produção de NO e PGE2.

Em estudo controverso, Liu et al. (2011) verificou *in vitro* que na concentração de 1nM o AC não foi capaz de suprimir a atividade de NFκB, inalterando a expressão gênica de COX-2, óxido nítrico, IL-1β, TNF-α e IL-12 nesta concentração estudada.

MIZOKAMI et al. (2012), no modelo *in vivo* de dor inflamatória induzida por ácido acético, verificou que o AC possui um efeito analgésico ao inibir citocinas como TNF-α, IL-1β, COX-2 e PGE2 (Mizokami et al,2012).

Em modelo de contração de artérias isoladas de ratos, Tirapelli et al. (2004) verificou que o pré-tratamento com AC reduziu as contrações induzidas por fenilefrina e cloreto de potássio (KCL) tanto no endotélio da aorta íntegra quanto nos anéis aórticos e causou o relaxamento do vaso. Isto esta relacionado ao fato do AC bloquear os canais de cálcio pela ativação da via óxido nítrico/Gaunosina monofosfato cíclico (NO/GMPc) e abertura dos canais de potássio. Também concluiu que este efeito vasorelaxante está relacionado a ativação da isoforma NO sintase neuronal e endotelial (TIRAPELLI, 2004).

Em modelo de colite experimental induzida por ácido acético, Paiva et al. (2002) concluiu que o ácido caurenóico é um eficaz anti-inflamatório que leva à uma redução da atividade da mieloperoxidase uma enzima presente nos leucócitos e utilizada de forma indireta a análise de migração de leucócitos, especialmente neutrófilos, para o local da inflamação, e diminuiu também a concentração de malondialdeido (MDA), um indicador da lipoperoxidação lipídica. Diante disso houve uma redução do infiltrado inflamatório, edema e lesão tecidual. (Paiva et al, 2002)

Sendo o AC um composto natural, presente em diversas plantas acessíveis à população mas com poucos trabalhos descrevendo seus efeitos e a LTA uma doença negligenciada que acomete milhares de pessoas e seu tratamento considerado um desafio, o presente trabalho apresenta os resultados de experimentos *in vitro* da ação imunomoduladora e leishmanicida do AC sobre macrófagos peritoneais de camundongos BALB/c infectados ou não com formas promastigotas de *Leishmania amazonensis.*

2. REFERÊNCIAS

AGÊNCIA NACIONAL DE VIGILÂNCIA EM SAÚDE, (ANVISA). **Dispõe sobre a priorização da análise técnica de petições, no âmbito da Gerência-Geral de Medicamentos da ANVISA, cuja relevância pública se enquadre nos termos desta Resolução.** Resolução RDC no 28, de 4 de abril de 2007. 2007.

AMBROSIO, S. R. et al. Antimicrobial activity of kaurane diterpenes against oral pathogens. **Z Naturforsch C,** v. 63, n. 5-6, p. 326-30, May-Jun 2008. ISSN 0939-5075 (Print) 0341-0382.

ARMIJOS, R. X. et al. Safety, immunogenecity, and efficacy of an autoclaved *Leishmania* amazonensis vaccine plus BCG adjuvant against New World cutaneous *Leishmania*sis. **Vaccine,** v. 22, n. 9-10, p. 1320-6, Mar 12 2004. ISSN 0264-410X (Print) 0264-410x.

ASHFORD, R. W.The leishmaniases as emerging and reemerging zoonoses. **Int J Parasitol**. 30(12-13),1269-1281,2000.

AWASTHI, A.; MATHUR, R. K.; SAHA, B. Immune response to *Leishmania* infection. **Indian J Med Res,** v. 119, n. 6, p. 238-58, Jun 2004. ISSN 0971-5916 (Print) 0971-5916.

BACCARIN, T. et al. Análise morfoanatômica das partes aéreas de Wedelia paludosa DC. (Acmela brasiliensis, Sphagneticola trilobata), Asteraceae. **Revista Brasileira de Farmacognosia,** v. 19, p. 612-616, 2009. ISSN 0102-695X.

BALUNAS, M. J.; KINGHORN, A. D. Drug discovery from medicinal plants. **Life Sci,** v. 78, n. 5, p. 431-41, Dec 22 2005. ISSN 0024-3205 (Print) 0024-3205.

BATES, P. A. Transmission of *Leishmania* metacyclic promastigotes by phlebotomine sand flies. **Int J Parasitol,** v. 37, n. 10, p. 1097-106, Aug 2007. ISSN 0020-7519 (Print) 0020-7519.

BATISTA, R. et al. Cytotoxicity of Wedelia paludosa D.C. extracts and constituents. **Revista Brasileira de Farmacognosia,** v. 19, p. 36-40, 2009. ISSN 0102-695X.

BATISTA, R.; CHIARI, E.; DE OLIVEIRA, A. B. Trypanosomicidal Kaurane Diterpenes from Wedelia paludosa. **Planta Med,** v. 65, n. 03, p. 283-284, //

04.01.2007 1999. ISSN 0032-0943.

BRASIL, M. D. S. **Manual de Vigilância da Leishmaniose Tegumentar Americana**. 2. Brasilia: 2007.

BRESCIANI, L. F. et al. Seasonal variation of kaurenoic acid, a hypoglycemic diterpene present in Wedelia paludosa (Acmela brasiliensis) (Asteraceae). **Z Naturforsch C,** v. 59, n. 3-4, p. 229-32, Mar-Apr 2004. ISSN 0939-5075 (Print) 0341-0382.

CARVALHO, P. B.; FERREIRA, E. I. *Leishmania*sis phytotherapy. Nature's leadership against an ancient disease. **Fitoterapia,** v. 72, n. 6, p. 599-618, Aug 2001. ISSN 0367-326X (Print) 0367-326x.

CAVALCANTI, B. C. et al. Genotoxicity evaluation of kaurenoic acid, a bioactive diterpenoid present in Copaiba oil. **Food Chem Toxicol,** v. 44, n. 3, p. 388-92, Mar 2006. ISSN 0278-6915 (Print) 0278-6915.

GARNIER, T.; CROFT, S. L. Topical treatment for cutaneous leishmaniasis. *Curr. Opin.* **Investig. Drugs**. 3(4), 538-544, 2002

GENARO, O. Leishmaniose Tegumentar Americana. In: Neves DP, Melo AL, Linardi PM, Vitor RWA. **Parasitologia Humana.** 11 ed. São Paulo: Atheneu, p.41-83, 2005.

GONTIJO, B.; CARVALHO, M. D. L. R. D. Leishmaniose tegumentar americana. **Revista da Sociedade Brasileira de Medicina Tropical,** v. 36, p. 71-80, 2003. ISSN 0037-8682.

GOTO, H.; LINDOSO, J. A. Current diagnosis and treatment of cutaneous and mucocutaneous *Leishmania*sis. **Expert Rev Anti Infect Ther,** v. 8, n. 4, p. 419-33, Apr 2010. ISSN 1478-7210.

GUPTA,N.;NOYAL,N. RASTOGI, A.K. *In vitro* cultivation and characterization of axenic amastigotes of *Leishmania.* **Trends Parasitol.** 17(3), 150-153, 2001.

IZUMI, E. et al. Terpenes from Copaifera demonstrated in vitro antiparasitic and synergic activity. **J Med Chem,** v. 55, n. 7, p. 2994-3001, Apr 12 2012. ISSN 0022-2623.

JI, J.; SUN, J.; SOONG, L. Impaired expression of inflammatory cytokines and chemokines at early stages of infection with Leishmania amazonensis. **Infect Immun**, 71, 4278-4288, 2003.

KAYE, P.; SCOTT, P. *Leishmania*sis: complexity at the host–pathogen interface. **Nat Rev Micro,** v. 9, n. 8, p. 604-615, 08//print 2011. ISSN 1740-1526. Disponível em: < http://dx.doi.org/10.1038/nrmicro2608 >.

KILLICK-KENDRICK, R. The biology and control of Phlebotomine sand flies. **Clinics in Dermatology,** v. 17, n. 3, p. 279 - 289, 1999. ISSN 0738-081X. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0738081X99000462 >.

LAUNOIS, P.; GUMY,A.; HIMMELRICH, H.; LOCKSLEY, R.M; LOUIS J.A.. Rapid Il-4 production by *Leishmania* homolog of mammalian RACK1-reative CD4+ T cells in resistance mice treated once with anti-IL-12R or IFN-γ antibodies at the onset of infection with *Leishmania major* instructs Th2 cell development, resulting in nonhealing lesions.**J. Immunol.** 168,4628-4635,2002

LIM, H. et al. Anti-inflammatory activity of the constituents of the roots of Aralia continentalis. **Arch Pharm Res,** v. 32, n. 9, p. 1237-43, Sep 2009. ISSN 0253-6269 (Print) 0253-6269.

LIMA-JUNIOR DS, COSTA DL, CARREGARO V *et al.* Inflammasome-derived IL-1beta production induces nitric oxide-mediated resistance to Leishmania. *Nat Med* 2013; **19**: 909-915.

LIMA, E. B. D. et al. Tratamento da Leishmaniose Tegumentar Americana. **Anais Brasileiros de Dermatologia,** v. 82, p. 111-124, 2007. ISSN 0365-0596.

LONARDONI, M. V.; SILVEIRA, T. G.; ALVES, W. A.; MAIA-ELKHOURY, A. N.; MEMBRIVE, U. A.; MEMBRIVE, N. A.; RODRIGUES, G.; REIS, N.; ZANZARINI, P. D.; ISHIKAWA, E.; TEODORO, U.. Human and canine American cutaneous leishmaniasis in Mariluz, Parana State, Brazil. **Cad Saude Publica.** 22(12), 2713-2716, 2006.

MACIEL, M. A. M. et al. Plantas medicinais: a necessidade de estudos multidisciplinares. **Química Nova,** v. 25, p. 429-438, 2002. ISSN 0100-4042.

MONTEIRO, W. M.; NEITZKE, H. C.; LONARDONI, M. V. C.; SILVEIRA, T. G. V.; FERREIRA, M. E. M. C. TEODORO, U. Distribuição geográfica e características

epidemiológicas da leishmaniose tegumentar americana em áreas de colonização antiga do Estado do Paraná, Sul do Brasil. **Cad Saúde Pública**. 24 (6), 1291-1303, 2008.

MINISTÉRIO DA SAÚDE. Secretaria de Vigilância em Saúde.Sistema de informação de Agravos e Notificação - SINAN.Casos confirmados Leishmaniose Tegumentar Americana.. Disponível em: < http://dtr2004.saude.gov.br/sinanweb/tabnet/dh?sinan/lta/bases/ltabr.def> Acesso em 21/01/2014.

MINISTÉRIO DA SAÚDE. Secretaria de Vigilância em Saúde. **Manual de Vigilância da Leishmaniose Tegumentar Americana / Ministério da Saúde, Secretaria de Vigilância em Saúde.** – 2. ed. atual. – Brasília : Editora do Ministério da Saúde. (Serie A. Normas e Manuais Técnicos) 180, 2007.

MOREL, C. M. Inovação em saúde e doenças negligenciadas. **Cadernos de Saúde Pública,** v. 22, p. 1522-1523, 2006. ISSN 0102-311X.

MUKBEL, R. M. et al. Macrophage killing of *Leishmania* amazonensis amastigotes requires both nitric oxide and superoxide. **Am J Trop Med Hyg,** v. 76, n. 4, p. 669-75, Apr 2007. ISSN 0002-9637 (Print)0002-9637.

NEVES, D. P.; MELO, A. L.; LINARDI, P. M.; VITOR, R. W. A. **Parasitologia Humana.** 12 ed. SãoPaulo: Editora Atheneu; 2011.

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, 12// 2002. ISSN 1537-1891. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1537189103000284 >.

PELISSARI, D. M. et al. **Treatment of Visceral *Leishmania*sis and American Cutaneous *Leishmania*sis in Brazil**. Epidemiologia e Serviços de Saúde. Brasilia. 20**:** 107-110 p. 2011.

PETERS, N. C.; EGEN, J.G.; SECUNDINO, N.; DEBRABANT, A.; KIMBLIN, N.; KAMHAWI, S.; LAWIER, P.; FAY, M.P.; GERMAIN, R.N.; SACKS, D. *In vitro* imaging reveals na essential role for neutrophils in leishmaniasis transmitted by sand flies. **Science** 321, 970-973, 2008.

RATES, S. M. K. Plants as source of drugs. **Toxicon,** v. 39, n. 5, p. 603-613, 5// 2001. ISSN 0041-0101. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0041010100001549 >.

REIS, L. C.; BRITO, M. E. F.; SOUZA, M. A.; PEREIRA, V. R. A. Mecanismo imunológico da resposta celular e humoral na Leishmaniose Tegumentar Americana. **Rev Pat Trop.** 35(2), 103-115, 2006.

REITHINGER, R. et al. Cutaneous *Leishmania*sis. **Lancet Infect Dis,** v. 7, n. 9, p. 581-96, Sep 2007. ISSN 1473-3099 (Print) 1473-3099.

REY, L. **Parasitologia.** 3 ed. Rio de Janeiro: Guanabara Koogan, 182-225. 2001.

REVEIZ, L. et al. Interventions for American cutaneous and mucocutaneous *Leishmania*sis: a systematic review update. **PLoS One,** v. 8, n. 4, p. e61843, 2013. ISSN 1932-6203.

RODRIGUES, A. M.; HUEB, M.; SANTOS, T.A.R.R.; FONTES, C.J.F. Fatores associados ao insucesso do tratamento de leishmaniose cutanea com antimoniato de meglumina. **Rev Soc Bras Med Trop.** 39(2) 139-145, 2006

SACKS, D.; NOBEN-TRAUTH, N. The immunology of susceptibility and resistance to *Leishmania* major in mice. **Nat Rev Immunol,** v. 2, n. 11, p. 845-858, 11//print 2002. ISSN 1474-1733. Disponível em: < http://dx.doi.org/10.1038/nri933 >.

SANTOS, A. O. et al. Anti*Leishmania*l activity of diterpene acids in copaiba oil. **Mem Inst Oswaldo Cruz,** v. 108, n. 1, p. 59-64, Feb 2013. ISSN 0074-0276.

SANTOS, A. O. D. et al. Anti*Leishmania*l activity of diterpene acids in copaiba oil. **Memórias do Instituto Oswaldo Cruz,** v. 108, p. 59-64, 2013. ISSN 0074-0276.

SANTOS, S. C. **Caracterização cromatográfica de extratos medicinais de guaco [manuscrito] : Mikania laevigata Schultz Bip. Ex Baker e M. glomerata Sprengel e ação de M. laevigata na inflamação alérgica pulmonar.** Universidade do Vale do Itajaí, 2005.

SCAPINI, P..; LAPINET-VERA, A.; GASPERINI, S. The neutropil as a cellular source of chemokines. **Immunol Reve.** 177, 195-203,2000.

SEN, R.; CHATTERJEE, M. Recent advances in *Leishmania*sis treatment. **International Journal of Infectious Diseases,** v. 15, n. 8, p. e525 - e532, 2011. ISSN 1201-9712. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1201971211000865 >.

SCHRIEFER, A. L. F.; SOUSA, R. S.; GUIMARÃES, L. H.; GÓES-NETO, A., ALBERT SCHRIEFER, A. Papel do Parasita e do Hospedeiro na Expressão Clínica das Leishmanioses. **Gaz. Med. Bahia** 75(1), 46-56, 2005.

