PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA

MORGANA VITAL DE ARAÚJO

INVESTIGAÇÃO LEISHMANICIDA DE PROTÓTIPOS DE ORIGEM NATURAL E SINTÉTICOS

MACEIÓ-AL

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Biotecnologia como requisito parcial para obtenção do grau de Doutor.

Área de concentração: Biotecnologia em Saúde

Linha de pesquisa: Desenvolvimento de agentes profiláticos, terapêuticos e testes de diagnósticos.

Orientadora: Profª. Drª. Magna Suzana Alexandre Moreira

Coorientadora: Profª. Drª. Tânia Maria Sarmento Silva

MACEIÓ-AL

2016

Catalogação na fonte
Universidade Federal de Alagoas
Biblioteca Central
Divisão de Tratamento Técnico
Bibliotecário Responsável: Valter dos Santos Andrade

A663i Araújo, Morgana Vital de.
Investigação leishmanicida de protótipos de origem natural e sintéticos / Morgana Vital de Araújo. – 2016.
162 f. : il. tabs. e grafs.

Orientadora: Magna Suzana Alexandre Moreira.
Coorientadora: Tânia Maria Sarmento Silva.
Tese (doutorado na Rede Nordeste de Biotecnologia) – Universidade Federal de Alagoas. Instituto de Química e Biotecnologia. RENORBIO. Maceió, 2016.

Bibliografia: f.139-156.
Anexos: f.157-162.

1. Leishmaniose. 2. Leishmania. 3. Naftoquinona. 4. Solanum paladosum. 5. Retusin. 6. Flavonoides. 6. Atividade leishmanicida. I. Título.

CDU: 547.1:616.993.161

MORGANA VITAL DE ARAÚJO

"Investigação leishmanicida de protótipos de origem natural e sintéticos".

Tese apresentada ao Programa de Pós-Graduação em Biotecnologia da Rede Nordeste de Biotecnologia – RENORBIO, Ponto Focal Alagoas, Universidade Federal de Alagoas, como requisito parcial para a obtenção do Título de Doutora em Biotecnologia, Área de Concentração: Biotecnologia em Saúde.

Aprovada em: 02/03/2016.

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Dedico este trabalho ao meu pai Waldney Vitalino de Araújo "in memoriam". Ainda escuto sua voz falando "Doutora", "minha doutora". Tinha um imenso orgulho em saber que me tornaria doutora, talvez nem tendo noção do real significado, mas ficava feliz, pelo simples fato de saber que era o que me deixava feliz...

AGRADECIMENTOS

A DEUS, pelas melhores dádivas da vida, pela minha experiência e formação, pelo amor da minha família, pela concretização de mais uma etapa de vida;

À Profa. Dra. MAGNA SUZANA ALEXANDRE MOREIRA pela orientação, dedicação, competência ao longo deste trabalho e acima de tudo por sua amizade... Palavras jamais seriam capazes de descrever a admiração, carinho e gratidão que sinto... Agradeço por me ensinar a ser uma profissional melhor e pelas valiosas lições de vida;

À Profa. Dra. TÂNIA MARIA SARMENTO SILVA, pelo auxílio na coorientação. Agradeço pelo apoio e dedicação neste trabalho;

À MINHA MÃE ANA, por todo esforço, sacrifício e dias de sono perdidos... Por você! Tenho amor maior do mundo;

Aos meus amados irmãos MICHAEL e MAX, meus maiores exemplos de ser humana. O que sou hoje, sou por vocês! Minha eterna gratidão...

Aos meu sobrinho KARL MAX, nossos sorrisos jamais seriam os mesmos sem você, obrigada por tornar nossa caminhada mais amena;

A toda minha FAMÍLIA, meu bem maior! Fica difícil citar nomes, mas cito o nome de nossa amada Vó Marinalva Vital, representante e matriarca da nossa família, a esta, a imensidão do nosso amor... Jamais teria chegado até aqui, se não tivesse uma família tão linda e abençoada. "Uma família muito engraçada e também muito ouriçada", mas que representam um elo de amor que me mantém firme e feliz;

Ao meu namorado DOMINGOS NETO, pela paciência, apoio e amor em todos os momentos;

A todos que fazem partem da grande família que compõe o Laboratório de Farmacologia e Imunidade (LaFI). Como sempre falamos: Uma vez LaFIano, para sempre LaFIano! Éverton Tenório de Souza, Yolanda Karla Cupertino da Silva, Thays de Lima Matos Freire Dias e Ariane Priscila, vocês sempre farão parte dessa família, meu muito obrigada pelo apoio ao longo deste trabalho. À Eliane Aparecida Campesatto, Mariana da Silva Santos, Anderson Brandão Leite, Max Viana, Ana Carolina Santana Vieira, Thiago Matos, Rafael Omena e Amuzza Aylla Santos, obrigada pela companhia e colaboração nessa caminhada;

A professora e pesquisadora ALINE CAVALCANTI DE QUEIROZ, você foi peça primordial para minha formação. Me ensinou muito do que sei hoje, foi paciente, colaborou na maior parte do desenvolvimento desse trabalho, à você minha eterna gratidão;

Aos alunos e amigos de iniciação científica do Laboratório de Farmacologia e Imunidade (ICs-LaFI), Liliane Braga, Gicely Dias, Karoline Jatobá, Jão Kaycke e Jeferson Miguel, sem o apoio de vocês o laboratório não seria o que é hoje, obrigada pelo apoio durante meu doutoramento;

Aos amigos LUIZ HENRIQUE AGRA e CAROLINA BARBOSA BRITO DA MATTA, meus eternos companheiros, fomos imensamente felizes no mestrado e sinto falta de cada momento nosso, obrigada pelo apoio e companheirismo durante minha a vida acadêmica;

Aos companheiros de fluxo e amigos LUIZ ANTÔNIO e AMANDA EVELYN, quantos sábados e domingos eim?! As noites nem se fala! As lâminas de amastigota foram traumatizantes, Luiz pode falar bem disso (rsrs!). As fotos tiradas de lâminas pareciam intermináveis, Amanda é que sabe! Mas, o melhor de tudo isso é que sempre fazíamos brincando e felizes por estarmos juntos. Sobre nossas teorias? Levarei a filosofia para minha vida e jamais esquecerei cada momento juntos. Meu muitíssimo obrigada por tão singela amizade;

A todos os professores da Rede Nordeste de Biotecnologia, pelo ensino e colaboração na minha formação acadêmica;

Aos coordenadores do Laboratório de Imunopatologia Keizo Asami (LIKA)-UFPE, Prof. Dr. Luiz Carlos Alves e Dr. Fábio André Brayner dos Santos, pelo auxílio na execução da Microscopia Eletrônica de Varredura;

A todos os membros do Laboratório de Síntese de Substâncias Bioativas, Departamento de Ciência Molecular, Universidade Federal Rural de Pernambuco pela colaboração neste trabalho;

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), a Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES) e à Fundação de Apoio à Pesquisa do Estado de Alagoas (FAPEAL) pelo apoio financeiro e pela concessão da bolsa;

Enfim, gostaria de agradecer a todos que direto ou indiretamente contribuíram para a realização deste trabalho e que estiveram comigo por toda essa longa caminhada;

Meu muitíssimo obrigada!!

RESUMO

A leishmaniose está entre as doenças mais negligenciadas do mundo. Atualmente, os medicamentos disponíveis para o tratamento são limitados, de toxicidade elevada e cepas de *Leishmania* spp. têm apresentado resistência aos tratamentos preconizados. Assim faz-se necessário a busca de medicamentos mais eficazes e mais seguros. Dessa forma, este trabalho visa investigar a atividade leishmanicida de novos compostos de origem natural e sintéticos. Foram sintetizados duas séries de novos derivados 1,4-naftoquinonas. A série com substituintes bis-2-hidroxi-1,4-naftoquinona exibiram atividade significante contra promastigotas de *Leishmania amazonensis* e *Leishmania braziliensis,* seis destes compostos mostraram boa atividade sem efeito tóxico para célula hospedeira, além disso o composto 3a selecionado para o tratamento no ensaio *in vivo* de infecção com *Leishmania amazonensis* reduziu a lesão da orelha infectada, porém não reduziu a carga parasitária da orelha e do linfonodo drenante, além disso, o tratamento com o composto 3a não induziu alteração no peso do baço, nem alterações de alanino aminotransferase (ALT), aspartato aminotransferase (AST), creatinina e ureia. Compostos da série com substituintes 2-*N,N'*-dialquilamino-1,4-naftoquinona apresentaram atividade contra promastigotas de *L.chagasi* e *L. amazonensis,* com destaque para os derivados 1d, 1h e 1i que inibiram o crescimento de promastigotas de *L. amazonensis* acima de 50%, e os derivados 1d, 1e, 1f, 1h, 1k e 1n que exibiram pronunciada inibição do crescimento de promastigotas de *L.chagasi*, com mais de 70% de inibição. No ensaio contra as formas amastigotas de *L.chagasi*, os derivados 1a, 1b, 1c, 1d, 1h, 1i, 1k e 1m apresentaram significante atividade contra o crescimento das formas intracelulares do parasito. Os flavonoides SP1, SP2, SP3 e o extrato bruto de *Solanum paladosum* inibiram o crescimento de promastigotas de *L. amazonensis e L. chagasi*. Além disso, SP4 inibiu o crescimento de promastigotas de *L. amazonensis*. No que diz respeito à inibição do crescimento das formas intracelulares, SP1, SP3 e o extrato bruto foram estatisticamente significantes. Além disso, no ensaio *in vivo* de infecção com *L. amazonensis* os compostos SP1 e SP3 reduziram a carga parasitária da orelha infectado, porém não reduziram a carga parasitária do linfonodo. Na análise do ciclo celular da *Leishmania,* foi observado que SP1 e SP3 induziram mudança na fase S e G2/M do ciclo celular. Ademais, o composto SP3 na concentração de 100 µM induziu morte por apoptose e alterou a intensidade de autofagia, indicando atividade antiproliferativa. O composto 3,7,3',4'-tetra-O-methylquercetin (retusin) inibiu o crescimento de promastigotas e amastigotas de *L.chagasi e L. amazonensis*. No ensaio de infecção utilizando hamsters infectados com *L. chagasi*, o composto retusin diminuiu a carga parasitária no baço dos animais infectados. Entretanto, no ensaio *in vivo* de infecção de camundongos Balb/c com *L. amazonensis*, o composto retusin não reduziu a carga parasitária da orelha e do linfonodo drenante. Além disso, o ensaio de microscopia eletrônica de varredura revelou que o composto retusin induziu alterações morfológicas em promastigotas de *L. chagasi*. O composto retusin foi capaz de induzir morte por apoptose na concentração de 100 µM, provavelmente não dependente de caspases, além disso, inibiu topoisomerase de *Leishmania*. Os resultados indicam que os derivados 1,4-naftoquinonas e os compostos flavonoides de *S. paladosum* são ativos contra espécies de *Leishmania,* principalmente os compostos 1a, 1b, 1c, and 1m que foram ativos contra espécies de *Leishmania* nos ensaios *in vitro,* sem efeito deletério para célula hospedeira, e os flavonoides SP1, SP3 e retusin que foram ativos por via intraperitoneal no ensaio *in vivo,* indicando que estes são fortes candidatos a fármacos leishmanicidas.

Palavras-chave: Leishmaniose. *Leishmania*. Naftoquinona. *Solanum paladosum*. Retusin. Atividade leishmanicida.

ABSTRACT

Leishmaniasis is among the most neglected diseases in the world. Currently, the drugs available for its treatment are limited and highly toxic, so the search for more effective and safer medicines is necessary. Thus, this paper aims to investigate the leishmanicidal activity of new natural, and synthetic. Two series of new 1,4-naphthoquinone derivatives were synthesized. The series of bis-2-hydroxy-1,4-naphthoquinone substituents exhibited significant activity against *Leishmania amazonensis* and *Leishmania braziliensis* promastigotes, six compounds showed good activity without toxic effects against the host cell. Moreover, compound 3a, which was selected to an *in vivo* infection assay with *Leishmania amazonensis*, reduced the size of the infected ear, but did not reduce parasite burden of the ear and draining lymph node. Furthermore, treatment with 3a induced no change in spleen weight or changes in alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, and urea levels. The series of 2-*N*,*N'*-dialkylamino-1,4-naphthoquinone substituents showed activity against promastigotes of *L. chagasi* and *L. amazonensis*, especially 1d, 1h and 1i, which exhibited *L. amazonensis* promastigote growth inhibition over 50%, and 1d, 1e, 1f, 1h, 1k and 1n derivatives which inhibited the growth of *L.chagasi* promastigotes over 70%. In the assay against the amastigote intracellular forms, 1a, 1b, 1c, 1d, 1h, 1i, 1k and 1m derivatives presented significant activity. The flavonoids SP1, SP2, and SP3 and *S. palodosum* crude extract inhibited the growth of promastigotes and amastigotes of *L. amazonensis* and *L. chagasi*. Moreover, SP4 inhibited the growth of promastigotes *L. amazonensis*. In regard an intracellular forms growth inhibition, SP1, SP3, and crude extract were active significantly. Furthermore, in the *in vivo* assay of infection with *L. amazonensis*, the compounds SP1 and SP3 reduced the parasitic burden on the infected ear, but did not reduce parasite burden on draining lymph node. On *Leishmania* cell cycle analysis, it was observed that SP1 and SP3 induced modifications on S and G2/M phases of cell cycle. In addition, SP3 induced death by apoptosis, and changed autophagy intensity, suggesting anti-proliferative activity at the concentration of 100 µM. The compound 3,7,3',4'-tetra-O-methylquercetin (retusin) inhibited the growth of promastigotes and amastigotes of *L. amazonensis* and *L. chagasi*. In the infection assay using hamsters infected with *L. chagasi*, retusin decreased the parasite burden in the spleen of infected animals. However, in the *in vivo* assay of infection using Balb/c mice infected with *L. amazonensis*, retusin did not reduce the parasitic load in the ear and draining lymph node. Moreover, retusin induced morphological alterations in *L. chagasi* promastigotes observed by scanning electron microscopy. In addition, retusin induced death by apoptosis at the concentration of 100 µM, which is probably not dependent on caspases; besides retusin inhibited *Leishmania* topoisomerases. The results indicate that the 1,4-naphthoquinone derivatives and flavonoid compounds derived from *S. paladosum* are active against *Leishmania*, mainly 1a, 1b, 1c, and 1m, which were active against *Leishmania* in the *in vitro* assays, without induce damage in host cells; and SP1, SP3, and rutesin, which were active *in vivo* by intraperitoneal route, suggesting they can be useful lead compounds candidates for antileishmanial drugs.

Keywords: Leishmaniasis. *Leishmania*. Naphthoquinone. *Solanum paladosum*. Retusin. leishmanicidal activity.

PREÂMBULO

Esta tese está dividida em revisão bibliográfica e artigos (publicado e em submissão) originados da tese. O artigo 1 derivado da tese corresponde ao artigo publicado no periódico *Molecules*; os demais artigos derivados da tese correspondem aos artigos a serem submetidos subsequentemente. Nesses artigos, foram compilados os dados farmacológicos relativos à avaliação da atividade leishmanicida de compostos de origem natural e sintéticos, tendo sido escritos e revisados pela Ms. Morgana Vital de Araújo e pela Profª Drª Magna Suzana Alexandre Moreira. Desta forma, o artigo 1 originado da tese é referente ao manuscrito "Synthesis, leishmanicidal activity and theoretical evaluations of a series of substituted bis-2-hydroxy-1,4-naphthoquinones" que foi publicado no periódico "Molecules", visando à avaliação leishmanicida de uma série de derivados bis-2-hidroxi-1,4-naftoquinonas., bem como sobre os dados de planejamento, síntese dos derivados (estes útimos realizados no Laboratório de Síntese de compostos Bioativos –Departamento de Ciência Molecular/ UFRPE). O artigo 2 intitulado "Evaluation on the leishmanicidal activity of New 2-*N,N'*-dialkylamino-1,4-naphthoquinone Derivatives" será submetido ao periódico "Experimental Parasitology" Este artigo demonstra os resultados relativos ao efeito leishmanicida *in vitro* de derivados 2-*N,N'*-dialquilamino-1,4-naphthoquinona contra *Leishmania amazonensis* e *L. chagasi*. O artigo 3: "Flavonoid compounds: Leishmanicidal evaluation and investigation of the proposed mechanism of action" e 4 intitulado "3,7,3',4'-tetra-O-methylquercetin (retusin): Leishmanicidal evaluation and investigation of the proposed mechanism of action" serão submetidos ao periódico "Antimicrobial Agents and Chemotherapy". Estes artigos mostram os resultados farmacológicos obtidos da avaliação da atividade leishmanicida *in vitro* e *in vivo* de compostos flavonoides contra *Leishmania amazonensis* e *Leishmania chagasi* e propõe o mecanismo de ação leishmanicida dos compostos flavonoides e os resultados farmacológicos obtidos na avaliação da atividade leishmanicida *in vitro* e *in vivo* do composto "3,7,3',4'-tetra-O-methylquercetin contra *Leishmania amazonensis* e *Leishmania chagasi* e propõe o mecanismo de ação leishmanicida desse composto, respectivamente.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

ALP	Alopurinol
CD	Células dendríticas
DTNs	Doenças Tropicais Negligenciadas
FLU	Fluconazol
GPI	Fostatidil-glicosilinositol
GLT	Glucantime
IgG	Imunoglobulinas G
HIV	Vírus da Imunodeficiência Humana
IFN-$_Y$	Interferon gama
IL-10	Interleucina-10
IL-6	Interleucina-6
IL-5	Interleucina-5
IL-4	Interleucina-4
IL-12	Interleucina-12
IL-33	Interleucina-33
iNOS	Síntase de óxido nítrico 2
LC	Leismaniose Cutânea
LCD	Leismaniose Cutânea Difusa
LMC	Leismaniose Mucoutânea
LPG	Lipofosfoglicanos
LT	Leismaniose Tegumentar
LV	Leismaniose Visceral
moCDs	Monócitos inflamatórios
NK	Células *Natural Killer*
NO	Óxido Nítrico
OMS	Organização Mundial de Saúde
Sb^{3+}	Complexo Antimonial Trivalente
Sb^{5+}	Complexo Antimonial pentavalente
STQ	Sitamaquina

TGF-β	Fator de Crescimento de Transcrição
TLR	*Toll Like receptors*
TNF-$_{\alpha}$	Fator de Necrose Tumoral alfa

SUMÁRIO

1 INTRODUÇÃO

As doenças tropicais negligenciadas (DTNs) são um grupo diverso de doenças transmissíveis que prevalecem em condições tropicais e subtropicais em 149 países e afetam mais de um bilhão de pessoas (WHO, 2015). Estas doenças infecciosas acometem principalmente as pessoas mais pobres do mundo e além de causarem sofrimento, anemia, desnutrição, deformação e deficiência física em estágios crônicos, as DTNs levam a altos níveis de mortalidade, ocasinando prejuízos sociais e econômicos para as populações afetadas (HOTEZ et al., 2009; FEASEY et al., 2010).

Dentre as doenças negligenciadas, a leishmaniose é uma das mais impactantes, por ocupar o quinto lugar em prevalência no mundo, sendo endêmica em 98 países distribuidos em cinco continentes, África, Ásia, Europa, América e Oceania, com um total de 310 milhões de pessoas expostas aos riscos de infecção. Acredita-se que 12 milhões de pessoas sejam clinicamente afetadas pela doença e que 1,5 a 2,0 milhões de novos casos ocorram a cada ano (WHO, 2010).

A leishmaniose é uma doença causada por protozoários parasitos de mais de 20 espécies de *Leishmania* que são transmitidas aos seres humanos pela picada de flebotomíneos fêmeas infectadas e que podem causar uma variedade de manifestações clínicas. A leishmaniose tegumentar (LT) é a mais comum das leishmanioses e é uma das causas mais importantes de lesões de pele ulcerativas crônicas em todo o mundo. Clinicamente são reconhecidas 3 tipos: Leishmaniose cutânea (LC), leishmaniose cutânea difusa (LCD) e leishmaniose mucocutânea (LMC) (FEASEY et al., 2010).

Na LC, algumas semanas após a infecção, uma pápula pode se desenvolver e evoluir para um nódulo, em seguida, uma úlcera surge com um centro deprimido. A LCD é uma forma rara de LT, descrita em alguns países da América e África. Caracterizada por manifestações clínicas do tipo maculopapulonodulares, riqueza parasitária, com possível cronicidade da doença. Além disso, existe uma refratariedade aos tratamentos e semelhança com a hanseníase virchowiana. Já a LMC se espalha pela mucosa nasal causando destruição do septo nasal e palato, podendo causar desfiguração facial significativa ou até mesmo a morte por meio de comprometimento das vias aéreas (COSTA et al., 2009; REITHINGER et al., 2007).

A síndrome de leishmaniose mais grave é leishmaniose visceral (LV), também conhecida como kala-azar. É caracterizada por febre irregular, perda de peso, anemia, aumento do baço e do fígado, e é fatal se não for tratada. A leishmaniose foi descrita como uma infecção

oportunista em pacientes infectados pelo HIV (Vírus da Imunodeficiência Humana). Com a evolução da epidemia global de HIV, a associação HIV e leishmaniose tornou-se um significante problema no leste da África, Brasil e Índia. A maioria da morbidade e mortalidade no contexto da coinfecção HIV/*Leishmania* ocorre em casos de LV, porém, tem sido relatados também em pacientes com LC e LMC (NGOUATEU et al., 2012; GUIMARAES et al., 2009; KHANDELWAL et al., 2011; GUERRA et al., 2011).

O arsenal terapêutico disponível para tratamento das Leishmanioses é bastante restrito. Os derivados de antimônio pentavalente são os fármacos de primeira escolha em diversas regiões, porém em áreas endêmicas, como em Bihar, seu uso está quase obsoleto, por causa da resistência do parasito (SUNDAR, 2001). Os outros tratamentos eficazes para leishmaniose são anfotericina B deoxicolato, a paramomicina, anfotericina B, pentamidina e miltefosina. Além do problema de resistência do parasito, que também já foi observado no tratamento com pentamidina e miltefosina, os tratamentos disponíveis apresentam outras restrições, como elevada incidência de efeitos adversos e toxicidade (OLIVEIRA et al., 2011; MALTEZOU, 2008).

Um avanço significativo na terapia das leishmanioses foi o desenvolvimento de uma terapia por via oral com uso da metilfosina, uma alquilfosfocolina originalmente desenvolvida para o cancêr (CROFT et al., 2003). Porém**,** além da resistência observada em culturas de *leishmania* spp., nos casos de coinfecção LV/HIV não demonstrou eficácia semelhante a dos antimoniais, e quando testado em LC, os níveis de reicidiva, com o aparecimento de lesões logo após a suspensão do tratamento foram significantes (RITMEIJER et al., 2006; TROYA el al., 2007).

Assim, a quimioterapia das leishmanioses apresenta várias limitações, devido tanto à toxicidade dos antimoniais, quanto a baixa eficácia dos outros fármacos atualmente disponíveis, sendo que com exceção da miltefosina e do ambisome® (formulação da anfotericina B em lipossomas) nenhum produto realmente novo, foi lançado no mercado nos últimos 20 anos, para tratar esta doença. Portanto, a descoberta e desenvolvimento de novos candidatos a fármacos mais eficazes e com reduzida toxicidade continuam a ser um desafio para os pesquisadores. O desafio desta busca é a descoberta de fármacos de fácil administração (preferencialmente ativos por via oral), seletivos e de alto índice terapêutico, sendo ainda capazes de ativar adequadamente o sistema imune do hospedeiro infectado (FREITAS-JÚNIOR et al., 2012).

Neste sentido, o nosso grupo, em colaboração com químicos medicinais, reunimos expertise na busca destes novos protótipos de fármacos leishmanicida.

2 OBJETIVOS

2.1 Objetivo Geral

Investigar a atividade leishmanicida de novos compostos de origem natural e sintéticos.

2.2 Objetivos Específicos

Determinar o potencial citotóxico de novos compostos de origem natural e sintéticos para a célula hospedeira;

Avaliar a toxicidade *in silico* dos derivados bis-2-hidroxi-1,4-naftoquinonaa, através dos perfis "drug-score" e "drug-likeness" no programa OSIRIS®;

Realizar o "screening" farmacológico de novos compostos de origem natural e sintéticos sobre a viabilidade de formas promastigotas (atividade direta sobe o parasito) de *Leishmania* spp.;

Avaliar a ação de novos compostos de origem natural e sintéticos sobre macrófagos infectados *in vitro* com *Leishmania* spp., quanto à taxa de infecção e a multiplicação dos parasitos intracelulares;

Determinar o potencial terapêutico dos compostos mais ativos em modelos *in vivo* de infecção de leishmaniose tegumentar e visceral;

Avaliar a toxicidade hepática e renal em animais infectados e tratados com os compostos mais ativos;

Investigar possíveis mecanismos de ação de morte do parasito com o tratamento dos compostos mais ativos.

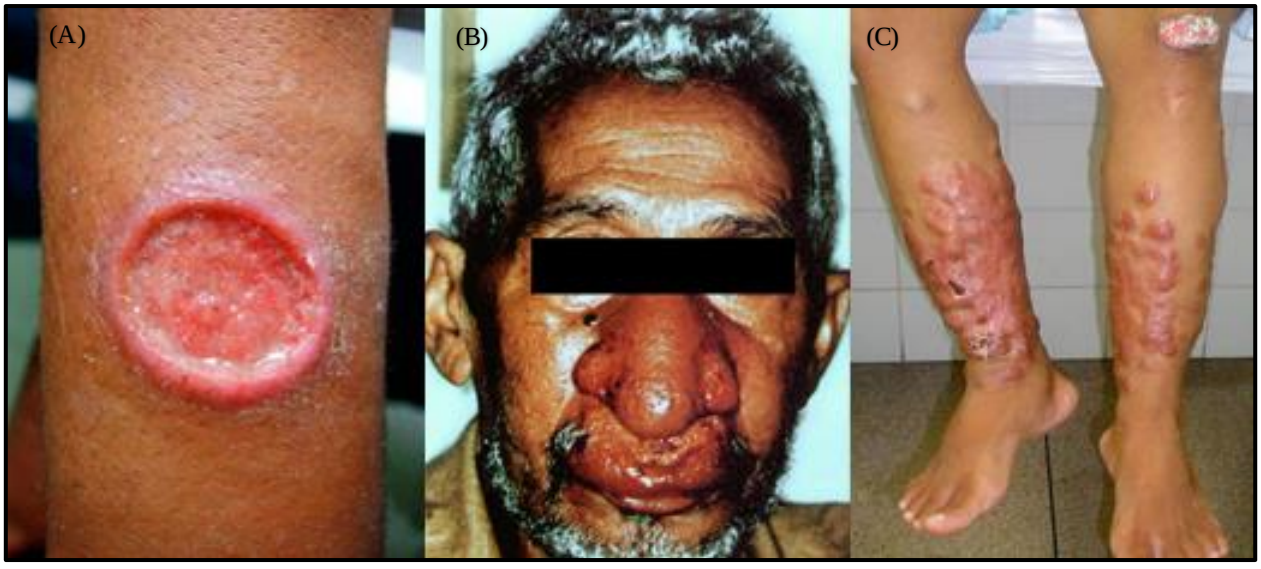
3 REVISÃO BIBLIOGRÁFICA

3.1 As leishmanioses

As leishmanioses são um grupo heterogêneo de síndromes clínicas causadas por protozoários do gênero *Leishmania* spp. (Kinetoplastida: Trypanosomatidae). A transmissão da leishmaniose ocorre através da picada da fêmea do flebotomíneo *Lutzomyia* (Diptera: Psychodidae) infectado com as diferentes espécies de *Leishmania*. As manifestações clínicas dependem da virulência da espécie infectante, susceptibilidade do hospedeiro e coinfecções, variando de uma lesão cutânea até uma visceralização da doença, muitas vezes fatal. (ANTINORI; SCHIFANELLA; CORBELLINO, 2012; MURBACK et al., 2011).

A intensidade e a natureza da resposta inflamatória levam, portanto, ao surgimento das lesões e determinam a sua extensão e duração. A baixa ou a ausência da resposta imune levam a uma doença crônica de difícil tratamento e, consequentemente, uma reatividade exacerbada leva a lesões desfigurantes envolvendo pele e mucosas. Portanto, a LT apresenta-se em 3 formas distintas, LC, LMC e LD (AWASTHI; MATHUR; SAHA, 2004; REITHINGER et al., 2007). A **figura 1** exemplifica as manifestações clínicas da LTA.

Figura 1. Manifestações clínicas da LT. (A) LC (B) LMC e (C) LCD.



Fonte: TEIXEIRA et al., 2013. (Foto de J. Costa, CPqGM/FIOCRUZ, Bahia).

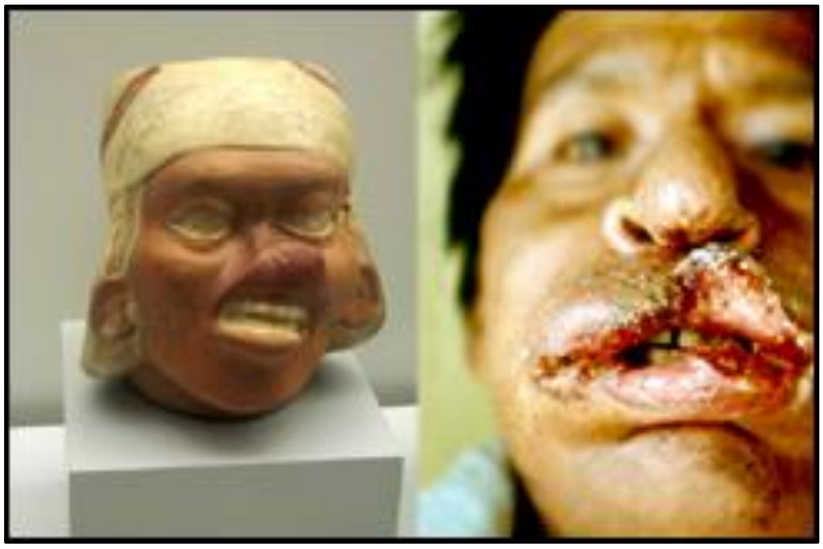
A LC é uma forma dermotrópica da doença. O período de incubação é bastante variável, podendo a infecção evoluir após um período de incubação de 1-12 semanas, em uma pápula que aumenta de tamanho e finalmente úlcera. A lesão típica é indolor e apresenta bordas salientes com fundo necrótico granuloso e úmido (AMATO et al., 2008). Esta lesão pode se curar espontaneamente, deixando uma cicatriz hipopigmentada ou desenvolver-se para outras formas da doença. Podem ocorrer múltiplas lesões metastáticas em áreas não contíguas do corpo (REITHINGER et al., 2007; AMEEN, 2010). Em casos de recidiva após a remissão clínica, novas lesões podem surgir nas bordas ou mesmo sobre a cicatriz de lesões resolvidas há meses ou anos (GOTO; LINDOSO, 2010).

A LMC é considerada a manifestação mais severa da LT. Compromete a mucosa, com destruição tissular progressiva da mucosa nasal e orofaringeana, podendo levar à perfuração do septo e ocasionar destruição de cartilagens e tecidos moles, com possível desfiguração da face e perda do septo, palato e pavilhão auditivo, devido à intensa resposta inflamatória (AMATO et al., 2008). O acometimento de faringe e laringe ocasiona dificuldade para deglutição e, assim, leva à desnutrição, além de eventualmente provocar aspiração e/ou obstrução de vias aéreas que causam importante risco de vida ao enfermo. Nos casos mais graves, o paciente pode chegar ao óbito por complicações respiratórias, devido ao envolvimento da traqueia (HEPBURN, 2000).