SILVA-LÓPEZ, R. E. D. Proteases de *Leishmania*: novos alvos para o desenvolvimento racional de fármacos. **Química Nova,** v. 33, p. 1541-1548, 2010. ISSN 0100-4042.

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. ISSN 0014-2999. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0014299904003784 >.

_____. Hypotensive action of naturally occurring diterpenes: A therapeutic promise for the treatment of hypertension. **Fitoterapia,** v. 81, n. 7, p. 690 - 702, 2010. ISSN 0367-326X. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0367326X10001279 >.

VAN ASSCHE, T. et al. *Leishmania*-macrophage interactions: insights into the redox biology. **Free Radic Biol Med,** v. 51, n. 2, p. 337-51, Jul 15 2011. ISSN 0891-5849.

WORLD HEARTH ORGANIZATION, (WHO) **Global Healt Observatory - Leishmaniasis - Situation and trends.** Disponível em: <http://www.who.int/gho/neglected_diseases/leishmaniasis/en/> Acesso em: 07 fev 2014.

YATSUDA, R. et al. Effects of Mikania genus plants on growth and cell adherence of mutans streptococci. **J Ethnopharmacol,** v. 97, n. 2, p. 183-9, Feb 28 2005. ISSN 0378-8741 (Print) 0378-8741.

3. OBJETIVOS

3.1 Objetivo Geral

Investigar a atividade imunomoduladora e leishmanicida *in vitro* do ácido caurenóico extraído da *Sphagneticola trilobata (L.) Pruski* sobre macrófagos peritoneais de camundongos BALB/c infectados ou não com *L. amazonensis,* bem como elucidar os mecanismos de ação.

3.2 Objetivos Específicos

- Verificar a ação leishmanicida *in vitro* direta do ácido caurenóico sob formas promastigotas de *L. amazonensis*;
- Verificar o potencial leishmanicida de macrófagos peritoneais de camundongos BALB/c infectados com *L. amazonensis* e tratados com ácido caurenóico *in vitro*;
- Determinar os níveis de citocinas (IFN-γ, TNF-α, IL-1β, IL-10, TGF- β e IL-12) em macrófagos peritoneais de camundongos BALB/c infectados ou não com *L. amazonensis* e tratados com ácido caurenóico *in vitro*;
- Quantificar os níveis de óxido nítrico no sobrenadante de cultura de macrófagos peritoneais de camundongos BALB/c infectados ou não com *L. amazonensis* e tratados com ácido caurenóico *in vitro*.
- Determinar os níveis de lipoperoxidação através da mensuração de malondealdeído (MDA) e capacidade antioxidante total (TRAP) em macrófagos peritoneais de camundongos BALB/c infectados ou não com *L. amazonensis;*
- Avaliar a participação do NLRP-12, iNOS e cNOS no efeito leishmanicida em macrófagos peritoneais de camundongos BALB/c infectados com *L. amazonensis;*

4. PRODUÇÃO CIENTÍFICA

4.1. Artigo 1

Immunomodulatory and antioxidant properties of Kaurenoic acid on macrophages *in vitro*

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Original Research Paper

Immunomodulatory and Antioxidant Properties of Kaurenoic Acid on Macrophages of BALB/c *in Vitro*

[1]Juliana Aparecida Macri, [1]Suelen Santos da Silva, [1]Milena Menegazzo Miranda, [1]Natalia Yoshie Kawakami, [2]Thiago Hideki Hayashida, [2]Tiago Bervelieri Madeira, [2]Suzana Lucy Nixdorf, [2]Vinicius Ricardo Acquaro Junior, [3]Sérgio Ricardo Ambrósio, [1]Waldiceu Aparecido Verri Jr, [1]Rubens Cecchini, [1]Ivete Conchon Costa, [2]Nilton Syogo Arakawa and [1]Wander Rogério Pavanelli

[1]Department of Pathological Sciences, Centre for Biological Sciences, State University of Londrina, Londrina, Brazil
[2]Department of Chemistry, Center of Exact Sciences, State University of Londrina, Brazil
[3]Center of Research in Science and Technology, University of Franca, UNIFRAN, Franca, SP, Brazil

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Corresponding Author:
Juliana Aparecida Macri,
Department of Pathological Sciences, Centre for Biological Sciences, State University of Londrina, Londrina, Brazil
Email: carolpanis@sercomtel.com.br

Abstract: Kaurenoic acid has been displaying anti-inflammatory effect described in different models. However, the per se immunomodulatory effects of kaurenoic acid remain to be investigated. Thus, the immunomodulatory and antioxidant effects of kaurenoic acid were investigated *in vitro* on peritoneal macrophages from BALB/c mice. Kaurenoic acid induced per se the production of pro-inflammatory cytokines such as TNFα, IL-1β and IFN-γ while also increased the levels of IL-10. There was also reduction of NO production and induction of anti-oxidant profile. Therefore, in addition to inhibiting inflammation, kaurenoic acid presents immunomodulatory effects per se.

Keywords: Immunomodulation, Kaurenoic Acid, Macrophages, *In Vitro* Study

Introduction

Kaurenoic Acid (ent-kaur-16-en-19-oic acid) (KA) is a tetracyclic diterpene that occurs naturally in several plants (Batista *et al*., 2005). Among the biological effects of this diterpene, studies have shown analgesic activity (Mizokami *et al*., 2012), anti-inflammatory effects in asthma models (Cho *et al*., 2010), antitumoral, antimicrobial and antiprotozoal actions (Costa-Lotufo *et al*., 2002; Wilkens *et al*., 2002; Izumi *et al*., 2012; Santos *et al*., 2013).

In fact, studies *in vitro* have demonstrated the ability of KA in inhibiting the expression of inducible Nitric Oxide Synthase (iNOS) in RAW 264.7 macrophages stimulated with LPS and the expression of Cyclooxygenase-2 (COX-2), consequently decreasing the Nitric Oxide (NO) and Prostaglandin E2 (PGE2) production. The proposed mechanism for immunomodulatory properties was related to inhibition of NFκB activation (Choi *et al*., 2011).

On the other hand, it was demonstrated that KA acts by inducing the activation of Nuclear factor erythroid 2-related factor 2 (Nrf2) which regulates expression of genes that are involved on antioxidant response without affecting NFκB activation (Lyu *et al*., 2011).

As the modulation of cytokines affects many physiological and pathological functions, including innate immunity, acquired immunity and aspects of the inflammatory response, it is important to investigate the action of KA related to possible oxidative stress and immune regulation considering *in vitro* non inflammatory conditions.

Materials and Methods

Kaurenoic Acid

The KA used in this manuscript was obtained from *Sphagneticola trilobata*. The crude extract was obtained from dried roots pulverized and extracted with dichloromethane and partitioned with *n*-hexane and ethyl acetate, all solvents were dried under reduced pressure. The hexane fraction was subjected to VLC by increasing gradient polarity, on the second fraction amorphous compound were washed with cold methanol (200 mg), this compound were analyzed by High Performance Liquid Chromatography (HPLC) methods yielding 96%

of purity. The identification was performed by 1H and 13 C NMR, EIMS and literature data (Da Costa *et al*., 1996).

The stock solution of KA was dissolved in DMSO (Invitrogen-Gibco®) at 2% in all experiments.

Cell Culture and Treatment In Vitro

Macrophages (5×10^5/mL) were obtained from the peritoneal cavity of BALB/c mouse by the injection of 2 mL of RPMI 1640 culture medium supplemented with fetal bovine serum 10% and cultured on 24 well plates for 2 h of adherence. The cells received KA (50, 70 or 90 µM) or medium for 24 h at 37°C and 5% CO_2. *DMSO* concentration did not exceed 0.01% in the wells. Reagents for cell cultures were purchased from Invitrogen-Gibco®. Female BALB/c mice used were obtained from the Fundação Osvaldo Cruz, FIOCRUZ, Curitiba, Brazil. Mice were kept under pathogen-free conditions and used according to protocols approved by Animals Ethics Committee of the Estate University of Londrina (protocol number 33064/2012.42).

Cytokine Production Determination by ELISA

The supernatants obtained from macrophage treated with KA (50, 70, 90 µM) by 24 hours were used for measurement of TNF-α, IFN-γ, IL-1β, TGF-β andIL-10using the technique of capture Enzyme-Linked Immune Sorbent Assay (ELISA) kit from Bioscience (USA). The concentration of cytokines was determined by reference to standard curve for serial dilutions and the optical absorbance measured at 450nm.

Total Antioxidant Capacity of Samples (Trapping Antioxidant Parameter-TRAP)

Samples (50 µL of cell supernatant) were analyzed by chemiluminescence method, for verifying the antioxidant profileas previously described by Repetto *et al*. (1996). Soluble vitamin E (Trolox) was employed as a standard antioxidant. The chemiluminescence curves were obtained using the Glomax luminometer (Promega) and the results are expressed in nM of Trolox.

Malondialdehyde (MDA)

MDA levels were determined using HPLC as previously described by Victorino *et al*. (2013) with slight modifications. The analyses were conducted with an Alliance e2695 HPLC (Waters, Milford, MA, EUA) equipped with a Security Guard ODS-C18 (4×3,0 mm, Phenomenex), C18 reverse phase column (Eclipse XDB-C18; 4,6×250 mm, 5 µm, Agilent) and a photo-diode array detector (Photodiode Array Detector (PDA, 2998)). Analyses were conducted in the Empower 2 software (Waters, Milford, MA, EUA). MDA standards were prepared using 1,1,3,3-Tetraetoxipropane (TEP). Aliquots containing 250 µL of the cells and supernatants were deproteinized by adding trichloric acid 20% and reacted with 1mL of thiobarbituric acid. The mobile phase was constituted with 70% 10 mM KH_2PO_4 buffer, pH 7.0 and 40% HPLC grade methanol. Readings were obtained at 532 nm, following an 8 min isocratic flow at the rate of 1 mL/min. Results were expressed in nM of MDA.

Determination of Nitrite Levels

The determination of nitrite supernatants collected from KA treated cells were used as estimates of the concentrations of Nitric Oxide (NO) by the Griess reagent accordingly to Panis *et al*. (2012) with some modifications. Briefly, supernatant aliquots were recovered and diluted in glycine buffer solution (45 g/L pH 9.7). It was added Cadmium granules previously activated with $CuSO_4$ 5 mM solution to the samples for 10 minutes under stirring. Aliquots of 200 µL were recovered into suitable tubes for determination of nitrite and the same volume of Griess reagent was added. After 10 min incubation at room temperature, the tubes were centrifuged at 10,000 rpm, 2 min, 25°C and added to 96-well micro plates in triplicate. Calibration curve was prepared by dilution of $NaNO_2$ and the absorbance was determined at 550 nm in a microplate reader.

Statistical Analyses

Statistical differences among groups were analyzed using a one-way Analysis Of Variance (ANOVA) follow Tukey test. Data are shown as the means ± Standard Error of the Mean (SEM) and significance was defined as $p<0.05$.

Results

Initially, the KA concentrations tested were evaluated about toxicity by MTT assay of peritoneal macrophages and the concentrations did not present interference on cell viability (data not show).

In attempt of evaluating the immunomodulatory properties of KA, we analyze cytokine production (IFN-γ, IL-1β, TNF-α, IL-10 and TGF-β) in BALB/c peritoneal macrophages treated during 24 h with KA.

We verified that, KA treatment increased the IFN-γ and IL-1β production of concentration dependent manner, with a significant increase at 70 and 90 µM (Fig. 1A and B). The TNF-α levels were increased by KA treatment only at the concentration of 70 µM (Fig. 1C).

On the other hand, the IL-10 levels were increased in all concentration tested (Fig. 1D). Therefore, KA treatment did not affect the levels of TGF-β (Fig. 1E).

In order to evaluate the role of KA about some oxidative stress parameters, we observed that this diterpene diterpene promoted increasing of total antioxidant capacity (TRAP) (Fig. 2A) in all the concentration used. This capacity was also observed in reduction of lipid per oxidation (malondialdehydeassay) (Fig. 2B) as well as in the nitrite dosage (Fig. 3).

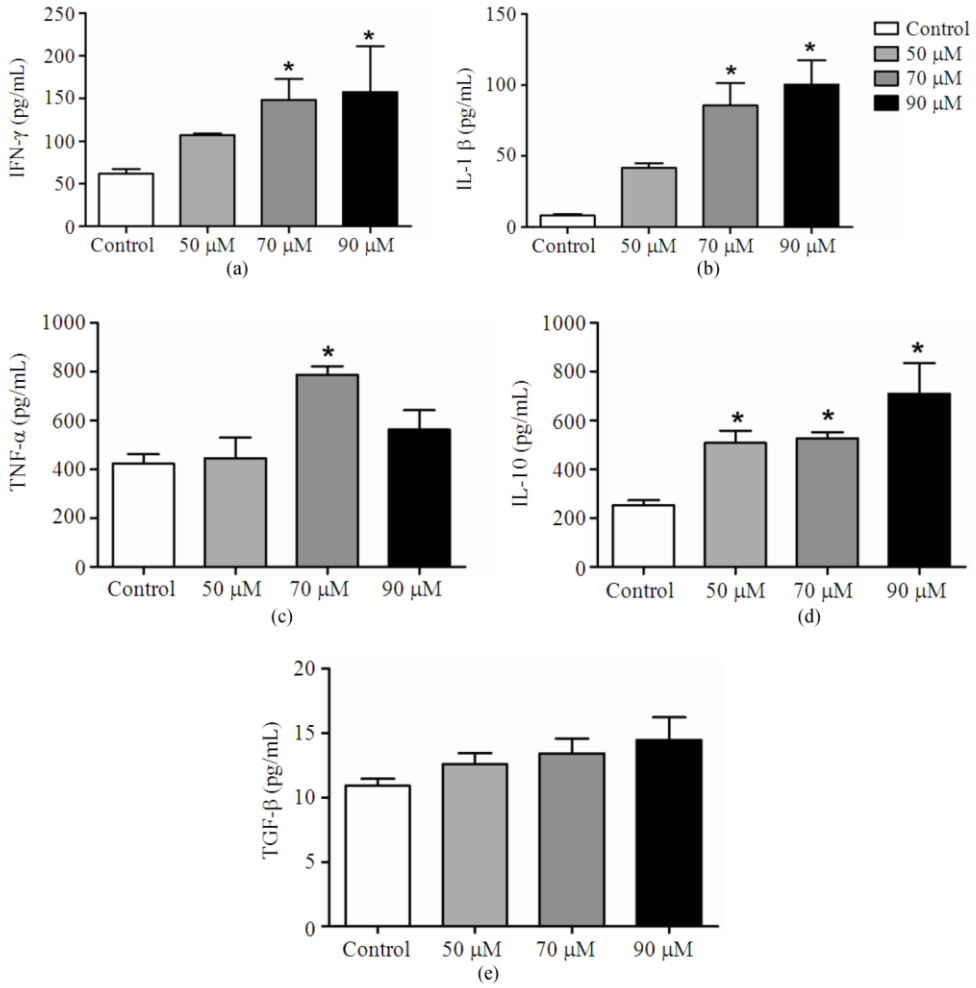

Fig. 1. Mapping the cytokine profile produced *in vitro* by macrophages treated with Kaurenoic acid (50, 70, 90 µM) for 24 h detected by ELISA. IFN-γ production (Panel A), IL-1βproduction (Panel B), TNF-α production (Panel C), IL-10 production (Panel D) and TGF-β production (Panel E). Data represent the mean ± SEM of three independent experiments

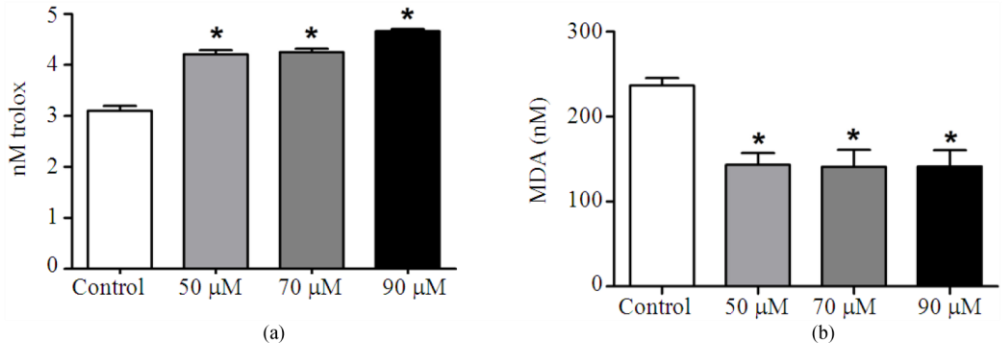

Fig. 2. Parameters about oxidative stress of macrophages treated with kaurenoic acid (50, 70, 90 µM) for 24 h. Total Antioxidant Capacity (TRAP) measurement by chemiluminescence (Panel A). Lipoperoxidation (malondialdehyde-MDA) levels measurement by High Performance Liquid Chromatography (HPLC) (Panel B). Data represent the mean ± SEM of three independent experiments

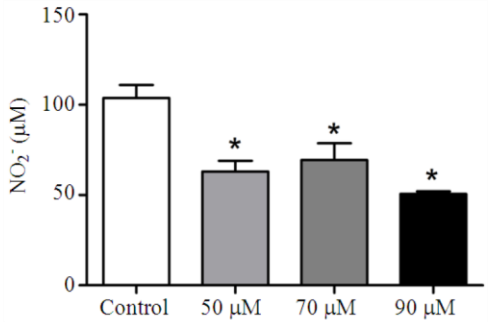

Fig. 3. Nitrite levels produced by macrophages treated with kaurenoic acid (50, 70, 90 µM) for 24 h. Data represent the mean ± SEM of three independent experiments

Discussion

Immunomodulation consists on the adjustment of the immune response by agents (endogenous or exogenous) that activate or suppress the immune response (Dutta, 2002). The class of immunomodulatory drugs presents a wide range of critical biological effects for a variety of therapeutic approaches including immunotherapies against cancer, infectious diseases, treatment of autoimmune disorders and allergies, transplant surgeries and regenerative medicine (Zimmerman, 2009; Purwada *et al*., 2013).

Macrophages, for being part of the first line of defense, play an important role in the early immune response mainly with the production of cytokines that will define the response pattern (Dinarello, 2000).

Therefore, one of the main targets of immunotherapy consists in modulating the secretion of cytokines that is responsible for the communication between cells that will determine the type, quality, amplitude, duration and outcome of the immune response (Bouabe, 2012).

In this present study, to evaluate the profile of immune response after the treatment of KA, we verified that this diterpene was able to activate the synthesis of pro-inflammatory cytokines: IFN-γ (Fig. 1A), IL-1β (Fig. 1B), TNF-α (Fig. 1C) demonstrating for the first time that KA presents its own effects independently of an inflammatory stimulus.

These results corroborate with earlier study that demonstrated that KA did not inhibit the expression of pro-inflammatory cytokines (Lyu *et al*., 2011) permitting to infer that KA presents immunomodulatory properties.

On the other hand, our results corroborate with the findings of Choi *et al*. (2011) that observed an inhibitory effect of KA on LPS induced in flammatory response.

Concerning of IL-10 function its known the ability to inhibit the production of other cytokines, such as TNF-α, IL-1β, IL-6 e IL-8 (Poole *et al*., 1995). In addition, the classic production of Reactive Oxygen Species (ROS) and Nitric Oxide (NO) is dependent of cytokines like IFN-, TNF-α and IL-1β. However, our findings reinforce this role of IL-10 since even with the IFN-γ, TNF-α and IL-1β synthesis, the parameters analyzed for ROS and NO were decreased, demonstrating the prevalence of IL-10 on pro-inflammatory cytokines (Fig. 1D).

Haddad and Fahlman (2002) demonstrated that IL-10 was described the anti-inflammatory cytokine with an antioxidant properties.

Additionally, Lyu *et al*. (2011) reported that KA induces the Nrf2 activation, which regulates inducible antioxidant responses attenuating oxidative stress, consequently the inflammatory response (Lee and Johnson, 2004; Kim *et al*., 2011).

This result suggested that KA can be used to control the damage provoked by inflammatory response by the modulation of the oxidants effects.

Corroborating that KA induces Nrf2 activation (Lyu *et al*., 2011), it induced an increase of total antioxidant capacity (TRAP) and reduced lipid peroxidation (malondialdehyde-MDA).

KA reduced the NO levels in LPS-stimulatedRAW264.7 (Choi *et al*., 2011). In the present study, KA reduced the basal NO levels in primary macrophage culture. Choi *et al*. (2011) data correlated with the inhibition of iNOS expression and activation of NFκB.

The controversial effects on *in vitro* inflammatory pathways previously demonstrated can be explained by differences of strategy study adopted like time accessing of substances produced, stimulus, concentration and treatments used.

Conclusion

This study evidenced the immunomodulatory property of Kaurenoic acid on *in vitro* primary macrophages culture. Therefore, it is important to consider the immunomodulatory effects of KA during non-inflammatory conditions.

Author's Contributions

All authors equally contributed in this work.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

References

Batista, R., F.C. Braga and A.B. Oliveira, 2005. Quantitative determination by HPLC of *ent*-kaurenoic and grandiflorenic acids in aerial parts of *Wedelia paludosa* D.C. Revista Brasileira de Farmacognosia, 15: 119-125. DOI: 10.1590/S0102-695X2005000200009

Bouabe, H., 2012. Cytokine reporter mice: The special case of IL-10. Scandinavian J. Immunol., 75: 553-567. DOI: 10.1111/j.1365-3083.2012.02695.x

Cho, J.H., J.Y. Lee, S.S. Sim, W.K. Whang and C.J. Kim, 2010. Inhibitory effects of diterpene acids from root of *Aralia cordata* on IgE-mediated asthma in guinea pigs. Pulmonary Pharmacol. Therapeut., 23: 190-199. DOI: 10.1016/j.pupt.2009.12.004

Choi, R.J., E.M. Shin, H.A. Jung, J.S. Choi and Y.S. Kim, 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

Costa-Lotufo, L.V., G.M. Cunha, P.A. Farias, G.S. Viana and K.M. Cunha *et al*., 2002. The cytotoxic and embryotoxic effects of kaurenoic acid, a diterpene isolated from *Copaifera langsdorffii* oleo-resin. Toxicon, 40: 1231-234. DOI: 10.1016/S0041-0101(02)00128-9

Da Costa, F.B., W. Vichnewski and W. Herz, 1996. Constituents of *Viguiera aspillioides* and *V. robusta*. Biochemical Systemat. Ecol., 24: 585-587. DOI: 10.1016/0305-1978(96)00057-9

Dinarello, C.A., 2000. Proinflammatory cytokines. Chest, 118: 503-508. DOI: 10.1378/chest.118.2.503

Dutta, R.C., 2002. Peptide immunomodulators versus infection; an analysis. Immunol. Lett., 83: 153-161. DOI: 10.1016/S0165-2478(02)00066-4

Haddad, J.J. and C.S. Fahlman, 2002. Redox- and oxidant-mediated regulation of interleukin-10: An anti-inflammatory, antioxidant cytokine? Biochemical Biophys. Res. Commun., 297: 163-176. DOI: 10.1016/S0006-291X(02)02094-6

Izumi, E., T. Ueda-Nakamura, V.F. Jr. Veiga, A.C. Pinto and C.V. Nakamura, 2012. Terpenes from Copaifera demonstrated *in vitro* antiparasitic and synergic activity. J. Med. Chem., 55: 2994-3001. DOI: 10.1021/jm201451h

Kim, K.H., J.H. Lyu, S.T. Koo, S.R. Oh and H.K. Lee *et al*., 2011. MyD88 is a mediator for the activation of Nrf2. Biochem. Biophys. Res. Commun., 404: 46-51. DOI: 10.1016/j.bbrc.2010.11.051

Lee, J.M. and J.A. Johnson, 2004. An important role of Nrf2-ARE pathway in the cellular defense mechanism. J. Biochem. Molecular Biol., 37: 139-43. DOI: 10.5483/BMBRep.2004.37.2.139

Lyu, J.H., G.S. Lee, K.H. Kim, H.W. Kim and S.I. Cho *et al*., 2011. ent-kaur-16-en-19-oic acid, isolated from the roots of *Aralia continentalis*, induces activation of Nrf2. J. Ethnopharmacol., 137: 1442-1449. DOI: 10.1016/j.jep.2011.08.024

Mizokami, S.S., N.S. Arakawa, S.R. Ambrosio, A.C. Zarpelon and R. Casagrande *et al*., 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. J. Nat. Products, 75: 896-904. DOI: 10.1021/np200989t

Panis, C., A.C. Herrera, V.J. Victorino, F.C. Campos and L.F. Freitas *et al*., 2012. Oxidative stress and hematological profiles of advanced breast cancer patients subjected to paclitaxel or doxorubicin chemotherapy. Breast Cancer Res. Treatment, 133: 89-97. DOI: 10.1007/s10549-011-1693-x.

Poole, S., F.Q. Cunha, S. Selkirk, B.B. Lorenzetti and S.H. Ferreira, 1995. Cytokine-mediated inflammatory hyperalgesia limited by interleukin-10. British J. Pharmacol., 115: 684-688. PMID: 7582491

Purwada, A., K. Roy and A. Singh, 2013. Engineering vaccines and niches for immune modulation. Acta Biomaterialia, 10: 1728-1740. DOI: 10.1016/j.actbio.2013.12.036

Repetto, M., C. Reides, G.M.L. Carretero, C. Marta and G. Griemberg *et al*., 1996. Oxidative stress in blood of HIV infected patients. Clin, Chimica Acta, 255: 107-117. DOI: 10.1016/0009-8981(96)06394-2

Santos, A.O., E. Izumi, T. Ueda-Nakamura, B.P. Dias-Filho and V.F. Veiga-Junior *et al*., 2013. Antileishmanial activity of diterpene acids in copaiba oil. Memórias do Instituto Oswaldo Cruz, 108: 59-64. DOI: 10.1590/S0074-02762013000100010

Victorino, V.J., C. Panis, F.C. Campos, R.C. Cayres and A.N. Colado-Simao *et al*., 2013. Decreased oxidant profile and increased antioxidant capacity in naturally postmenopausal women. Age, 35: 1411-1421. DOI: 10.1007/s11357-012-9431-9

Wilkens, M., C. Alacón, A. Urzúa and L. Mendonza, 2002. Characterization of the bactericidal activity of the natural diterpene kaurenoic acid. Planta Medica, 68: 452-454. DOI: 10.1055/s-2002-32086

Zimmerman, T., 2009. Immunomodulatory agents in oncology. Update Cancer Therapeut., 3: 170-181. DOI: 10.1016/j.uct.2009.03.003

4.2. Artigo 2

Kaurenoic acid isolated from *Sphagneticola trilobata* exerts antileishmanial activity by up-regulating NO levels and the pro-inflammatory mediators NLRP-12 and IL-1β in a cNOS-dependent mechanism.

Artigo a ser submetido a revista: Parasite immunology.

FULL ARTICLE

Kaurenoic acid isolated from *Sphagneticola trilobata* exerts antileishmanial activity by up-regulating NO levels and the pro-inflammatory mediators NLRP-12 and IL-1β in a cNOS-dependent mechanism

Juliana Aparecida Macri [1†], Carolina Panis [1†], Milena Menegazzo Miranda [1†], Suelen Santos da Silva [1†], Natalia Yoshie Kawakami[1] , Thiago Hideki Hayashida[2], Tiago Bervelieri Madeira [3], Suzana Lucy Nixdorf [3], Vinicius Ricardo Acquaro Junior [3], Luciana Pizzatti [4], Rubens Cecchini [1], Waldiceu Aparecido Verri Jr [1], Ivete Conchon Costa [1], Nilton Syogo Arakawa [2], Wander Rogério Pavanelli [*, 1]

1 Departamento de Ciências Patológicas - Centro de Ciências Biológicas, Universidade Estadual de Londrina, 86051990 Londrina, Brazil

2 Departamento de Ciências Farmacêuticas - Centro de Ciências de Saúde, Universidade Estadual de Londrina, 86039440 Londrina, Brazil

3 Departamento de Química, Centro de Ciências Exatas, Universidade Estadual de Londrina, Brazil

4 Departamento de Bioquimica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

[†]*authors equal contribution*

*Author to whom correspondence should be addressed Tel: + 55 43 3371 4539. Fax: +55 43 3371 4387. E-mail: wanderpavanelli@uel.br or wanderpavanelli@yahoo.com.br

ABSTRACT

Leishmania (Leishmania) amazonensis (*L. amazonensis*) infection can cause severe local and diffuse injuries in humans, a condition clinically known as American Cutaneous Leishmaniasis (ACL). Nowadays, there is no effective therapeutic approach for ACL, which remains a neglected disease. Here we propose the use of the diterpene kaurenoic acid [*ent*-kaur-16-en-19-oic acid] in an *in vitro* experimental model of leishmaniasis. Ours results showed that kaurenoic acid exhibit direct antileishmanial effect on *L. amazonensis* promastigotes. This effect was better observed during the infection of susceptible macrophages, which kaurenoic acid caused significant reduction in the percentage of infected macrophages besides diminished intracellular number of amastigotes. In addition, the kaurenoic acid treatment restablished the production of nitric oxide (NO), subverting the NO-depleting escape mechanism of *L.amazonensis*. Moreover, we identified the production of NO induced by kaurenoic acid is dependent on cNOS activity and correlated with increased the levels of active IL-1β and by upregulated the expression of the inflamossome-activating component NLRP-12. These findings identify a possible concentration-dependent mechanism by which kaurenoic acid acts for enhancing the *in vitro* leishmanicidal capability of macrophages against *L. amazonensis*.

key words: American Cutaneous Leishmaniosis, Kaurenoic acid, Immune-modulatory property, NLRP-12.

Introduction

American Cutaneous Leishmaniasis (ACL) is caused by the protozoa *Leishmania* spp. that displays distinct clinical manifestations depending upon the parasite species and the immune status of the host. Therefore, the disease may appear on host as cutaneous, mucocutaneous or diffuse forms (1).

The treatment of this severe disease is based on chemotherapy with the antimonials sodium stibogluconate (Pentostam®) and antimonate N-methyl-glucamine (Glucantime®). In lack of response, second-line drugs as amphotericin B or pentamidines are used (2). However, these drugs frequently exhibit high toxicity, which has been related to its restricted use. An aggravating factor is the existence of several reports of chemoresistant patients to such treatments (2-5). Therefore, the development of new therapeutic approaches for this neglected disease is urgent and remains an unsolved challenge.

Nowadays, several natural compounds have been investigated as potential sources of leishmanicidal activity. An interesting molecule to this aim is the kaurenoic acid [*ent*-kaur-16-en-19-oic acid], a diterpene obtained from several brazilian plants (6, 7). This molecule has been reported as presenting a wide of biological activities such as antiprotozoal(8, 9), antimicrobial (10), antinociceptive (11), vasorelaxant, hypotensive (12, 13) and anti-inflammatory activities (11, 14, 15). Moreover, some immunomodulatory properties of kaurenoic acid have been reported in this models.