A LCD é uma variante rara da LT, caracteriza-se por múltiplos nódulos não ulcerosos (≥10) difundidos por todo o corpo sem envolvimento visceral e desfigurantes. A doença é resistente à quimioterapia e caracterizado por relapsos. A disseminação supostamente ocorre devido à anergia imunológica de alguns indivíduos aos antígenos do parasito (VERMA et al., 2012; AMEEN, 2010; GOTO; LINDOSO, 2010).

Registros de LT existem há milhares de anos no velho mundo e de acordo com os locais em que ocorriam tinham diferentes denominações, como Ferida de Balkh, Botão-de-Bagdá, Botão-do-Oriente. No Novo Mundo, as civilizações pré-colombianas produziram peças de cerâmica, conhecidas como Huacos, representando as lesões destrutivas da LMC. Acredita-se que LV também seja antiga, mas tenha sido historicamente confundida com a Malária até o século XIX, sendo conhecida como Febre Dum-dum ou Kalazar (COX, 2002; BARI, 2006). A **figura 2** representa uma comparação entre um Huaco de cera e um indivíduo com LMC.

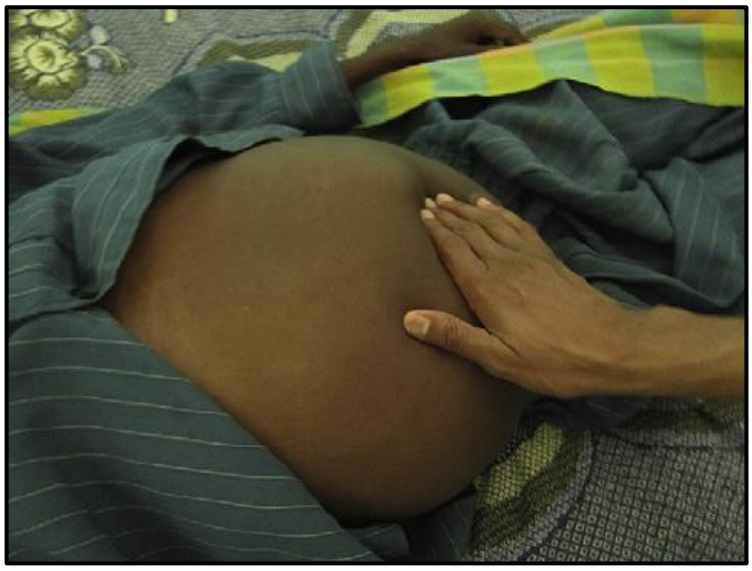
Figura 2. Comparação entre um Huaco de cera e um indivíduo com LMC.



Fonte: WHO, 2015.

A forma mais grave dentre as leishmanioses é a visceral. A LV é uma doença sistêmica, fatal que afeta fígado, baço e medula óssea. Os sinais e sintomas incluem febre, perda de peso, hepatoesplenomegalia e pancitopenia. Em alguns casos o indivíduo pode permanecer assintomático por décadas. Devido ao comprometimento do sistema imunológico, coinfecções bacterianas e virais (principalmente HIV), como pneumonia e diarreia podem surgir. O óbito ocorre pelas coinfecções, sangramento massivo ou anemia severa (CHAPPUIS; SUNDAR; HAILU, 2007; CLEM, 2010). A **figura 3** exemplifica um paciente com LV e hepatoesplenomegalia (Gondar, Ethiopia).

Figura 3. Paciente com LV.



Fonte: GRIENSVEN; DIRO, 2012.

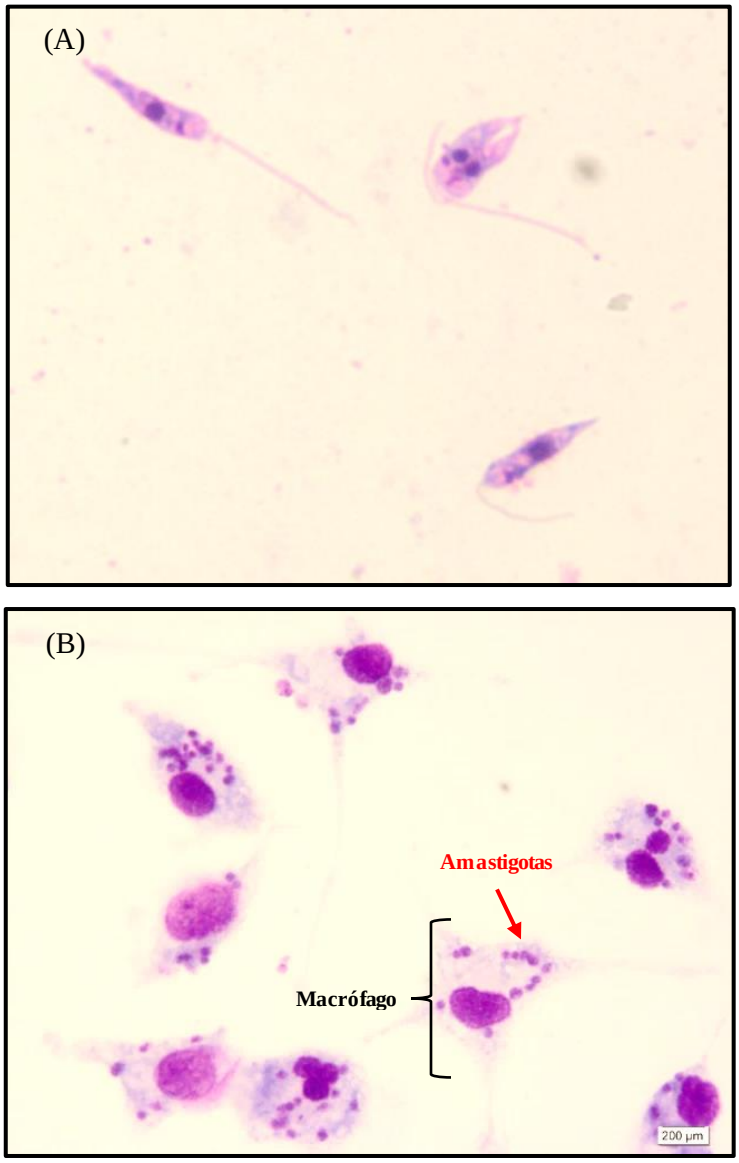
3.2 O parasito *Leishmania* e sua transmissão

Cerca de 15 espécies diferentes são causadoras de LT. Na Ásia, África e Europa são conhecidas: *L.* (*L.*) *major*, *L.* (*L.*) *tropica*, *L.* (*L.*) *aethiopica*, e algumas cepas de *L.* (*L.*) *infantum*, e no Novo Mundo as principais espécies causadoras são *Leishmania* (*Viannia*) *braziliensis*, *L.* (*L.*) *amazonensis*, *L.* (*V.*) *guyanensis*, *L.* (*V.*) *panamensis*, *L.* (*L.*) mexicana, *L.* (*L.*) *pifanoi*, *L.* (*L.*) *venezuelensis*, *L.* (*V.*) *peruviana*, *L.* (*V.*) *shawi*, e *L.* (*V.*) *lainsoni*. A maior diversidade é encontrada na região amazônica, sendo a *L* (*V.*) *braziliensis* a mais prevalente, seguido por *L.* (*L*) *amazonensis* e *L.* (*V.*) *guyanensis* ((BANULS et al., 2007; GOTO; LINDOSO, 2012; WHO, 2010).

A LV é causada por *L. donovani* e *L. infantum* (também conhecida como *L. chagasi* na América do Sul). Excepcionalmente, tem sido observado visceralização de espécies tipicamente associada com a leishmaniose cutânea. Este achado tem sido relatado com *L. tropicalis* no Oriente Médio e *L. amazonensis* na América do Sul. Além disso, em indivíduos infectado com o HIV, a visceralização de um número de espécies dermatotrópicos foi documentada (CHAPPUIS; SUNDAR; HAILU, 2007; MURRAY et al., 2005, BELO et al., 2013; ALVAR et al., 2008).

Existem duas formas evolutivas: a forma amastigota com estrutura arredondada ou ovalada sem flagelo encontra-se no interior de células do sistema mononuclear fagocitário de hospedeiros vertebrados, como monócitos, histiócitos e macrófagos; e a forma promastigota, que é alongada, extracelular, possui flagelo livre e longo emergindo do corpo do parasito e pode ser encontrada no tubo digestivo dos flebotomíneos (ROBERTS, 2006). A **figura 4** mostra as duas formas evolutivas de *Leishmania* sp.

Figura 4. Formas evolutivas de *Leishmania* sp. (A) Promastigotas (B) Amastigotas.

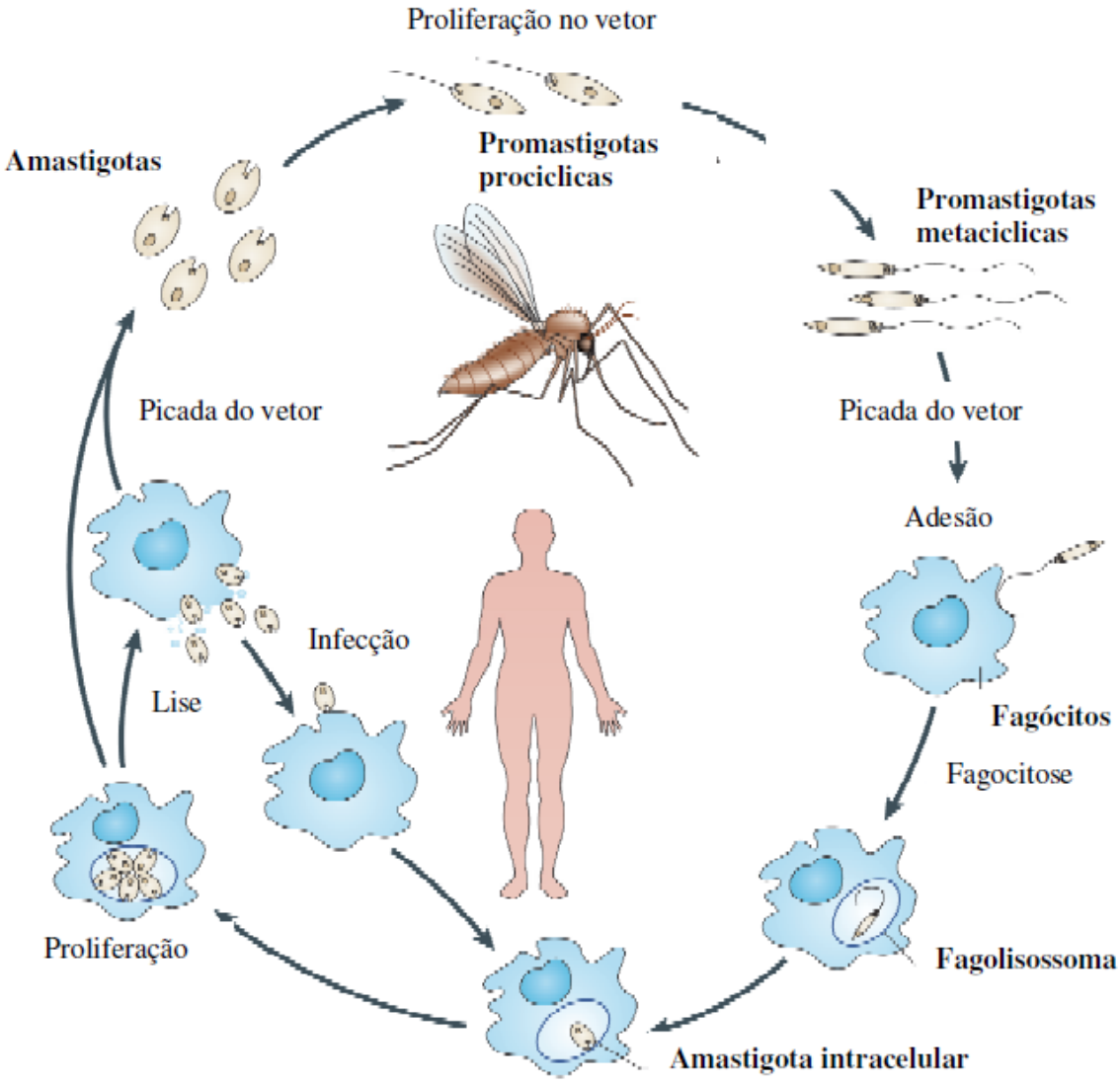


Fonte: Autora, 2015.

As leishmanias são protozoários heteroxenos e durante o seu ciclo biológico assumem duas formas morfológicas distintas: promastigota no flebotomíneo e amastigota no hospedeiro mamífero (MURRAY et al., 2005). Durante o repasto sanguíneo em um mamífero infectado, as fêmeas de flebotomíneos ingerem formas amastigotas. No trato digestório do inseto, as formas amastigotas se transformam em promastigotas flagelados móveis que se proliferam através de divisão binária. Após uma semana do repasto sanguíneo, ocorre o processo de metaciclogênese com o surgimento de parasitos não proliferativos e altamente infectantes, as promastigotas metacíclicos. Estes se acumulam nas porções anteriores da hipofaringe do inseto e durante o novo repasto sanguíneo são regurgitados na derme do hospedeiro (OSÓRIO; FORTEA et al., 2007; KAYE; SCOTT, 2011).

As promastigotas metacíclicos são recobertas com um denso glicocálix composto de macromoléculas fixadas por âncoras de fostatidil-glicosilinositol (GPI). Os constituintes mais abundantes são os lipofosfoglicanos (LPG) com âncoras de GPI e enzimas como a protease gp63, moléculas essenciais para a virulência do parasito (AWASTHI; MATHUR; SAHA, 2004). A promastigota converte o C3b, componente do sistema complemento, em iC3B através da ação da gp63a, favorecendo a pronta fagocitose através da interação com os receptores de complemento CR1 e CR3 de células dendríticas, neutrófilos e principalmente macrófagos (SHARMA; SINGH, 2010). Os parasitos passam por um remodelamento e diferenciação celular levando a formas ovais amastigotas não flageladas por um processo de autofagia mediado por cisteína peptidases (WILLIAMS et al., 2006). Essas amastigotas intracelulares ficam dentro de vacúolos parasitóforos e multiplicam-se por divisão binária. Pode ocorrer lise celular causada pela replicação das formas amastigotas, estas podem então infectar outros fagócitos e/ou serem sugadas pela fêmea do vetor em um novo repasto, completando o ciclo (MURRAY et al., 2005; SHARMA; SINGH, 2010). A **figura 5** mostra o ciclo de vida do parasito *Leishmania* sp.

Figura 5. Ciclo de vida do parasito *Leishmania* sp.



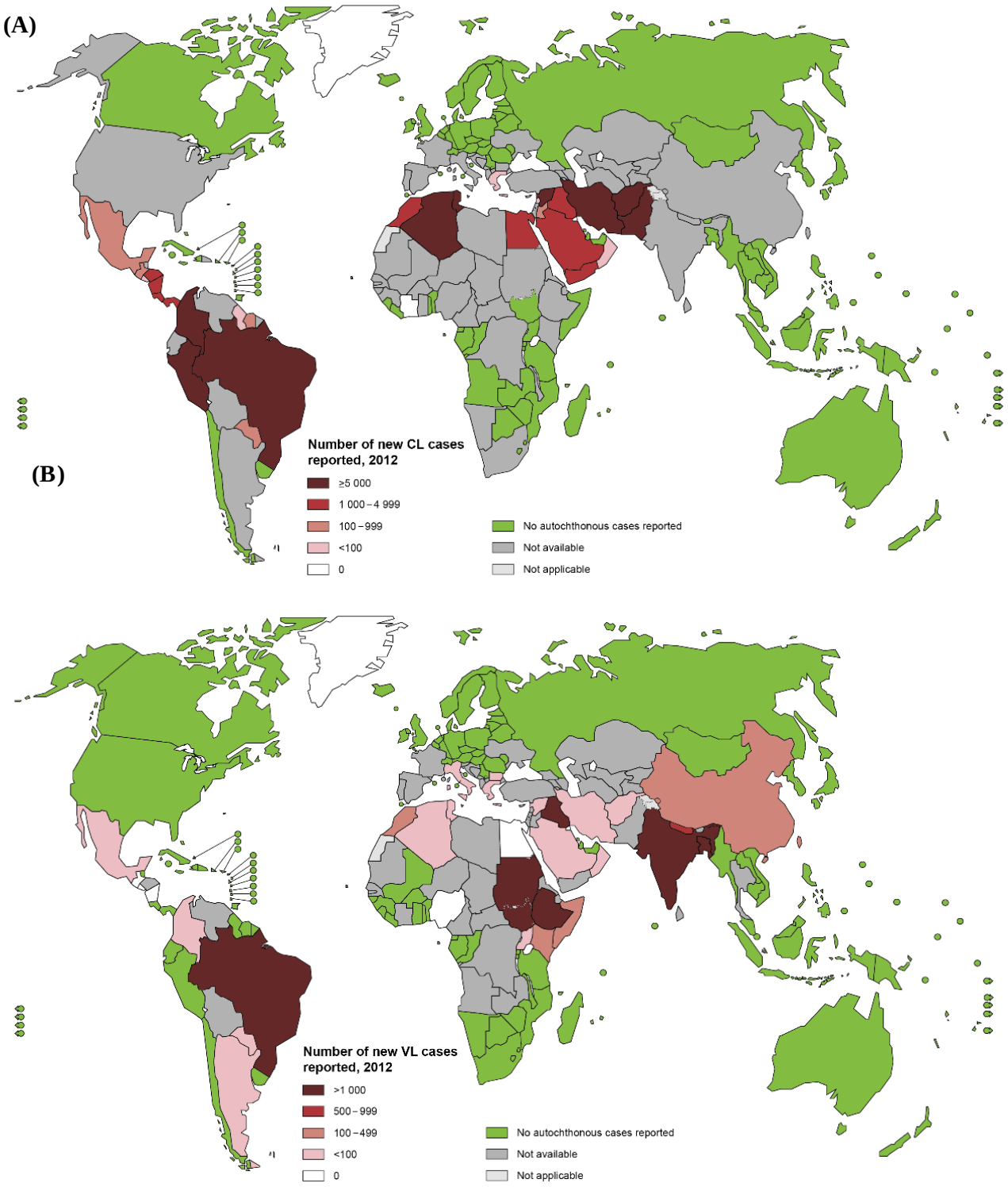
Fonte: KAYE; SCOTT, 2011.

3.3 Epidemiologia

A leishmaniase ocorre em cinco continentes e é endêmica em 98 países. A Organização Mundial da Saúde (OMS) estima que 310 milhões de pessoas estão em risco de contrair leishmaniose e que ocorram anualmente 1,5 a 2 milhões de casos em todo o mundo. Cerca de 58 mil casos de LV e 220.000 LT são reportados oficialmente a cada ano. No entanto, apenas cerca de dois terços dos países informam dados de incidência, e esses dados são excassos em alguns países endêmicos, como por exemplo a África. Baseado em avaliações da sub-notificação, 0,2 a 0,4 milhões de novos casos de LV e 0,7 a 1,2 milhões de novos casos de LT

são estimados para ocorrer a cada ano (WHO, 2010; ALVAR et al., 2012). A **figura 6** mostra a distribuição geográfica de LT e LV, segundo dados da OMS.

Figura 6. Distribuição geográfica das leishmanioses em 2012. (A) LT (B) LV



Fonte: WHO, 2015.

LV humana é causada principalmente por duas espécies de parasitos, *L. infantum* que é o agente causador da doença no Mediterrâneo, Oriente Médio, Ásia Central, China e América Central e do Sul e *L. donovani* na Índia e na África Oriental. LV também pode ser causada por *L. tropica* no Velho Mundo e *L. amazonensis* no Novo Mundo, e é fatal em 85-90% dos casos não tratados e até 50% dos casos tratados (GILL; BEECHING, 2009). Aproximadamente 90% da LT ocorrem no Afeganistão, Paquistão, Síria, Arábia Saudita, Argélia, República Islâmica do Irã, Brasil e Peru (KASSI et al., 2008).

Após a malária e tripanossomíase Africana (doença do sono), a leishmaniose é a terceira doença mais importante transmitida por vetores e está em nono em termos de peso global dentre as doenças infecciosas e parasitárias, representando mais de 57.000 mortes por ano (HOTEZ et al., 2004). A mortalidade associada a LT não é significante. No entanto, a morbidade, sob a forma de deformação, com subsequente estigmas sociais que surgem a partir de lesões cutâneas e das cicatrizes resultante da doença é muito importante. Em áreas endêmicas, muitas pessoas tem a crença de que o LT pode ser transferido através do contato físico (KASSI et al., 2008), resultando em restrição de participação social. Indiscutivelmente, tão importante como os efeitos na saúde e economia, são os impactos negativos sobre a qualidade de vida e saúde mental resultante do estigma social (HOTEZ, 2008). A LT é endêmica no norte da Argentina. O maior número de casos notificados (53,1%) e a maior incidência foram encontradas no norte da província de Salta, o que representa apenas 0,7% da população do país, sendo o foco primário de LC e de LMC (AMPUERO et al., 2005; GIL et al., 2010; MARCO et al., 2005; SALOMÓN et al., 2001; SOSA-ESTANI et al., 2001).

A leishmaniose é endêmica em todos os países do sul da Europa, com aproximadamente 700 casos de infecções humanas registradas a cada ano (DUJARDIN et al., 2008). A incidência média anual de leishmaniose na Espanha é de 0,45 casos/100000 habitantes. Entre 2000 e 2010, foram registrados 2739 pacientes hospitalizados com diagnóstico de leishmaniose na Espanha, dos quais 30,5% eram crianças com menos de 14 anos de idade, e 36,1% eram adultos coinfectados com HIV (SUÁREZ et al., 2012).

Em Madrid, ao longo da costa do Mediterrâneo e nas Ilhas Baleares, a incidência é um pouco maior com 12 a 25 casos registrados por ano, uma incidência de 0,5/100000, e esse padrão epidemiológico é o mesmo para o resto do país. Algumas cidades ao sudoeste de Madrid (Espanha) sofreram um surto de leishmaniose de 2009 até dezembro de 2012, e os números de infecções humanas aumentaram consideravelmente. Foram registrados 446 casos de leishmaniose, dos quais 35,9% eram LT e 64,1% eram LV (ARCE, et al., 2013). A

epidemiologia deste surto foi inesperada, por ser uma área urbana e por ter afetado apenas 14,1% de crianças com menos de 14 anos de idade. Além disso, apenas 15% das pessoas infectadas foram indivíduos imunocomprometidos (11% imunossuprimidos e 4% com HIV) (ARCE et al., 2013). Tais estatísticas mostraram um perfil epidemiológico diferente para a leishmaniose humana na Espanha, pois em estudos anteriores os mais acometidos eram crianças menores de 14 anos e pacientes com HIV (SUÁREZ et al., 2012).

No período de 2009 a 2013, foram registrados no Brasil 115.164 casos de LT e 18.560 de LV. O maior número de casos de LTA está concentrado na região Norte (39,7% dos casos registrados no país), seguido da região Nordeste (34,9% dos casos registrados no país). Já com relação à LV, a região Nordeste é a que apresenta maior percentual de casos notificados no Brasil (50,8%) (SINAM, 2015). O país é responsável por 90% dos casos notificados nas Américas e é o terceiro maior foco de LV global. No Brasil, a LV é uma doença que exige a notificação compulsória, cujos medicamentos para o tratamento são fornecidos exclusivamente pelo governo e são liberados somente depois do relato do caso para o Sistema Brasileiro de Informações Reportável de Doenças (ARAÚJO et al., 2013; BELO et al., 2013).

No estado de Alagoas, entre 2011 e 2014, foram notificados 175 casos confirmados de LTA e 110 de LV, sendo que 56% dos casos de LTA ocorreram nos municípios de Colônia Leopoldina, Joaquim Gomes, Novo Lino, União dos Palmares e Tanque D´arca. Do total de casos de LTA, 159 foram tratados com antimônio pentavalente e 69,1% dos pacientes correspondem ao sexo masculino. Em relação a LV, 73 também utilizaram antimônio pentavalente e 59,1% dos pacientes pertencem ao sexo masculino. Neste mesmo período, foram registrados que 92,6% dos casos de LTA apresentaram lesão cutânea e 7,4% lesão mucosa, sendo que 3,4% apresentaram as formas cutâneas e mucocutânea concomitantemente. Em alagoas, a zona residencial tem importância na epidemiologia do estado, sendo a zona rural de forte influência para a leishmaniose. Dos casos notificados de 2011 a 2014, 65,22% dos infectados com LV e 73,14% dos infectados com LT residiam em área rural (SINAM, 2015).

A LV tende a ser altamente focalizado entre os grupos baixo poder socioeconômico. Em Bihar, na Índia, 83% dos domicílios com altas taxas de infecção pertenciam aos mais pobres (BOELAERT et al., 2009). A LT tem sido menos estudada do ponto de vista econômico, apesar de ser citada a ligação entre pobreza e doença (ALVAR; YACTAYO; BERN, 2006). No entanto, algumas avaliações econômicas têm surgido desde 2006, sugerindo intervenções para o diagnóstico precoce e para o tratamento altamente eficaz. O impacto financeiro da LT para o sistema de saúde é considerável. Os custos médicos e não médicos associados a um foco

ultrapassou US $ 385.000 para o tratamento de 1524 pacientes em um único hospital, excluindo os custos indiretos dos pacientes (por exemplo, custos de viagem ou a perda de tempo de trabalho assalariado) (VEGA et al., 2007).

A OMS estima que, se as tendências atuais continuarem, os investimentos podem diminuir ao longo do tempo para LV e LT. Este dado é preocupante, tendo em vista que atualmente o investimento para doenças negligenciadas, já é inferior a outras doenças e os números de casos continuam alarmantes. Portanto, o combate ao vetor, busca de novas terapias e tratamento dos doentes e hospedeiros é urgente. Atualmente é investido menos de um dólar por dia para doenças negligenciadas, esse investimento representara apenas 0,1 por cento da despesa corrente nacional de saúde dos países de renda baixa e média afetados por doenças tropicais. A OMS ressaltou que os governos de países em que pessoas ficam cegas, desfiguradas e morrem por causa dessas enfermidades negligenciadas, precisam reconhecer o grande potencial em retorno humano e econômico do combate ao problema (WHO, 2015).

3.4 O parasito e sua interação com o sistema imune do hospedeiro mamífero

A leishmaniose é um perfeito exemplo da interação parasito-hospedeiro. Através de anticorpos IgG e de fibronectinas, ocorre o fenômeno de adesão das promastigotas metacíclicos à superfície dos macrófagos, que possuem receptores para a porção Fc das imunoglobulinas G (IgG) presentes no local. As promastigotas aderem a algumas moléculas de superfície como CR1, CR3 e C3b antes de serem internalizados (AWASTHI et al., 2004). Alguns parasitos sobrevivem à ação lítica do complemento, e um fator de virulência que contribui para a resistência ao complemento é a atividade proteolítica da glicoproteína de massa molecular 63 kDa (gp63), pois facilita a adesão do parasito à células fagocíticas do sistema imune inato (JOSHI et al., 2002; KAYE; SCOTT, 2011).

Um dos principais desafios que promastigotas metacíclicos enfrentam quando entram no hospedeiro mamífero é estabelecer residência intracelular em macrófagos sem disparar suas defesas antimicrobianas inatas. Em 2003, Laskay e colaboradores a partir de um estudo com *L. major* sugeriram que os neutrófilos poderiam atuar como "Cavalos de Tróia", colaborando assim para a sobrevida do parasito (LASKAY et al., 2003).

O princípio de "cavalo de Tróia" revela que após promastigotas metacíclicas serem depositadas na derme numa mistura de secreções salivares imunomoduladores e

proteofosfoglicanos derivados do parasito, neutrófilos seriam recrutados, a partir de danos do tecido resultante pelo trauma mecânico da picada que liberaria citocinas, como a interleucina-33 (IL-33), e facilitaria esse recrutamento. Os neutrófilos então ficariam em torno das promastigotas metacíclicos, envolvendo muitos em vacúolos não leishmanicidas. Com a morte do neutrófilo, ocorreria a libertação de promastigotas metacíclicos que podem ser pré-condicionados para a sobrevivência em outras células, como células dendríticas (CD) e/ou macrófagos. Além disso, os monócitos inflamatórios (moCDs) recrutados, facilitariam o tráfego do parasito para o linfonodo drenante. Portanto, a replicação ao longo prazo e perpetuação do patógeno envolve neutrófilos, macrófagos ou moCDs, dependendo das espécies de parasitos, porém os neutrófilos seriam responsáveis pela fagocitose inicial por serem de curta duração e os macrófagos dentre poucos dias passam a predominar na região. Não se sabe se os neutrófilos estão envolvidos na absorção da amastigota após a infecção inicial (LASKAY et al., 2003; AWASTHI et al., 2004; KAYE; SCOTT, 2011) **(Figura 7).**

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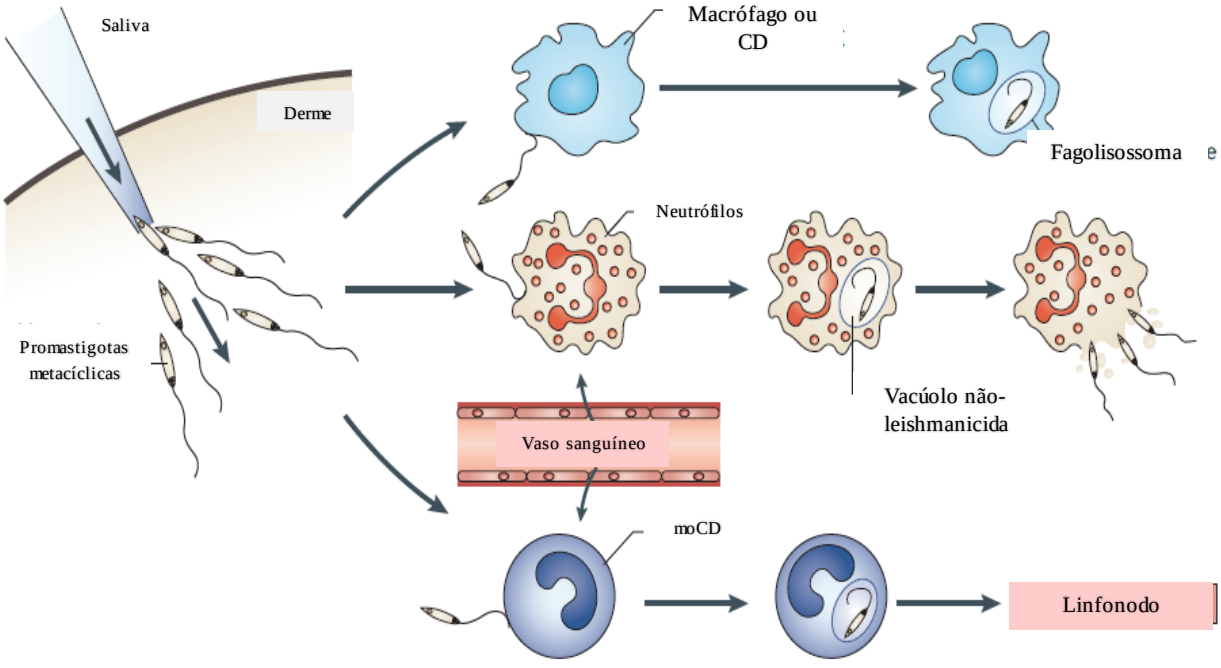
Figura 7. Células envolvidas na captação do parasito *Leishmania* sp.