The antiprotozoal activity of this diterpene involves its direct action, by altering the cell membrane integrity and the mitochondrial membrane depolarization on promastigote and amastigote forms of *L. amazonensis* (16) and epimastigote forms of *Trypanosoma cruzi* (17) . This direct activity of kaurenoic acid against the protozoa is not enough to reflect its overall potential as a therapeutic leishmanicidal drug, since *Leishmania* spp. parasites replicate intracellularly in macrophages and have several depleting mechanisms of microbicidal

molecules that are not dependent of the direct action of antileishmanicidal drugs (18-21). The macrophage is the main target cell for perpetuating the *Leishmania* infection; thus, their use on experimental model is crucial for reveal the reliable therapeutic potential of some componds.

In this study we proposed to evaluate the *in vitro* effect of the kaurenoic acid on susceptible macrophages from BALB/c mice infected with *L. amazonensis* promastigote forms. Thus, we performed assays to investigate effect of kaurenoic acid on direct parasites or modulatory actions on infected macrophages, as well as investigated a putative mechanism of action of this compound.

MATERIALS AND METHODS

Protozoan

L. amazonensis (MHOM/BR/1989/166MJO) was used in promastigote forms, kept in culture medium 199 (Invitrogen-GIBCO®), and supplemented with 10% fetal bovine serum (Invitrogen-GIBCO®), 1M Hepes, 0.1% human urine, 0.1% L-glutamine, 10µg/mL penicillin and streptomycin (Invitrogen- GIBCO®), and 10% sodium bicarbonate (complete medium for promastigotes—CMP). Cell cultures were incubated at 25°C in 25cm^2 flasks.

Animals

Female BALB/c mice weighing approximately 25-30g and aged 6-8 weeks old were obtained from the Fundação Osvaldo Cruz, FIOCRUZ, Curitiba, Brazil. Mice were kept under pathogen-free conditions and used according to protocols approved by the Institutional Animal Care.

The procedures were approved by the Ethics Committee of the Universidade Estadual de Londrina (protocol number 33064/2012.42). Every effort was made to minimize the number of animals used and their suffering.

Plant Material

The kaurenoic acid used in this manuscript was obtained from *Sphagneticola trilobata.* The crude extract kaurenoic acid (1.2 g) was obtained from dried roots pulverized and subjected to extraction with dichloromethane and then partitioned with n-hexane and ethyl acetate, with subsequent drying of the solvent. After analysis by CCDC, the hexane fraction was subjected to CLV in increasing polarity gradient yielding 10 subfractions. We performed the process of washing with cold methanol in the second subfraction, isolating amorphous crystals (200 mg). The kaurenoic acid isolated and used in the assay presented with 96% purity (by HPLC) and their identification was performed by 1H and 13C NMR, EIMS and comparison with authentic standard and literature data. Kaurenoic acid was dissolved in DMSO (Invitrogen- Gibco®) at 2% in all experiments.

Viability of Promastigotes

The viability of *L. amazonensis* promastigote forms treated with kaurenoic acid was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (22). Promastigotes forms (10^6/100µl) were incubated with different concentrations of kaurenoic acid (10, 30, 50, 70 e 90µM) or with kaurenoic acid solvent (0.2% DMSO/mL) and maintained in culture for 24, 48 and 72 hours at 25º C. Thereafter, was added 10µL of MTT (5mg/mL) and incubated for an additional time 4 hour at 24°C. For read in spectrophotometer we used 300µL of solution of dimethyl sulfoxide (DMSO) and this content was transferred to 96 well-plates in a final volume of 100µL each well and the absorbance was determined at 550 nm. The results were expressed as percentage of MTT reduction having parameter the control group, which was assigned to 100% reduction of formazan (100% of viable promastigotes).

Kinetics of Cellular Proliferation

Promastigote forms (10^6/mL) incubated in CMP were treated with different concentrations of kaurenoic acid (10, 30, 50, 70 and 90 µM/mL) or with kaurenoic acid solvent (0.2%

DMSO/mL) and cultured for 5 days at 25°C. Promastigotes were counted in a Neubauer chamber after 24, 48, 72 and 120 hours.

Phagocytic Assay

Macrophages (5×10^5/mL) were obtained from the peritoneal cavity by the injection of 2mL of RPMI 1640 culture medium (Invitrogen- GIBCO®) supplemented with fetal bovine serum 10% (Invitrogen-GIBCO®) and cultured on 24- well plates containing 13mm diameter glass coverslips. Cells were pre-incubated with 200µL RPMI medium for 2 hour for adherence, and incubated with promastigotes forms (5:1) for 2 hour. Treated with kaurenoic acid (50, 70 or 90µM/mL) or medium for 24 hours at 37°C and 5% CO_2. The cells were stained with Giemsa to establish the phagocytic index of infection (by percentege) and the parasites/macrophages (mean). The supernatant was utilized to measure the levels of malondialdehyde (MDA), total antioxidant capacity of plasma (TRAP), nitric oxide (NO) and cytokines.

Mensurement of total antioxidant capacity of samples (Trapping Antioxidant Parameter-TRAP)

Samples (50 uL of supernatant with cells) from phagocytic assay were analyzed as previously described to Repetto et al.(23) using the chemiluminescence method. Soluble vitamin E (Trolox) was employed as a standard antioxidant. The chemiluminescence curves were obtained using the Glomax luminometer (Promega), and the results are expressed in nM of Trolox.

Mensurement of lipid perocidation malondialdehyde (MDA)

MDA levels were determined using high performance liquid chromatography (HPLC) as previously described by Victorino et al. (2012) (24) with slight modifications. The analyses were conducted with an Alliance e2695 HPLC (Waters, Milford, MA, EUA) equipped with a

SecurityGuard ODS-C18 (4 x 3,0 mm, Phenomenex), C18 reverse phase column (Eclipse XDB-C18; 4,6 x 250 mm, 5 µm, Agilent) and a photo-diode array detector (Photodiode Array Detector – PDA, 2998). Analyses were conducted in the Empower 2 software (Waters, Milford, MA, EUA). MDA standards were prepared using 1,1,3,3-tetraetoxipropane (TEP). Aliquots containing 250 uL of the cells+supernatants were deproteinized by adding trichloric acid 20% and reacted with 1 mL of thiobarbituric acid. The mobile phase was constituted with 70% 10 mM KH_2PO_4 buffer, pH 7.0, and 40% HPLC grade methanol. Readings were obtained at 532 nm, following an 8 min isocratic flow at the rate of 1 mL/min. Results were expressed in nM of MDA.

Determination of nitrite levels

The determination of nitrite supernatants collected from phagocytic tests were used as estimates of the concentrations of nitric oxide (NO) by the Griess reagent accordingly to Panis et al. (2011) with some modifications (25). Briefly, the supernatant aliquots were desproteinized by adding 50 µL of $ZnSO_4$ 75 mM solution and 70µL of NaOH, shaked and centrifuged at 10000 rpm, 5 minutes, 25° C. The limpid supernatant was recovered and diluted in glycine buffer solution (45g/L pH 9.7). Cadmium granules were rinsed in distilled sterile water and added to a $CuSO_4$ 5mM solution in glycine-NaOH buffer (15 g/L, pH 9.7) during 5 minutes and the copper-coated cadmium granules were used within 10 minutes. Activated granules were added to glycine buffer diluted supernatant and stirred during 10 minutes. Aliquots of 200 µL were recovered in appropriated tubes to nitrite determination and the same volume of Griess reagent was added. After an incubation of 10 minutes at room temperature, tubes were centrifuged at 10000 rpm, 2 minutes, 25°C and added to 96 wells microplates in triplicate. Calibration curve was prepared by dilution of $NaNO_2$ and the absorbance was determinate at 550 nm in a microplate readers.

Cytokine Determination

The supernatants obtained from phagocytic assay were used for to establish the levels of IL-1β, IL-12, TNF-α, IFN-γ, TGF-β and IL-10 using the technique of capture Enzyme-Linked Immune Sorbent Assay (ELISA) by kit from eBioscience (USA). The concentration of cytokines was determined by reference to standard curve for serial dilutions, and the optical absorbance measured at 450nm.

Immunocytochemistry Labeling for NLRP-12 and iNOS

Immunocytochemistry for NLRP-12 and inducible nitric oxide synthase (iNOS) were performed on coverslip-adherent cells (cell prepared as the protocol in item phagocytic assay) using the labeled streptavidin biotin method by LSAB KIT (DAKO Japan, Kyoto, Japan) without microwave accentuation. The coverslips were incubated with 10% Triton X-100 solution during 1 hour, washed 3 times in PBS and treated for 40 min at room temperature with 10% BSA. In addition, coverslips were incubated overnight at 4°C with the primary antibody (anti-NALP12 rabbit polyclonal antibody dilute 1:300, Abcam, catalog number ab93113 and anti-iNOS rabbit monoclonal antibody diluted 1:200, BD Biosciences, catalog number 610599). After secondary antibody treatment (2 hours, room temperature), horseradish peroxidase activity was visualized by treatment with H_2O_2 and 3,3'-diaminobenzidine (DAB) for 5 min. At the last step, the sections were weakly counterstained with Harry's hematoxylin (Merck). For each case, negative controls were performed by omitting the primary antibody. Intensity and localization of immunoreactivities against primary antibody used were examined on all coverslips using a photomicroscope (Olympus BX41, Olympus Optical Co., Ltd., Tokyo, Japan). For the image analysis study, photomicroscopic colour slides of representative areas (objective lens x 40) were digitally acquired. For determining a semi-quantitative scoring,

images were evaluated by using the color de-convolution tool from the Image J software (NIH, USA). Pixels were categorized as previously described by Chaterjee et al., 2013 as high positive (3+), positive (2+), low positive (1+) and negative (0).

cNOS inhibition assay

Peritoneal macrophages received infection and treatment as described in item phagocytic assay. However, for this test, before treatment with kaurenoic acid, the cells were incubated with NG-nitro-L-arginine methyl ester (L-NAME) 20µM for 2 hours at 36°C and 5% CO_2 (26). The supernatant was utilized to measure NO levels (by determination of nitrite levels, previously described).

Statistical analysis

Data were analyzed using the PrismGraphPad statistical software (GraphPad Software, Inc.,USA, 500.288). Significant differences between treatments were determined by one way ANOVA, followed by the Tukey's test for multiple comparisons. Statistical significance was accepted when $P < 0.05$.

RESULTS

In the first set of experiments, the antileishmanial effect of kaurenoic acid was investigated on the promastigote forms of *L. amazonensis.* The concentrations 50, 70 and 90µM promoted a reduction of aproximately 34 % of promastigote viability in MTT assay in all periods tested (Figure 1A).

We also verify a reduction of 22.8%, 45.75% and 51.07% in the proliferation of promastigotes in 50, 70 and 90μM respectively by 120 hours (Figure 1 B). The solvent (DMSO 2%) did not interfere in promastigotes *L. amazonensis* viability and proliferation (Figures 1 A and B).

To investigate the immunodulatory effects of kaurenoic acid on infected macrophages, we challenged these cells with promastigote forms of *L. amazonensis* for 2 hours. After this period and subsequent phagocytosis cells were treated with kaurenoic acid for 24 hours. The count of macrophages and amastigotes were performed for establish the phagocytic index by means of % of infection and the parasites/macrophages number.

Regarding the percentage of infected macrophages incubated with kaurenoic acid, all tested concentrations induced a significant reduction (27% approximately) when compared to the infected untreated macrophages (Figure 2A). Moreover, the mean of amastigotes per macrophage was significantly decreased at the concentrations of 70 and 90uM, with 21.5% and 20.3% of reduction, respectively (Figure 2B).

There are several reports about the antioxidant and immunomodulatory capacity of this diterpene in other models. However, parasites of the genus *Leishmania* also exhibit influence on the oxidative status after phagocytosis. To assess the involvement of kaurenoic acid in modulating the respiratory burst in this model, we measured the oxidative stress status of macrophages treated with this compound. To reach this goal we quantified the levels of the total antioxidant capacity (TRAP), malondialdehyde (MDA) and NO levels of supernatants of phagocytic assay.

It was observed an increase in TRAP of macrophages infected with *L. amazonensis* and treated with 50 and 70 μM of kaurenoic acid (Figure 3A). However, the treated cells did not differ of control cells about of MDA levels (Figure 3B).

When assessed levels of nitrite, our results showed that macrophages infected with *L. amazonensis* displayed decreased levels of NO. Interestingly, the treatment with kaurenoic acid reestablished NO basal levels in all concentrations tested (Figure 3C).

Several studies have demonstrated the importance of cytokines during the early stages of experimental leishmaniasis, suggesting that some cytokines may drive the clinical manifestation of ACL by modulating the resistance or susceptibility to infection (30). The IL-1β, IL-12, TNF-α and IFN-γ are cytokines essential for the development of an effective immune response against *Leishmania* spp., leading to activation of macrophages burst and its microbicidal effects against this parasite (21, 31-33).

In order to verify the immunomodulatory action of kaurenoic acid on influence of NO levels of infected macrophages we measured the levels of IL-1β, IL-12, TNF-α, IFN-γ, TGF-β and IL-10. Only levels of active IL-1β was exhibited significantly augmented at 70 and 90 μM (Figure 4A and Fig. S1).

The production of this cytokine is dependent of active inflammasome complex. The structural diversity of agents that activate the inflamassome, combined with lack of information about inflammasome complex in leishmaniasis we tested NLRP-12 one member of subfamily NLRP able to activate this complex. As shown in Figure 4B, kaurenoic acid was able to up-regulate NLRP-12 expression in macrophages infected with *L. amazonensis* at the concentration of 90μM.

For assessing the iNOS and cNOS role as responsible on for the augment in NO production indiced by 90μM of kaurenoic acid, iNOS expression was evaluated by imunocitochemistry whereas cNOS participation was investigated by its selective inhibition by L –NAME 20μM.

The expression of iNOS was not affected by any concentration of kaurenoic acid (Figure 5A). On the other hand, cNOS blockage showed a substantial reduction in NO production, indicating

that the augmented NO previously observed at 90 μM of kaurenoic acid is dependent on cNOS activity (Figure 5B).

DISCUSSION

Previous *in vitro* studies demonstrated that kaurenoic acid have direct antileishmanial activity on promastigote and amastigote forms of *L. amazonensis* (17, 34, 35). The proposed mechanism was related to significant increases in plasma membrane permeability and mitochondrial membrane depolarisation of the protozoan.

In the model proposed herein kaurenoic acid showed directly inhibits the viability and proliferation of *Leishmania amazonensis* promastigote forms (Figure 1). However, the immunomodulatory activity in experimental leishmaniasis model is still unknown, which reinforces the need for investigating the leishmanicidal effects of kaurenoic acid in a macrophage-based system, once the parasites of the genus *Leishmania* are obligatory intracellular microrganisms (20). This way, we performed a series of methodological approaches aiming to unravel if kaurenoic acid could enhance the leishmanicidal acitivity of susceptible macrophages, as well as the putative mechanism enrolled in this phenomenon.

The infected macrophages treated with kaurenoic acid are more effective on the phagocytic activity of intracellular forms of *L. amazonensis* (Figure 2), suggesting that this diterpene could revert the microbicidal machinery on macrophages active after infection, which depleted by *Leishmania* spp.(18, 21, 31, 36).

NO production is the main antileishmanial molecules produced in early macrophage response activated against intracellular parasites is the respiratory burst.