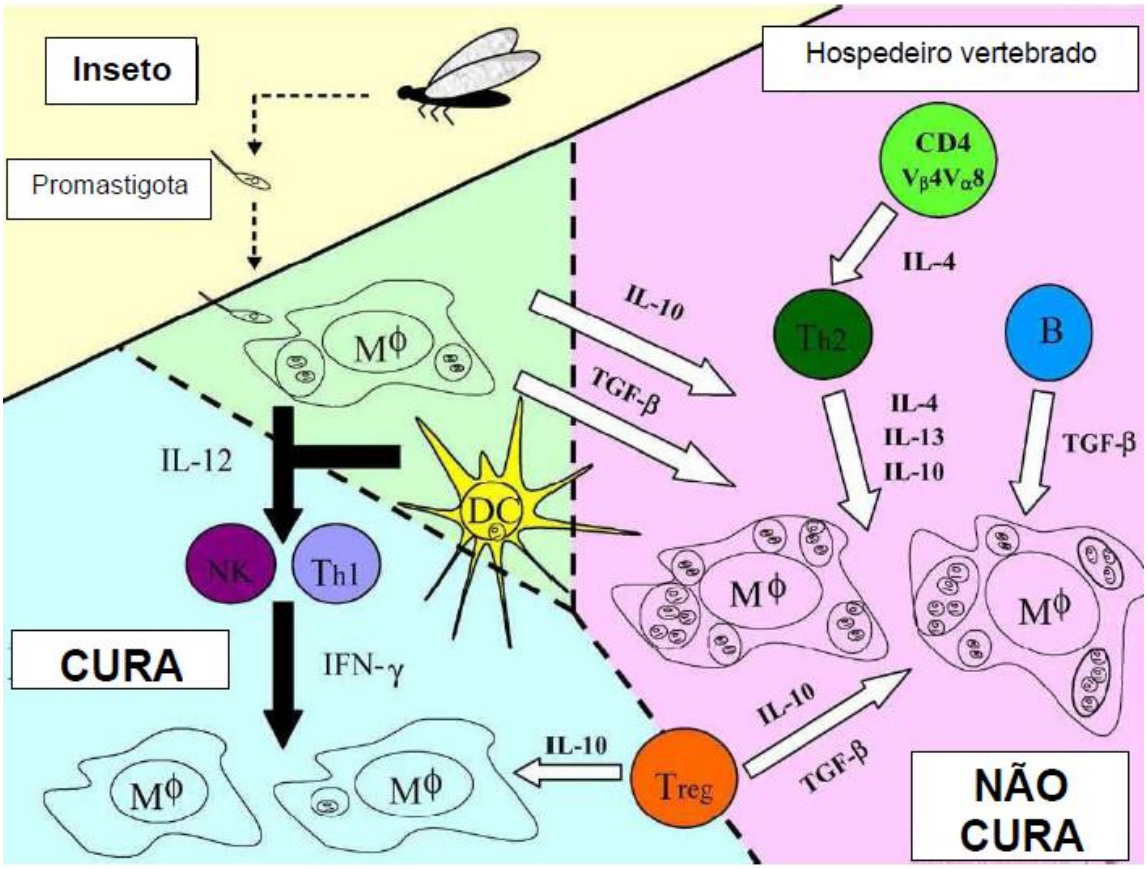


Fonte: KAYE; SCOTT, 2011.

As células do sistema imune inato possuem receptores que reconhecem padrões moleculares do parasito e podem ativar fatores de transcrição desencadeando a produção de citocinas e quimiocinas. Dentre os receptores, estão os da família *Toll Like receptors* (TLR). Na infecção por *Leishmania*, receptores do tipo TLR-2 presentes nos macrófagos e CD podem ser ativados através de moléculas de LPG encontrados em abundância no parasito (DE VEER et al., 2003). Os TLR-2 também podem sinalizar a ativação do fator de transcrição NF-k B em células *Natural Killer* (NK) para produção de interferon gamma (IFN-γ) e Fator de necrose tumoral alfa (TNF-α) (BECKER et al., 2003). Essas citocinas são importantes para ativar os macrófagos e destruirem os parasitos intracelulares via produção de radicais de oxigênio e nitrogênio. Receptores TLR-4 também estão envolvidos no controle da leishmaniose e recentemente foi mostrado a ativação para a produção de IL-12 pela interação de TLR-9 e *Leishmania*. A interleucina-12 (IL-12) é uma citocina importante para ativar NK e linfócitos T a produzirem IFN-γ (ABOU FAKHER et al., 2009).

A resposta imune do hospedeiro vertebrado contra a *Leishmania* é mediada por uma resposta celular, pois o parasito escapa da resposta humoral do hospedeiro por residir dentro dos fagolisossomos dos macrófagos (CUNNINGHAM, 2002). Células "T helper" tem um papel importante na resposta imune antileishmania do hospedeiro vertebrado. Existem duas subpopulações funcionalmente distintas de linfócitos T CD4+, Th1 e Th2, que foram identificadas baseado no perfil de citocinas promovido pelo próprio estímulo antigênico. A resistência do hospedeiro mamífero é associada à ativação seletiva e à diferenciação das células T helper CD4+ efetoras, que se diferenciam em células Th1 produtoras de IFN-γ, IL-12 e TNF-α, dentre outras (PETERS; SACKS, 2006). A indução de IL-12 por células apresentadoras de antígenos e de IFN-γ por célula T tem sido considerada essencial para controlar o progresso da doença (SINGH et al., 2012). Dois mediadores descritos e envolvidos na morte do parasito por macrófagos ativados com IFN-γ e TNF-α são os intermediários reativos de nitrogênio como o óxido nítrico (NO) e o peroxinitrito (KANE; MOSSER, 2000; LINARES et al., 2001; KAYE; SCOTT, 2011). Por outro lado, a suscetibilidade ao parasito é relacionada com a resposta imune Th2, por meio de células T produtoras de citocinas, tais como a interleucina-4 (IL-4), interleucina-5 (IL-5), interleucina-6 (IL-6), interleucina-10 (IL-10), dentre outras (PETERS; SACKS, 2006) **(Figura 8)**.

Figura 8. Esquema mostrando as possíveis vias de resposta imune do hospedeiro vertebrado à *Leishmania* sp.



Fonte: ALEXANDER; BRYSON, 2005.

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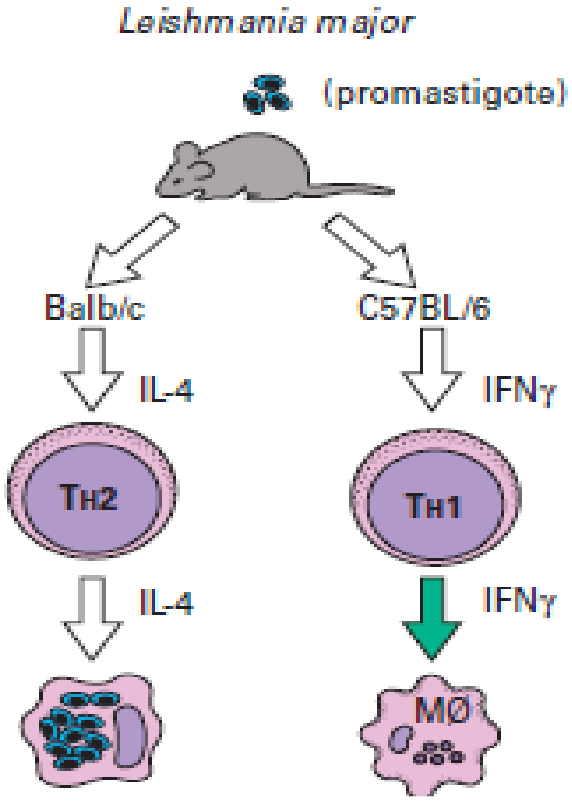
A imunidade mediada por células para controlar protozoários depende da espécie e localização do animal infectado e complexidade do ciclo de vida no hospedeiro. Por exemplo, em camundongos C57Bl6 infectados com *L. major*, a diferenciação de células Th1, com indução de IFN-y e NO, é crucial para a proteção contra a doença. Já linhagens de camundongos Balb/c infectados com *L. major*, desencadeiam resposta Th2, manifestando níveis elevados de IL-4, IL-13, IL-10, e desenvolvendo assim, uma progressiva e fatal doença (SHER; PEARCE; KAYE, 2003; SCOTT, 2004; KAYE; SCOTT, 2011) **(Figura 9).**

Figura 9. De senvolvimento de resposta imune com infecção por *L. major*.



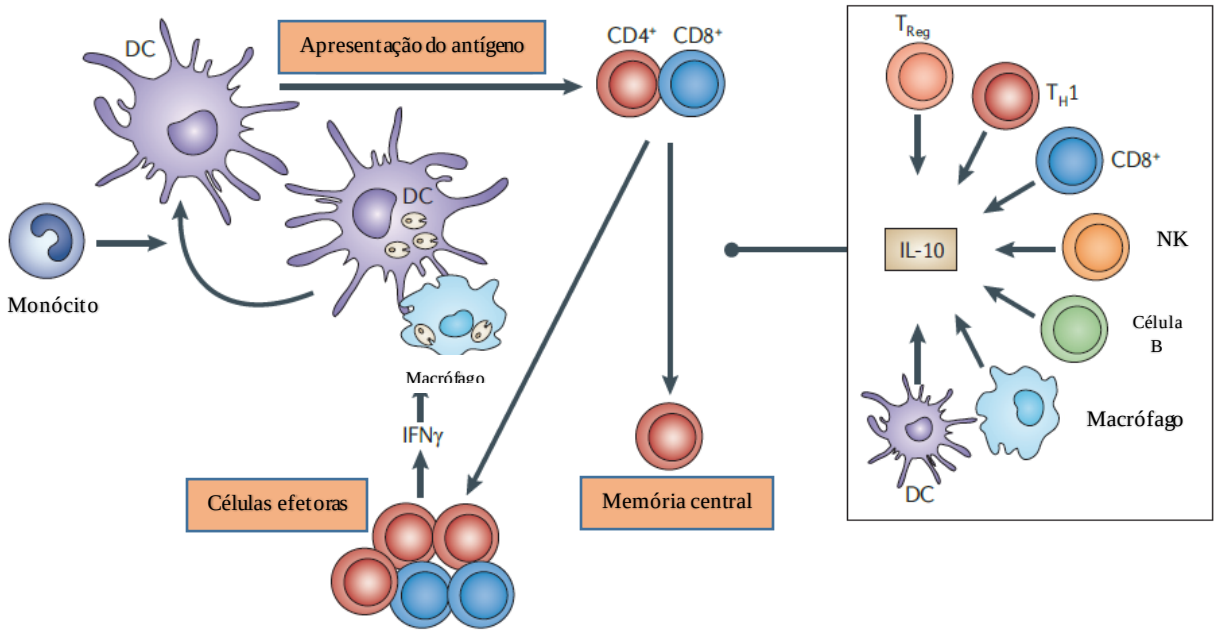
Fonte: MALE et al, 2006.

O papel das células T CD8 + não está bem elucidado. Na LT tem sido menos citada e na LV estudos iniciais indicam serem importantes no controle da doença. Estudos iniciais com *L. major* indicam que as células T CD8+ não foram importantes para o controle da infecção primária, mas participaram da resistência à reinfecção. No entanto, quando os camundongos foram infectados com baixa carga parasitária, as células T CD8+ pareciam ser essenciais para a resolução de infecção primária, devido a capacidade do IFN-y promover uma resposta do tipo Th1. Portanto, células T CD8+ que reconhecem antígenos de *Leishmania* são também ativadas e produzem IFN-y, além de serem capazes de desenvolver uma memória central. Entretanto, os fatores que determinam quando as células T CD8+ são protetoras e quando elas promovem a doença ainda devem ser elucidados (UZONNA; JOYCE; SCOTT, 2004; KAYE; SCOTT, 2011).

A produção de IL-10 tem um grande papel na resposta imune e, permite que alguns parasitos escapem da destruição. Estudos tem mostrado que a IL-10 pode vir de uma variedade

de fontes após infecções de *Leishmania*, incluindo células T reguladoras (Treg) (BELKAID et al., 2002), Th1 (NYLEN et al, 2007), T CD8+ (BELKAID, 2001), células B (RONET et al., 2010) *natural killer* (MAROOF et al., 2008) DCs (SVENSSON et al., 2004), macrófagos (MILES et al., 2005) e neutrófilos (CHARMOY et al., 2007). Qual destes é mais importante como uma fonte de IL-10 ainda não está claro, mas pode depender de diferenças entre os parasitos e as fases da infecção (Figura 10).

Figura 10. Componentes celulares da resposta imune antileishmaniose.



Fonte: KAYE; SCOTT, 2011.

As citocinas como IL-4, IL-10 e fator de crescimento de transcrição- β (TGF-β) inibem a ativação dos macrófagos e a produção de NO por estas células e consequentemente a destruição dos parasitos intracelulares é reduzida (BALESTIERI et al., 2002). Tanto TGF-β como IL-10 podem ser produzidas por Treg, além de outras células. As Treg regulam negativamente o sistema imune e uma subpopulação destas células apresenta o fenótipo TCD4+, CD25+ e fator de transcrição Foxp3^{+} durante a infecção por *L. amazonensis*, estas células foram capazes de produzir TGF-β e IL-10, que juntas inibiram a produção de IFN-y (JI et al., 2005). As células Treg foram encontradas em lesões com LT e foram capazes de produzir IL-10. Outro estudo evidenciou a presença de Foxp3 nas lesões dos pacientes com LC, LMC, e LCD. Os autores sugeriram que as células Treg poderiam está regulando a resposta imune via

Faz/FasL, levando as células T efetoras à apoptose (CARNEIRO et al., 2009). Por outro lado, é necessário enfatizar que IL-10 e TGF-β podem exercer atividade "regulatória" negativa sobre a ativação linfocitária e sobre seus mecanismos microbicidas de macrófagos e não serem prvenientes de Treg, e sim de células do sistema imune inato ou de células Th2. De fato, em camundongos infectados com *L. major*, mostrou-se que a IL-10 é a principal citocina produzida na fase cônica, e é secretado por linfócitos Th1 CD4+ CD25-Foxp3, produtoras também de IFN- y (ANDERSON et al., 2007).

Uma melhor compreensão sobre as células do sistema imune, que têm papéis centrais na etiologia de infecções por *Leishmania*, patologia, desenvolvimento da doença e resistência. Assim, os estudos com o sistema imune, nos leva a compreender o papel das células T regulatórias e de mémoria, o que poderá produzir importantes avanços que levarão a novas ideias para o tratamento, tendo em vista que fármacos imunomoduladores tem atividade contra o parasito *Leishmania* (KAYE; SCOTT, 2011).

3.5 A coinfecção LV-HIV/AIDS

O HIV é um retrovírus que infecta as células do sistema imunitário, destroem ou prejudicam a sua função. À medida que a infecção progride, pode desencadear um quadro de deficiência imune, que aumenta a susceptibilidade a infecções que normalmente seriam controladas em pessoas com resposta imune competente. Uma importante infecção oportunista que surgiu em pacientes infectados com HIV é a LV (KOVACS; MASUR, 2000).

A Organização Mundial de Saúde relata a presença de LV em 65 países, dos quais 34 também relataram coinfecção com o HIV (DESJEUX, 2004). Foi relatado que entre 1,5 a 9% de pacientes com HIV desenvolvem LV (GUERIN et al., 2002). As maiores taxas de prevalência são encontradas na África Central, América do Sul, Europa e subcontinente indiano. Na Europa, de 25 a 70% dos casos adultos de LV acontecem em pacientes com HIV (SUNDAR, 2003). Na Espanha, a LV é a quarta doença parasitária oportunista em pacientes portadores de HIV/AIDS. Nesse país, um estudo mostrou que 91,7% dos pacientes na faixa etária de 21 a 60 anos com LV atendidos em um grande hospital, também encontravam-se infectados pelo HIV (DE LA ROSA, 2002; PINTADO, 2001). Em algumas áreas da Etiópia, 35% de todos os pacientes com leishmaniose são coinfectadas com o HIV (WHO, 2009).

No Brasil, nos últimos anos, a coinfecção VL-HIV/AIDS tem apresentado uma tendência crescente, mas a dimensão exata ainda não foi referida. Mortalidade de casos em pacientes com coinfecção VL-HIV/AIDS é consideravelmente maior do que em pacientes apenas com VL no Brasil. No entanto, estudos sistemáticos para avaliar a epidemiologia e mortalidade relacionada à VL-HIV/AIDS em países endêmicos são escassos (SOUSA-GOMES et al., 2011).

A interação entre Leishmaniose/HIV leva a uma letalidade até cinco vezes maior entre os coinfectados e existe uma maior dificuldade de diagnóstico (PINTADO et al., 2001). A interação da Leishmania e o HIV em uma mesma célula induzem a transcrição e liberação do vírus na circulação, mediante aumento de TNFα e IL-1α. A redução do número de linfócitos T $CD4^{+}$, leva à falta de reconhecimento dos antígenos da *Leishmania* para estimular linfócitos B, isto leva a uma falha na ativação das células B, que acontece em imunocompetentes. Isto explica elevada frequência de resultados falsos negativos em pacientes coinfectados (ZHAO et al., 2004).

Doentes com depleção de células T CD4+ também mostram envolvimento de órgãos atípicos, parasitemia frequente, produção de imunoglobulina G específica abaixo do ideal, reduzida capacidade de resposta à quimioterapia, e previsivelmente elevada taxa de recaída, uma vez que o tratamento é descontinuado (FERNANDEZ-GUERRERO et al., 2004; FERNANDEZ COTARELO et al., 2003). Na Europa, descreveu-se um novo tipo de transmissão associada ao compartilhamento de agulhas entre usuários de drogas injetáveis. Este fato modificou o ciclo de vida da LV, convertendo-a em uma doença antroponótica entre usuários de drogas intravenosas, onde o próprio homem é o reservatório do parasito (CRUZ et al., 2002).

Casos de LT associada ao HIV são pouco frequentes. Porém, casos de coinfecção LT/HIV têm sido relatados, com pacientes apresentando nódulos leprosos em diversas regiões do corpo, além de esplenomegalia leve. Além disso, biópsias de medula óssea e pele foram realizadas e encontradas *L. tropica* (JAFARI et al., 2010). No Irã *L. infantum* foi isolado de um paciente com coinfecção LC/AIDS, neste, ocorreram lesões cutâneas disseminadas. (MOHEBALI, 2013). Caso de coinfecção LC/HIV também foi descrita no Brasil (SILVA et al., 2014).

O tratamento em pacientes coinfectados continua a ser um grande desafio, considerando baixa resposta terapêutica, o que consequentemente aumenta casos fatais com coinfecção, além da alta frequência de graves reações adversas (COTA; SOUSA; RABELLO. 2011). A

toxicidade do antimônio é aumentada em pacientes com HIV, e efeitos adversos como insuficiência renal, pancreatite e miocardite são comumente relatados (ALVAR et al., 2008; ALEXANDRINO-DE-OLIVEIRA et al., 2010).

No Brasil, a anfotericina B desoxicolato foi recomendada como o fármaco de primeira escolha para pacientes coinfectados. No entanto, devido aos resultados desfavoráveis, o Ministério da Saúde revisou as Diretrizes Terapêuticas em 2013, incluindo anfotericina B lipossomal como uma indicação para terapêutica para coinfecção Leishmania/HIV. A OMS considera a coinfecção VL-HIV/AIDS uma prioridade, porém a identificação dos casos e sua notificação precisam ocorrer com rapidez, para que o registro de casos e óbitos de coinfecção seja melhorado, já que esses números ainda são subestimados (WHO, 2015).

3.6 Tratamento das leishmanioses

3.6.1 Antimoniais

Embora conhecidos desde a antiguidade, séculos antes da era cristã, para diversos fins terapêuticos, os sais de antimônio foram introduzidos pela primeira vez em 1912 para o tratamento das leishmanioses pelo cientista brasileiro Gaspar Vianna, que observou que o tártaro emético, um complexo antimonial trivalente (Sb^{3+}) era eficaz contra LTA. Entretanto, o uso clínico do Sb^{3+} foi interrompido por causa dos seus severos efeitos colaterais, dando lugar aos complexos de antimônio pentavalente (Sb^{5+}), que são compostos mais eficazes e cerca de 10 vezes menos tóxicos que seus análogos Sb^{3+}. Assim, em 1936 Schmidt iniciou o uso clínico do estibogluconato de sódio, e em 1946 foi introduzido o antimoniato de meglumina ou N-metilglucamina, que são os antimoniais utilizados até hoje para o tratamento da leishmaniose (RATH et al., 2003, LEE; GILBERT, 2000).

Os Sb^{5+} passam por uma dissociação para que o antimônio exerça sua atividade (FRÉZARD et al., 2001). Os antimoniais pentavalentes exercem pouco efeito sobre as formas promastigotas que crescem em culturas axênicas. Este acentuado contraste entre as atividades *in vitro* e *in vivo* desses compostos, sugere que, para que exerça a atividade leishmanicida, é necessária a redução de Sb^{5+} para Sb^{3+}. Foi proposto que o Sb^{5+} seria uma pró-droga, que se torna ativa e tóxica após redução no organismo a Sb^{3+} e que, dessa forma, interferiria no metabolismo dos tióis, levando a um efluxo de tióis e a inibição da tripanotiona redutase (TR)

provocando estresse oxidativo, além da ejeção de zinco, que possuem papéis importantes na função metabólica do parasito, fazendo então com que ocorra a morte por apoptose do parasito (WYLLIE; CUNNINGHAM; FAIRLAMB, 2004; DEMICHELI et al., 2008). Algumas pesquisas mostram que somente as formas amastigotas são capazes de reduzir Sb^{5+} a Sb^{3+}. Por outro lado, alguns grupos têm sugerido que a redução do Sb^{5+} ocorra no macrófago. Estas hipóteses não são incompatíveis, sendo possível que a redução ocorra em ambos os locais (SHAKED-MISHAN et al., 2001; SERENO et al., 1997).

Duas importantes proteínas tem sido descritas, por serem capazes de mediarem a redução do antimônio Sb^{5+} a Sb^{3+} dentro da célula, são elas redutase tiol dependente (TDR1) e arsenato redutase (ACR2) (DENTON et al., 2004). Transportadores de fosfato e aquaporina-1 na membrana plasmática da amastigota são capazes de transportar metaloides trivalentes para dentro da célula, embora a redução em forma trivalente ocorre apenas numa quantidade menor em fagolisossomas do macrófago (GOURBAL et al., 2004). É possível ainda que o antimônio (tanto o trivalente quanto o pentavalente) tenha ação na quebra do DNA da *Leishmania*. Acredita-se que haja inibição da via glicolítica e da oxidação dos ácidos graxos do parasito (RATH et al., 2003), o que leva à redução do trifosfato de adenosina (ATP) e do trifosfato de guanosina–GTP (SINGH; SIVAKUMAR, 2004).

Estudos têm evidenciado que há uma importante participação da atividade imunológica na resposta à terapia com o antimônio. Na infecção do macrófago *in vitro*, o antimônio pentavalente age como leishmanicida. No entanto, em modelo animal, a ação do antimônio depende de citocinas e de subtipos de células T. Há relato, inclusive, de aumento do TNF-α em pacientes submetidos ao tratamento com N-metilglucamina (KOCYIGIT et al., 2002).

Após administração endovenosa ou intramuscular, o antimoniato de metilglucamina é rapidamente absorvido e, praticamente 90% do antimônio é excretado nas primeiras 48 h pelos rins. Em consequência, faz-se necessária a administração de doses elevadas do fármaco, em regime contínuo, para garantir um elevado teor de antimônio nos tecidos e, assim, obter a eficácia do tratamento (RATH et al., 2003). Essas doses elevadas e contínuas levam ao acúmulo do antimônio, principalmente no sangue e em órgãos vascularizados como rins e baço, sendo descrita importante afinidade do antimônio trivalente pelo eritrócito. Um ano após a suspensão da medicação, o antimônio foi detectado nos cabelos de pacientes que a receberam (RATH et al., 2003).

Entre as possíveis reações ao tratamento destacam-se risco de insuficiência respiratória e a parada cardíaca súbita (RIBEIRO et al., 1999). A alteração mais frequente ao eletrocardiograma é o prolongamento do intervalo QT (inclusive do QT corrigido - QTc) e a inversão da onda T (NAME et al., 2005). Torsades de pointes, uma taquiarritmia ventricular polimórfica potencialmente fatal, frequentemente ocorre em associação com o prolongamento do QT ou do QT corrigido, podendo se apresentar como síncope, palpitações, convulsões, tonteira e até mesmo como morte súbita. No entanto, se o episódio de torsades de pointes for de curta duração, pode ser assintomático. A anemia e a hiperpotassemia são fatores que podem predispor a alterações cardíacas. Pacientes que apresentam alterações, mesmo leves, da condução elétrica devem ser tratados em regime hospitalar (OWENS; NOLIN, 2006).

O antimonial não deve ser prescrito a gestantes. Ao atravessar a barreira placentária, o antimônio pode impregnar o tecido nervoso do feto e levar ao retardo mental (MAYRINK et al., 2006). Há um relato de uma paciente gestante tratada com glucantime, que teve o parto no quinto dia após o tratamento, ocorrendo óbito do recém-nascido após um dia de vida (SILVEIRA et al., 2003). Crianças abaixo de 5 anos tratadas com glucantime tiveram baixa porcentagem de cura das lesões em LC (PALACIO et al., 2001). Esses resultados podem ser explicados porque a quantidade de fármaco encontrada no soro das crianças de 3-6 anos é bem menor, comparada à dose de glucantime (20mg/kg/dia), isso resulta em baixa eficácia do fármaco nessa faixa etária (CRUZ et al., 2007). Entretanto, crianças entre 5 e 14 anos que usaram glucantime, não apresentaram diferenças nos resultados de cura se comparados com os adultos (SCHUBACH ADE et al., 2005). Além disso, não deve ser indicado a hepatopatas e nefropatas, devido seu potencial hepatóxico e nefrotóxico (DEMICHELI et al., 2005).

Os relatos de pacientes não responsivos às drogas tem aumentado, devido ao surgimento de cepas resistentes de parasitos (CROFT et al., 2006). Os maiores problemas de resistência aos antimoniais tem sido relatados na região de Bihar (Índia), cerca de 50 a 65% dos pacientes com LV não respondem bem ao tratamento com os antimoniais. A razão para o aparecimento de resistência é difundido, pois ocorre o uso indevido do fármaco. Sb^{5+} está disponível gratuitamente na Índia e é de fácil acesso. Intervalos livres do fármaco são dados com a crença de que eles vão impedir a toxicidade renal. Em muitas ocasiões, a dose diária de fármaco é dividida em duas injeções, a serem dadas duas vezes por dia. Estas práticas presumivelmente levam a uma tolerância progressiva do parasito aos Sb^{5+}. Tem sido observado que apenas uma minoria de pacientes (26%) foram tratados de acordo com as diretrizes prescritas, esses fatos

apontam para a má gestão de fármacos leishmanicidas em Bihar como um contributo significativo para o desenvolvimento de resistência ao fármaco (SUNDAR, 2001).

3.6.2 Pentamidina

As amidinas são compostos orgânicos caracterizados pela presença dos grupamentos C-N e C=N, que proporcionam propriedades específicas amina e azometina, respectivamente. Esses compostos apresentam atividades biológicas variadas, como antitripanossomatídea, antifúngica, antibacteriana, antiviral e antitumoral (SOARES-BEZERRA; LEON; GENESTRA, 2004). A pentamidina (lomidina®) é uma molécula de grande interesse no tratamento de leishmaníase visceral e mucocutânea refratária a antimoniais pentavalentes (AMATO et al, 2000). A alta toxicidade deste fármaco, com relatos de morte repentina, é um fator limitante de seu emprego terapêutico. Pode causar mialgia, dor no local da injeção, náuseas, cefaleia e, menos comumente, resulta em um gosto metálico, sensação, dormência e queimação, hipotensão e diabetes mellitus insulino-dependente irreversível (SUNDAR; CHATTERJEE, 2006). O número de pacientes não responsivos ao tratamento com pentamidina tem aumentado, principalmente em casos de coinfeções com HIV, e em casos de toxicidade, como hipoglicemia reversível e nefrotoxicidade (BASSELIN et al., 2002).

A entrada da pentamidina na célula ocorre através de transportador de alta afinidade dependente de prótons. Sabe-se que a pentamidina liga-se ao DNA mitocondrial (kDNA) da *Leishmania*, podendo assim afetar os processos de replicação e transcrição e, consequentemente provocar a desintegração de moléculas de DNA que compõe o genoma mitocondrial (BASSELIN et al., 1997). Outros dados mais recentes vieram a confirmar que a mitocôndria é um importante alvo da pentamidina em *Leishmania* ocorrendo alterações no potencial de membrana mitocondrial (BASSELIN et al., 2002; BRAY et al., 2003). Outro mecanismo de ação aceito é a inibição da topoisomerase I (SINGH AND DEY, 2007) e II (MISHRA; SAXENA; SINGH, 2006).

Outra hipótese, diz respeito a interferência de diamidinas aromáticas (ex. berenil e pentamidina) sobre sistemas de transporte poliamínicos, biomoléculas de importância em vários processos bioquímicos da fisiologia celular (BASSELIN et al, 2000). O mecanismo molecular está associado à inibição não-competitiva da recaptura de poliaminas (ex. espermidina, espermina e putrescina) e inibição direta da S-adenosilmetionina descarboxilase (SAMDC), enzima envolvida na biossíntese da espermidina, sendo que estudos quantitativos de relação

estrutura atividade (QSAR) mostraram que a inibição da recaptaçao é proporcional à distância entre grupos aminos dos substituintes amidino. Outro mecanismo proposto é a inibição direta da S-adenosilmetionina descarboxilase (SAMDC), enzima envolvida na biossíntese da espermidina (REGUERA et al, 2005).

3.6.3 Anfotericina B

A anfotericina B é um antibiótico poliênico, com atividade fungicida e leishmanicida, derivado de uma cepa de *Streptomyces nodosus*. Pertence ao grupo dos fármacos leishmanicidas de segunda geração e é usada no caso de falhas no tratamento com compostos antimoniais. Apesar de sua elevada toxicidade e do fato de também requerer administração parenteral, a anfotericina B tem sido proposta como agente terapêutico de escolha para leishmaniose visceral em alguns estados da Índia. Além disso, a anfotericina B é a única opção empregada para o tratamento de LV no período gestacional (AMATO et al, 2000; SERENO et al, 2000).

O metabolismo dos esteróis na *Leishmania* é particular, sendo o ergosterol o mais importante esterol de membrana no parasito o que o diferencia da célula hospedeira, onde o colesterol é o componente predominante na membrana plasmática. A anfotericina tem uma alta afinidade pelo episterol, que é um percursor do ergosterol (SINGH et al., 2006), o que leva a uma alteração na composição da membrana, produzindo poros e alterando a permeabilidade, o que conduz a escape de íons e consequentemente morte do parasito (BALANA-FOUCE et al., 1998).

A anfotericina B é administrado por via endovenosa, sendo seu uso limitado devido a toxicidade apresentando efeitos como anafilaxia, trombocitopenia, anemia, dor muscular, febre, tremor, calafrio, disfunção renal (KHAW, PANOSIAN, 1995). Novas formulações de anfotericina B associada a lipídeos tem melhorado as propriedades farmacocinéticas e a biodisponibilidade, facilitando sua absorção pelas células do reticuloendotelial e reduzindo os efeitos secundários, além de melhorar a eficácia.

As formulações de anfotericina B são anfotericina B desoxicolato (Fungizone®) e formulações lipossomais (por exemplo, AmBisome®). Lipossomas pequenos residem na corrente sanguínea durante muito tempo, enquanto que as partículas lipídicas maiores são rapidamente fagocitadas por fagócitos mononucleares, por exemplo macrófagos. Assim, são raidamente distribuídos, acumula-se rapidamente no fígado, e atinge a sua concentração terapêutica mais rápido do que antimoniais, e o efeito aumenta, devido à sua meia-vida longa.

A anfotericina B lipossomal tem custo elevado, o que pode dificultar o seu uso em saúde pública (ADLER-MOORE; PROFFITT, 2002, JAIPRAKASH et al., 2015).

A anfotericina B lipossomal é um tratamento altamente eficaz e seguro para LV em áreas endêmicas de *L. infantum* e *L. donovani* no Sul da Ásia (Índia, Bangladesh, Nepal). Recentemente, a OMS recomendou como tratamento de primeira escolha no controle da leishmaniose em algumas áreas do estado de Bihar onde a falha terapêutica é alta com o uso de antimoniais pentavalentes. (WHO, 2010). São necessárias pesquisas adicionais para melhor definir a indicação de anfotericina B lipossomal para outras formas de leishmaniose (LC, LMC, LCD) e para projetar ideal regime de tratamento para os doentes coinfectados pelo HIV (BALASEGARAM et al., 2012).

3.6.4 Miltefosina

A miltefosina é um alquilfosfocolina desenvolvida originalmente com agente antineoplásico, utilizado para o tratamento tópico de metástases cutâneas de câncer de mama (UNGEZ, DEMENZ et al, 1989). Em 2002, foi aprovada para o tratamento da leishmaniose visceral após alguns ensaios clínicos bem sucedidos na Índia, a miltefosina, este é o único fármaco por via oral para o tratamento da leishmaniose (SUNDAR et al., 2002).

O mecanismo de ação da metilfosina ainda não é totalmente esclarecido, mas são possíveis mecanismos de ação, bloqueio da proliferação da *Leishmania*, a inibição da biossíntese de fosfatidilcolina, alteração da composição de esterol e fosfolipídeo, ativação de imunidade celular, ou inibição da transdução de sinal e a homeostase do cálcio (CROFT; SEIFERT; DUCHENE, 2003, SUNDAR et al., 2003). Além disso, tem sido relatado que a atividade da miltefosina pode ser aumentada por acúmulo intracelular, que é regulada pelos transportadores do fármaco. Pesquisadores também descobriram que ele estimula morte por apoptose em *L. donovani*, mas o mecanismo exato não é conhecido. Miltefosina tem sido mostrado que estimula a produção de sintase de óxido nítrico 2 (iNOS2), causando a geração de NO, que ajuda a matar o parasito dentro do macrófago (WADHONE et al., 2009).

O conhecimento de resistência experimental de *Leishmania* à miltefosina é limitado a defeitos na internalização do fármaco e aumento do efluxo do fármaco (PEREZ-VICTORIA et al., 2006). Parasitos resistentes a esses composto já foram obtidos *in vitro* em laboratório foi observado e caracterizado o fenótipo de *Leishmania major* resistente a miltefosina (KIMBRA et al., 2015) Além disso, estudos já demonstraram a existência de linhagens de *L. donovani*

resistentes a miltefosina (PEREZ-VICTORIA, 2003). Resistência clínica generalizada foi também demonstrada, em dois casos isolados de pacientes coinfectados com HIV e *L. infantum* e tratados com miltefosina (COJEAN et al., 2012, HENDRICKX et al., 2014).

Ademais, Soto et al. (2004) demostraram que apesar de bem tolerada, a administração de miltefosina em pacientes com LC não foi tão eficaz no tratamento tanto de infecções por *L. braziliensis* na Colômbia, como de *L. panamensis* na Guatemala (SOTO et al., 2004). Entretanto, resultados de um ensaio clínico recentemente publicado, mostrou que a utilização de miltefosina em pacientes na Bahia infectados com *L. braziliensis*, levou a taxas de cura superiores aos pacientes tratados com antimonial pentavalente (MACHADO et al., 2010). Os principais efeitos colaterais associados são efeitos gastrintestinais, mas a maior limitação da miltefosina é o seu potencial teratogênico, particularidade que impede o uso em mulheres grávidas (CROFT e COMBS, 2003; SINGH e SIVAKUMAR, 2004).

3.6.5 Paramomicina

Paromomicina (Aminosidina) é um antibiótico aminoglicosideo, extraído de *Streptomyces rimosus*, com amplo espectro de ação e atualmente também é utilizado para o tratamento da leishmaniose. É utilizado para o tratamento de LV e LT, embora seja mais eficaz para LT (SUNDAR; CHAKRAVARTY, 2008, SUNDAR et al., 2009). Paromomicina está disponível como injeção intramuscular para tratar infeções sistémicas e como uma formulação de pomada para o tratamento de infecções locais da pele, ou seja para o tratamento de LC (HAMILTON, 2008),

Seu mecanismo de ação antibacteriana está bem documentado, o fármaco inibe a síntese de proteínas através de sua interação com as subunidades do DNA ribossomal (JHINGRAN et al., 2009). Porém, o mecanismo de ação leishmanicida ainda não foi totalmente elucidado. No entanto, tem-se observado no citoplasma e na mitocôndria de *L. donovani* uma diminuição da dissociação de Mg^{+}, interferindo assim no potencial de membrana, inibição da síntese de proteínas e alteração do metabolismo dos lipídeos. Além disso pode induzir alterações conformacionais no RNA e disfunção respiratória em promastigotas de *L. donovani* (MAAROUF et al., 1997).

Com o uso da paromomicina foram observados algumas reações adversas e efeitos secundários, incluindo elevação das transaminases hepáticas, nefrotoxicidade, ototoxicidade, dor no local da injeção, náuseas, cólicas abdominais e diarreia (SUNDAR et al., 2007, KHAN;

KUMAR, 2011). Evidências experimentais mostraram promastigotas de *L. donovani* resistentes a paromomicina (MAAROUF et al., 1997). Verificou-se ser eficaz na Índia, Quênia, e mais recentemente na Tunísia, mas foi menos eficaz no Sudão e Colômbia (THAKUR et al., 2000).

3.6.6 Outros fármacos e associações

Outros fármacos estão sendo utilizados para o tratamento da leishmaniose de maneira individual ou combinados para potencializar seu efeito e a adesão ao paciente, entre eles temos a Sitamaquina (STQ), Alopurinol (ALP) e Fluconazol (FLU).

STQ é um 8-aminoquinolina em estudos para terapia de LV por via oral. Estudos clínicos de fase II na Índia e África mostraram eficácia para LV, porém efeitos adversos renais foram relatados após 21 dias de tratamento (JHA et al., 2005, WASUNNA et al., 2005). Estudos clínicos de Fase II estão em andamento e os de Fase III estão sendo planejadas na Índia (CHAPPUIS et al., 2007). Um recente estudo mostrou que o seu possível alvo é a succinato desidrogenasse causando estresse oxidativo em promastigotas de *L. donovani* (CARVALHO et al., 2011).

O ALP é um análogo de purinas sendo utilizado como um substrato de várias enzimas na rota de salvamento de purinas em tripanosomas e é seletivamente incorporado nos nucleotídeos intermediários e ácidos nucleicos nos parasitas, formando um análogo altamente tóxico do ATP que é incorporado no RNA (ácido ribonucleico) dos parasitos, inibindo a síntese proteica. Sua atividade leishmanicida foi descrita há mais de 30 anos (KOUTINAS et al., 2001; CROFT; COOMBS, 2003). Em um estudo de fase II, utilizado concomitantemente com o Glucatime®, mostrou-se eficaz a combinação para tratar LC em cães, mostrando também que esta combinação em cães é eficaz em prevenir recaídas (TORRES et al., 2011).

O FLU é um antifúngico triazólico que, devido à similaridade entre as membranas celulares dos fungos e *Leishmania*, tem sido utilizado para ser usado como fármaco no tratamento da leishmaniose (ALNAIM et al., 2007). Em um estudo realizado com o uso de 200 mg/kg de FLU durante 6 semanas em pacientes infectados com *L. major* foi observado uma cura de 79% dos pacientes (ALRAJHI et al., 2002). Em um outro estudo realizado com hamsters foi observado que a combinação de FLU com Sb^{5+} não demonstrou ser uma boa alternativa terapêutica para o tratamento, mostrando que existe uma potencial interação farmacocinética entre estes dois fármacos. O seu mecanismo de ação se dá através da inibição da 14α-lanoesterol

demetilase que é uma enzima utilizada para a síntese de ergosterol, consequentemente, haverá a inibição da formação da membrana celular (ALNAIM et al., 2007; PACE, 2014).

Para limitar a extensão da resistência aos derivados do antimônio, prevenir a emergência da resistência à paramomicina ou à miltefosina, e melhorar a eficácia do tratamento para leishmaniose, uma alternativa é fazer uso de esquemas associados. A associação de estibogluconato de sódio e paromomicina foi segura e eficaz em testes preliminares realizados na Índia e África (MELAKU et al., 2007). A politerapia através da combinação entre anfotericina B Lipossomal, paromomicina e /ou miltefosina também foi altamente eficaz (98% -99%), segura e agora foi incluída nas recomendações da OMS para o tratamento da doença no subcontinente indiano (BRYCESON, 2001, VAN-GRIENSVEN; DIRO, 2012).

4 ALVOS LEISHMANICIDAS

Uma etapa para o processo de descoberta de fármacos leishmanicidas é pesquisar e/ou selecionar o alvo terapêutico. Sistemas enzimáticos de vias metabólicas e bioquímicas de *Leishmania* spp., tal como via da biossíntese de esteróis e estigmasterol, enzimas do glicossoma, bem como aquelas envolvidas na via das poliaminas, sistema tripanotiona, biossíntese do folato, e enzimas especiais como topoisomerase, metacaspases, são alvos terapêuticos no desenvolvimento de novos fármacos (CHAWLA; MADHUBALA, 2010; SINGH et al., 2012).

Os alvos mais importantes são enzimas, que desempenham papéis específicos na regulação dos mecanismos metabólicos e bioquímicos, sendo indispensáveis para a sobrevivência ou vitais para controlar uma particular via de sinalização do parasito. Um alvo molecular importante são metacaspases, que induzem morte celular programada na *Leishmania* (DAS et al., 2013).

Metacaspases de *Leishmania* são expressas em promastigotas e amastigotas e são fundamentais para a divisão nuclear e do cinetoplasto. Também tem sido reportado uma superexpressão de metacaspases tornando o parasito *Leishmania* sensível a H_2O_2, induzindo assim a morte celular programada através desta via. Assim, a indução de metacaspases de *Leishmania*, pode ser usada como um eficiente agente antileishmania (MESLIN et al., 2011).

Proteases de parasitos são alvos enzimáticos atrativos, pois estão envolvidos na replicação, metabolismo, sobrevivência e patologia. Existe um total de 154 peptidases presente no genoma da *Leishmania*, compreendendo principalmente cisteína, serina e metaloprotease.

Cisteína protease desempenha papel fundamental na modulação da resposta imune, degradação de várias proteínas hospedeiras, autofagia e diferenciação de promastigotas em amastigotas, modificando a resposta imune para sua própria vantagem (MOTTRAM; COOMBS; ALEXANDER, 2004; OLIVIER et al., 2012). Portanto, a inibição de cisteína protease pode ser uma opção terapêutica para combater a infecção por *Leishmania* (LIMA; REIS; COSTA, 2013).

A metaloprotease gp63 localizada na superfície da promastigota, é essencial para invasão e sobrevivência mediada pelo sistema complemento. Foi demonstrado que a inibição de metaloproteases pade ser importante para o tratamento de doenças parasitárias (BANGS et al., 2001). Uma protease envolvida em processos de invasão e ativação do macrófago durante a interação entre o parasito e a célula hospedeira é a serina protease. (CHOUDHURY et al., 2010, GUEDES et al., 2010, SWENERTON et al., 2011, MUNDAY et al., 2011). A serina protease de *L. amazonensis* aumenta a susceptibilidade à infecção por ativação da resposta imune Th2 (GUEDES et al., 2010, SWENERTON et al., 2011). Portanto, cisteina protease, serina e metaloprotease são identificadas como potenciais alvos terapêuticos contra *Leishmania*.

Os tripanossomatídeos sintetizam ergosterol. Assim, neste parasito, etapas da biossíntese de esteróis que são divergentes em relação à síntese realizada por células de mamíferos, que sintetizam o colesterol, são intensamente estudadas como alvo quimioterápico (MELOS, 2012).

Uma diferença principal entre a biossíntese do ergosterol e colesterol, é a presença da cadeia 24-alquil no ergosterol e o estigmasterol. Este é introduzido pela enzima 24-C Metiltransferase (24-SMT), que adiciona um resíduo de metileno à cadeia lateral insaturado do esterol usando S-adenosilmetionina (SAM) como um cofator, levando à formação de esteróis (NES, 2000). Uma série de azaesteróis foi investigada frente à *Leishmania* spp e *T.cruzi*, apresentando atividade significante sobre a inibição da enzima esterol 24-C metiltransferase, destacando-se o 22,26-azaesterol como potente inibidor (RODRIGUES et al., 2002). Mais recentemente, derivados esteroidais com grupamentos amido e amino em substituição ao anel piperidina do 22,26-azaesterol foram sintetizados e avaliados quanto a inibição da enzima esterol 24-C-metiltransferase, mostrando atividade inibitória frente à *T. cruzi, T. brucei* e *L. donovani*, com melhores resultados para os derivados substituídos no nitrogênio amínico. (LORENTE et al., 2005).

Nos tripanossomatídeos, o metabolismo parcial de carboidratos, incluindo a glicólise, ocorre em organelas específicas denominadas glicossomas. A biogênese destas organelas e a

correta separação de enzimas glicolíticas é essencial para esses parasitos e envolvem proteínas chamadas peroximas, que apresentam, por sua vez, baixo nível de identidade com o humano. Desta forma, as peroximas constituem-se, também, um alvo em potencial (MELOS, 2012).

Dentre as enzimas da via glicolítica de tripanossomatídeos, a que vem despertando maior interesse é a gliceraldeido-3-fosfato desidrogenase (GADPH). Esta enzima catalisa a fosforilação oxidativa de D-gliceraldeido-3-fosfato em 1,3-bisfosfoglicerato na presença de NAD^+ e fosfato inorgânico. As formas tripomastigotas de *T. cruzi*, por exemplo, são altamente dependentes da glicólise para a produção de ATP, e como muitas enzimas glicolíticas apresentam características próprias, revelam-se como importantes alvos de novos agentes antiparasitários (MELOS, 2012).

Poliaminas como putrescina, espermidina e espermina, além de serem importantes para o crescimento e diferenciação do parasito, atenuam a peroxidação lipídica. Atuam, também, no empacotamento do DNA e são necessárias em grandes quantidades nas células em processo de multiplicação (DAS et al., 2013). Na síntese destas moléculas, duas enzimas são de fundamental importância: a ornitina descarboxilase e a S-adenosil L-metionina descarboxilase. As poliaminas funcionam como substrato para a tripanotiona redutase na síntese da tripanotiona. Os tripanossomatídeos utilizam a via tripanotiona/tripanotiona redutase para realizar o balanço redox de fundamental importância para esses parasitos. Nos mamíferos este balanço redox é realizado via glutationa/glutationa redutase, sendo equivalente ao sistema tripanotiona/tripanotiona redutase dos tripanossomatídeos (HEBY; PERSSON; RENTALA, 2007).

A arginase, assim como o iNOS, utilizam a L-arginina como substrato. A arginase catalisa a hidrólise de L-arginina em ureia e ornitina. Este último, é utilizado pela *Leishmania* spp. para produzir poliaminas, que são importantes para o crescimento e diferenciação celular do parasito, logo, a inibição da arginase acarreta no impedimento da produção de ornitina e, consequentemente, afeta a biossíntese de poliaminas. NOS é expresso em macrófagos e essa produção é uma importante resposta microbicida dessas células. Arginase e NOS atuam por competição, assim, a arginase regula negativamente os níveis de NO produzidos pela iNOS consumindo o substrato arginina, logo diminuindo e eficiência da produção de óxido nítrico pelo macrófago e, desta forma, impedindo uma melhor resposta microbicida natural (MORI; GOTOH, 2000, SHIMIZU et al., 2002).

Topoisomerases de DNA (Topo) são enzimas essenciais na replicação, transcrição, recombinação e reparo do DNA, mantendo assim, a estabilidade genômica durante a recombinação de DNA. Como a inibição destas enzimas pode ocorrer a morte celular.

Inibidores de topo estão entre as mais eficazes substâncias nas terapias do cancêr e infecções por bactérias. Além disso, diferenças estruturais importantes foram encontrados entre Topo dos membros da família Trypanosomatidae e Topo de hospedeiros vetebrados. Tais diferenças tornam estas proteínas um importante alvo para a concepção de medicamentos (BALAÑA-FOUCE et al., 2014). Nesse contexto, o conhecimento e a identificação dos alvos são importantes na busca de novos fármacos contra várias doenças.

5 ARTIGO I DERIVADO DA TESE

Artigo 1: Publicado

Molecules **2014**, *19*, 1-x manuscripts; doi:10.3390/molecules190x0000x

OPEN ACCESS

molecules

ISSN 1420-3049

www.mdpi.com/journal/molecules

Article

Synthesis, leishmanicidal activity and theoretical evaluations of a series of substituted bis-2-hydroxy-1,4-naphthoquinones

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Abstract: A series of eight substituted bis-2-hydroxy-1,4-naphthoquinone derivatives was synthesized through condensing lawsone with various aromatic and aliphatic aldehydes under mild acid conditions. These compounds were evaluated for antileishmanial activity *in vitro* against *Leishmania amazonensis* and *Leishmania braziliensis* promastigotes; six compounds showed good activity without significant toxic effects. The compound with the highest activity was used with *Leishmania amazonensis* for an *in vivo* assay.

Keywords: Naphthoquinones, Knoevenagel, Bis-2-Hydroxy-1,4-naphthoquinones, Antileishmanial activity, *Leishmania braziliensis*, *Leishmania amazonensis*

1. Introduction

Quinones are a well-known class of compounds with a broad natural distribution; they exhibit a diverse spectra of biological activities, including antitumor [1,2], molluscicidal [3], leishmanicidal [4,5], bactericidal, fungicidal [6,7] and trypanocidal functions [8], and they act as inhibitors of the reverse transcriptase enzyme of HIV-1 [9] and topoisomerase II human [10]. The conjugated 1,4-dicarbonyl or 1,2-dicarbonyl moiety in the most well-known *para*- and *ortho*-quinone molecular structures confers specific properties and a reactivity that facilitates participation in redox processes, which are likely related to these substances' action mechanisms [11,12].

Studies that have considered natural compounds with leishmanicidal activity have highlighted diospyrin, which is a bis-naphthoquinone isolated from *Diospyros montana* (Ebenaceae) bark; plumbagin isolated from *Plumbago* species (Plumbaginaceae); and lapachol,

which is a prenylated hydroxynaphthoquinone isolated from *Tecoma* species (Bignoniaceae). These compounds represent a class of quinones with an action mechanism that involves generating oxygen free radicals, which affects the parasites' defense mechanism and renders the parasites defenseless [13].

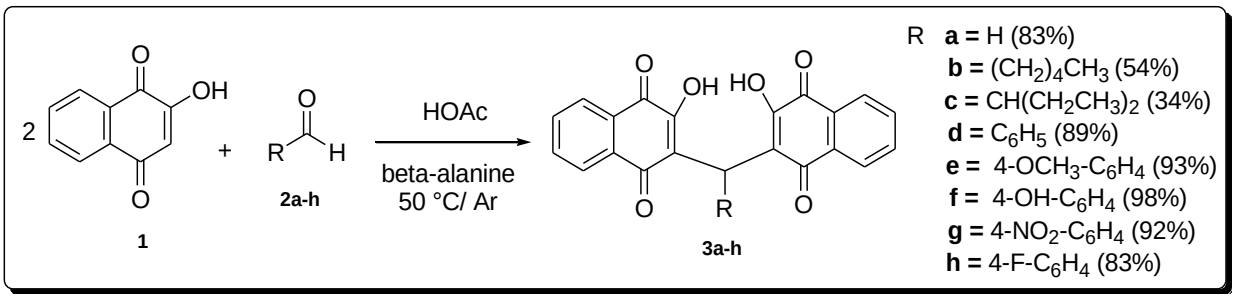
Dimeric structures with two 2-hydroxy-1,4-naphthoquinone groups have been studied primarily as intermediates in benzoxanthene derivative synthesis [14,15,16]; however, few studies have investigated the biological activities of these compounds. Mazumder and co-workers [17] studied the inhibitory activity of the HIV integrase enzyme using a series of bis-2-hydroxy-1,4-naphthoquinones and reported good results.

In this study, a series of bis-2-hydroxy-1,4-naphthoquinones (bis-lawsones) were synthesized using a simple and quick method. These compounds were evaluated *in vitro* for antileishmanial activity against *Leishmania amazonensis* and *Leishmania braziliensis* promastigotes. The most active compound was used for an *in vivo* assay with *Leishmania amazonensis*.

2. Results and Discussion

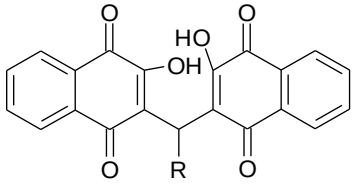
Compounds **3a-h** (Scheme 1) were obtained from a condensation reaction between two lawsone molecules with the corresponding aldehyde using β-alanine and acetic acid in an inert atmosphere at 50 °C. The compounds **3b** and **3c** obtained from the aliphatic aldehydes **2b** and **2c** showed lower yields due to the formation of the corresponding alkene through a condensation side reaction that yielded the corresponding 2-alkenyl derivative [15-16].

Scheme 1. Synthesis of compounds 1-3h



Primarily, the bis-lawsone analog cytotoxicity was determined using the MTT method [18] and J774 cell line. The host cells were treated with bis-lawsone analog and compared with the vehicle (DMSO). **Table 1** shows the results for the bis-lawsone analog and pentamidine experiments (reference drug). The compounds **3c** and pentamidine showed the same deleterious activity to the host cell, as evidenced by the MTT assay, which presented the maximum cytotoxicities 71.7 ± 3.8 and 78.0 ± 3.8 % as well as LC_{50} values at 67.4 ± 2.1 and 73.0 ± 6.0 µM, respectively. After 48 h of incubation, the other compounds did not affect the J774 cell line viability at 100 µM.

Table 1. Determination of the cytotoxicity of bis-lawsone analogs (3a-h) against macrophages (MTT assay).

Compounds	Chemical structure (R=) 	IC_{50} (µM) [a]	Maximum Cytotoxicity (%)[b]
Pentamidine	-	73.0 ± 6.0	78.0 ± 3.8***
3a	H	> 100	NT
3b	n-Pentyl	> 100	NT
3c	$CH(Et)_2$	67.4 ± 2.1	71.7 ± 3.8***
3d	Ph	> 100	NT
3e	(4-OMe)Ph	> 100	NT
3f	(4-OH)Ph	> 100	NT
3g	(4-NO_2)Ph	> 100	NT
3h	(4-F)Ph	> 100	NT

[a]Inhibitory Concentration 50 (IC_{50}) calculated by concentration-response curves toxic. [b]Mean ± standard error of the mean maximum cytotoxicity in triplicates of a representative experiment. The values of maximum effect were considered significant when * $p < 0.05$, ** $p < 0.01$ compared to the DMSO group; NT: substance presents no significant lethal activity to cell until the concentration of 100 µM in compared to DMSO group.

Theoretical toxicity analysis was performed using the OSIRIS program, which is available at http://www.organic-chemistry.org/, to analyze their overall drug score and drug likeness potential as well as toxicity risks (mutagenic, irritant, tumorigenic and reproductive effects) [19]. Comparing the compounds 3a-h to the available drugs currently used for Leishmaniasis treatment, the results show that the bis-lawsone derivatives had no toxicity effect

(Figure 1). Except **3c**, these results are consistent with the MTT assay results (**Table 1**). Notably, the toxicity predicted herein is neither a fully reliable toxicity prediction nor a guarantee that these compounds are completely free of a toxic effect. However, the data reinforce the promising profiles for these compounds, which were also detected *in vitro*, for further experimental investigation. Further, the bis-lawsone analogs' drug-like profiles (drug likenesses and drug-score values) using the OSIRIS program (**Figure 2**).

Figure 1. *In silico* toxicity risk (tumorigenic, irritant and reproductive effects) for lawsone dimers. Theoretical toxicity risks calculated using the Osiris program. The toxicity profile scale for the side effects included low (1), medium (0.8) and high (0.6).

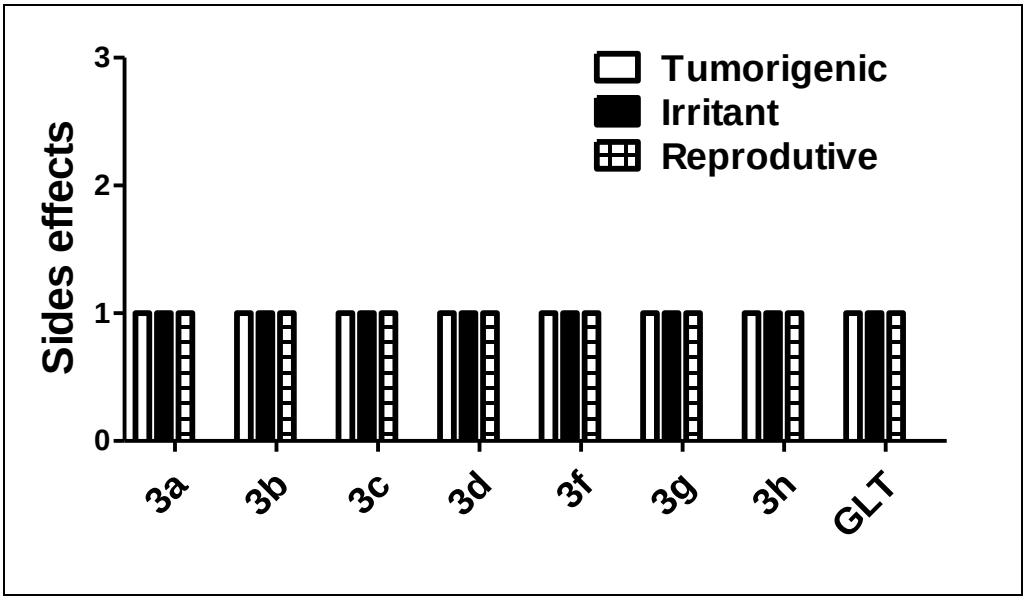
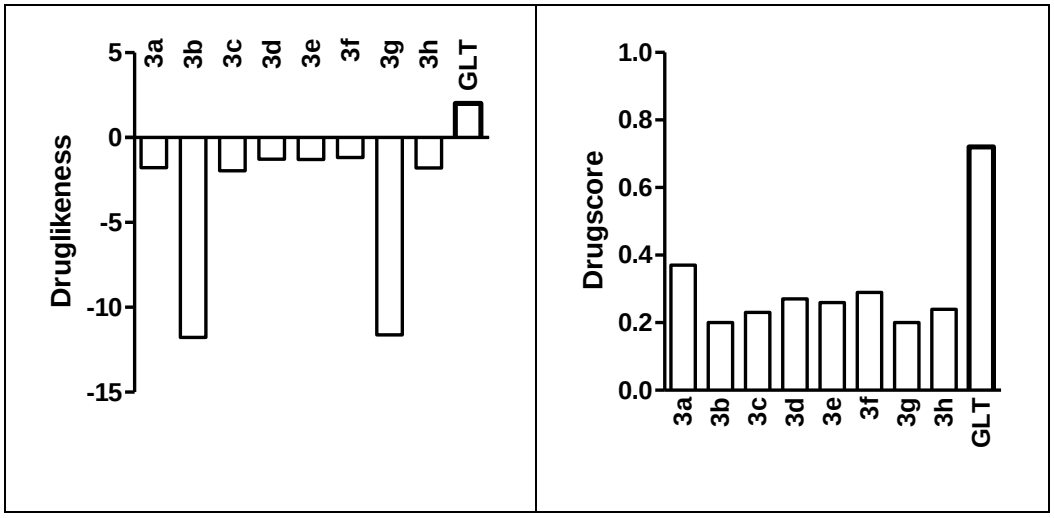


Figure 2. *In silico* comparison of the drug-like profile (drug likeness and drug score values) for the bis-lawsone analogs and Glucantime (GLT). These parameters were calculated using the Osiris program as described in the experimental section.



To establish the leishmanicidal profile, the bis-lawsone analogs were evaluated *in vitro* against the forms of *L. amazonensis* and *L. braziliensis*. As a parameter for antileishmanial activity, the maximum effect and IC_{50} value (*i.e.*, the sample concentration that reduces survival/viability of the parasites by 50%) were used (**Table 2**). As shown, the compounds **3a** and **3c** were highly active against both *Leishmania* species, presenting the effects 72.8% ± 1.0% and 75.3% ± 1.3% against *L. amazonensis* and killed promastigotes of *L. braziliensis* in the proportion of 88.4% ± 0.9% and 90.4% ± 0.7%, respectively. In addition, **3b**, **3e**, **3f**, **3g** and **3h** exhibited high antileishmanial activities against *L. braziliensis* promastigotes with the maximum effects 93.0% ± 0.1%, 61.6% ± 7.5%, 91.7% ± 0.3%, 91.7% ± 1.3% and 88.7% ± 0.3%, respectively. Moreover, the bis-lawsone analogs **3a** (IC_{50} value 0.9 ± 0.08 µM), **3b** (IC_{50} value 5.2 ± 0.1 µM), **3e** (IC_{50} value 0.9 ± 0.04 µM) and **3h** (IC_{50} value 0.8 ± 0.03 µM) were as potent as pentamidine (with the efficacy 91.0% ± 0.1% and IC_{50} value 0.8 ± 0.06 µM) for this *Leishmania* species. In contrast, the derivative **3d** did not present activity against promastigote forms of *L. braziliensis* until 100 µM; however, it showed a considerable effect (85.0% ± 3.8%) and great potency (IC_{50} value 0.3 ± 0.1 µM) against *L. amazonensis*.