Our data showed that NO levels did not undergone the action of inhibition by *L. amazonensis* on cells treated with the diterpene (Figure 3C).

Thus, aiming to investigate the mechanisms of NO production triggered by kaurenoic acid, we further mapped the cytokine profiling of treated macrophages and the putative mechanisms enrolled in the immunomodulatory effects.

Leishmania spp. protozoa is able in downregulation of pro-inflammatory cytokines of initial immune response (19,20). In this model, the IL-12, TNF-α, IFN-γ, TGF-β and IL-10 levels showed no difference when compared to the infected control (Fig. S1). The cytokines TNF-α and IFN- γ are classically related to the activation of the NO pathway by inducible nitric oxide synthase (iNOS), resulting in sustained NO production. However, we identify IL- 1β has only stimulus for the production of this molecule microbicide (Figure 4A), which agrees with with evidence that this pro-inflammatory citokine is sufficient for trigger this pathway in leishmaniasis (32).

IL-1β is a pro-inflammatory cytokine that become active after its cleavage by the inflamassoma complex. This complex requires several acessory molecules for trigger this process successfully (37). Most of the inflammasomes are compound by a NOD-like receptor (NLR) sensor molecule, namely NLRP (NOD-, LRR- and pyrin domain-containing or CARD-containing) (38).

A recent study have shown the involvement of the IL-1β-inflammasome-NLRP-3 complex in host resistance to *Leishmania* infection (32).

The inflamossome complex can be formed by several types of NLRPs, most of them are completely unknown in leishmaniasis. Analysis of NLRP-12 expression by immunocytochemistry showed that this inflammassome component was not expressed in untreated infected macrophages (Figure 4B). Altogheter, these findings suggested a linkage for the augmented NO, enhanced IL-1β production and upregulated NLRP-12 expression in infected macrophages treated with kaurenoic acid at 90μM.

The upregulation of sustained NO production is dependent on the activation of Nitric Oxide Synthases (NOS). The main known mechanism for NO production started by intracellular parasites on macrophages is the enhancement and re-synthesis of inducible NOS (iNOS) (39). Constitutive NOS (cNOS) activation is an alterantive route for NO production (40, 41), underinvestigated in parasitic infections as leishmaniasis. Considering the augment in NO induced by kaurenoic acid at 90μM, we identify the cNOS participation in this process (Figure 5).

Some studies have previously reported cNOS involvement during kaurenoic acid treatment on vasorelaxant effect (12). Despite evaluate only in 24 hours, we believe that in our study the microbicide activity of cNOS-derived NO may be possibly by the reaction with superoxide anion yielding the killing molecule peroxinitrite (42).

Our results demonstrated the involment of NO as mechanism of the leishmanicidal activity induced by kaurenoic acid on *L. amazonensis* infected macrophages. The mechanism disclosed herein may be mediated by kaurenoic acid-driven cNOS activation by active IL-1β, putatively processed by NLRP12-inflammossome complex. The use of kaurenoic acid presents a therapeutic potential against ACL, but further in *in vivo* effects are necessary, as well chemical studies for developing molecular analogs of kaurenoic acid with enhanced antileishmanial potential.

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Juliana Aparecida Macri: Conducted and analyzed the experiments: Viability of promastigotes, kinetics of cellular proliferation, phagocytic assay and Writing;

Carolina Panis: Conducted and analyzed the experiments: TRAP, Determination of nitrite levels and immunocytochemistry. Besides, participated of designed research study and contributing in discussion and manuscript writing;

Milena Menegazzo Miranda: Conducted and analyzed the experiment: cNOS inhibition assay and participated of designed research study. Besides axillary writing and discussion of the manuscript.

Suelen Santos da Silva: Conducted and analyzed the experiment Cytokine Determination by ELISA and participated of designed research study. Besides assisting in the writing and discussion of the manuscript.

Natalia Yoshie Kawakami: Assistance in the experiments, maintenance of animals and culture of *L. amazonensis*;

Thiago Hideki Hayashida: Assistence on kaurenoic acid extraction;

Tiago Bervelieri Madeira: Performed experiments about MDA;

Suzana Lucy Nixdorf: Performed experiments about MDA;

Vinicius Ricardo Acquaro Junior: Performed experiments about MDA;

Waldiceu Aparecido Verri Junior: Contributed with citokyne kits and Support on discussion about the date and writing of manuscript;

Luciana Pizzatti: Contributed with immunocytochemistry kits;

Rubens Cecchini: Contributed with TRAP, Determination of nitrite levels kits;

Ivete Conchon Costa: Contributed with discussion about the date;

Nilton Syogo Arakawa: Performed kaurenoico acid extraction;

Wander Rogério Pavanelli: Designed the research study and discussion about the date.

References

1. Kaye P and Scott P. Leishmaniasis: complexity at the host–pathogen interface. *Nat Rev Micro* 2011; **9**: 604-615.

2. BRASIL MDS. *Manual de Vigilância da Leishmaniose Tegumentar Americana*. Brasilia2007.

3. Goto H and Lindoso JA. Current diagnosis and treatment of cutaneous and mucocutaneous leishmaniasis. *Expert Rev Anti Infect Ther* 2010; **8**: 419-433.

4. Silva-López REd. Proteases de Leishmania: novos alvos para o desenvolvimento racional de fármacos. *Química Nova* 2010; **33**: 1541-1548.

5. Oliveira LF, Schubach AO, Martins MM *et al.* Systematic review of the adverse effects of cutaneous leishmaniasis treatment in the New World. *Acta Trop* 2011; **118**: 87-96.

6. Baccarin T, Czepula AI, Ferreira RA and Lucinda-Silva RM. Análise morfoanatômica das partes aéreas de Wedelia paludosa DC. (Acmela brasiliensis, Sphagneticola trilobata), Asteraceae. *Revista Brasileira de Farmacognosia* 2009; **19**: 612-616.

7. Batista R, Brandão GC, Braga FC and Oliveira AB. Cytotoxicity of Wedelia paludosa D.C. extracts and constituents. *Revista Brasileira de Farmacognosia* 2009; **19**: 36-40.

8. Batista R, Chiari E and de Oliveira AB. Trypanosomicidal Kaurane Diterpenes from Wedelia paludosa. *Planta Med* 1999; **65**: 283-284.

9. Batista R, Humberto JL, Chiari E and de Oliveira AB. Synthesis and trypanocidal activity of ent-kaurane glycosides. *Bioorg Med Chem* 2007; **15**: 381-391.

10. Wilkens M, Alarcón C, Urzúa A and Mendoza L. Characterization of the Bactericidal Activity of the Natural Diterpene Kaurenoic Acid. *Planta Med* 2002; **68**: 452-454.

11. Mizokami SS, Arakawa NS, Ambrosio SR *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.

12. Tirapelli CR, Ambrosio SR, Costa FBd, Coutinho ST, Oliveira DCRd and Oliveira AMd. Analysis of the mechanisms underlying the vasorelaxant action of kaurenoic acid in the isolated rat aorta. *European Journal of Pharmacology* 2004; **492**: 233 - 241.

13. Tirapelli CR, Ambrosio SR, Oliveirada AM and Tostes RC. Hypotensive action of naturally occurring diterpenes: A therapeutic promise for the treatment of hypertension. *Fitoterapia* 2010; **81**: 690 - 702.

14. Lim H, Jung HA, Choi JS, Kim YS, Kang SS and Kim HP. Anti-inflammatory activity of the constituents of the roots of Aralia continentalis. *Arch Pharm Res* 2009; **32**: 1237-1243.

15. Choi RJ, Shin EM, Jung HA, Choi JS and Kim YS. Inhibitory effects of kaurenoic acid from Aralia continentalis on LPS-induced inflammatory response in RAW264.7 macrophages. *Phytomedicine* 2011; **18**: 677-682.

16. Izumi E, Ueda-Nakamura T, Veiga VF, Jr., Pinto AC and Nakamura CV. Terpenes from Copaifera demonstrated in vitro antiparasitic and synergic activity. *J Med Chem* 2012; **55**: 2994-3001.

17. Santos AO, Izumi E, Ueda-Nakamura T, Dias-Filho BP, Veiga-Junior VF and Nakamura CV. Antileishmanial activity of diterpene acids in copaiba oil. *Mem Inst Oswaldo Cruz* 2013; **108**: 59-64.

18. Van Assche T, Deschacht M, da Luz RA, Maes L and Cos P. Leishmania-macrophage interactions: insights into the redox biology. *Free Radic Biol Med* 2011; **51**: 337-351.

19. Faria MS, Reis FC and Lima AP. Toll-like receptors in leishmania infections: guardians or promoters? *J Parasitol Res* 2012; **2012**: 930257.

20. Handman E and Bullen DV. Interaction of Leishmania with the host macrophage. *Trends Parasitol* 2002; **18**: 332-334.

21. Sacks D and Noben-Trauth N. The immunology of susceptibility and resistance to Leishmania major in mice. *Nat Rev Immunol* 2002; **2**: 845-858.

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

23. Repetto M, Reides C, Gomez Carretero ML, Costa M, Griemberg G and Llesuy S. Oxidative stress in blood of HIV infected patients. *Clin Chim Acta* 1996; **255**: 107-117.

24. Victorino VJ, Panis C, Campos FC *et al.* Decreased oxidant profile and increased antioxidant capacity in naturally postmenopausal women. *Age (Dordr)* 2013; **35**: 1411-1421.

25. Panis C, Herrera AC, Victorino VJ *et al.* Oxidative stress and hematological profiles of advanced breast cancer patients subjected to paclitaxel or doxorubicin chemotherapy. *Breast Cancer Res Treat* 2012; **133**: 89-97.

26. Lee KS, Lee DK, Jeoung D *et al.* Differential effects of substrate-analogue inhibitors on nitric oxide synthase dimerization. *Biochem Biophys Res Commun* 2012; **418**: 49-55.

27. Mukherjee A, Roy G, Guimond C and Ouellette M. The gamma-glutamylcysteine synthetase gene of Leishmania is essential and involved in response to oxidants. *Mol Microbiol* 2009; **74**: 914-927.

28. Thomson L, Denicola A and Radi R. The trypanothione-thiol system in Trypanosoma cruzi as a key antioxidant mechanism against peroxynitrite-mediated cytotoxicity. *Arch Biochem Biophys* 2003; **412**: 55-64.

29. Bocedi A, Dawood KF, Fabrini R *et al.* Trypanothione efficiently intercepts nitric oxide as a harmless iron complex in trypanosomatid parasites. *Faseb j* 2010; **24**: 1035-1042.

30. Nylen S and Gautam S. Immunological perspectives of leishmaniasis. *J Glob Infect Dis* 2010; **2**: 135-146.

31. Babior BM. Phagocytes and oxidative stress. *Am J Med* 2000; **109**: 33-44.

32. Lima-Junior DS, Costa DL, Carregaro V *et al.* Inflammasome-derived IL-1beta production induces nitric oxide-mediated resistance to Leishmania. *Nat Med* 2013; **19**: 909-915.

33. Cunningham AC. Parasitic adaptive mechanisms in infection by leishmania. *Exp Mol Pathol* 2002; **72**: 132-141.

34. Santos AO, Ueda-Nakamura T, Dias Filho BP, Veiga Junior VF, Pinto AC and Nakamura CV. Effect of Brazilian copaiba oils on Leishmania amazonensis. *J Ethnopharmacol* 2008; **120**: 204-208.

35. Dos Santos AO, Ueda-Nakamura T, Dias Filho BP, da Veiga Junior VF and Nakamura CV. Copaiba Oil: An Alternative to Development of New Drugs against Leishmaniasis. *Evid Based Complement Alternat Med* 2012; **2012**: 898419.

36. Balestieri FM, Queiroz AR, Scavone C, Costa VM, Barral-Netto M and Abrahamsohn Ide A. Leishmania (L.) amazonensis-induced inhibition of nitric oxide synthesis in host macrophages. *Microbes Infect* 2002; **4**: 23-29.

37. Latz E, Xiao TS and Stutz A. Activation and regulation of the inflammasomes. *Nat Rev Immunol* 2013; **13**: 397-411.

38. Ting JP, Lovering RC, Alnemri ES *et al.* The NLR gene family: a standard nomenclature. *Immunity* 2008; **28**: 285-287.

39. Panaro MA, Brandonisio O, Acquafredda A, Sisto M and Mitolo V. Evidences for iNOS expression and nitric oxide production in the human macrophages. *Curr Drug Targets Immune Endocr Metabol Disord* 2003; **3**: 210-221.

40. Huang Z, Hoffmann FW, Fay JD *et al.* Stimulation of unprimed macrophages with immune complexes triggers a low output of nitric oxide by calcium-dependent neuronal nitric-oxide synthase. *J Biol Chem* 2012; **287**: 4492-4502.

41. Connelly L, Jacobs AT, Palacios-Callender M, Moncada S and Hobbs AJ. Macrophage endothelial nitric-oxide synthase autoregulates cellular activation and pro-inflammatory protein expression. *J Biol Chem* 2003; **278**: 26480-26487.

42. Pacher P, Beckman JS and Liaudet L. Nitric oxide and peroxynitrite in health and disease. *Physiol Rev* 2007; **87**: 315-424.

Legends of Figures

Figure 1. Kaurenoic Acid inhibits the viability and proliferation of *L. amazonensis* promastigote forms. MTT assay in promastigote forms of *L. amazonensis* treated with Kaurenoic Acid (10, 30, 50, 70, 90 μM) or DMSO (2%) for 24, 48 and 72h (Panel A). Kinetics of *L. amazonensis* promastigotes forms proliferation after treatment with Kaurenoic Acid (10, 30, 50, 70 and 90μM) or DMSO (2%) for 24, 48, 72 and 120h (Panel B). Data represent mean ± SEM of three independent experiments. [*Significantly different from control ($P < 0.05$ compared with control group-promastigotes in culture medium). (One-way ANOVA followed by Tukey's test)]. (300 X 300 DPI).

Figure 2. The *In vitro* treatment of macrophages infected with *L. amazonensis* by kaurenoic acid reduces the percentage of infection and the number of amastigotes inside macrophages on phagocytic activity analysis. Percentage of infected macrophages after 24h of incubation with acid kaurenoic (50, 70, 90 μM) (Panel A); Amount of amastigotes by macrophages macrophages after 24h of incubation with acid kaurenoic (50, 70, 90 μM) (Panel B). Data represent the mean ± SEM of six independent experiments. [*Significantly different from infected cells ($P<0.05$). (One-way ANOVA followed by Tukey's test)]. (300 X 300 DPI)

Figure 3. The antileishmanial activity exerted in macrophages treated with kaurenoic acid is associated with reversion on infection-depleted NO production. Total antioxidant capacity (TRAP) measurement by chemiluminescence (Panel A) Lipoperoxidation (malondialdehyde-MDA) levels measurement by high performance liquid chromatography (HPLC) (Panel B) and nitrite levels (Panel C) was evaluated in supernadant or macrophages infected with *L. amazonensis* and treated with kaurenoic acid (50, 70, 90μM) for 24h. Data represent the mean ± SEM of three independent experiments. [*Significantly different from infected cells (P<0.05).

Significantly different from control cells (P<0,05). (One-way ANOVA followed by Tukey's test)]. (300 X 300 DPI)

Figure 4. Kaurenoic acid promotes the production of active IL-1β in association with the up-regulation of the inflammasome component NLRP-12. *In vitro* IL-1β production by peritoneal macrophages infected with *L. amazonensis* and treated with Kaurenoic Acid (50, 70, 90μM) for 24h by ELISA (Panel A). Immunocitochemistry scoring and labeling NLRP-12 on macrophages infected with *L. amazonensis* and treated with Kaurenoic Acid (50, 70, 90μM) for 24h (Panel B). Data represent the mean ± SEM of three independent experiments. [*Significantly different from infected cells (P<0.05). (One-way ANOVA followed by Tukey's test)]. (300 x 300 DPI)

Figure 5. Kaurenoic acid potentiates the synthesis of NO in infected macrophages in a cNOS-dependent manner. Immunocitochemistry scoring and labeling for nitric oxid induzible (iNOS) peritoneal macrophages infected with *L. amazonensis* and treated with Kaurenoic Acid (50, 70, 90μM) for 24h (Panel A). Determination nitrite levels of peritoneal macrophages infected with *L. amazonensis* and blocked with L-NAME at a concentration of 20μM and treated with Kaurenoic Acid (50, 70, 90μM) for 24h (Panel B). Data represent the mean ± SEM of three independent experiments. [*Significantly different from infected cells (P<0.05). (One-way ANOVA followed by Tukey's test)]. (300x 300 DPI)

Figure Supporting information 1. Mapping the cytokine profiling produced *in vitro* by macrophages infected with *L. amazonensis* and treated with kaurenoic acid (50, 70, 90μM) for 24h detected by ELISA. IL-12 production (Panel A), TNF-α production (Panel B), IFN-γ

production (Panel C), TGF-β production (Panel D) and IL-10 production (Panel E). Data represent the mean ± SEM of three independent experiments. (300 x 300 DPI)

FIGURES

Figure 1.

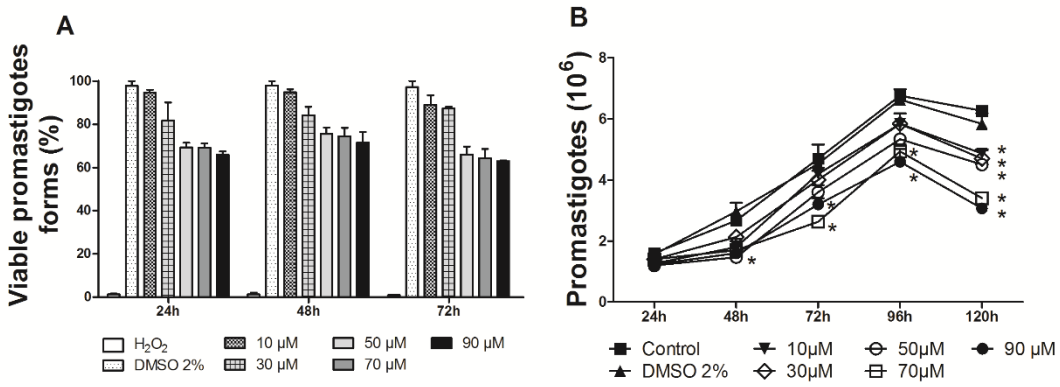


Figure 2.

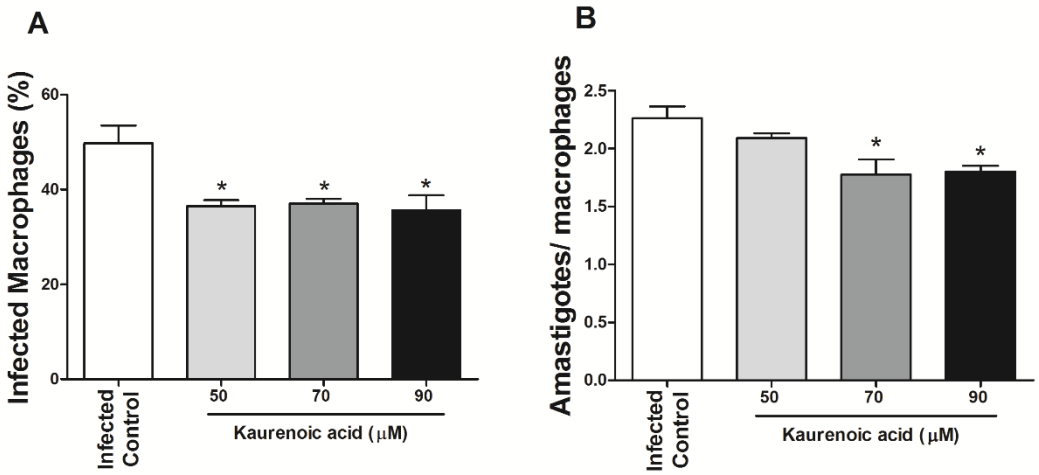
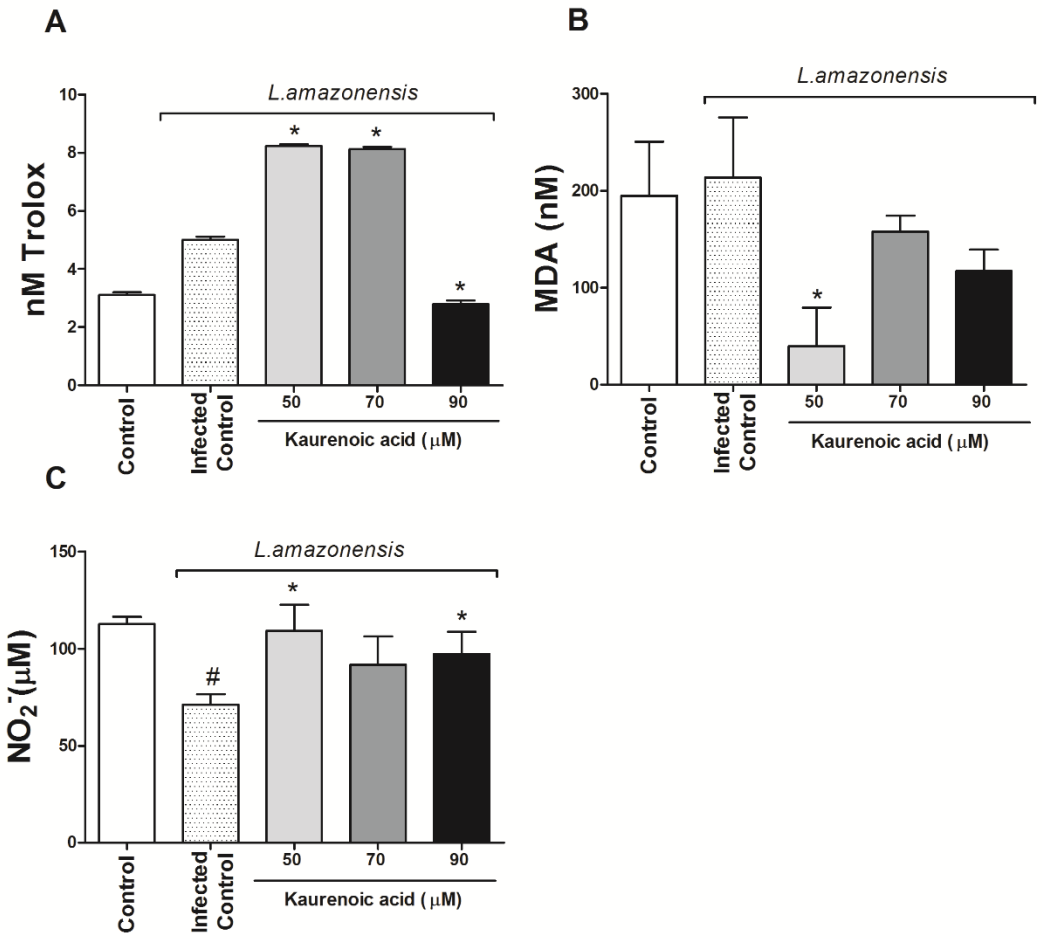

A
Infected Macrophages (%)
60
40
20
0
*
*
*
Infected Control
50
70
90
Kaurenoic acid (μM)
B
Amastigotes/ macrophages
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Infected Control
50
70
90
Kaurenoic acid (μM)

Figure 3.


A
L.amazonensis
nM Trolox
10
8
6
4
2
0
*
*
*
Control
Infected Control
50
70
90
Kaurenoic acid (μM)
B
L.amazonensis
MDA (nM)
300
200
100
0
*
Control
Infected Control
50
70
90
Kaurenoic acid (μM)
C
L.amazonensis
NO2- (μM)
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Infected Control
50
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90
Kaurenoic acid (μM)

Figure 4.

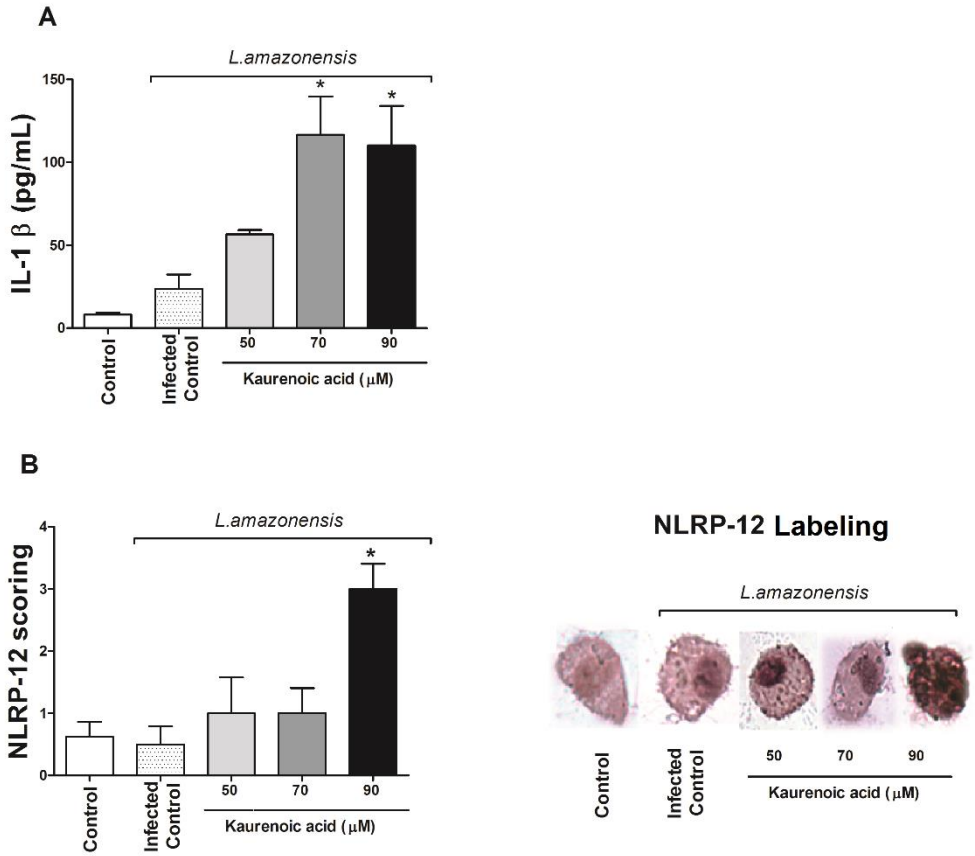
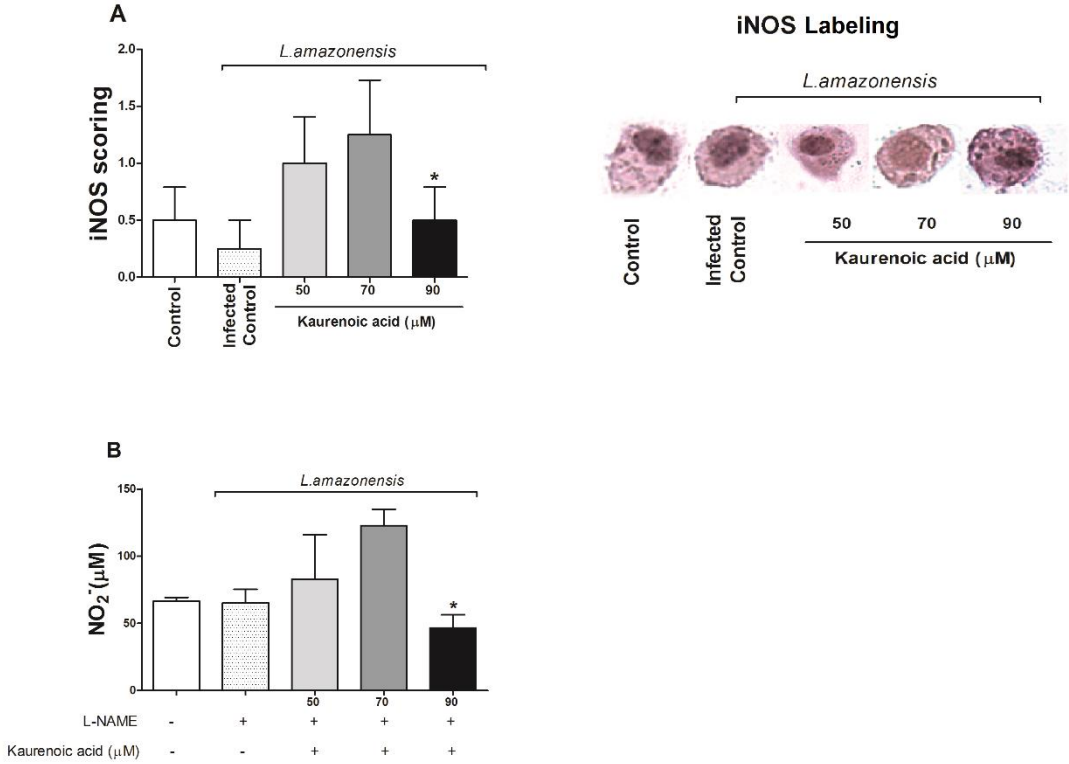

A
L.amazonensis
IL-1 β (pg/mL)
150
100
50
0
*
*
Control
Infected Control
50
70
90
Kaurenoic acid (μM)
B
L.amazonensis
NLRP-12 scoring
4
3
2
1
0
*
Control
Infected Control
50
70
90
Kaurenoic acid (μM)
NLRP-12 Labeling
L.amazonensis
Control
Infected Control
50
70
90
Kaurenoic acid (μM)

Figure 5.