Table 2. Leishmanicidal effect of bis-lawsone analogs (**3a-h**) against the growth of promastigotes of *L. amazonensis* and *L. braziliensis.*

Compounds	*L. amazonensis* Promastigotes		*L. braziliensis* Promastigotes	
	IC_{50} (µM) [a]	Maximum Effect (%)[b]	IC_{50} (µM) [a]	Maximum Effect(%)[b]
Pentamidine	2.3 ± 0.8	85.4 ± 0.4**	0.8 ± 0.06	91.0 ± 0.1**
3a	**71.0 ± 1.1**	**72.8 ± 1.0****	**0.9 ± 0.08**	**88.4 ± 0.9****
3b	5.2 ± 0.1	57.2 ± 3.8**	**5.2 ± 0.1**	**93.0 ± 0.1****
3c	**0.4 ± 0.1**	**75.3 ± 1.3****	**34.7 ± 4.3**	**90.4 ± 0.7****
3d	**0.3 ± 0.1**	**85.0 ± 3.8****	> 100	NT
3e	> 100	44.6 ± 2.4**	**0.9 ± 0.04**	**61.6 ± 7.5****
3f	68.7 ± 15.1	55.4 ± 4.7**	**38.7 ± 2.0**	**91.7 ± 0.3****
3g	7.7 ± 1.2	55.6 ± 3.2**	**2.8 ± 0.1**	**91.7 ± 1.3****
3h	0.6 ± 0.2	51.0 ± 5.5**	**0.8 ± 0.03**	**88.7 ± 0.3****

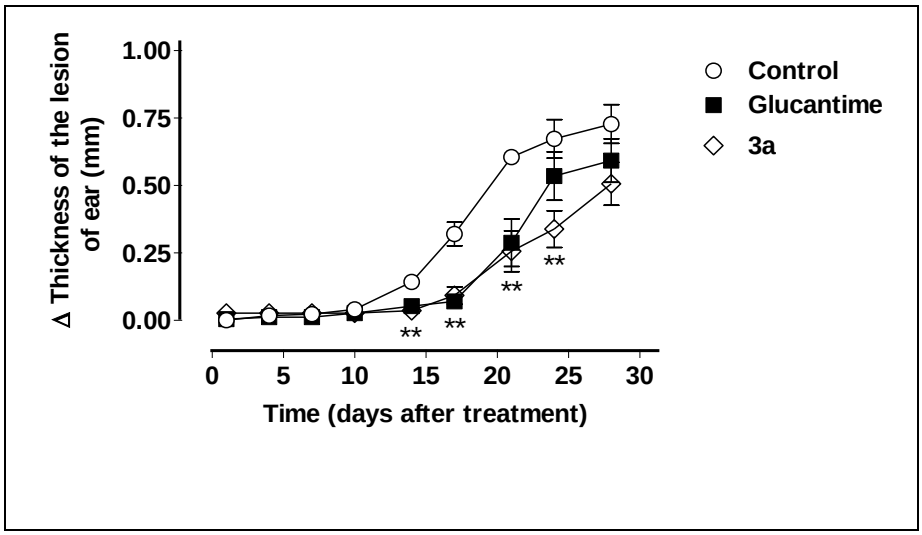
[a]Inhibitory Concentration 50 (IC_{50}) was calculated by concentration-response curves toxic and expressed as mean ± standard error of the mean. [b]Maximum Effect (ME) is expressed as mean ± standard error of maximum toxicity average of triplicates of a representative experiment. The values of maximum effect were considered significant when * $p < 0.05$, ** $p < 0.01$ compared to the 0.1% DMSO group; NT: substance presents no significant inhibitory activity for the parasite to the concentration of 100 □M compared to DMSO group.

These results indicate a correlation between the leishmanicidal activity and alkyl side-chain moiety hydrophobic character for the compounds in the series **3a-c**. The compounds with lower polarity generally exhibit lower activities (**Table 2**); however, the exception to this trend is the compound **3d**, likely due to an oxidative pathway at the free phenyl ring 4-position. The capacity for further oxidation exhibited by the derivative **3f** with a free *p*-hydroxyl seems to support this assumption. Moreover, the electron-withdrawing substituents in the phenyl ring, as demonstrated for **3h**, seems more selective compared with the 4-methoxy donor (**3e**) in *L. amazonensis*; both show a similar profile in *L brasiliensis*.

Considering the *in vitro* results, the compound **3a** was also used to evaluate the *in vivo* leishmanicidal activity against *L. amazonensis*. Intraperitoneal treatment with **3a** at 30 µmol/kg/day x 28 days decreased the lesion size for the infected ear on the third week after the treatment began **(Figure 3)**; it did not decrease the parasite load in the infected ear and draining lymph node, similar to the Glucantime treatment at same dose **(Figure 4)**. In addition, *in vivo*

treatment with **3a** neither induces a change in the spleen weight **(Figure 5)** nor altered alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CREA) and urea **(Figure 6)** in the animals' plasma.

Figure 3. *In vivo* efficacy of 3a and meglumine antimoniate treatments (30 µmol/kg/dia x 28 days, i.p.) in BALB/c mice infected with *L. amazonensis*. The lesion sizes were monitored weekly. The values are the mean lesion sizes for five mice from each group, and the bars represent the standard error of the mean.



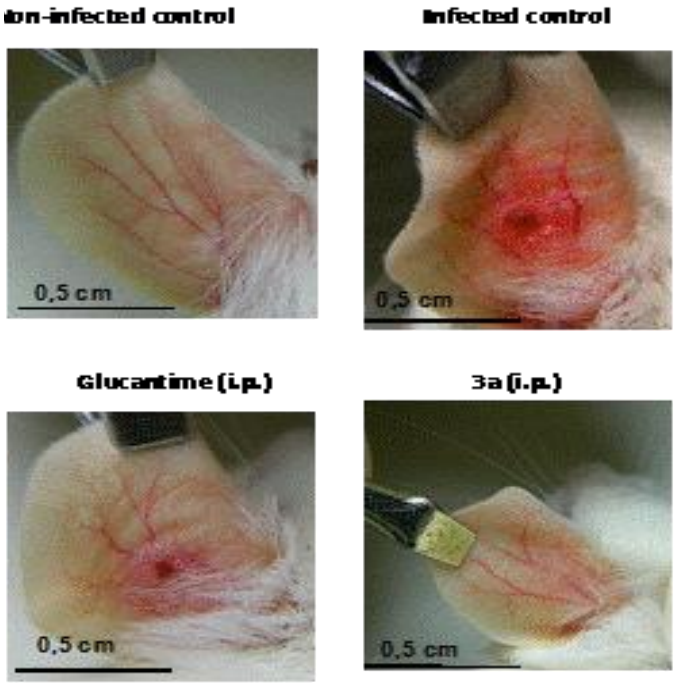


Figure 4. Parasite burden throughout the course of the 3a and meglumine antimoniate treatments (30 µmol/kg/dia x 28 days, i.p.) in BALB/c mice infected with *L. amazonensis*. (A) Log_{10} of the parasites load in the infected ear. (B) Log_{10} of the parasites load in the draining lymph node. The infected ear and draining lymph node parasite loads were determined using a quantitative limiting-dilution assay. Values are the mean parasites load for five mice from each group, and the bars represent the standard error of the mean. *P < 0.05, **P < 0.01 vs. control.

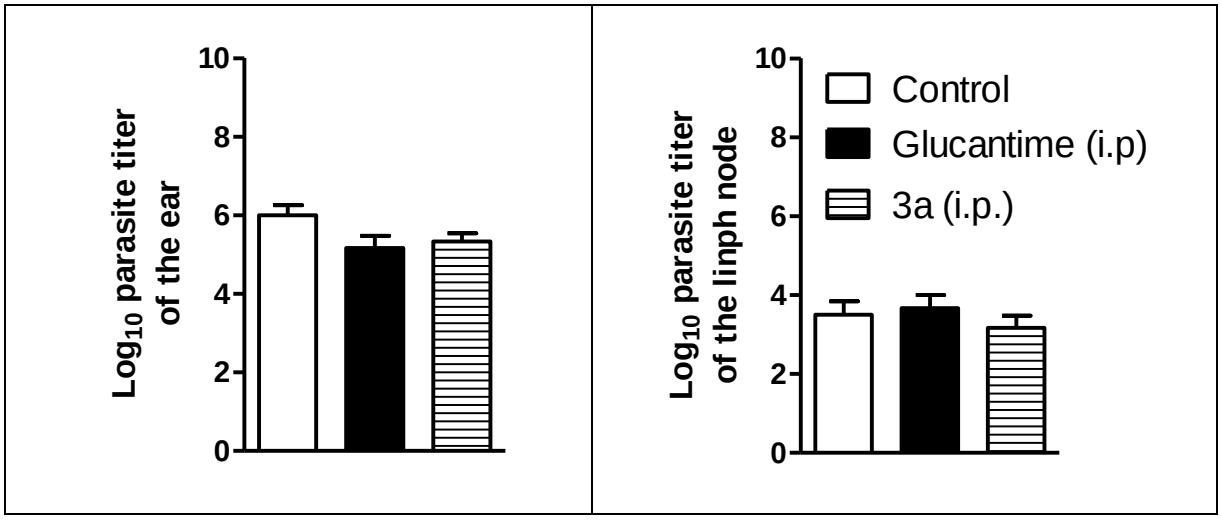


Figure 5. The *in vivo* effect from the 3a and meglumine antimoniate treatments (30 µmol/kg/dia x 28 days, i.p.) on the spleen weights of the BALB/c mice infected with *L. amazonensis*. The spleen weight was determined on last day of treatment. The values are the mean lesion size for five mice from each group, and the bars represent the standard error of the mean. *P < 0.05, **P < 0.01 vs. control.

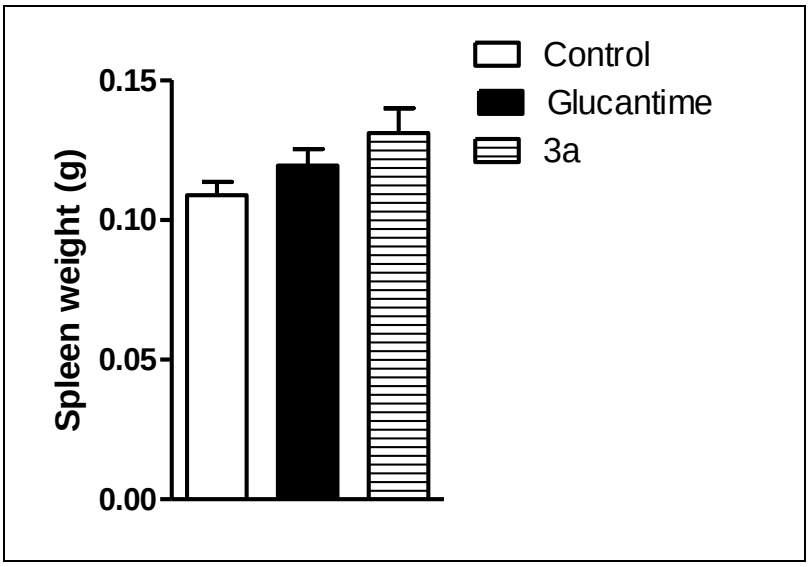
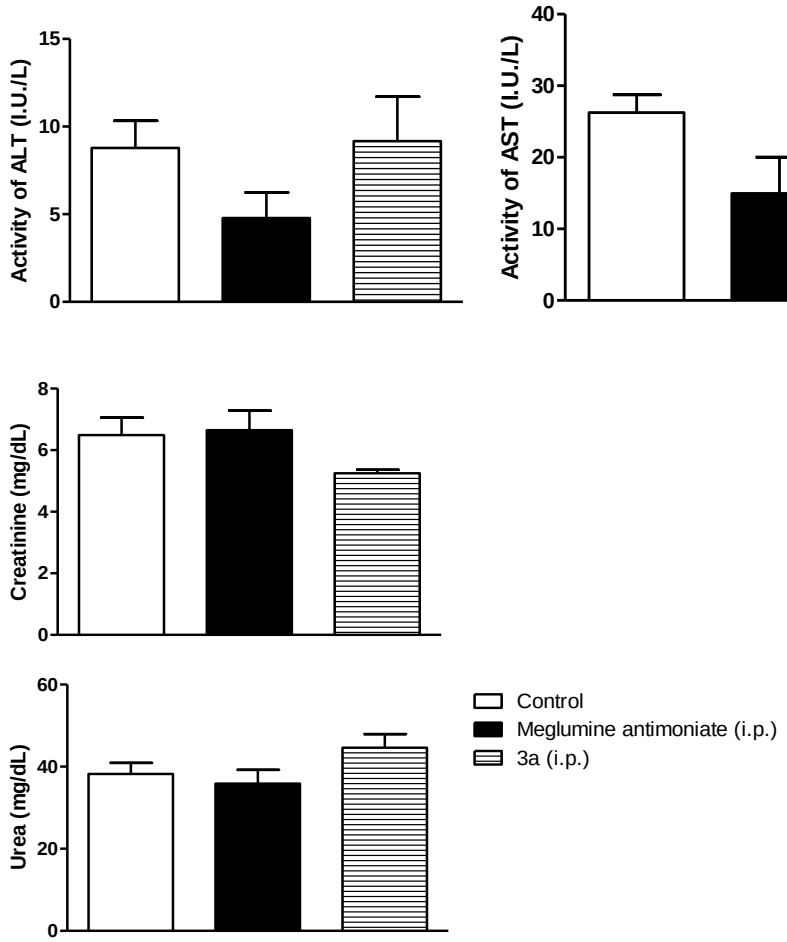


Figure 6. The *in vivo* effect from the 3a and meglumine antimoniate treatments (30 µmol/k/dia x 28 days, i.p.) on the serum ALT (A), AST (B), creatinine (C) and urea (D) levels in BALB/c mice infected with *L. amazonensis*. The lesion sizes were monitored weekly. The values are the mean lesion size for five mice from each group, and the bars represent the standard error of the mean. *P < 0.05, **P < 0.01 vs. control.



Among the known biological activities of compounds with a quinone structure, stands out for its antiprotozoal activity. For example, three naphthofuranquinones C-allyl lawsone derivatives were synthesized that were active against *Trypanosoma cruzi* trypomastigotes [8]. When used as therapeutic agents, quinonoidal ligand cytotoxic activity operates through various mechanisms, such as redox cycling, intercalation, inducing DNA strand breaks, arylation, alkylation via quinone methide formation and free radical generation [20].

Leishmaniasis is a public health issue and is among the five most prevalent parasitic diseases worldwide. According to the World Health Organization, anthropozoonosis leishmaniasis affects 12 million people with an annual incidence of approximately 2 million new cases, and most are present in undeveloped countries, such as Brazil. The standard

leishmaniasis treatment includes antimonials, amphotericin B and pentamidine, but these compounds are often associated with serious side effects [21] Discovery and development of new therapeutic agents is a priority due to the increasing prevalence of drug resistance in *Leishmania*, toxicity towards currently used drugs and the lack of an effective prophylactic vaccine against disease [22].

Naphthoquinone antileishmanial activity has also been observed. Lapachol, isolapachol and dihydrolapachol present significant activity; isolapachol acetate was most active against promastigotes, with the IC_{50} values 1.6 μg/ml and 3.4 μg/ml for *L. amazonensis* and *L. braziliensis*, respectively [23]. A series of monomeric and dimeric naphthoquinones with potential for treating *Leishmania* infections was identified *in vitro* against extracellular *L. donovani, L. infantum*, *L. enriettii* and *L. major* promastigotes. Several naphthoquinones are active at microgram concentrations (EC_{50} 0.9-17.0 μg/mL). When tested against a panel of human cancer cell lines and murine bone marrow culture-derived macrophages as mammalian host cell controls, compounds with anti-leishmanial activity showed moderate (EC_{50} 25.0μg/mL) to pronounced (EC_{50} 10.0 μg/mL) toxic effects [24]. Our study also verified that most of the naphthoquinones tested noticeably inhibited extracellular parasite growth (IC_{50} 0.8-38.7 μM) of *L. braziliensis*.

While the naphthoquinone mechanism used to kill these parasites is a matter of debate, biochemical experiments with a panel of quinone structures provide initial insights; remarkable data by Fry and Pudney clearly show an interaction between atovaquone and the cytochrome Bl-c complex in *Plasmodium* spp. [25, 26] reviewed the importance of ubiquinone as a potential target for coenzyme Q analogs, such as naphthoquinones, in parasitic diseases. This theory supports the hypothesis by Croft and co-workers (1985, 1992), [27, 28] wherein killing *Leishmania* through a series of previously tested monomeric naphthoquinones involves generating free radicals when the drug interacts with the respiratory chain. In addition, the bis-lawsone analogs exhibit leishmanicidal activity against *L. donovani* promastigotes (with the IC_{50} values 2 to 14 μM) and inhibit leishmanial DNA topoisomerase-I [29]. Moreover, Plyta et al. [30] showed that 1,4-naphthoquinones bearing at least one phenolic hydroxyl group are potent topoisomerase enzyme inhibitors.

3. Experimental Section

3.1. Chemistry

All reagents and solvents were obtained from commercial suppliers without further purification. The reaction progress was monitored using thin layer chromatography on a silica gel TLC aluminum sheet. The melting points were determined using a Kofler hot stage apparatus and are uncorrected. FTIR spectra were obtained in a BOMEM MB-Series 100 spectrophotometer or Bruker IRS66 using KBr discs. The NMR spectra were recorded in a Varian Unitty Plus 300 or Varian UNMRS 400. Elemental analyses were performed using a CE EA1110 CHNS-O analyzer.

3.1.1. General procedure for the synthesis of compounds 3a-h

A solution of 1 mmol of lawsone **1** (174 mg), 0.5 mmol of the corresponding aldehyde **2a-h**, 15 mg of β-alanine and 2.5 mL of glacial acetic acid was stirred at 50 ° C in argon atmosphere and reflux system for periods of 90 min at 5 h according to aldehyde used (the progress of reaction was monitored by TLC). The isolation of the compounds was done by adding crushed ice followed (except for compounds **3b** and **3c**) of the vacuum filtration of the formed precipitates which were washed with water and dried at room temperature. For compounds **3b** and **3c** after adding ice was performed extraction with portions of ethyl acetate followed by removal of the solvent under reduced pressure in and subsequent purification by chromatography column with silica gel as stationary phase and methylene chloride as mobile phase.

2,2'-(methylene)-bis[3-hydroxy-1,4-naphthalenedione] (3a). Yield: 83% yellow solid. mp: 229-232 °C. IR (KBr) ν_{max}/cm^{-1}: 3452, 3070, 1678, 1610, 1573, 1458, 1350, 1323, 1265, 1215, 975, 937, 771, 736, 466; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 3.74 (s, 2H), 7.78 (dt, 2H, *J* 7.5/ 1.8Hz), 7.83 (dt, 2H, *J* 7.5/1.5 Hz), 7.97 (m, 4H); ^{13}C NMR(DMSO-d_6, 75.4 MHz) δ 17.9, 122.0, 125.7, 125.9, 129.9, 132.0, 133.2, 134.6, 155.1, 180.8, 183.6. Anal. Calcd. $C_{21}H_{12}O_6$: C, 70.00; H, 3.36. Found:C, 69.03; H, 3.82.

2,2'-(hexylidene)-bis[3-hydroxy-1,4-naphthalenedione] (3b). Yield: 54%, orange solid. mp: 205-8°C. IR (KBr) ν_{max}/cm^{-1}: 3433, 2928, 2855, 1667, 1628, 1585, 1566, 1458, 1366, 1281, 953, 737; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 1.32 (t, 3H, *J*6.6Hz), 1.72 (m, 6H), 3.05 (m, 2H), 5.83 (t, 1H, *J* 8.1Hz), 8.17 (dt, 2H, *J* 7.5/ 1.5 Hz), 8.27 (dt, 2H, *J* 7.8/ 1.5 Hz), 8.38 (dd, 2H, *J* 7.5/ 1.5 Hz), 8.46 (dd, 2H, *J* 7.8/ 1.5 Hz); ^{13}C NMR (DMSO-d_6, 75.4 MHz) δ 14.0, 22.1, 27.6, 28.8, 29.1, 31.4, 123.7, 125.0, 125.7, 130.6, 132.0, 133.1, 133.9, 162.6, 182.9, 183.6. Anal. Calcd. $C_{26}H_{22}O_{6}.(2H_2O)$: C, 66.95; H, 5.63.Found: C, 68.96; H, 5.74

2,2'-(2-ethylbutylidene)-bis[3-hydroxy-1,4-naphthalenedione] (3c). Yield: 34%, orange solid. mp 210-1°C. IR (KBr) ν_{max}/cm^{-1}: 3438, 2962, 2930, 1649, 1597, 1461, 1364, 1283, 729; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 0.71 (t, 6H, *J* 7.2 Hz), 1.16 (m, 2H),1.31 (m, 2H), 2.8 (m, 1H), 5.20 (d, 1H, *J* 12.4Hz), 7.63 (t, 2H, *J* 7.2Hz), 7.72 (t, 2H, *J*7.6Hz), 7.84 (d, 2H, *J* 7.6Hz), 7.90 (d, 2H, *J* 7.6Hz); ^{13}C NMR (DMSO-d_6, 75.4 MHz) δ 10.0, 22.0, 33.7, 35.6, 123.4, 125.2, 125.9, 130.3, 132.4, 132.8, 134.2, 160.6, 182.7, 183.5. Elemental Anal. Calcd. $C_{26}H_{22}O_6$: C, 72.55; H, 5.15. Found: C, 67.50; H, 5.40.

2,2'-(phenylmethylene)-bis[3-hydroxy-1,4-naphthalenedione] (3d). Yield: 89%, yellow solid. mp 215-7°C, IR (KBr) ν_{max}/cm^{-1}: 3450, 1674, 1597, 1570, 1361, 1284, 1222, 1111, 10,56, 729; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 6.69 (s, 1H), 7.11 (m, 5H), 7.67 (dt, 2H, *J* 7.5/ 1.2Hz), 7.76 (dt, 2H, *J* 7.5/ 1.2Hz), 7.88 (dd, 2H, *J* 7.5/ 1.2Hz), 7.97 (dd, 2H, *J* 7.5/ 1.2Hz); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 37.7, 123.1, 125.4, 125.6, 126.0, 127.6, 128.2, 129.9, 132.2, 133.1, 134.7, 140.8, 156.3, 181.2, 183.5. Anal. Calcd. for $C_{27}H_{16}O_6$: C, 74.31; H, 3.70. Found:C, 74.16; H, 4.28.

2,2'-(4-methoxyphenylmethylene)-bis[3-hydroxy-1,4-naphthalenedione] (3e). Yield: 93%, yellow solid. mp 222-3°C, IR (KBr) ν_{max}/cm^{-1}: 3394, 1666, 1639, 1593, 1512, 1458, 1361, 1338, 1276, 1261, 1238, 1045, 1018, 721; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 3.69 (s, 3H), 5.94 (s, 1H), 6.75 (d, 2H, *J* 8.7Hz), 7.14 (d, 2H, 8.7Hz), 7.77 (dt, 2H, *J* 7.5/ 1.5Hz), 7.82 (dt, 2H, *J* 7.5/ 1.5Hz), 7.92 (dd, 2H, *J* 7.5/ 1.5Hz), 7.98 (dd, 2H, *J* 7.5/ 1.5Hz); ^{13}C NMR (DMSO-d_6, 75.4MHz) δ 37.3, 54.9, 113.1, 123.6, 125.6, 126.1, 129.4, 129.9, 132.2, 132.7, 133.2, 134.7, 155.9, 157.3, 181.3, 183.7. Anal. Calcd. $C_{28}H_{18}O_7$: C, 72.10; H, 3.89. Found: C, 70.49; H, 3.98.

2,2'-(4-hydroxyphenylmethylene)-bis[3-hydroxy-1,4-naphthalenedione] (3f). Yield: 98%, yellow solid. mp 203-6°C, IV (KBr) ν_{max}/cm^{-1}: 3352, 1647, 1593, 1512, 1458, 1365, 1276,

1045, 1010, 972, 902, 725; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 5.92 (s, 1H), 6.58 (d, 2H, *J* 8.7Hz), 7.01 (d, 2H, *J* 8.7Hz), 7.76, (dt, 2H, *J* 7.5/ 1.2Hz), 7.81 (dt, 2H *J* 7.5/1.8Hz), 7,92 (dd, 2H, *J* 7.5/1.2Hz), 7.97 (dd, 2H, *J* 7.5/1.8Hz); ^{13}C NMR (DMSO-d_6, 75.4 MHz) δ 37.2, 114.6, 123.8, 125.6, 126.8, 129.3, 129.8, 130.8, 132.2, 133.2, 134.7, 155.3, 155.9, 181.3, 183.9. Anal. Calcd. $C_{27}H_{16}O_7(2H_2O)$: C, 66.40; H, 4.14 Found: C, 67.91; H, 4.38.

2,2'-(4-nitrophenylmethylene)-bis[3-hydroxy-1,4-naphthalenedione] 3g. Yield: 92%, yellow solid. mp 143-6°C, IR (KBr) ν_{max}/cm^{-1}: 3433, 2924, 1670, 1597, 1570, 1512, 1350, 1280, 111, 732; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 6.08 (s, 1H), 7.53 (d, 2H *J* 6.3 Hz), 7.78 (dt, 2H *J* 5.7/ 0.9Hz), 7.83 (dt, 2H, *J* 5.7/0.9Hz) 7.93 (dd, 2H, *J* 5.7/ 0.9Hz), 8.00 (dd, 2H, *J* 5.7/ 0.9Hz), 8.07 (d, 2H, *J* 6.6Hz); ^{13}C NMR (DMSO-d_6, 75.4 MHz)δ 37.6, 121.8, 122.8, 125.7, 126.1, 129.3, 130.0, 132.2, 133.2, 134.7, 145.5, 150.0, 156.9, 181.2, 183.3. Anal. Calcd. $C_{27}H_{15}NO_8$: C, 67.36; H, 3.14; N, 2.91. Found: C, 65.79; H, 3.63; N, 3.63.

2,2'-(4-fluorophenylmethylene)-bis[3-hydroxy-1,4-naphthalenedione] (3h). Yield: 83%, yellow solid. mp 202-4°C; IR (KBr) ν_{max}/cm^{-1}: 3414, 3348, 1666, 1625, 1593, 1508, 1458, 1365, 1342, 1276, 1230, 1161, 1041, 833, 725; ^{1}H NMR (DMSO-d_6, 400 MHz) δ6.00 (s, 1H), 7.00 (m, 2H), 7.28 (m, 2H), 7.77 (m, 4H), 7.91 (d, 2H,*J* 7.2 Hz), 7.97 (d, 2H, 6.4 Hz); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 37.7, 114.5, 123.5, 126.0, 126.5, 130.3, 132.6, 133.5, 135.1, 137.4, 156.5, 159.8, 162.2, 181.6, 184.0. Anal. Calcd. $C_{27}H_{15}FO_6$: C, 71.37; H, 3.33. Found: C, 71.16; H, 3.44.

3.2. Biological evaluation of naphthoquinones

3.2.1. Parasite culture

L. braziliensis promastigotes (MHOM/BR/87/BA788) were obtained from Dra. Valéria de Matos Borges at the Gonçalo Moniz Research Center, Fiocruz_BA. *L. amazonensis* promastigotes (MHOM/BR/77/LTB0016) were obtained from Dr. Eduardo Caio Torres dos Santos at the Oswaldo Cruz Institute – Fiocruz_RJ. The parasites were maintained *in vitro* in Schneider's medium supplemented with 10% FBS and 2% human urine at 27º C in BOD incubator.

3.2.2. J774.A1 murine macrophage culture

The adherent-phenotype macrophage line was cultured in Dulbecco's Modified Eagle's medium (DMEM, Sigma) supplemented with 10% FBS at 37ºC, 95% humidity and 5% CO_2.

3.2.3. Cytotoxicity against host cells

To evaluate the cytotoxicity activity against the J774 cell line, the host cells were plated in 96-well vessels at 2 x10^5 cells per well in a complete culture medium with 10% FBS at 37 ºC. After 1 h, the wells were washed with HBSS to remove non-adherent cells, leaving approximately 1 x 10^5 adherent macrophages. The cells were cultured in DMEM complete medium supplemented with 10% FBS. The compounds and pentamidine were added at serial concentrations (0.1 – 100 µM). The cells were also cultured in media free from compounds, a vehicle (basal growth control) or media with DMSO 0.1% (vehicle control). The positive control (dead cells) was obtained through cellular lyses with 1% of Triton 100X in DMEM complete medium. After 48 h, the cytotoxicity was evaluated using the MTT assay [18]. The data obtained from the experiments were expressed as the mean ± S.E.M., and the significant differences between the treated and vehicle groups were evaluated using ANOVA and Dunnett hoc tests**.**

3.2.4. *In vitro* activity against *Leishmania braziliensis*

The parasites were maintained *in vitro* in Schneider's medium supplemented with 10% FBS and 2% human urine. Novel bis-lawsone analog stock solutions and pentamidine (reference leishmanicidal drug) were prepared in DMSO immediately before use. The cytotoxicities of the bis-lawsone analogs and pentamidine against the promastigotes were determined. Stationary phase *L. braziliensis* promastigotes were plated in 96-well vessels (Nunc) at 1x10^5 cells per well in Schneider´s medium supplemented with 10% FBS and 2% human urine. Each compound solution was added at increasing concentrations (0.1-100 mg to the extract and its phases or 0.1 – 100 µM to the isolates and pentamidine). The cells were also cultured in a medium free of compounds, a vehicle (basal growth control) or with DMSO 0.1% (vehicle control). After 48 h, the extracellular load for *L. braziliensis* promastigotes was estimated by counting the promastigotes in Schneider's medium using a CELM automatic cell counter (model CC530) [31]. The data obtained from experiments were expressed as the mean

± S.E.M., and the significant differences between the treated and vehicle groups were evaluated using ANOVA and Dunnett hoc tests.

3.2.5. *In vivo* activity against *Leishmania amazonensis*

This study was approved (protocol no. 2013.02) by the Ethics Committee for Animal Experimentation of the Federal University of Alagoas (Brazil). All animals received humane care in compliance with the 'Principles of laboratory animal care' formulated by the National Society for Medical Research and the 'Guide for the care and use of laboratory animals' prepared by the National Academy of Sciences (Washington, DC). Next, $1x10^5$ stationary promastigotes (5 days of culture in Schneider's medium) of *L. amazonensis* (MHOM/BR/77/LTB0016) were subcutaneously inoculated into the right ear dermis of 6-week-old female BALB/c mice weighing ca. 20 g. and were later intraperitoneally treated with 3a or meglumine antimoniate at 30 µmol/kg x 28 days. The lesion size was measured using a paquimeter [32]. The parasite loads of infected ears and draining lymph nodes were determined using a quantitative limiting-dilution assay [33]. Complex toxicity was also evaluated through biochemistry dosages in plasma. The experimental data were expressed as the mean ± S.E.M., and the significant differences between the treated and vehicle groups were evaluated using ANOVA and Dunnett hoc tests.

3.2.6. In silico screening

Bis-lawsone analogs were submitted to *in silico* screening using the program OSIRIS, which is available at http://www.organic-chemistry.org/ to analyze their overall drug score and drug likeness potential as well as toxicity risks (mutagenic, irritant, tumorigenic and reproductive effects) [19] of the bis-lawsone analogs.

4. Conclusions

The present study demonstrates synthesis and leishmanicidal evaluation of a series of substituted bis-2-hydroxy-1,4-naphthoquinones prepared from lawsone. The *in vitro* cytotoxic activities of the derivatives synthesized were evaluated against *L. braziliensis* with a maximum effect greater than 60%. The bis-lawsone analogs **3a, 3b, 3e** and **3h** present efficacies and potencies similar to the reference drug pentamidine without cytotoxicity to the host cells.

Finally, **3a** presented the activity 30 μmol/kg x 28 days (i.p.); the *L. amazonensis* lesion size on the infected ear of BALB/c mice decreased, but the number of parasites in the infected ear and draining lymph nodes did not decrease. In summary, these findings show that **3a**, **3e** and **3h** are antileishmanial drug candidates and suggest a useful starting point for rationally designing new agents against leishmaniasis. Clearly, further studies are necessary for exciting advances in the medicinal use of drug candidates in this class of secondary products.

Acknowledgments

The authors are grateful to CAPES, CNPq (404344/2012-7), FAPEAL, INCT-INOFAR/CNPq (573.564/2008-6), INCT Amb Tropic and the Ministry of Environment (License MMA/CGEN 18/2007), FACEPE-PRONEM (APQ-1232-1.06/10) and CENAPESQ.

Author Contributions

Conceived and designed the experiments: MVA, ACQ, CBBM. Performed the experiments: MVA, ACQ, CBBM, ABL, AES, PSOS. Analyzed the data: MVA, ACQ, PSOS, JAAF, MSA. Contributed reagents/materials/analysis tools: PSOS, JAAF, MSA, TMSS, CAC. Wrote the paper: MVA, ACQ, MSA, TMSS, CAC.

Conflicts of Interest

The authors declare no conflict of interest.