A
L.amazonensis
iNOS scoring
2.0
1.5
1.0
0.5
0.0
*
Control
Infected Control
50
70
90
Kaurenoic acid (μM)
iNOS Labeling
L.amazonensis
Control
Infected Control
50
70
90
Kaurenoic acid (μM)
B
L.amazonensis
NO2-(μM)
150
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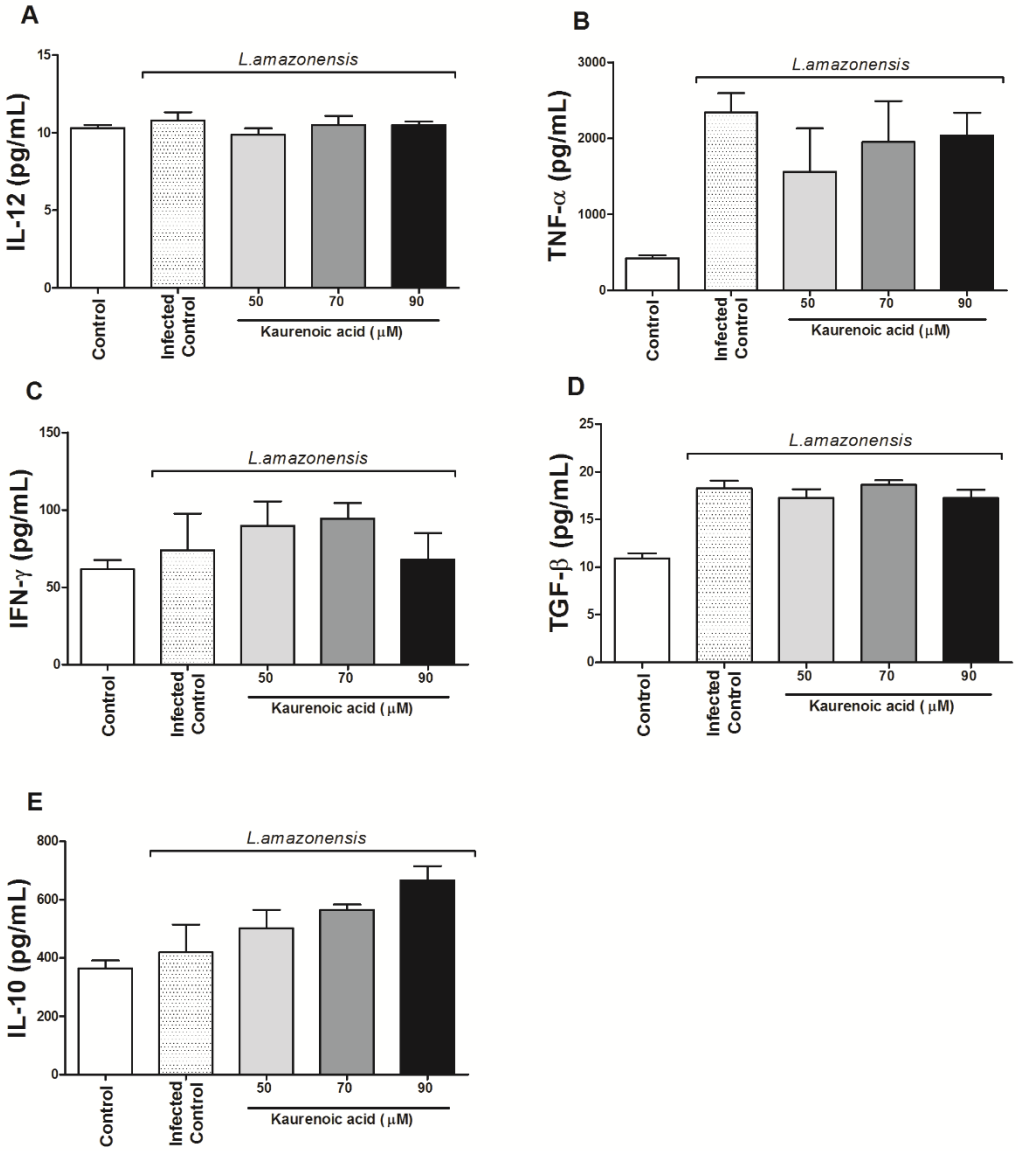
70
90
L-NAME - + + + +
Kaurenoic acid (μM) - - + + +

Figure Supporting information 1.



5. ANEXOS

5.1. Anexo 1- Normas da revista *American Journal of Immunology*.

Online Submission of Manuscript

Microsoft Word or PDF formats may be submitted online to Science Publications for initial evaluation. For online submission of manuscripts authors should go to "Online Submission".

Conflict of Interest

A conflict of interest exists when judgement regarding the research is influenced by factors such as financial gain or personal relationships. All authors are required to disclose any financial, personal or other associations that may influence or be perceived to influence, their work.

Ethics

Authors must give assurance that no part of manuscript reporting original work is being considered for publication in whole or in part elsewhere. The corresponding author must affirm that all of the other authors have read and approved of the manuscript.

When reporting experiments on human subjects, authors should indicate whether the procedures followed were in accordance with any ethical standards set by a governing committee responsible for human experimentation (ie, if applicable, a university review board) and with the Helsinki Declaration of 1975, as revised in 2000. If doubt exists whether the research was conducted in accordance with the Helsinki Declaration, the authors must, in a separate document, explain the rationale for their approach, and, if presented before a review body, demonstrate that the institutional review body explicitly approved the doubtful aspects of the study.

Studies using human subjects are required to state in the manuscript that all human subjects were provided with the approved informed consent.

When reporting experiments on animals, indicate whether the institution’s or the National Research Council’s guide for, or any national law on, the care and use of laboratory animals was followed.

Cover Letter & Copyright Form

All manuscripts should be submitted with a pre-defined cover letter & copyright form.

Style of Manuscript

The manuscript should be written in clear, concise and grammatically correct English. It is recommended that you ask colleagues to read over your paper prior to submission to ensure it is of a high standard and conforms to a high level of scientific writing. Times New Roman font style with 10 font size should be used. Manuscripts that do not conform to these requirements and the following manuscript format may be returned to the author for correction. The entire manuscript should be typed single spaced, with margins of 1 inch each side. All pages should be numbered consecutively in the bottom centre. Indent new paragraphs. The style of heading and subheading should be as follows

The first heading should be centred, bold and in uppercase letters.

Order of Manuscript

The manuscript should be presented in the following order.

Title Page

This should contain the title of the contribution (capitalize first letter of each word in the title) and the name(s) and address(es) of the author(s). The full postal address, e-mail address, telephone and facsimile number(s) of the author who will receive correspondence and check the proofs should be included.

Abstract

All manuscripts must include a brief but informative Abstract. It should not exceed 300 words and should describe the scope, hypothesis or rationale for the work and the main findings. The abstract should allow the reader to quickly have a clear idea about the rational for the work, the experiments conducted and the results of those experiments before reading the rest of the manuscript. Both common and scientific names should be included; the authorities are not given if they appear in the title. References to the literature and mathematical symbols/equations should not be included.

Keywords

Key words (3-5) should be provided below the Abstract to assist with indexing of the article.

Introduction

Aquaculture is the cultivation and harvesting of aquatic organisms, including finfish, shellfish and aquatic plants in marine or freshwater. The industry has grown at an average rate of 8.9% per year since 1970 and continues to grow more rapidly than all other animal food-producing sectors.....

2. The first sub-heading should be left justified, bold and title case.

1.1 Water Quality Guidelines. The availability of a high quality water supply greatly influences the success of any aquaculture operation and is the first factor considered during site selection (Ackefors et al., 1994). Tables 3- 5 present water quality criteria for the culture of fish, crustaceans and molluscs. The tolerance limits of the various water quality parameters depend on the species cultivated (Pillay, 1992).....

3. Sub-sub-headings should be left justified, bold, italics and title case.

1.1.1 Dissolved Gases In water, The dissolved gases of biological and ecological importance are oxygen and carbon dioxide, which originate from diffusion from the atmosphere and photosynthesis and respiration of aquatic organisms.

4. Sub-sub-sub-headings should be left justified, bold, italics and title case.

1.1.1.1 Dissolved Oxygen. The level of dissolved oxygen (DO) available to organisms in an aquaculture system is the most critical water quality parameter because it is essential to the metabolism of the majority of cultured fish and crustaceans (Stickney, 1994). Fish.....

The introduction should articulate the problem being addressed. It should provide sufficient background information on the subject allowing the reader to have more

insight into what will be presented in the rest of the paper. The aims of the manuscript should be clearly stated.

Materials and Methods

This section should be concise but provide sufficient detail of the material used and equipment and the procedure followed to allow the work to be repeated by others.
The sources of the laboratory procedures should be cited and any changes that were made must be noted. Information on the equipment model, manufacturer's name and address including the city, province/state and country should be provided. The procedures should be written in the past tense.

Results

Results should be presented in a logical sequence in the text, tables and figures. Repetitive presentation of the same data in tables and figures should be avoided. The results should not contain material appropriate to the Discussion. All tables, graphs, statistical analyses and sample calculations should be presented in this section.

Discussion

The results should be discussed in relation to any hypotheses advanced in the Introduction. Comment on results and indicate possible sources of error. Place the study in the context of other work reported in the literature. Only in exceptional cases should the "Results and Discussion" sections be combined. Refer to graphs, tables and figures by number (for example Figure 5 or Table 5. This helps tie the data into the text in a very effective manner.

Conclusion

The main conclusions of the experimental work should be presented. The contribution of the work to the scientific community and its economic implications should be emphasized.

Acknowledgment

The source of financial support must be acknowledged. Authors must declare any financial support or relationships that may pose conflict of interest in the covering letter submitted with the manuscript. Technical assistance may also be acknowledged.

References

All publications cited in the text should be presented in a list of references following the text of the manuscript.

A. Citation in Text

Use the author/date system of references. In the text refer to the authors' name (without initials) and year of publication.

1. Examples for a single author

Peterson (1993) has shown that. This is in agreement with the results obtained by several authors (Kramer, 1994; Smith, 1995; Brown, 1999).

2. Examples for two authors

Smith and White (1999) reported that….

This was later found to be incorrect (Amir and Ahmed, 2000).

3. Examples for three or more authors (use the first author's name and then et al.)

Moore et al. (1990) stated that ….

Similar results were reported recently (Smith et al., 2003).

B. List of References

The list of references should include only those cited in the manuscript and arranged alphabetically by authors' names. Titles of journals should be given in full. 'In press' can only be used to cite manuscripts actually accepted for publication in a journal. Citations such as 'manuscript in preparation' or 'manuscript submitted' are not permitted. Authors must provide Digital Object Identifier (DOI) number for all references. If there is no DOI for any reference, author may provide its URL/direct accessible web link for verification purpose. References without DOI or internet link are not acceptable. The following format should be adhered to.

Journal Papers

Calik, P., P. Yilgora, P. Ayhanb and A.S. Demir, 2004. Oxygen transfer effects on recombinant benzaldehyde lyase production. Chemical Engineering and Science, 59 (22-23):5075-5083. DOI:10.1016/j.ces.2004.07.070

Hernández-Herrero, M.M., G. Duflos, P. Malle and S. Bouquelet, 2003. Collagenase activity and protein hydrolysis as related to spoilage of iced cod (Gadus morhua). Food Research International, 36(2):141-147.DOI:10.1016/s0963-9969(02) 00129-1.

Text Book

Navabi, Z., 1998. Analysis and Modeling of Digital Systems. 2nd Ed. McGraw Hill, New York. ISBN: 0070464790, pp: 632.

Berg, J.M., L.T. John and L. Stryer, 2007. Biochemistry. 5th Ed. W.H. Freeman, New York. ISBN-13: 978-0716787242, pp.580.

Book Chapter

Katz, R.H., 1986. Computer-Aided Design Databases. In: New Directions for Database Systems, Ariav, G. and J. Clifford, (Eds.), Intellect Books, Norwood, NJ, pp: 110-123. ISBN: 0893913448.

Ashie, I.N.A. and T.C. Lanier, 2000. Transglutaminases in Seafood Processing. In: Seafood Enzymes Utilization and Influence on Postharvest Seafood Quality, Haard, N.F. and B.K. Simpson (Eds.), Marcel Dekker Inc, New York, NY, pp: 271-275. ISBN: 0-8247-0326-X.

Conference Proceedings

Magott, J. and K. Skudlarski, 1989. Combining Generalized Stochastic Petri Nets and PERT Networks For The Performance Evaluation Of Concurrent Processes. Proceedings of the 3rd International Workshop on Petri Nets and Performance Models, Dec. 11-13, IEEE Xplore Press, Japan, pp: 249-256. DOI: 10.1109/PNPM.1989.68558

Baird-Parker, A.C. and M.A.H. Baillie, 1974. The Inhibition of Clostridium botulinum by Nitrite and Sodium Chloride. Proceedings of the International Symposium on Nitrite in Meat Products, Sep.10-14, Zeist, the Netherlands, pp: 268. ISBN-10:9022004635.

Government Publications (Some will still have specific authors – find them if you can)

Forastieri, V., 1999. The ILO Programme for Occupational Safety and Health in Agriculture. International Labour Organization, Geneva, Switzerland.

Sheng, T.C., 1989. Soil Conservation for Small Farmers in the Humid Tropics. FAO Soils Bulletin No. 60. Food and Agriculture Organization of the United Nations. Rome, Italy.

United Nations, 2001. Indicators of Sustainable Development: Guidelines and Methodologies. United Nations Press, New York, USA.

Online Publications

Lal, R., 1995. Sustainable Management of Soil Resources in the Humid Tropics. United Nations University Press, Tokyo, Japan.
http://www.unu.edu/unupress/unupbooks/uu27se/uu27se00.htm (Accessed on March 17, 2011)

Mörner, J., R. Bos and M. Fredrix, 2002. Guidance on Alternative Strategies for Sustainable Pest and Vector Management. World Health Organization, Geneva, Switzerland.
http://www.who.int/water_sanitation_health/resources/en/Organicpescont.pdf
(Accessed on February 13, 2011)

Rice, R.A. and J.R.Ward, 1996. Coffee, Conservation and Commerce in the Western Hemisphere. Natural Resources Defence Council, Weadon Progressive and The Smithsonian, Washington, D.C.
http://www.nrdc.org/health/farming/ccc/cptinx.asp (Accessed on January 3, 2012)
Generic Website
UNEP, 2002. Cleaner Production Assessment in Industries. Production and Consumption Branch. United Nations Environment Program.
http://www.unepie.org/pc/cp/understanding_cp/cp_industries.htm (Accessed on February 13, 2011)

FLO, 2002. The World Coffee Crisis. Fair Trade Labeling Organization.http://www.fairtrade.net/pdf/english/Coffee.pdf (Accessed on May 6, 2011)

FAO, 2002. Statistical Database, Food and Agriculture Organization. http://apps.fao.org/default.htm (Accessed on March 27, 2012)

Thesis

Alkoaik, F., 2005. Fate of plant pathogens and pesticides during composting of greenhouse tomato plant residues. Unpublished dissertation in partial fulfilment of the requirements for the degree of Doctor of Philosophy, Dalhousie University, Halifax, Nova Scotia, Canada.

Tables

Tables should be self-contained and the data should not be duplicated in figures. Tables should be numbered consecutively in Arabic numerals. Each table should be presented on a separate page with a comprehensive but concise legend above the table. Tables should be double-spaced and vertical lines should not be used to separate columns. Column headings should be brief, with units of measurement in parentheses. All abbreviations should be defined in footnotes. Use superscript letters (not numbers) for footnotes and keep footnotes to a minimum. *, **, *** should be reserved for P values.

Figures

Only necessary illustrations should be included. All illustrations (line drawings and photographs) are classified as figures. Figures should be cited in consecutive order in the text. Figures should be sized to fit within the column (82 mm) or the full text width (171 mm). Line figures should be supplied as sharp, black and white or color diagrams, drawn with a computer graphics package. Photographs should be sharp and magnifications should be indicated on photographs using a scale bar. Graphics should be supplied as high resolution (at least 300 d.p.i.) electronic files. Digital images supplied as low-resolution cannot be used. The legend should incorporate definitions of any symbols used and all abbreviations and units of measurement should be explained so that the figure can be understood without reference to the text.

Abbreviation and Units

SI units (metre, kilogram, etc.), as outlined in the latest edition of Units, symbols and Abbreviations: A Guide for Medical and Scientific Editors and Authors (Royal Society of Medicine Press, London), should be used wherever possible. Statistics and measurements should always be given in figures; that is, 10 mm, except where the number begins the sentence. When the number does not refer to a unit measurement, it is spelt out, except where the number is greater than nine. Use only standard abbreviations. The word ‘Figure' should be shortened to Fig. unless starting a sentence.

Galley Proofs

Once the final review is completed, the author will be required to resubmit the revised manuscript using a journal template. The final Galley Proof will be sent via e-mail as an Acrobat PDF (Portable Document Format) file and should be returned within 3 days of receipt with the signed copyright form and payment of publication fees. Alterations to the text and figures (other than the essential correction of errors) are unacceptable at proof stage and authors may be charged for excessive alterations. Acrobat Reader will be required in order to read the PDF. This software can be downloaded from the following website: http://get.adobe.com/reader/

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The page charges are currently $525 for an article under 8 pages and $75 per additional page.

5.2. Anexo 2 - Normas da revista *Parasite Immunology*

Manuscript Submission

All papers must be submitted via ScholarOne Manuscripts. Authors may wish to consult an appropriate member of the Editorial Board prior to submission of material. On submission you will need: (1) the type of your paper (original paper, brief definitive report, Review) (2) the full title of your paper (limited to 120 characters), (3) the abstract (limited to 200 words), (4) up to six keywords, (5) the full names and affiliations of all authors, (6) details of preferred and/or non-preferred reviewers (optional), (7) a cover letter, (8) a manuscript keyword, (9) to answer questions regarding the Woods Hole Immuno-Parasitology Prize, human tissues and animal experimentation, (10) to complete and upload a Conflict of Interest form for each author, (11) to adhere to the ARRIVE guidelines, and (12) to upload the appropriate files and forms. Submissions should be prepared using double-line spacing, a minimum of 1 inch margins, and 12 point Times New Roman font. The preferred file type formats for the text and tables of your manuscript are .doc, .docx, .rtf, .ppt, .xls; please do not save them as portable document files (PDF) files. Ideally, illustrations should be uploaded in native format of PICT if created on a Mac, or in native format or WMF if created in Windows. Files saved as PS, EPS, GIF and TIF may also be used. Avoid using tints if possible; if they are essential to the understanding of the figure, try to make them coarse. Detailed information on our digital illustration standards is available on-line at: http://authorservices.wiley.com/bauthor/illustration.asp.

Each submission should include a title page which should contain: the paper title, full names and affiliations of all authors, and should indicate an author for use for correspondence (including an email address). For each author, disclose potential conflicts of interest, including all relevant financial interests (e.g. employment, significant share ownership, patent rights, consultancy, research funding) in any company or institution that might benefit from the publication (or state 'none'). Authors do not need to report the sums concerned. Please can you therefore add a small paragraph entitled "Disclosures" and then include any relevant details or "none" if there is nothing to disclose, if you have not already done so. For indexing purposes a short number of 'key words' should be included. If you are not a native English-language speaker, we strongly recommend that you have your manuscript professionally edited

before submission. A list of companies that will edit your manuscript for a fee can be found here http://authorservices.wiley.com/bauthor/english_language.asp. Professional editing is not compulsory, but will mean that reviewers are better able to read and assess your manuscript. Use of one of these companies does not guarantee acceptance or preference for publication in this journal.

Manuscript Types and Style

Original Papers

The text should be preceded by a short abstract not exceeding 200 words. The abstract of a paper in Parasite Immunology is the face of the paper turned to the world. It is not a prècis of the paper, but is a clear, direct account of what was done, why it was done, and why the outcome matters. This is not the place for details or data, or for speculation. Avoid abbreviations, write sparingly, but simply and clearly as to a general audience, and give the highlights only. You can make some simple changes to your title and abstract to improve your article's ranking in search engines: guidelines on this can be found here. Please review your article's title and abstract in the light of these suggestions to improve your paper prior to submission and maximise its potential readership.

The abstract should be followed by these sections: Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Legends to Tables and Figures, Tables and Figures. Pages should be numbered consecutively in arabic numerals. Please also ensure that the lines in your manuscript are numbered. Acknowledgements should include an indication of the source of funding for the work and the contribution of individual authors (see “Authorship” below).

References should be cited in the Vancouver style. In the text they should be numbered in superscript in the order in which they appear. The reference list should follow the numbering sequence used in the text and should include: the names and initials of all authors; the full title of the article; the source of reference using abbreviations for journal titles as shown in Index Medicus, the year, volume number and first and last pages. For references with more than six authors, the first three should be listed, followed by et al. For references cited from books, the title of the book should be

followed by the names and initials of the editors, the edition, the place of publication, the publisher, the year of publication and the first and last pages.

Examples:

1) Rogerson SJ, Novakovic S, Cooke BM, Brown GV. Plasmodium falciparum-infected erythrocytes adhere to the proteoglycan thrombomodulin in static and flow-base systems. Exp Parasitol 1997; 86 8-18.

2) Parks DR, Lanier LL, Herzenberg LA. In Handbook of Experimental Immunology, ed. Weir DM, Oxford: Blackwell Science Ltd; 1986; 29

Authors are responsible for the accuracy of their references.

Spelling should conform to The Concise Oxford Dictionary of Current English and units of measurement, symbols and abbreviations with those in Units, Symbols and Abbreviations(1977) published and supplied by the Royal Society of Medicine, 1 Wimpole Street, London W1M 8AE. This specifies the use of SI units.

Tables and figures should be referred to in the text together with an indication of their approximate position. Colour illustrations are encouraged; there is no charge to the author for the inclusion of colour illustrations.

Brief Definitive Reports

The journal publishes studies that are not required to be written up as conventional papers. They should not be structured like original papers but should contain brief accounts of background studies, methods used, results and discussion. References should be kept to a minimum and there should be no more than one table and one figure. Maximum length accepted is four pages of typescript. They will be given some priority in publication as the schedule permits.

Review Articles

We would be happy to receive Review articles with a limit of 5000 words that are structured in the same format as Original Articles.

Letters to the Editor

These are published if they comment usefully on material published in previous issues, and should not exceed 400 words.

Meeting Reports

Reports on meetings or parts of meetings concerned with parasite immunology will be published. Again, these reports will be commissioned, but the editors will be pleased to receive reports that might be used.

Authorship

All authors must fulfil the following three criteria:

• Have made a substantial contribution to research design, or the acquisition, analysis or interpretation of data;

• Have drafted the paper or revised it critically;

• Have approved the submitted and final versions.

In the Acknowledgments section of the paper all authors must indicate their specific contributions to the work described in the manuscript. Some examples include

• X performed the research;

• Y designed the research study;

• Z contributed essential reagents or tools;

• A analysed the data;

• B wrote the paper.

An author may list more than one contribution, and more than one author may have contributed to the same element of the work. E.g. 'A performed the research, A and C analysed the data and wrote the paper, E contributed the knockout mice for the study and G designed the research study and wrote the paper'.

Supporting Information

Online Supporting Information can include additional explanatory notes, data sets, videos, lists, figures or tables that will not be published in the print edition of the journal and which are ancillary to, rather than central to, the article. Supporting Information must be approved by the Editors and should be supplied as a single PDF file headed by the title of the paper and the authors' names, addresses and contact information. Supporting Information will be published exactly as supplied and it is the author's responsibility to ensure that the material is logically laid out, adequately described, and in a format accessible to readers. Animations and other moving images or sound files in standard formats must be supplied as separate files. Figures and tables in

Supporting Information should be referred to in the main text and labelled Fig. S1, Fig. S2 or Table S1, etc., in the order cited. Full guidelines and information on acceptable file formats may be found athttp://authorservices.wiley.com/bauthor/suppmat.asp.

Display of Sequences

Prepare sequences as figures, not tables. This will ensure that proper alignment is preserved.

Microarray Databases

Parasite Immunology supports the efforts of the Microarray Gene Expression Data Society to standardize the presentation of microarray data, and we recommend that authors follow their guidelines and checklist (http://www.mged.org/Workgroups/MIAME/miame_checklist.html). In addition, the journal strongly recommends the supplemental microarray data be deposited in a public database such as Gene Expression Omnibus (or GEO, athttp://www.ncbi.hlm.nih.gov/geo/) or Array Express (http://www.ebi.ac.uk/arrayexpress/) or submitted for peer-review with the initial submission of the manuscript.

Ethical Policy and Guidelines

Parasite Immunology encourages its contributors and reviewers to adopt the standards of theInternational Committee of Medical Journal Editors. The Editors reserve the right to reject a paper that does not meet these standards.

Parasite Immunology will not consider papers that have been accepted for publication or published elsewhere. Copies of existing manuscripts with potentially overlapping or duplicative material should be submitted together with the manuscript, so that the Editors can judge suitability for publication.

Please click here to read the Ethical Policies of Parasite Immunology.

For more detailed ethical guidelines please visit:http://authorservices.wiley.com/bauthor/publicationethics.asp

All papers to Parasite Immunology are checked for potential plagiarism using CrossCheck plagiarism detection software. By submitting your manuscript to Parasite Immunology you accept that your manuscript will be screened for plagiarism against previously published works.

Animal Experimentation

The Editors will not allow any papers to be published that describe experiments on living animals which may reasonably be presumed to have inflicted unnecessary pain or discomfort upon them. Experiments on living vertebrates or Octopus vulgaris should conform in principle to the legal requirements in the UK. Whenever appropriate, a statement should be included indicating that experiments were performed in accordance with local/national guidelines.

ARRIVE Guidelines

Authors are expected to comply with the ARRIVE guidelines for reporting research before submission of a manuscript. While the guidelines refer to animal experiments, most of the elements are common to all forms of research communication and adherence will strengthen the transparency and completeness of reporting.

Data Sharing

Parasite Immunology supports the efforts to encourage open sharing of publication-related data.Parasite Immunology adheres to the beliefs that authors should include in their publications the data, algorithms, or other information that is central or integral to the publication or make it freely and readily accessible; use public repositories for data whenever possible; and make patented material available under a license for research use. For more information, see the NAS website:http://books.nap.edu/books/0309088593/html/1.html

Distribution of Reagents

The Editors of Parasite Immunology have adopted the policy that any readily renewable resources mentioned in a journal article not already obtainable from commercial sources shall be made available to all qualified investigators in the field. The policy stems from the long-standing scientific principle that authenticity requires reproducibility. Publication in Parasite Immunology constitutes a de facto acceptance of this policy. Included are reagents that can be easily provided; specifically, nucleic acid sequences, cDNA and genomic clones, cell lines, and monoclonal antibody clones. Small amounts (sufficient for the replication of any in vitro work reported) of novel protein reagents are also considered easily transferable.

Although the Editors appreciate that many of the reagents mentioned in Parasite Immunology are proprietary or unique, neither condition is considered adequate grounds for deviation from this policy. Suitable material transfer agreements can be drawn up between the provider and requester, but if a reasonable request is turned down and submitted to the Editor-in-Chief, the corresponding author will be held accountable. The consequence for noncompliance is simple: the corresponding author will not publish in Parasite Immunology for the following three years.

Disclosures and Conflict Interest

Authors are required to disclose financial interests in any company or institution that might benefit from their publication. All authors must complete and sign the Conflict of Interests Form. The completed form must be returned to the editorial office. A competing interest exists when a primary interest (such as patients' welfare or the validity of research) might be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors of aParasite Immunology article when they have a financial interest that may influence their interpretation of their results or those of others.

Financial interests are the easiest to define and they have the greatest potential to influence the objectivity, integrity or perceived value of a publication. They may include any or all, but are not limited to, the following:

• Personal financial interests: Stocks or shares in companies that may gain or lose financially through publication; consultant fees or fees from speakers bureaus other forms of remuneration from organisations that may gain or lose financially; patents or patent applications whose value may be affected by publication.

• Funding: Research support from organisations that might gain or lose financially through publication of the paper.

• Employment: Recent, present or anticipated employment of you or a family member by any organization that may gain or lose financially through publication of the paper.

Any such competing interest that authors may have should be declared. The aim of the statement is not to eradicate competing interests, as they are almost inevitable. Papers will not be rejected because there is a competing interest, but a declaration on whether or not there are competing interests will be added to the paper.

• Consultancy work. All authors must disclose competing interests, or state “none” via the Journal's ScholarOne Manuscripts website.

All sources of funding must be disclosed in the Acknowledgments section of the paper. List governmental, industrial, charitable, philanthropic and/or personal sources of funding used for the studies described in the manuscript. Attribution of these funding sources is preferred. Examples:• This work was supported by a grant from the National Institutes of Health, USA (DKxxxx to AB).

• This work was supported by the Crohn's and Colitis Foundation of Canada (grant to AB and CD).

• This work was supported by a grant from Big Pharma Inc. (to AB) and equipment was donated by Small Pharma Inc. EF received a graduate studentship award from the University of xxxxx.

For papers where there are no competing interests, all authors must include the statement ‘Competing interests: the authors have no competing interests.’ We will also ask reviewers to provide a statement of competing interests.

5.3. Anexo 3 - Aprovação do Comitê de Ética

Universidade Estadual de Londrina

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 43/2013 Londrina, 08 de Março de 2013.

Prezado Pesquisador,

A CEUA/UEL reunida em 19 de Fevereiro de 2013 avaliou o projeto de pesquisa intitulado **"Uso de compostos naturais e sintéticos como alternativas terapêuticas para o tratamento da Leishmaniose"**, processo CEUA nº 33064.2012.42, do Centro de Ciências Biológicas, desenvolvido sob sua responsabilidade. Esclarecidos os aspectos metodológicos solicitados, o projeto de pesquisa está **aprovado** para execução entendendo-se que os princípios éticos postulados pelo Conselho Nacional de Controle de Experimentação Animal estão respeitados.

Serão utilizados 210 camundongos BALB/C com idade de 6 a 8 semanas e peso de 25 gramas, provenientes do Biotério da FIOCRUZ de Curitiba-PR. O projeto tem como objetivo investigar os mecanismos de ação dos compostos naturais e sintéticos na interação parasito/hospedeiro, em busca de encontrar alternativas terapêuticas para a Leishmaniose. Para isto será avaliada a atividade antileishmanicida *in vitro* dos compostos naturais e sintéticos, pelo teste de cultura com formas promastigotas de *Leishmania sp* e pelo teste em cultura de macrófagos de camundongos tratados com os compostos. Também será avaliada atividade antileishmanicida *in vivo*, pela infecção com *Leishmania sp* nos camundongos e tratamento com os compostos em diferentes concentrações. Será realizado o teste de análise de intradermorreação de Montenegro nos camundongos para confirmar a infecção pela *Leishmania sp*. 240 horas após infecção, os animais serão eutanasiados e os órgãos serão coletados para análise da carga parasitária, análise histopatológica, e análise imunohistoquímica para anticorpo anti-NO, anti-CD4 e anti-CD8, o soro será obtido para dosagem de óxido nítrico, dosagem de citocinas IL-4, IL-10, IL-17 e IFN, a determinação da expressão das citocinas também será avaliada por imunoblotting, e os parâmetros de estresse oxidativo como medida da capacidade antioxidante plasmática e quantificação dos níveis de glutationa reduzida das amostras também serão avaliadas. O projeto está previsto para ser desenvolvido em 30 meses após aprovação da CEUA.

Cumpre orientar que caso pretendam-se quaisquer alterações no protocolo de aula prática aprovado, deve-se submeter o novo protocolo à apreciação da CEUA/UEL anteriormente à execução das modificações. Sem mais para o momento, subscrevo-me. Cordialmente,

Profa. Dra. Minan Siliane Batista de Souza
Vice-Coordenadora da CEUA-UEL

Ilmo. Sr.
Prof. Dr. Wander Rogério Pavanelli
Coordenador do Projeto
Departamento de Ciências Patológicas/Parasitologia
Centro de Ciências Biológicas
Com copia para Sra. Egle Maria de Sousa (Chefe da DCA/PROPPG) e Diretor (a) do Centro de Ciências Biológicas