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6 ARTIGO II DERIVADO DA TESE

Artigo 2: Em submissão

Evaluation on the leishmanicidal activity of new 2-*N*,*N*'-dialkylamino-1,4-naphthoquinone derivatives

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Abstract: Parasites of the *Leishmania* genus are the causative agents of leishmaniasis in humans, a disease that affects more than 12 million people worldwide. In this study was evaluated *in vitro* leishmanicidal activity of new 2-*N*,*N*'-dialkylamino-1,4-naphthoquinone derivatives, covering a series of fourteen 2-*N*-morpholino-, 2-*N*-thiomorpholino, 2-*N*-piperidino, 2-*N*-(N^4-methyl)-piperazino naphthoquinones (1a-n) derived from nor-lapachol and lawsone, belong to some other di-alkyaminoderivatives. At the cytotoxicity assay on peritoneal macrophages, the compounds possessing larger alkyl groups and *N*-methyl-piperazino moiety (1d, 1h, 1i and 1k), showed toxic effects similar to the standard drug used pentamidine. However, the other compounds of the series showed no deleterious effect on the host cell. Meanwhile, these cytotoxic derivatives (1d, 1h and 1i) had pronounced leishmanicidal activity against *L. amazonensis* promastigotes, and treatments with six other compounds (1d, 1e, 1f, 1h, 1k and 1n) had significant effect leishmanicidal against *L. chagasi* promastigotes. In the assay against *L. chagasi* amastigotes, eight compounds (1a, 1b, 1c, 1d, 1h, 1i, 1k and 1m) showed significant activity. Moreover, the compounds (1a, 1b, 1c, and 1m) showed effect against amastigotes of *L. chagasi* and not being toxic to the host cell. These data show the derivatives as promising substances for research leishmanicidal activity.

Keywords: leishmaniasis. Naphthoquinone. *L. chagasi. L. amazonensis.*

1. Introduction

More than 30 *Leishmania* species (Protozoa, Trypanosomatidae) are known worldwide, 21 of which may be transmitted to humans by the bite of infected female phlebotomine sandflies (Diptera, Psychodidae) causing leishmaniasis (Kaye and Scott, 2011). This disease is considered a neglected tropical disease with high morbidity and mortality in the tropics and subtropics (Santos et al., 2008). According to the World Health Organization (WHO), leishmaniasis is endemic in 98 countries and 350 million people are at risk of contracting this disease (WHO, 2010). Considering the officially reported cases, approximately 58 000 cases of visceral leishmaniasis and 220.000 cutaneous cases are diagnosed each year (Stockdale and Newton, 2013).

The different parasite species can lead to cutaneous leishmaniasis (CL), mainly caused by *Leishmania major, Leishmania tropica, Leishmania mexicana, Leishmania braziliensis*, and *Leishmania amazonensis* species, or visceral leishmaniasis (VL) primarily caused by

Leishmania donovani and *Leishmania chagasi*. (Berman, 1997). CL is a group of diseases characterised by a spectrum of clinical manifestations, which range from small cutaneous nodules to mucosal tissue destruction. VL, on the other hand, is the most severe and debilitating form of the disease, characterized by prolonged fever, splenomegaly, hypergammaglobulinemia, and pancytopenia and can lead to death if left untreated (Bern et al., 2008).

The treatment for leishmaniasis is limited, and include pentavalent antimonials (Sb^v), pentamidine, amphotericin B (AmB) and its liposomal formulations, paromomycin and miltefosine. Antimonials have been used in the treatment of the majority of cases of leishmaniases for more than 60 years worldwide, but their side effects are frequent and can be serious. Moreover, parasite resistance has caused an increase in treatment failure (Hadighi et al., 2006; Lira et al., 1999).

Pentamidine is a drug used for patient refractory to Sb^v. Recently, pentamidine resistance was also described by the literature (Bray et al., 2003) as well as difficulties in treating immune-depressed patients (i.e., HIV), in whom conventional drugs are less efficient and higher drug doses and a long treatment period are commonly necessary (Escobar et al. 2001). Paromomycin is used in combination with other drugs for VL and in topical treatment for CL (Croft and Coombs, 2003; de Morais-Teixeira et al., 2015).

AmB is used as a prototype leishmanicidal drug due to its excellent efficacy, with great commercial success. It possesses selective killing activity against *Leishmania* parasites, however, toxic side effects, in particular hematological intolerance and nephrotoxicity, produced by AmB at therapeutic doses, have often limited its clinical application (Dupont, 2002). The investigations of newer, less-toxic formulations of AmB have led to the development of commercial preparations for therapeutic use such as liposomal AmBisome. Although liposomes and lipid complexes have succeeded in reducing the adverse effects of AmB, their lower stability and high cost restrict their clinical utility (Hiemenz and Walsh, 1996; Wong-Beringer et al., 1998).

Miltefosine since its registration in 2002 remains the only oral agent used for the treatment of all types of leishmaniasis. The U.S. Food and Drug Administration (FDA) recently (March, 2014) approved Impavido (miltefosine) for the treatment of cutaneous, visceral, and muco-cutaneous leishmaniasis. However, cases of resistance *in vitro* have already been reported and its teratogenicity is a problem (Bhandari et al., 2012; Perez-Victoria et al., 2006).

Therefore, treatment for leishmaniasis is limited to a small number of drugs and is far from ideal. Hence, the development of new drugs is necessary. Naphthoquinones have been reported to possess leishmanicidal activity (Araújo et al., 2014; Teixeira et al., 2001; Ali et al., 2012), thus, the present study aims to evaluate the leishmanicidal activity of new 2-*N*,*N*'-dialkylamino-1,4-naphthoquinone derivatives.

2. Material and methods

2.1. Substances. Naphthoquinone derivatives were synthesized by the Laboratory of Bioactive Compounds Synthesis, Molecular Sciences Department, Federal Rural University of Pernambuco, and published in the online magazine chemistry (David et al., 2015). The biological evaluation was conducted at the Laboratory of Pharmacology and Immunity, Institute of Biological Sciences and Health, Federal University of Alagoas, They were solubilized in 0.1% dimethyl sulfoxide (DMSO), forming the stock solution I (100 µM). During the experiments, serial dilutions were performed to the desired concentrations.

2.2. Parasites. Two strains of *Leishmania* were used in the present study: *Leishmania amazonensis* [MHOM/BR/77/LTB0016] and *Leishmania chagasi* [MCAN/BR/89/BA262]. They were maintained *in vitro* as proliferating promastigotes at 26°C in Schneider's medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum, gentamycin (1 mg/L), *L*-glutamine (2 mM), and 2% sterile human urine.

2.3. Macrophages. Peritoneal macrophages were obtained from swiss mice (weighing 20–25 g) (protocol no. 2015.01) through peritoneal lavage with 10 mL of cold PBS (Phosphate-Buffered Saline; 137 mM NaCl, 8 mM Na2HPO4, 2.7 mM KCl, 1.5 mM KH2PO4, pH 7.0). The peritoneal exudate cells were centrifuged at 1500 rpm for 5 min and the pellet was resuspended in culture medium. Cells were counted and plated into 96-well plates for the cytotoxicity assay and in 48-well plates for the intracellular amastigote assay.

2.4. Cytotoxicity assay in macrophages. Peritoneal macrophages (protocol no. 2015.01) were seeded ($3x10^5$ cells/well) in 96-well plates with 100 mL of media. Cells were allowed to attach to the bottom of the well for 24 h at 37°C and then treated with 1, 10, and 100 µM of naphthoquinones or pentamidine previously diluted in RPMI-1640 medium with dimethyl sulfoxide (DMSO). The plates were maintained in a 5% CO_2 incubator at 37°C for 48 h. Cells were also cultured in media free from compounds, a vehicle (basal growth control), or media with 0.1% DMSO (vehicle control). Thereafter, the supernatant was removed and cells were

incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (100 mL/well) for 1 h in the dark at 37°C. The MTT solution was removed, cells were resuspended in 100 mL of DMSO, and the absorbance was measured using an ELISA reader at 550 nm (Hussain et al., 1993).

2.5. Leishmanicidal Assay. Promastigotes of *L. amazonensis* and *L. chagasi* in RPMI culture phenol red –free medium plus 10% FBS were seeded into flat bottom 96 well plates at a concentration of 10^5 parasites / well (50 µL / well) and incubated for 48 hours at 26°C in the presence of different concentrations of compounds (100, 10, 1, 0.1, 0.01, and 0.001 µM). After the incubation period, 20 µL of MTT (5mg / mL) were added to each well. The plate was then incubated at 37°C in a CO_2 incubator for 2 hours. After this period 120 µL of isopropyl alcohol were added to each well to solubilize formazan crystals and the plates were kept at room temperature for one hour, reading was then performed on ELISA reader at 550 nm. The absorbance obtained by the cell control, untreated, was considered as 100% viable cells (Mosmann, 1983; Denizot and Lang, 1986).

2.6. Intracellular Amastigote Assay. Peritoneal cavity macrophages of swiss mices were seeded on glass coverslips (13 mm diameter) in 48-well plates and incubated at 37°C with 5% CO_2 for 12 hours for adhesion. Macrophages were infected with *L. chagasi* promastigotes using a ratio of 1:10 at 37°C overnight in isolated experiments. Non-internalized promastigotes were removed by washing (three times) with PBS. Infected macrophages were treated with different concentrations of compounds (0.1, 1 and 100 µM) and maintained at 37°C in 5% CO_2 for 48 h. The glass coverslips were fixed with methanol, stained with May-Grünwald-Giemsa, and intracellular amastigotes were counted (one hundred macrophages were evaluated per glass coverslip). Data was expressed as infection index (percentage of infected macrophages multiplied by the average number of amastigotes per macrophage) (Nunes et al., 2005).

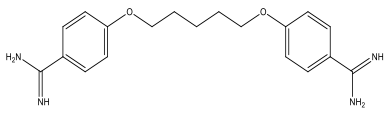
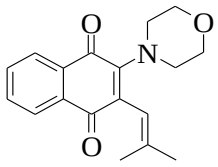
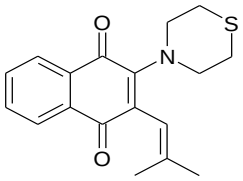
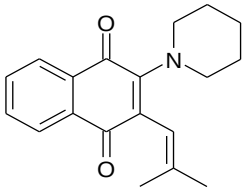
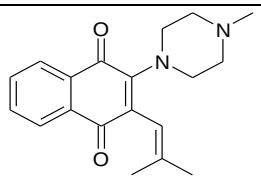
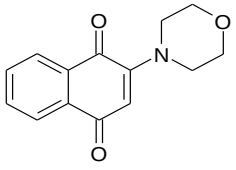
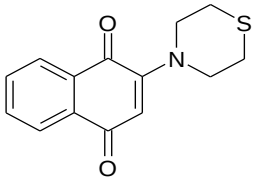
2.7. Statistical analysis. Data were expressed as the mean ± standard error of the mean (S.E.M) and significant differences between the treated and control groups were evaluated using ANOVA and Dunnett post-hoc tests by Graph Pad Prism 5.0 software, and the 95% confidence intervals were included.

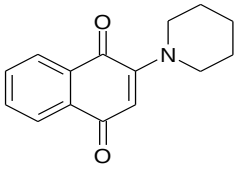
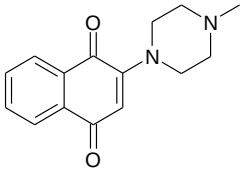
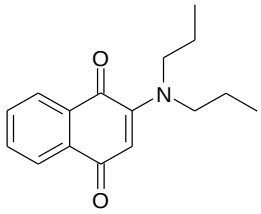
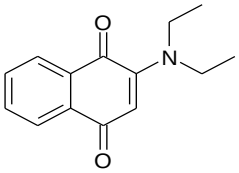
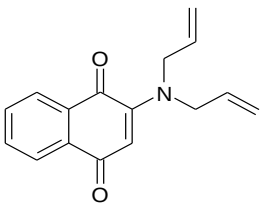
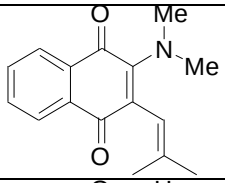
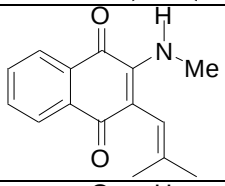
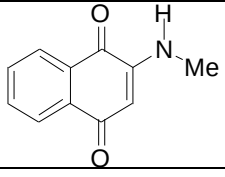
3. Results

Initially it was investigated the cytotoxicity of the compounds on peritoneal macrophages. The results revealed that only the compounds (1d), (1h), (1i), and (1k) showed toxic effects similar to the standard drug used pentamidine with maximum cytotoxicity of 100

± 0.0% (Table 1). Other compounds showed no deleterious effect on the host cell. The vehicle used to solubilize all substances (0.1% DMSO) showed no significant toxic effects on the cell when compared to the negative control (only cells grown in culture medium). Thus, the toxicity of substances can not be attributed to the presence of DMSO in culture (data not shown).

Table 1. Determination of the cytotoxicity of naphthoquinone derivatives (1a-n) against macrophages (MTT assay)

Treatment	Chemical structure (R=)	IC_{50} (µM) [a]	Maximum Cytotoxicity (%)[b]
Pentamidine		54.5 ± 4.5	100 ± 0.0***
(1a)		> 100	ND
(1b)		> 100	ND
(1c)		> 100	ND
(1d)		44.2 ± 0.2	99.7 ± 0.4***
(1e)		> 100	ND
(1f)		> 100	ND

(1g)		> 100	ND
(1h)		9.0 ± 2.1	100 ± 0.0***
(1i)		34.7 ± 2.6	100 ± 0.0***
(1j)		> 100	ND
(1k)		22.7 ± 4.7	100 ± 0.0***
(1l)		>100	ND
(1m)		>100	ND
(1n)		>100	ND

[a] Inhibitory Concentration 50 (IC_{50}) calculated by concentration-response curves toxic. [b]Mean ± standard error of the mean maximum cytotoxicity in triplicates of a representative experiment. The values of maximum effect were considered significant when * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to the 0.1% DMSO group; ND: Not determined Maximum Cytotoxicity to cell until the concentration of 100 µM compared to DMSO group.

In order to verify a possible direct leishmanicidal activity on the parasite, it calculated the 50% inhibitory concentration (IC_{50}) of the growth of promastigotes and the maximum effect of the compounds and pentamidine against promastigotes of *L. amazonensis* and *L. chagasi.* The results of the evaluation of the direct activity on *L. amazonensis* promastigotes evidenced that all the compounds showed leishmanicidal effects, except (1a), (1b) and (1i). Belong the most actives are the compounds (1d), (1h), and (1k), which showed maximal effect (ME) against *L. amazonensis* promastigotes exceeding 50%, with ME of 51 ± 0.1%, 73.8 ± 7.3%, and 67.6 ± 7.9%, and IC_{50} of 97.7 ± 0.3 µM, 40.2 ± 6.8 µM, and 46.5 ± 9.0 µM, respectively (Table 2).

During the evalution of the leishmanicidal activity against *L. chagasi* promastigotes, it was observed that derivatives 1d, 1e, 1f, 1h, 1i, 1k, and 1n had significant activity. Highlighting the compounds 1d, 1e, 1f, 1h, 1k, and 1n presenting ME of 94.6 ± 6.3%, 89.7 ± 4.9%, 72.7 ± 5.0%, 95.9 ± 5.8%, 79.5 ± 0.8% and 74.7 ± 2.9%, and IC_{50} of 28.3 ± 1.4 µM, 44.5 ± 2.5 µM, 65.8 ± 3.0 µM, 25.7 ± 3.8 µM, 39 ± 8.0 µM, and 55.0 ± 3.5 µM (Table 2).

Table 2. Leishmanicidal effect of naphthoquinone derivatives (1a-n) against the growth of promastigotes of *L. amazonensis* and *L.chagasi* (MTT assay).

Treatment	*L. amazonensis* promastigotes		*L. chagasi* promastigotes	
	IC_{50} (µM)	Efficacy (%)	IC_{50} (µM)	Efficacy (%)
Pentamidine	7.2 ± 0.9	100 ± 0.0***	4.2 ± 0.7	100 ± 0,0***
(1a)	> 100	NA	> 100	NA
(1b)	> 100	NA	> 100	NA
(1c)	> 100	30.1±8.8*	>100	NA
(1d)	97.7 ± 0.3	51 ± 0.1***	28.3 ± 1.4	94.6 ± 6.3***
(1e)	> 100	36.6 ± 4.3*	44.5 ± 2.5	89.7 ± 4.9***
(1f)	> 100	35.1 ± 2.0*	65.8 ± 3.0	72.7 ± 5.0***
(1g)	> 100	35.7 ± 3.8*	> 100	NA
(1h)	40.2 ± 6.8	73.8 ± 7.3***	25.7 ± 3.8	95.9 ± 5.8***
(1i)	>100	NA	> 100	38,.7 ± 2.1**
(1j)	>100	42.4 ± 1.5**	>100	NA
(1k)	46.5 ± 9.0	67.6 ± 7.9***	39 ± 8.0	79.5 ± 0.8***
(1l)	>100	38.7 ± 0.1*	>100	NA
(1m)	>100	41 ± 4.3**	>100	NA
(1n)	>100	28.8 ± 1.0*	55.0 ± 3.5	74.7 ± 2.9***

Data are reported as the mean ± standard error of the mean, S.E.M. Differences with *$p < 0.05$, **$p < 0.01$ and ***$p < 0.01$ were considered significant in relation to the 0.1% DMSO group. IC50 is the concentration required to give 50% inhibition; NA: compound is not active.

Based on the viability of macrophage cells, *L. amazonensis* and *L. chagasi* promastigotes, it was possible to calculate the selectivity index (SI) of compounds (Table 3). Thus, when comparing the SI between macrophage and *L. amazonensis* promastigote, the compounds didn't show selectivity for *L. amazonensis.* However, when compared the SI between macrophage and *L. chagasi* promastigote, pentamidine showed selectivity for *L. chagasi* 13 times more active. Compounds (1a), (1b), (1c), (1g), (1j), (1l), and (1m) showed no deleterious effect on the macrophage. Compounds (1e), (1f), and (1n) are probably selective for *L. chagasi*, however, as their IC_{50} values were not determined for macrophages, it is not possible to calculate the SI for *L. chagasi.*

Table 3. Selectivity Index (SI) to macrophages x *L. amazonensis* and macrophages x *L. chagasi* of the compounds (1a-n).

Treatment	macrophages x *L. amazonensis* [1]IS para *L. amazonensis*	macrophages x *L. chagasi* [1]IS para *L. chagasi*
Pentamidine	7.6	13.0
(1a)	-	-
(1b)	-	-
(1c)	-	-
(1d)	0.45	1.56
(1e)	-	>2.25
(1f)	-	>1.52
(1g)	-	-
(1h)	0.22	0.35
(1i)	<0.35	<0.35
(1j)	-	-
(1k)	0.5	0.58
(1l)	-	-
(1m)	-	-
(1n)	-	>1.82

The results refer to: [1]selectivity index for *L. chagasi* calculated from the ratio of IC_{50} values of macrophage and *L. chagasi* amastigotes. (-): Substance does not have a deleterious effect on the macrophage or *Leishmania*.

The results of the evaluation of leishmanicidal activity against intracellular forms of *L. chagasi* revealed that (1a), (1b), (1c), (1d), (1h), (1i), (1k), and (1m) presented statistically activity, with ME of 87.7 ± 1.8%, 66.9 ± 1.2%, 92.5 ± 0.5%, 100 ± 0.0%, 100 ± 0.0%, 100 ± 0 0%, 100% ± 0.0, and 71.8 ± 5.2%, respectively (Table 4). The compounds (1d), (1h), (1i), and (1k) induced 100% of amastigotes death, moreover, proved to be toxic at the highest concentration tested, it also occurred death of infected cell. These data confirm cell viability assay data which showed that the compounds (1d), (1h), (1i), and (1k) were toxic to the host cell.

Compounds (1a), (1b), (1c), and (1m) showed ME of 87.7 ± 1.8%, 66.9 ± 1.2%, 92.5 ± 0.5%, and 71.8 ± 5.2% with IC_{50} of 26.5 ± 1.5 µM, 49.3 ± 10.8 µM 57.3 ± 7.3 µM, and 6.5 ±

0.5 µM, respectively (Table 4), against amastigotes of *L. chagasi*. These compounds, besides not being toxic to the host cell at the highest concentration tested (data obtained from the amastigote and direct viability tests on the host cell), showed excellent inhibitory activity against the *L. chagasi* forms (Figure 1 – anexo A).

ANEXO A: Figure 1. Photographs of the Leishmanicidal assay against the growth of amastigotes of *L. chagasi*. Viewing through optical microscope with objective of 100X. Staining May-Grunwald / Giemsa.

Table 4. Leishmanicidal effect of naphthoquinone derivatives (1a-n) against the growth of amastigotes of *L. chagasi.*

Treatment	IC_{50} (µM) [a]	Efficacy (%)[b]
Pentamidine	30.7 ± 1.4	100 ± 0.0***
(1a)	26.5 ± 1.5	87.7 ± 1.8***
(1b)	49.3 ± 10.8	66.9 ± 1.2***
(1c)	57.3 ± 7.3	92.5 ± 0.5***
(1d)	38.7 ± 3.5	100 ± 0.0***
(1e)	>100	NA
(1f)	>100	NA
(1g)	>100	NA
(1h)	51 ± 0.3	100 ± 0.0***
(1i)	19 ± 6.4	100 ± 0.0***
(1j)	>100	NA
(1k)	43.5 ± 3.0	100 ± 0.0***
(1l)	>100	NA
(1m)	6.5 ± 0.5	71.8 ± 5.2***
(1n)	>100	NA

Data are reported as the mean ± standard error of the mean, S.E.M. The efficacy values were considered significant when * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.01$ compared to the 0.1% DMSO group. IC_{50} is the concentration required to give 50% inhibition; NA: compound is not active.

4. Discussion

Naphthoquinones (NQs) are natural substances found in different families of plants, including Bignoniaceae and Verbanaceae. These substances are aromatic cyclic α,β-dienones with a basic skeleton of naphthalene (Silva et al., 2013). Lapachol, α-lapachone, and β-lapachone are well known naphthoquinones used in medicinal chemistry studies (de Moura et al., 2001; da Silva Jr et al., 2010). Such compounds, besides being obtained from natural sources, can be easily synthesized (da Silva Jr et al., 2010) and these qualities favour the synthesis of several other substances with potential pharmacological activities, including the development of drug candidates against neglected and other types of diseases (Castro et al., 2013).

NQs are also susceptible to oxidative reactions as well as to the addition of electrophiles via Michael-addition. This includes for example dimeric NQs like diospyrin, which result from oxidative coupling of monomers and may also exhibit antiprotozoal activities (Babula et al., 2009; Schmidt et al., 2012). NQs and other quinones may disturb the cellular redox status and thereby provoke the generation of reactive oxygen species (ROS) by redox cycling. Because their redox metabolism significantly differs from that of other eukaryotic cells, protozoans of the Trypanosomatidae family are strongly impaired by ROS (Babula et al., 2009; Schmidt et al., 2012; Sun et al., 2003).

The fourteen substances used in this study were synthesized from suitable 2-methoxy-nor-lapachol and 2-methoxy-lawsone, by direct displacement of the methoxyl group with suitable amines (David et al., 2015). Cell viability assay was conducted with peritoneal macrophages, through MTT assay. This method measures the metabolic activity of cells and quantifies the metabolic reduction of MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5 diphenyl tetrazolium bromide) carried by the mitochondrial enzyme succinate dehydrogenase associated with NADPH and NADH, which results in the production of formazan crystals (purplish color and insoluble in water), which are quantified by spectrophotometry (Mosmann, 1983). Hence, it is a significant method in the scientific community to measure cell death by apoptosis and necrosis. The compounds possessing larger alkyl groups and *N*-methyl-piperazino moiety (1d, 1h, 1i and 1k, see table 1) showed toxic effect, but the other compounds showed no deleterious effect on the host cell. These compounds have structural features including large apolar substituents at the amino moiety at 2-position (1i and 1k), di-n-propyl- and di-allyl-, respectively) (Camara et al., 2008; Silva et al., 2008; Esteves-Souza et al., 2008; Cavalcante et

al., 2008; Fechine et al., 2007; Silva et al., 2007; Silva et al., 2007; Esteves-Souza et al., 2007; Lins et al., 2007; Silva et al., 2006; Silva et al., 2006; Vasconcellos et al., 2006; Silva et al., 2006; Cunha et al., 2006; Antunes et al., 2006; Silva et al., 2006; Silva et al., 2005), or presents the extra amine nitrogen at the piperazine nucleus (1d and 1h), capable of salt formation, because this nitrogen is more basic than the direct ring-attached ones. It is noteworthy that even in the absence of the butenyl side chain compound (1h) is more cytototoxic than (1d) (9 versus 44 Mm), suggesting different roles of mechanisms of action (the free C-3 position like these of compound (1h) is reported as an effective Michael acceptor) (Camara et al., 2008).

Among the known biological activities of compounds with a quinone structure, it is of paramount importance to emphasize their antiprotozoal activity. For example, three naphthofuran quinone C-allyl lawsone derivatives were active against *Trypanosoma cruzi* trypomastigotes (Da Silva Júnior et al., 2012). Out of thirty-eight compounds evaluated by Jardim et al. (2015) against *Trypanosoma cruzi*, six were considered more potent against trypomastigotes than the standard drug benznidazole (Jardim et al., 2015). Other studies have also shown activity against Trypanosoma (Naujorks et al., 2015; Ellendorff et al., 2015). When used as therapeutic agents, quinonoid ligand cytotoxic activity operates through various mechanisms, such as redox cycling, intercalation, inducing DNA strand breaks, arylation, alkylation via quinone methide formation, and free radical generation (Moore, 1977).

Studies have also showed the leishmanicidal activity of naphthoquinones. In the study of Araújo et al. (2014) a series of eight substituted bis-2-hydroxy-1,4-naphthoquinone derivatives were tested *in vitro* against *Leishmania amazonensis* and *Leishmania braziliensis* promastigotes, presenting significant inhibitory activity of the growth of promastigotes (Araujo ey al., 2014). Souza-Silva (2014) examined the leishmanicidal effect of epoxy-α-lapachone on *L.* (*V.*) *braziliensis* and *L.* (*L.*) *amazonensis* and their results showed that the epoxy-α-lapachone has an antiparasitic effect on *Leishmania* in both morphological phases and can potentially affect both species (Souza Silva- 2014).

A study suggested death against *L. Braziliensis* by induction of apoptosis (Costa et al., 2014). A naphthoquinones series was evaluated against the three most important human trypanosomatid pathogens (*Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, and *Leishmania donovani*), and two compounds showed good activity, despite concomitant mammalian cytotoxicity. Furthermore, a subset also inhibited the glycolytic TbGAPDH enzyme *in vitro*, and were selected to undergo anticancer assays. Intriguingly, they displayed antitumor activity and were less toxic to noncancerous cells. The observed cytotoxic potency

was ascribed to a multitarget mechanism of action accounting for hGAPDH inhibition and mitochondrial toxicity (Prati et al., 2015). All these studies show naphthoquinones as promising substances against protozoa and other diseases. Although some of the most proeminent compounds in this study showed undesirable cytotoxic activity, the compounds (1a, 1b, 1c, and 1m) showed effect against amastigotes of *L. chagasi* and not being toxic to the host cell. The common feature among them is the presence of a 3-alkenyl side chain, suggesting that derivatives of norlapachol were more promising substances for continuous research of the leishmanicidal activity.

Acknowledgments

The authors would also like to thank the CAPES, CNPq, MCT, FINEP, INCT-INOFAR (573.564/2008-6), CNPQ (479822/2013-1), CNPQ (404344/2012-7), FACEPE and FAPEAL (Pronem 20110722-006-0018-0010) FACEPE-PRONEM (APQ-0741-1.06/14) and CENAPESQ *for providing financial assistance for this research project. Moreover, the authors would like to thank several colleagues working at the UFAL for constructive criticism of and assistance with this project.*

5. Conclusion

Treatment with derivatives (1d), (1h), and (1k), had pronounced leishmanicidal activity against *L. amazonensis* promastigotes, and treatment with (1d), (1e), (1f), (1h), (1k), and (1n) had significant leishmanicidal activity against *L.chagasi* promastigotes. In the activity assay against *L. chagasi* amastigotes, the derivatives (1a), (1b), (1c), and (1m) showed significant activity, without deleterious effects to the host cell. These data show the derivatives as promising substances for designing new prototypes of antileishmanial drugs.

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7 ARTIGO III DERIVADO DA TESE

Artigo 3: Em submissão

Flavonoid compounds: Leishmanicidal evaluation and investigation of the proposed mechanism of action

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Abstract: The present study evaluated the *in vitro* and *in vivo* leishmanicidal activity and proposed a possible mechanism of action of flavonoids isolated from *Solanum paladosum*. These compounds evaluated for antileishmanial activity *in vitro* against *Leishmania chagasi* and *Leishmania amazonensis* promastigotes and showed good activity. *In vitro* activity against *L. amazonensis* amastigotes also presented significant activity without significant toxic effects. *In vivo* assay, the selected compounds reduced the parasite load in the ear, but did not reduce the parasitic load on the draining lymph node. The SP1 and SP3 compounds induced change in the S phase of the cell cycle. Additionally composed SP3 induce death by apoptosis and induction of autophagy. The results support that flavonoids derived from *S. paladosum* can become lead molecules for the design of new prototypes of antileishmanial drugs.

Keywords: leishmanicidal. *Solanum paladosum*. *L. chagasi*. *L. amazonensis*.

1. Introduction

Leishmaniases are a complex of diseases caused by protozoans of the *Leishmania* genus affecting human beings. Leishmaniasis is considered a neglected disease with a major impact among the poorest individuals, mainly in developing countries (1, 2). It is the second main cause of death among parasitic diseases caused by protozoa, falling behind only with malaria (3).

In spite of being a disease that affects millions of people worldwide, the current available treatment for leishmaniasis is restricted to few drugs of first choice such as pentavalent antimonials, miltefosine, amphotericin B with its derivatives, and paromomycin. The main problem of conventional treatments is the intrinsic or acquired resistance by the protozoan. Other restrictions related to the available drugs are high incidence of adverse effects and toxicity (4). The clinic symptoms are diverse and are associated with antigenic differences among *Leishmania* species, despite host genetic and immunologic factors (5).

Due to the lack of better therapies, identification of novel drugs, compounds, and targets is of interest to researchers worldwide. Natural products are potential sources of new agents for the treatment of neglected tropical diseases, especially those caused by protozoan parasites (6). Various secondary plant metabolites like quinones, alkaloids, terpenes, saponins, phenolics and their derivatives are beneficial for human beings due to their antiparasitic properties and highly selective mode of action. Furthermore, the skeletons of many metabolites have been successfully utilized to design pharmacologically more active compounds (7).

Many flavonoids occur widely in nature and NMR data can help to identify new flavonoids isolated from natural sources. The genus *Solanum* has been studied and several constituents were isolated from their species (8, 9). This is the largest and most complex genus of the Solanaceae family, with about 1400 species and 5000 epithets in ecological systems established by tropical and subtropical regions of the world and with South America as the center of its diversity and distribution (10).

Some *Solanum* species have been reported to have anti-inflammatory, sedative, anti-spasmodic, anti-epileptic (11), hypoglycemic, anti-obesity and decreasing cholesterol levels (12, 13), leishmanicidal, and trypanosomidal activity (14, 15). In addition, studies demonstrated anticancer activity against hepatoma cells (Hep3B) and cytotoxic activity against tumor cells of lung lines (H441, H520, H661, and H69) (16, 17).

Considering the necessity to identify new alternatives for the treatment of leishmaniasis and the discovery of potential therapeutic agents from natural sources, the present study aims to investigate the leishmanicidal activity and suggest of action of flavonoids isolated from *Solanum paladosum*.

2. Experimental Section

Chemistry

Parasites. Two strains of *Leishmania* were used in the present study: *Leishmania amazonensis* [MHOM/BR/77/LTB0016] and *Leishmania chagasi* [MCAN/BR/89/BA262]. They were maintained *in vitro* as proliferating promastigotes at 26°C in Schneider's medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum, gentamycin (1 mg/L), L-glutamine (2 mM), and 2% sterile human urine.

Animals. Swiss mice (six to eight weeks old) were obtained from the Central Animal House of the Federal University of Alagoas in order to obtain peritoneal macrophages. BALB/c mice (six to eight weeks old) were obtained from breeding stocks maintained at the Multidisciplinary Center for Biological Investigation on Laboratory Animal Science (UNICAMP) to be used in *in vivo* assay. These studies were approved by the Ethics Committee for Animal Experimentation of the Federal University of Alagoas (Brazil) (protocol no. 2015.01 and protocol no. 2013.02, respectively).

Macrophages. Peritoneal macrophages were obtained from swiss mice (weighing 20–25 g) through peritoneal lavage with 10 mL of cold PBS (Phosphate-Buffered Saline; 137 mM NaCl, 8 mM Na2HPO4, 2.7 mM KCl, 1.5 mM KH2PO4, pH 7.0). The peritoneal exudate cells were centrifuged at 1500 rpm for 5 min and the pellet was resuspended in culture medium. The cells were counted and plated into 96-well plates for the cytotoxicity assay and in 48-well plates for the intracellular amastigote assay.

Cytotoxicity assay in macrophages. Peritoneal macrophages were seeded ($3x10^5$ cells/well) in 96-well plates with 100 mL of media. Cells were allowed to attach to the bottom of the well for 24 h at 37 °C and then treated with1, 10 and 100 µM of flavonoids or pentamidine previously diluted in RPMI-1640 medium with dimethyl sulfoxide (DMSO). The plates were maintained in the 5% CO2 incubator at 37 °C, for 48 h. The cells were also cultured in media free from compounds, a vehicle (basal growth control), or media with 0.1% DMSO (vehicle control).Thereafter, the supernatant was removed and cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (100 mL/well) for 1 h in the dark at 37 °C. The MTT solution was removed, cells were resuspended in 100 mL of DMSO, and the absorbance was measured using an ELISA reader at 550 nm (18).

Leishmanicidal Assay. *L. amazonensis* and *L. chagasi* promastigotes (2×10^6 cells/mL) were maintained *in vitro* in Schneider's medium supplemented with 10% FBS and 2% human urinein the presence of various concentrations of flavonoids and pentamidine (0.1, 1.0, 10 and 100 µM) for 48 h at 26 °C. Cells were also cultured in a medium free of compounds (basal growth control), or with 0.1% DMSO (vehicle control). Promastigotes were adjusted to a concentration of 10^2 in PBS and viable parasites counted in a Neubauer's chamber (19).

Intracellular Amastigote Assay. Peritoneal cavity macrophages of swiss mices were seeded on glass coverslips (13 mm diameter) in 48-well plates and incubated at 37 ˚C with 5% CO_2 for 12 hours for adhesion. Macrophages were infected with *L. amazonensis* and *L. chagasi* promastigotes using a ratio of 1:10 at 37 °C overnight in isolated experiments. Non-internalized promastigotes were removed by washing (three times) with PBS. Infected macrophages were treated with different concentrations of compounds (0.1, 1.0 and 100 µM) and maintained at 37 ˚C in 5% CO_2 for 48 h. The glass coverslips were fixed with methanol, stained with May-Grünwald-Giemsa and intracellular amastigotes were counted (one hundred macrophages were evaluated per glass coverslip). Data was expressed as infection index (percentage of infected macrophages multiplied by the average number of amastigotes per macrophage) (20).

***In vivo* activity against *Leishmania amazonensis*.** Female BALB/c mice, 6 to 8 weeks old, were purchased from the Federal University of Ribeirão Preto (Brazil) and maintained in a regulated animal care facility. A murine model closely resembling human pathology was previously described [23]. Briefly, the right ear dermis of BALB/c mice was inoculated with stationary-phase promastigotes of *L. amazonensis* with 10^5 parasites in 10 mL of sterile saline, using a 27.5-gauge needle. After the second week postinfection, a group of mice (n =5) was treated intraperitoneally with SP1, SP3 or meglumine antimoniate (positive control) at 100 mg/kg/day, diluted in water for administration. A negative control group was treated orally with water by injection (n =5). Lymph nodes and ears of mice BALB/c were collected after euthanasia and macerated with Schneider's medium. Supernatants were grown in 48-well plates and the parasite load was observed. Animal blood was also collected and centrifuged in order to obtain serum to be used in cytokine assays (21).

Cell cycle analysis.The cell cycle assay was performed according to the instructions of the Cell Cycle Kit Muse®. *L. amazonensis* promastigotes (10^5 cells/mL) were treated with pentamidine, SP1 and SP3 at a concentration of 100 µM for 48 h. After the incubation period, cells were centrifuged and the pellets were resuspended in 1 mL of PBS supplemented with 1% FBS to wash cellsand then fixed by incubation in 70% ethanol for 3 hours at -20 ˚C. After fixation, 200 µL of each cell suspension was centrifuged, washed with PBS, and pellets were resuspended in 200 of Cell Cycle reagent Muse®. After incubation for 30 min at room temperature and protected from light**,** data acquisition was carried out by flow cytometry on Muse® Cell Analyzer and analysed using Muse™ 1400 Analysis software.

Analysis of phospholipid externalizationin *L. amazonensis* promastigotes by flow cytometry. The quantification of the percentage of parasites undergoing apoptosisor necrosis was performed according to the instructions of the Annexin Muse® & Dead Cell Kit. Exponential-phase *L. amazonensis* promastigotes (10^5 cells/mL) were incubated with pentamidine, SP1, and SP3 (10 µM and 100 µM for 48 h). After the incubation period, cells were centrifuged and resuspended in PBS supplemented with 1% FBS to wash the cells. Thereafter, 100 µL of each cell suspension were incubated with 100 µL of the reagent containing Annexin V-PE and 7-AAD. After there is incubation for 30 min at room temperature protected from light. Data acquisition was carried out by flow cytometry on Muse® Cell Analyzer and analysis was performed using Muse® 1400 Analysis software.

Determination of Caspase-like proteases. To determine the percentage of caspase-positive cells, we used the Muse® Multicaspase kit, performed according to the manufacturer's instructions. *L. amazonensis* promastigotes were grown to 10^5 cells/mL and then treated with pentamidine, SP1, and SP3 at a concentration 100 µM and incubated at 26°C. After 48h, 1 mL of the culture was pelleted and resuspended in 1mL of PBS buffer supplemented with 1% FBS. Muse® Multicaspase Reagent followed by7-AAD were added. Data acquisition was carried out using a Muse® Cell Analyzer and analyzed using MuseT 1400 Analysis software.

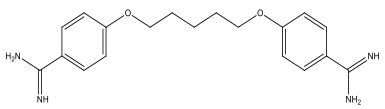
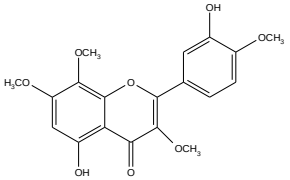
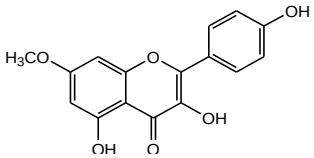
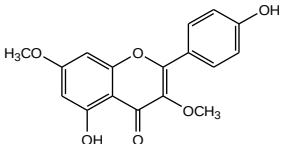
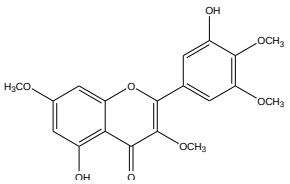
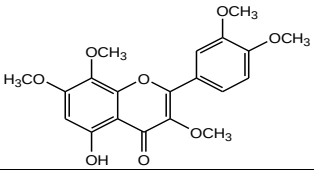
Induction of autophagy. The determination of autophagy induction was performed according to the instructions of the Muse® Autophagy LC3-based antibody kit. *L. amazonensis* promastigotes (5x 10^4 cells/mL) were incubated with pentamidine, SP1, and SP3 at a concentration of 100 µM for 48 h. After the incubation period, the contents of each well were transferred to microcentrifuge tubes, centrifuged and the pellets were resuspended in PBS supplemented with 1% FBS to wash the cells. Subsequently, the content was treated with Reagent Autophagy A for 3 hours at 37 ° C. Thereafter, each cell suspension was centrifuged and the pellet was resuspended in 200 µM of solution containing reagent Autophagy B and anti-LC3 Alexa Fluor®555. After 30 min of incubation on ice and protected from light, the tubes were centrifuged and the pellets were resuspended in assay buffer. Data analysis was performed using Muse® 1400 Analysis software.

Statistical analysis. Data were expressed as the mean ± S.E.M. and significant differences between the treated and control groups were evaluated using ANOVA and Dunnett *post-hoc* tests by Graph Pad Prism 5.0 software, and the 95% confidence intervals were included.

3. Results

Murine peritoneal macrophages were treated with the flavonoid compounds (1, 10 and 100 µM) to test their potential toxic effects on mammalian cells using the MTT method (18). Table 1 shows the results for flavonoid compounds and pentamidine (reference standart drug). The compounds SP3 and pentamidine showed the same deleterious activity to the host cell, as evidenced by the MTT assay, which presented maximum cytotoxicities of 47.9 ± 8.2% and 72.1 ± 4.8%, respectively. However, the flavonoid SP3 has IC_{50} values (concentrations causing 50% inhibition of growth of the macrophages) greater than the maximum concentration tested, but the standard pharmacological pentamidine showed IC_{50} of 68.2 ± 3.8 µM.

Table 1. Determination of the cytotoxicity of flavonoid compounds against macrophages (MTT assay)

Substances	Chemical structure (R=)	IC_{50} (µM) [a]	Maximum Cytotoxicity (%)[b]
Pentamidine		68.2 ± 3.8	72.1 ± 4.8**
crude extract	-	> 100	ND
SP1		> 100	ND
SP2		> 100	ND
SP3		> 100	47.9 ± 8.2*
SP4		> 100	ND
SP5		> 100	ND

[a] Inhibitory Concentration 50 (IC_{50}) calculated by concentration-response curves toxic. [b]Mean ± standard error of the mean maximum cytotoxicity in triplicates of a representative experiment. The values of maximum effect were considered significant when * $p < 0.05$, ** $p < 0.01$ compared to the 0.1% DMSO group; ND: Not determined Maximum Cytotoxicity to cell until the concentration of 100 µM compared to DMSO group.

An initial screening was carried out to evaluate and compare the *in vitro* leishmanicidal profiles of the flavonoid compounds and pentamidine, against the promastigote forms of *L. chagasi* and *L. amazonensis*. The maximum effects and the IC_{50} values (concentrations causing 50% inhibition of growth of the promastigotes) were used as parameters for leishmanicidal activity (Table 2). After 48 h of incubation, the compounds SP1, SP2, SP3, SP4 and the crude extract were highly active against *L. chagasi* promastigotes, presenting effects of 81.2 ± 8.8%, 83.2 ± 5.6%, 98.8 ± 2%, 90.6 ± 2.7 and 81.2 ± 7.2%, respectively. Moreover, the compounds SP2 (IC_{50} value 7.6 ± 1.6 µM), SP3 (IC_{50} value 7.5 ± 1.5 µM), SP4 (IC_{50} value 1.0 ± 0.1 µM) and crude extract (IC_{50} value 3.0 ± 0.4 µM) were as potent as pentamidine (with efficacy 95.8 ± 4.2% and IC_{50} value 4.6 ± 0.4 µM) for *L. chagasi* promastigotes **(Table 2)**.

In addition, the crude exctract and the isolated compounds SP1, SP2, and SP3, exhibited antileishmanial activity against *L. amazonensis* promastigotes with maximum effects of 84.3 ± 5.5%, 82.6 ± 7.6%, 48.8 ± 1.7% and 83.7 ± 2.6%, respectively. The crude extract and compounds SP1 and SP3 exhibited IC_{50} of 63.0 ± 2.3 µM, 44.0 ± 6.0 µM and 54.2 ± 2.2 µM, they were as potent and effective as pentamidine (with efficacy 90.4 ± 5.5% and IC_{50} value 24.3 ± 5.3 µM) for *L. amazonensis* promastigotes. In contrast, the compound SP5 did not present activity against promastigote forms of *L. chagasi* and *L. amazonensis*. Moreover, SP4 did not exhibit activity against promastigote forms of *L. amazonensis* up to 100 µM **(Table 2)**.

Table 2. Leishmanicidal effect of flavonoid compounds against the growth of promastigotes of *L. chagasi* and *L. amazonensis.*

Substances	*L. chagasi* Promastigotes		*L. amazonensis* Promastigotes	
	IC_{50} (µM)	Efficacy (%)	IC_{50} (µM)	Efficacy (%)
Pentamidine	4.6 ± 0.4	95.8 ± 4.2***	24.3 ± 5.3	90.4 ± 5.5***
crude extract	3.0 ± 0.4	81.2 ± 7.2***	63.0 ± 2.3	84.3 ± 5.5***
SP1	49.7 ± 6.6	81.2 ± 8.8***	44.0 ± 6.0	82.6 ± 7.6***
SP2	7.6 ± 1.6	83.2 ± 5.6***	> 100	48.8 ± 1.7**
SP3	7.5 ± 1.5	98.8 ± 2.2***	54.2 ± 2.2	83.7 ± 2.6***
SP4	1.0 ± 0.1	90.6 ± 2.7***	> 100	NA
SP5	> 100	NA	> 100	NA

Data are reported as the mean ± standard error of the mean, S.E.M. Differences with **p < 0.01 and ***p < 0.01 were considered significant in relation to the 0.1% DMSO group. IC_{50} is the concentration required to give 50% inhibition; NA: compound is not active.

The activity of compounds against promastigotes and amastigotes can differ, depending on the targets of antileishmanial action, which may be selective for one of two developmental forms. Overall, promastigotes may be more sensitive than intracellular amastigotes, because amastigotes are adapted to survive in hostile intracellular environment as well as due to the fact the compounds have direct contact with promastigotes; in contrast, to have anti-amastigote activity, substance must be capable of crossing the membrane of the host cell. In this study, crude extract, SP1, and SP3 inhibited amastigote growth, with a maximum efficacy of 85.6± 0.0%, 72.2 ± 13.7%, and 88.8 ± 0.9%, respectively. The crude extract and compounds SP1 and SP3 exhibited IC_{50} of 23.3 ± 4.5 µM, 34.0 ± 9.6 µM, and 10.5 ± 2.5µM. The compounds have similar inhibitory activity against promastigotes and amastigotes.

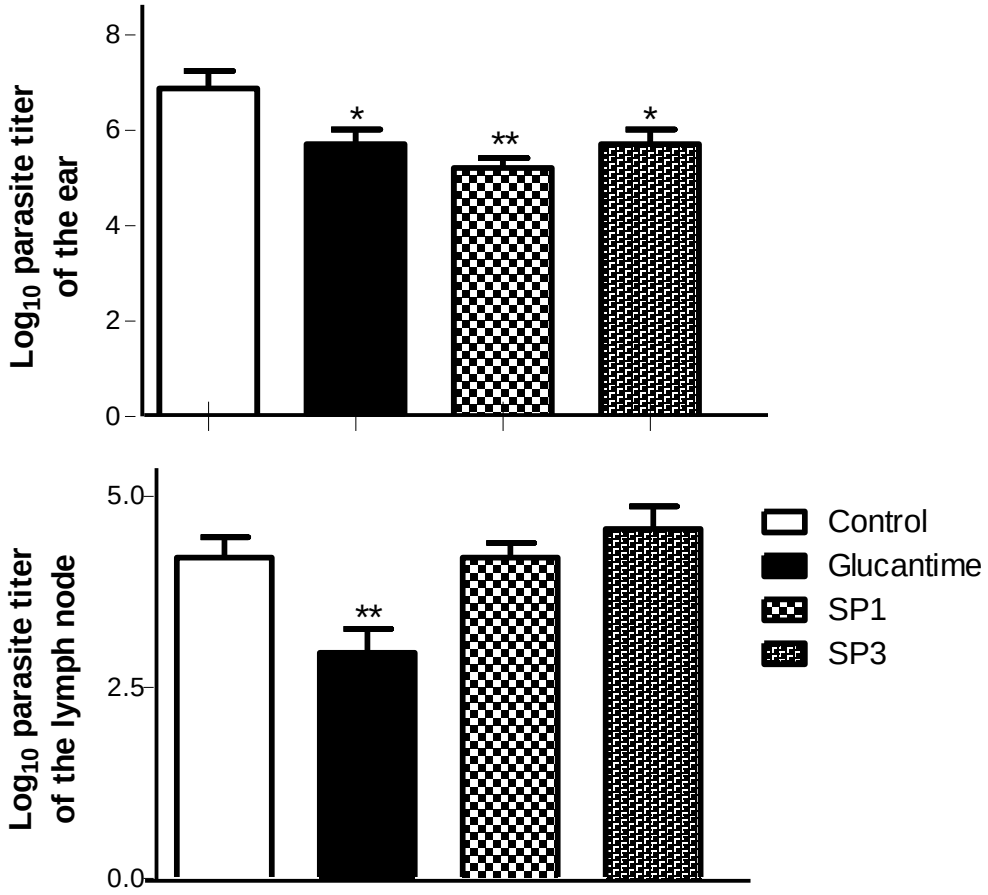
Table 3. Leishmanicidal effect of flavonoid compounds against the growth of amastigotes of *L. amazonensis.*

Substances	*L. amazonensis* amastigotes	
	IC_{50} (µM) [a]	Efficacy (%)[b]
Pentamidine	6.4 ± 0.1	99.91 ± 0.04***
crude extract	23.3 ± 4.5	85.6 ± 0.0**
SP1	34.0 ± 9.6	72.2 ± 13.7***
SP3	10.5 ± 2.5	88.8 ± 0.9***

Data are reported as the mean ± standard error of the mean, S.E.M. [a]IC_{50} is the concentration required to give 50% inhibition. [b]The efficacy values were considered significant when * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.01$ compared to the 0.1% DMSO group.

Considering the *in vitro* results, SP1 and SP3 flavonoids were selected to be evaluated *in vivo* for leishmanicidal activity against *L. amazonensis*. Intraperitoneal treatment with SP1 and SP3 at 100 mg/kg/day x 28 days decreased the parasite load in the infected ear, but it did not decrease the parasite load in the draining lymph node. Glucantime was able to reduce the parasite load on both the infected ear and the draining lymph node **(Figure 1)**. It was used as a standard drug for being the treatment of first choice recommended by the World Health Organization, despite the literature showing that there are already strains resistant to this drug.

Figure 1. Parasite load in the ear and draining lymph node were determined at 4 weeks post-infection via a limiting dilution assay.

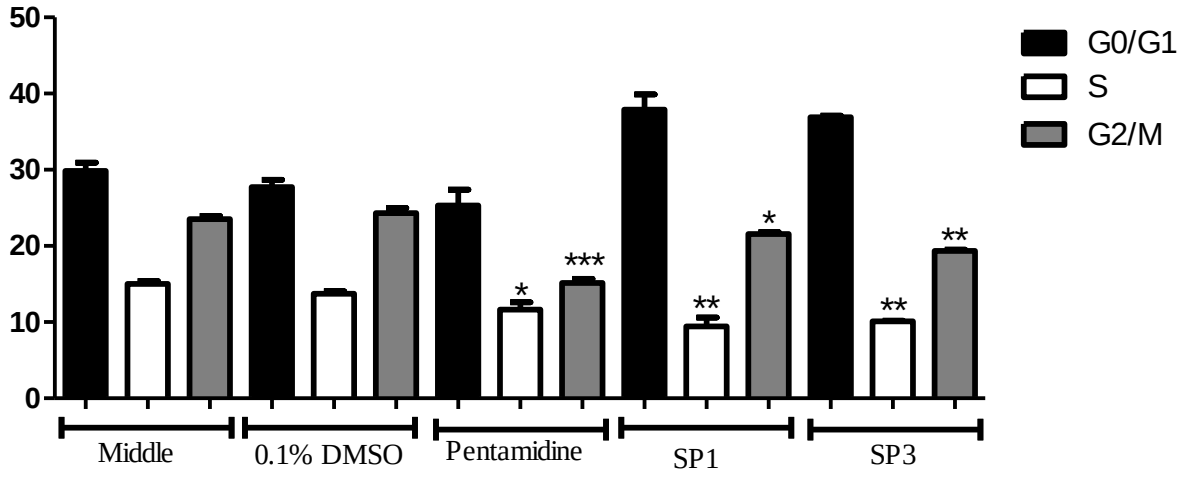


(A) Ear parasite load data (normal distribution following log transformation) represent the mean ± SEM (n = 5) (B) Lymph node parasite load data were not normally distributed, even after log transformation, and are expressed as the median ± SEM (n = 5) (Dunnett's test, **p<0.01, *p<0.05).

Once characterized the leishmanicidal activity *in vitro* and *in vivo* of flavonoids SP1 and SP3, other studies were conducted to investigate the mode of action of these leishmanicidal compounds. *Leishmania* cell cycle was analyzed by flow cytometry after treatment with the compounds SP1, SP3, and crude extract at a concentration of 100 µM for 48 hours **(Figure 2)**. The analysis of the cell population in different cell cycle phases was possible after staining with 7-AAD and subsequent analysis by flow cytometry. In **Figure 2**, it was observed that SP1 and SP3 were able to induce changes in the cell cycle of *L. amazonensis* promastigotes after 48 h

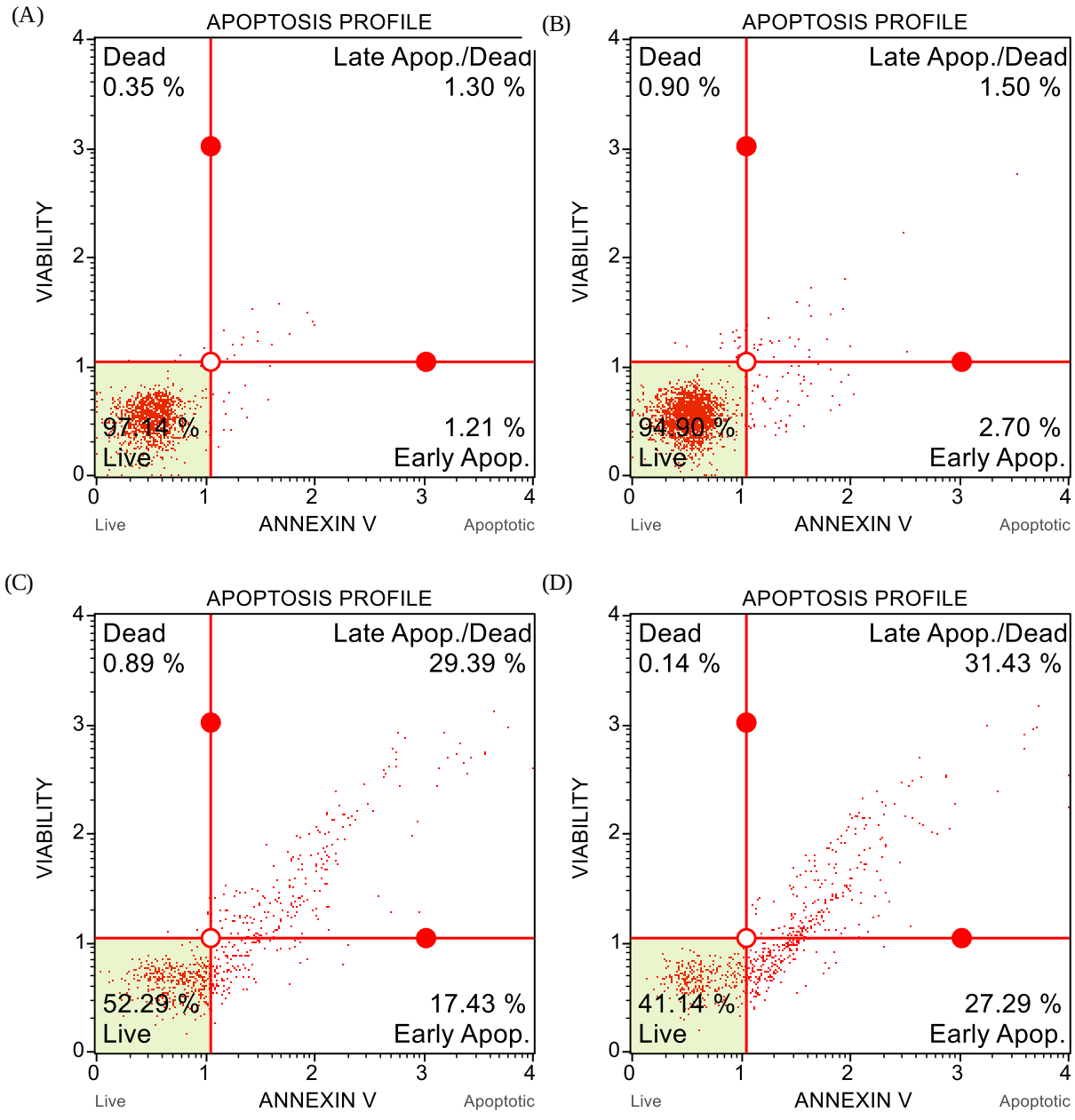
of treatment. SP1 and SP3 at the concentration of 100 µM induced a significant decrease in the proportion of cells in S phase compared with the DMSO control.

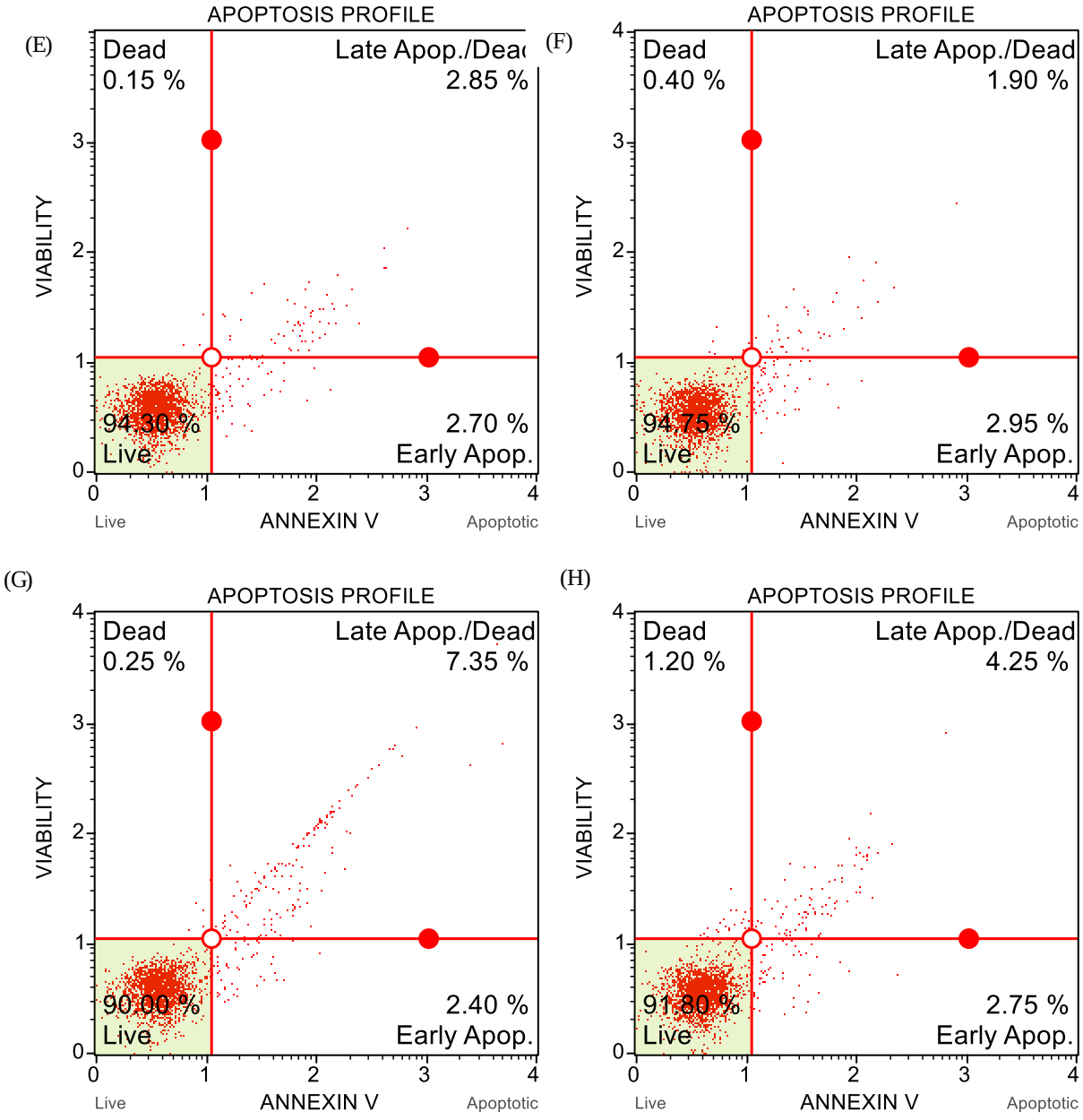
Figure 2. Cell cycle of *L. amazonensis* promastigotes. Analysis using flow cytometry after treatment with pentamidine (10 µM), SP1 and SP3 (100 µM) for 48h. Values represent means ± SEMs for three samples.



In order to determine the manner in which the flavonoids exert their antiproliferative activity flow cytometric analysis using AnnexinV-PE was performed to investigate the externalization of phospholipids. As demonstrated in **Figure 3**, the SP3 flavonoid at the concentration of 100 µM induced cell death through induction of apoptosis in *L. amazonensis* promastigotes after 48 h of incubation. SP3 induced 7.3 % of late apoptosis. Furthermore, it was found that apoptosis triggered by the treatment with SP3 is not dependent on caspase activation, as shown in **Figure 4**. The SP1 flavonoid did not induce cell death through induction of apoptosis in *L. amazonensis* promastigotes after 48 h of incubation at the concentrations of 10 and 100 µM **(Figure 3)**.

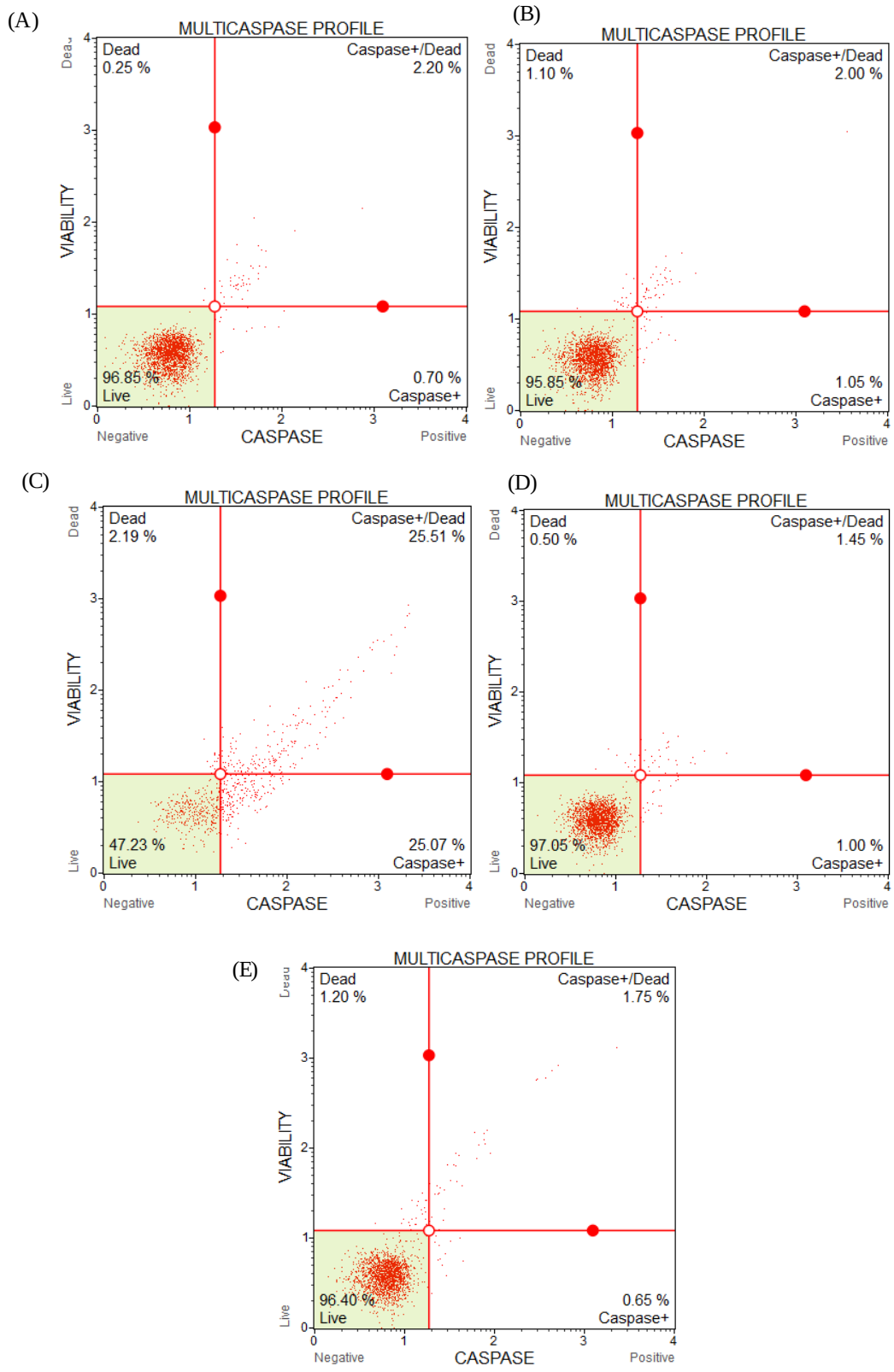
Figure 3. Phospholipid externalization of *L. amazonensis* promastigotes. Analysis using flow cytometry after treatment with pentamidine, SP1 and SP3 (10 and 100 µM) for 48 h.





(A) Middle **(B)** DMSO 0.1% **(C)** Pentamidine 100 µM **(D)** Pentamidine 10 µM **(E)** SP01 100 µM **(F)** SP01 10 µM **(G)** SP03 100 µM **(H)** SP03 10 µM.

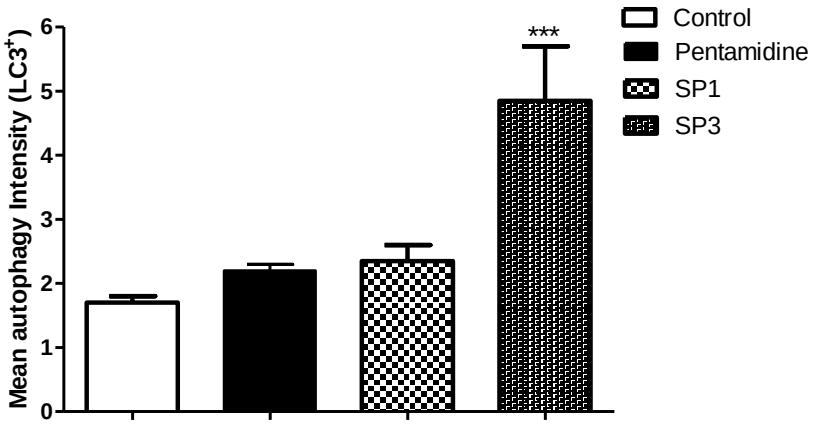
Figure 4. Determination of the presence of active caspase-like proteases in *L. amazonensis* promastigotes. Analysis using flow cytometry after treatment with pentamidine, SP1 and SP3 (100 µM) for 48 h.



(A) Middle **(B)** DMSO 0.1% **(C)** Pentamidine 100 µM **(D)** SP01 100 µM **(E)** SP03 100 µM.

It was evaluated the induction of autophagic cell death in *L. amazonensis* promastigotes treated with SP1and SP3 at a concentration of 100 µM for 48 hours analysis via flow cytometry. Treatment with SP3 altered the mean of autophagy intensity in promastigotes, showing that the antiproliferative activity of these compounds probably also the result of an exacerbated autophagy process **(Figura 5)**.

Figure 5. Determination of the presence of autophagic $LC3^{+}$ in *L. amazonensis* promastigotes incubated with pentamidine, SP1 and SP3 (100 µM) for 48 h and analyzed by flow cytometry. Values represent means ± SEMs for three samples.



4. Discussion

Considering the efficacy of drugs available for the treatment of leishmaniasis as well as their side effects and the resistance developed by parasites, research using natural products, mainly due to the properties of bioactive compounds found in the crude extracts of medicinal plants, may lead to the discovery of new therapy with appropriate efficiency which are cheap and safe for patients (22). Hence, the purpose of this research was to study the antileishmanial effects of flavonoid compounds isolated from the crude extract of *Solanum paladosum* and investigate their mechanism of action.

The Solanaceae family consists of about 3000 species in 106 genus with worldwide distribution (10). It is a family of great economic importance, with different species used for food, such as *Solanum tuberosum* (potato), *Capsicum frutescens* (malageta pepper), *Solanum*

lycopersicum (tomato); and ornamentals, *Brunfelsia* sp., *Petunia* spp.; and medicinal purposes, *Atropa belladonna* and *Mandragora officinailis* (23, 24). The genus *Solanum* is the largest of the Solanaceae family, and it is known for the prevalence of alkaloids in its composition. However, different secondary metabolites with therapeutic and commercial importance are produced by this genus, such as glycoalkaloids and polyphenolic compounds, which possess different pharmacological actions. In addition, flavonoids are frequent substances in the *Solanum* genus (25, 26).

Studies using flavonoids have shown significant leishmanicidal activity. Wong et al. (2014) and Wong et al. (2007) tested flavonoids dimers and found potent leishmanicidal activity. In the study of Ramírez-Macías et al. (2012) it was identified flavonoids which showed inhibitory activity against the growth of *L. infantum* and *L. braziliensis*, with greater selectivity index for these species (27, 28, 29). Another study, which was investigated *in vivo* and *in vitro* leishmanicidal activity of flavonoids compounds, these showed activity in both assays (30), indicating flavonoids are likely to become strong antileishmanial drug candidates.

Previous studies using *Solanum* species have demonstrated various pharmacological activities, including activity against trypanosomatids, such as those causing leishmaniasis and Chagas disease (14, 15). In this study, flavonoids showed pronounced leishmanicidal activity against promastigotes and amastigotes of *L. amazonensis*, data that corroborates with the study of Abreu-Miranda et al. (2013) that tested glycoalkaloids isolated from *S. lycocarpum* against promastigotes of *L. amazonenensis* (15). In another study, compounds isolated from *S. acueastrum* were tested against promastigotes and amastigotes of *L. major* and also showed significant leishmanicidal activity (31).

After the *in vitro* screening, it was performed an *in vivo* assay in which BALB/c mice were infected with *L. amazonensis* and treated with the SP1 and SP3 flavonoids. These compounds were able to reduce the parasitic load in the animal's ear, but did not reduce the number of parasites found in the lymph node. In another study, it was observed a lesion reduction in of BALB/c mice infected with *L. major* and treated with all the *S. acueastrum* compounds, indicating a protective effect against cutaneous disease. These results are similar to those presented in this study since the tested compounds were able to reduce the parasitic load on the site of injury (31).

Although a screening for leishmanicidal activity is important to select promising substances, understanding the mechanism of action by which they inhibit parasite growth or cause parasite death, it is necessary to find specific targets. In the cell cycle assay, SP1 and SP3 significantly decreased the proportion of promastigotes at *S* phase, which is a strong indicative which these compounds have action in DNA replication. Thus, we will continue this study to evaluate the inhibitory effects of theses flavonoids on validated chemotherapeutic targets which act on *S* phase of cycle cell, such as topoisomerase.

Morphological, biochemical or molecular changes lead to parasite death or prevent Parasite proliferation (32). The cell death pathways can be generally classified into apoptosis, necrosis, and exacerbation of autophagic processes (33). To determine the manner in which the flavonoids tested exerts their antiproliferative activity, flow cytometry analyzes were performed using annexin V-PE to investigate the externalization of phospholipids, typical characteristic of apoptosis in *Leishmania* (34). Lipids are essential for the structural and functional integrity of cells. As the predominant constituents of cellular membranes, lipids compartmentalize cellular functions and are involved in various aspects of signal transduction (35). Some changes in lipid distribution generally triggers a physiological event such as the clearance of apoptotic cells or the internalization of viruses by host cells. So the externalization of phospholipids, as phosphatidylserine, may be indicative of apoptotic death. In this study, the SP3 flavonoid at the concentration of 100 µM caused cell death through induction of apoptosis in *L. amazonensis* promastigotes after 48 h of incubation. During apoptosis, the asymmetric lipid in the plasma membrane is likely to be lost, resulting in drastic changes in the phospholipid composition of leaflets (36, 37, 38, 39). Binding of annexin V to the cell surface of *Leishmania* parasites is therefore likely to be a consequence of changes in the plasma membrane lipid arrangement.

Unlike a necrotic cell which presents membrane rupture and release of intracellular content, a cell that undergoes apoptosis is rapidly phagocytosed by tissue cells or phagocytes, such as macrophages. Death by apoptosis can be triggered by various stimuli ranging from stress on intracellular organelles to receptor-mediated signaling. An important characteristic of this process is the activation of several cysteine proteases, known as caspases. A proteolytic cleavage of different cell substrates by caspases determine the main characteristics of the process of cell death by apoptosis (40). The pharmacological manipulation of apoptosis and caspases may have clinical application and is a new frontier in the development of novel approaches to treat human diseases. However, in our study, we observed that SP3 caused death

by apoptosis not dependent on caspases pathway. Thus, we concluded that apoptosis triggered by SP1 and SP3 was a result of other mechanisms.

During the programmed cell death known as autophagy type II, there is premature degradation of organelles and preservation of cytoskeletal elements to later stages (41). In this study, the anti-LC3 Fluor®555 Alexa conjugate was used to evaluate the induction of autophagic cell death in promastigotes treated with SP1 and SP3 at the concentration of 100 µM for 48 hours.

In conclusion, it was demonstrated in this work that this flavonoids showed excellent activity against *Leishmania* and were able to diminish the lesion to the animal's ear in *in vivo* assay. In addition, in evaluating possible mechanisms of action, the flavonoid SP1 and SP3 were able to reduce the *S* phase of the cell cycle, and that the antiproliferative activity of the flavonoid SP3 occurs also due to induction of late apoptosis and autophagy induction. The results support that flavonoids derived from *S. paladosum* can become lead compounds for the design of new prototypes of antileishmanial drugs.

Acknowledgments

The authors would also like to thank the CAPES, CNPq, MCT, FINEP, INCT-INOFAR (573.564/2008-6), CNPQ (479822/2013-1), CNPQ (404344/2012-7), FACEPE and FAPEAL (Pronem 20110722-006-0018-0010) FACEPE-PRONEM (APQ-1232-1.06/10) and CENAPESQ *for providing financial assistance for this research project. Moreover, the authors would like to thank several colleagues working at the UFAL for constructive criticism of and assistance with this project.*

Conflicts of Interest

The authors declare no conflict of interest.

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8 ARTIGO IV DERIVADO DA TESE

Artigo 4: Em submissão

3,7,3',4'-tetra-O-methylquercetin (retusin): Leishmanicidal evaluation and investigation of the proposed mechanism of action

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Abstract: Leishmaniasis is among the world's most neglected diseases. Currently available drugs for treatment present drawbacks, urging the need for more effective, safer, and cheaper drugs. This study evaluated the leishmanicidal activity and propose the mechanism of action of flavonoid 3,7,3',4'-tetra-O-methylquercetin (retusin). *In vitro* assays against promastigotes and amastigotes of *L. chagasi* and *L. amazonensis* the retusin compound showed significant activity. In the *in vivo* assay in hamsters infected with *L. chagasi,* retusin decreased the parasite load of the spleen of infected animals and retusin treated. However, the *in vivo* assay with mice Balb /c infected with *L. amazonensis* was not able to reduce the parasite load in the ear and in the lymph node draining. In addition, the retusin induced death for apoptosis at the concentration of 100 µM, probably not dependent on caspases. Retusin was able to inhibit relaxation activity of LcTopI at concentrations of 100 up 3.125 µM (IC_{50} = 2.23±0.8). These results indicate retusin compound can become lead molecule for designing new prototypes antileishmanial.

Keywords: Leishmaniasis. *L. chagasi. L. amazonensis.* Retusin.

1. Introduction

Leishmaniasis is an infectious disease caused by parasites of the genus *Leishmania* in the family Trypanosomatidae. In humans, the clinical forms of leishmaniases are broadly categorized into cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL) (1). CL is a group of diseases with a varied spectrum of clinical manifestations, which range from small cutaneous nodules to mucosal tissue destruction. Conversely, VL is the most severe and debilitating form of the disease, characterized by prolonged fever, splenomegaly, hypergammaglobulinemia, and pancytopenia eventually resulting in death if not treated (2).

Leishmania occurs in five continents and is endemic in 98 countries, with an estimated 350 million people at risk of contracting leishmaniasis. Approximately 58,000 cases of visceral leishmaniasis and 220,000 cutaneous cases are officially reported each year (1, 3).

The evidence base for neglected tropical diseases (NTDs) is acknowledged to be particularly problematic. Leishmaniasis, like many other NTDs, occurs in a focal distribution and in remote locations, making extrapolation from official data sources difficult. Furthermore, the available treatment is limited to a small number of drugs, and in many cases have not responded to the clinical need of the patient (4, 5).

For many years, pentavalent antimonials have been the recommended drugs for VL and CL. They have variable efficacy against VL and CL, and require injectable administration. Due to side effects such as high cardiotoxicity (6), pancreatitis (7, 8), and nephrotoxicity (9), patients should be hospitalized and monitored, such as the treatment may need to be suspended. Despite the toxicity and several cases of parasite resistance, antimonials remain the first-line treatment.

Due to the increasing resistance to antimonials, amphotericin B deoxycholate is used as an alternative drug for VL; this drug, however, is highly toxic and require careful and slow IV administration. Lipid formulations of amphotericin B have reduced toxicity, a better half-life, and a high level of efficacy. The main limitations are its high cost, administration route, and lack of stability at high temperature (11).

Another drug used as second choice is pentamidine that has high toxicity combined with decreased efficacy in patient treatment, which suggests resistance (12). However, it is still valuable for combined therapies. Paromomycin is an aminoglycoside antibiotic with antileishmanial activity, but it is more used in topical treatment for CL and associated with other drugs for VL, as miltefosine (13). Miltefosine is the most recent antileishmanial drug on the market and the first effective oral treatment against VL, being recommended as first-line drug for childhood VL. Although its toxicity is not very high, its teratogenicity is a problem (14).

Thus, the development of new drugs is necessary and naturals products are an excellent proposed, such as flavonoids, which it has been cited with leishmanicidal activity. Therefore, the present study aims to investigate the leishmanicidal activity and propose the mechanism of action of flavonoid 3,7,3',4'-tetra-O-methylquercetin (retusin).

2. Experimental Section

Parasites. Two strains of *Leishmania* were used in the present study: *Leishmania amazonensis* [MHOM/BR/77/LTB0016] and *Leishmania chagasi* [MCAN/BR/89/BA262]. They were

maintained *in vitro* as proliferating promastigotes at 26°C in Schneider's medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum, gentamycin (1 mg/L), L-glutamine (2 mM), and 2% sterile human urine.

Animals. Swiss mice (six to eight weeks old) were obtained from the Central Animal House of the Federal University of Alagoas in order to obtain peritoneal macrophages. BALB/c mice (six to eight weeks old) were obtained from breeding stocks maintained at the Multidisciplinary Center for Biological Investigation on Laboratory Animal Science (UNICAMP) and Golden Syrian hamsters (*Mesocricetus auratus*) were obtained from Oswaldo Cruz Foundation (Rio de Janeiro, Brazil) to *in vivo* assays. These studies were approved by the Ethics Committee for Animal Experimentation of the Federal University of Alagoas (Brazil) (protocol no. 2015.01, protocol no. 2013.02 and protocol no. 2014.56, respectively).

Macrophages. Peritoneal macrophages were obtained from swiss mice (weighing 20–25 g) through peritoneal lavage with 10 mL of cold PBS (Phosphate-Buffered Saline; 137 mM NaCl, 8 mM Na2HPO4, 2.7 mM KCl, 1.5 mM KH2PO4, pH 7.0). The peritoneal exudate cells were centrifuged at 1500 rpm for 5 min and the pellet was resuspended in culture medium. Cells were counted and plated into 96-well plates for the cytotoxicity assay and in 48-well plates for the intracellular amastigote assay.

Cytotoxicity assay in macrophages. Peritoneal macrophages were seeded ($3x10^5$ cells/well) in 96-well plates with 100 mL of media. Cells were allowed to attach to the bottom of the well for 24 h at 37 °C and then treated with 1, 10, and 100 µM of retusin and pentamidine previously diluted in RPMI-1640 medium with dimethyl sulfoxide (DMSO). The plates were maintained in 5% CO2 incubator at 37 °C, for 48 h. Cells were also cultured in media free from compounds, a vehicle (basal growth control), or media with 0.1% DMSO (vehicle control). Thereafter, the supernatant was removed and cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (100 mL/well) for 1 h in the dark at 37 °C. The MTT solution was removed, cells were resuspended in 100 mL of 0.1% DMSO, and the absorbance was measured using an ELISA reader at 550 nm (15).

Leishmanicidal Assay. *L. amazonensis* and *L. chagasi* promastigotes (2×10^6 cells/mL) were maintained *in vitro* in Schneider's medium supplemented with 10% FBS and 2% human urine in the presence of various concentrations of retusin and pentamidine (0.1, 1, 10 and 100 µM) for 48 h at 26 °C. Cells were also cultured in a medium free of compounds, a vehicle (basal growth control) or with 0.1% DMSO (vehicle control). Promastigotes were adjusted to a concentration of 10^2 in PBS and viable parasites counted in a Neubauer's chamber (16).

Intracellular Amastigote Assay. Peritoneal cavity macrophages of swiss mices were seeded on glass coverslips (13 mm diameter) in 48-well plates and incubated at 37◦C with 5%CO2 for 12 hours for adhesion. Macrophages were infected with *L. amazonensis* and *L. chagasi* promastigotes using a ratio of 1:10 at 37 °C overnight in isolated experiments. Non-internalized promastigotes were removed by washing (three times) with PBS. Infected macrophages were treated with different concentrations of retusin (0.1, 1 and 100µM) and maintained at 37 °C in 5% CO_2 for 48 h. The glass coverslips were fixed with methanol, stained with May-Grünwald-Giemsa, and intracellular amastigotes were counted (one hundred macrophages were evaluated per glass coverslip). Data was expressed as infection index (percentage of infected macrophages multiplied by the average number of amastigotes per macrophage) (17).

Experimental studies with *L. amazonensis*-infected BALB/c mice. Female BALB/c mice, 6 to 8 weeks old, were purchased from the Multidisciplinary Center for Biological Investigation on Laboratory Animal Science (UNICAMP, Campinas-SP, Brazil) and maintained in a regulated animal care facility. A murine model closely resembling human pathology was previously described. Briefly, the right ear dermis of BALB/c mice was inoculated with stationary-phase promastigotes of *L. amazonensis* with 10^5 parasites in 10 mL of sterile saline, using a 27.5-gauge needle. After the second week postinfection, a group of mice (n=5) was treated intraperitoneally with retusin or meglumine antimoniate (positive control) at 100 µmol/kg/day, diluted in water for administration. A negative control group was treated orally with water by injection (n=5). Lymph nodes and ears of BALB/c mice were collected after euthanasia and macerated with Schneider's medium (18). Supernatants were grown in 48-well plates and parasite burden was estimated by a limiting dilution assay (19).Animal blood was also collected and centrifuged in order to obtain serum to be used in cytokine assays.

Experimental studies with *L.* (L.) *infantum chagasi*-infected hamsters. Young male Golden Syrian hamsters (*Mesocricetus auratus*) (88–145 g) were infected intraperitoneally (i.p.) with *L. chagasi* promastigotes (10^7/animal). 45 days post-infection, hamsters were treated for 15 consecutive days with retusin at a concentration of 100 µmol/kg/day (n = 5/group) or pentavalent antimonial (Glucantime) at a concentration of 100 µmol/kg/day (n = 5/group). Animals were euthanized (by overdose anesthesia) on day 60 post-infection (20, 21). A tissue sample of the spleen and liver was removed, weighed and used for analysis of parasite load. Parasite burden was estimated by a limiting dilution assay. The results were expressed as log10 values of the number of parasites per gram of tissue (19).

Biochemical parameters. To spectrophotometric evaluation of the biochemical parameters, the standard diagnostic kits Labtest® were used. The assays were performed according to the protocol provided in the kit. Blood samples were obtained by centrifugation (1500 rpm x 10 min) of whole blood without anticoagulant and frozen. The following biochemical parameters were analyzed: alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea, and creatinine.

Scanning electron microscopy (SEM): These studies were processed in the pharmacology and immunity laboratory (UFAL) and blades were be read in LIKA-Fiocruz-PE. For morphological analysis *L. chagasi* promastigotes incubated at 100 µM of the flavonoid retusin were processed for 48 hours. After 48 h of treatment, cells were washed and fixed for 72 hours in a solution containing 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) and made to adhere to glass coverslips precoated with poly-L-lysine. After 20 min, the coverslips were washed with 0.1M cacodylate buffer to remove non-adherent cells and post-fixed for one hour in a solution containing 1% osmium tetroxide (OsO4), 0.8% potassium ferricyanide, and 5 mM CaCl2 in 0.1M cacodylate buffer. Then cells were washed in 0.1M sodium cacodylate buffer, dehydrated in increasing ethanol series and subjected to drying through the critical point dryer HCP-2 (Hitachi), metallized with 20 nm of gold in the sputter JFC -1100 (Jeol) and visualized by the scanning electron microscope JEOL T-200 (22).

Analysis of phospholipids externalization. Double staining with annexin V-PE and 7-AAD was performed to measure the effects of pentamidine or retusin (100 µM and 10 µM) on the plasma membrane of *Leishmania* promastigote cells. The expression of phospholipidsin the outer membrane of treated and untreated *L. amazonensis* promastigote cells was monitored by labeling with annexin V-PE, and staining with 7-AAD was used to measure the permeability of the plasma membrane. *L. amazonensis* promastigotes were grown to $1x10^5$ cells/ml and then treated with pentamidine or retusin (100 µM and 10 µM) and incubated at 26°C. After 48h, 1 mL of the cell suspension was pelleted and resuspended in 1mL of PBS buffer supplemented with 1% FBS and annexin V-PE and 7-AAD were added. Cells were incubated for 30 min in the dark prior to analysis by flow cytometry on Muse® Cell Analyzer and analyzedusing the Muse® 1400 Analysis software.

Determination of Caspase-like proteases. To determine the percentage of caspase-positive cells, we used the Muse® Multicaspase kit, performed according to the manufacturer's instructions. *L. amazonensis* promastigotes were grown to $1x10^5$ cells/ml and then treated with pentamidine or retusin (100 µM and 10 µM) and incubated at 26 ˚C. After 48h, 1 mL of the cell

suspension was pelleted and resuspended in 1mL of PBS buffer supplemented with 1% FBS. MuseR Multicaspase Reagent and subsequently 7-AAD were added. Data acquisition was carried out using a Muse® Cell Analyzer and analyzed using the Muse® 1400 Analysis software.

Determination of the presence of autophagic LC3^{+}. To determine the percentage of caspase-positive cells, we used the MuseT Autophagy LC3-antibody based kit, performed according to the manufacturer's instructions. *L. amazonensis* promastigotes were grown to 1x10^{5} cells/ml and then treated with pentamidine or retusin (100 µM) and incubated at 26°C. After 48h, 1 mL of the cell suspension was pelleted and resuspended in 1mL of PBS buffer supplemented with 1% FBS. Cells were permeabilized and subsequently anti-LC3 Alexa Fluor® 555 conjugated antibody was added. Data acquisition was carried out using a Muse® Cell Analyzer and analyzed using the Muse® 1400 Analysis software.

Analysis of the cell cycle progression. Promastigotes of *L. amazonensis* (1x10^{5} cells) were treated with retusin 100 µM for 48. Cells were then fixed in chilled 70% ethanol for 3h. After the cells were washed with PBS, the resultant pellet was resuspended in 7-AAD and incubated in the dark for 30 min. Data acquisition was carried out using a Muse® Cell Analyzer and analyzed using the Muse® 1400 Analysis software.

Preparation of nuclear extracts of *Leishmania chagasi* promastigotes containing topoisomerase I (LcTopI). Nuclear extracts of were prepared from 1-5x10^{9} cells/ml 3-day *Leishmania* promastigotes by a modification of previous method of Jean-Moreno and collaborators (2006). Briefly, promastigotes were pelleted by centrifugation (1.500g; 5 min; 4°C), and resuspended in Hepes buffer (Hepes-KOH, 10 Mm, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol [DTT], 0.2 mM PMSF, 0.2 X PIC). After centrifugation, as above, promastigotes were resuspended in 800 µl HEPES buffer and incubated in ice for 10 min, and then lysed by 1 cycle of sonication of 1 min (power output = 100%). Nuclei were pelleted by centrifugation (16.000g; 5 min; 4°C), and post nuclear supernatant fraction was removed. The nuclei pellet was resuspended in 100 µl of TOPI extraction buffer (Tris-HCl, 12.5 mM. pH 7.5, 10 mM MgCl2, 50 mM KCl, 30 µg ml^{-1} bovine serum albumin (BSA), 5% glycerol, 0.5 mM DTT, 0.2 mM PMSF and PIC at the standard concentration) and lysate on ice by repeated passage through a 25-gauge needle using a Hamilton syringe. After centrifugation of the nuclear lysate (16.000g; 30 min; 4°C), the supernatant fraction was recovered and mixed with a one-third volume TOPI extraction buffer containing 18% (wt/v) PEG and 18% (v/v) glycerol. The lysate was incubated on ice for 30 min and centrifuged (16.000g; 30min; 4°C). The clear supernatant fraction (nuclear extract) containing LcTopI was used in TOPI activity assay (23).

TopI activity assay. The activity assay ATP-independent relaxation of supercoiled DNA, a reaction catalyzed exclusively by TopI, was measured with modifications as described Walker and Saraiva (2004). Was prepared an standard reaction mixture for *L. chagasi* nuclear TopI (LcTopI), containing a final volume of 20 µL: 25 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 105 mM/ml BSA, 0.5 mM DTT, 5% glycerol and 0.5 µg supercoiled pBR322 plasmid DNA and 1-5 µL of undiluted nuclear extract. This reactions was incubated at 37 °C for 30 min, following addition of 4 µl stop buffer-gel loading (25% SDS, 25% Bromphenol Blue–Xylene, 40% sacarosa, 1% SDS). The production of relaxation intermediates by LcTopI were analyzed by electrophoresis at 25 V in 16 h run, on 2% agarose gels in TBE buffer (90 mM Tris-borate, 2 mM EDTA) and then stained with 10 µl EtBr. EtBr-stained gels were photographed under UV illumination using Vilber Lourmat ECX 20.M Transsidelight with Coupled camera the darkroom Vilber Lourmat E1/2 C8-48 nm. One unit of TopI activity is defined as the amount of enzyme required to catalyzed 50% relaxation of supercoiled plasmid DNA in 30 min at 37 °C (24).

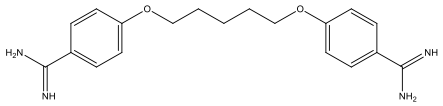
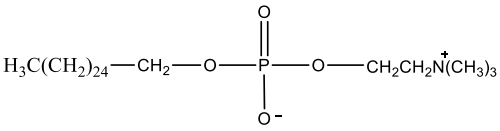
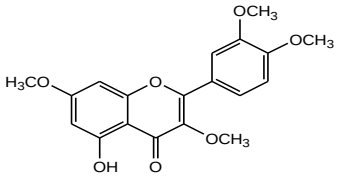
TOPI inhibition assays (IC_{50} assays). LcTopI activities were measured at a minimum of eight different concentrations of retusin relative to uninhibited control. The concentration of retusin required to cause 50% inhibition of TopI activity in the standard assays (IC_{50}) was determined by analysis of supercoiled substrate and relaxed DNA product bands on digital gel images using Gel Analyzer 2010® software from the percent relaxation of supercoiled DNA substrate and GraphPrisma 6.0® software. 2 µL of each concentration of retusin was pre-incubated with 5µL of LcTopI for 15 min in ice, then was added 0.5 µg supercoiled pBR322 plasmid DNA and 13 µL of mixing 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 105 mM/ml BSA, 0.5 mM DTT, 5% glycerol and incubated in 30 min at 37 °C. 4 µl stop buffer-gel loading (25% SDS, 25% Bromphenol Blue–Xylene, 40% sacarosa, 1% SDS) was added to stop the reaction.

Statistical analysis. Data were expressed as the mean ± S.E.M. and significant differences between the treated and control groups were evaluated using ANOVA and Dunnett *post-hoc* tests by Graph Pad Prism 5.0 software, and the 95% confidence intervals were included.

3. Results

Murine peritoneal macrophages were treated with retusin and pentamidine (1, 10 and 100 µM) to test the potential toxic effect of these substances on mammalian cells using the MTT method (15). Pentamidine showed deleterious activity to the host cell, as evidenced by the MTT assay, which presented maximum cytotoxicity of 72.1 ± 4.8% and IC_{50} of 68.2 ± 3.8 µM. However, retusin and mitefosine didn't show deleterious activity to the host cell, thus becoming a promising substance for studies (Table 1).

Table 1. Determination of the cytotoxicity of pentamidine, miltefosine and retusin against macrophages (MTT assay)

Substances	Chemical structure (R=)	IC_{50}(µM) [a]	Maximum Cytotoxicity (%)[b]
Pentamidine		68.2 ± 3.8	72.1 ± 4.8**
Miltefosine		> 100	ND
Retusin		> 100	ND

[a]Inhibitory concentration 50 (IC_{50}) calculated by concentration-response curves toxic. [b]Mean ± standard error of the mean maximum cytotoxicity in triplicates of a representative experiment. The values of maximum effect were considered significant when * $p < 0.05$, ** $p < 0.01$ compared to the 0.1% DMSO group; ND: Not determined Maximum Cytotoxicityt o cell until the concentration of 100 µM compared to DMSO group.

An initial screening was carried out to evaluate and compare the *in vitro* leishmanicidal profiles of retusin and pentamidine against the promastigote forms of *L. chagasi* and *L. amazonensis* (Table 2 and fugure 1). The maximum effects and the IC_{50} values (concentrations causing 50% inhibition of promastigote growth) were used as the parameters for leishmanicidal activity. After 48 h of incubation, retusin was active against *L. chagasi* promastigotes, presenting maximum efficacy of 85.5 ± 5.0% and IC_{50} of 65.8 ± 2.9 µM. Moreover, pentamidine and mitefosine were also actives against *L. chagasi* promastigotes, with efficacy of 97.1 ± 5.0% (IC_{50} value of 4.0 ± 0.4 µM), and 96.7 ± 0.4% (IC_{50} value of 21.5 ± 1.3 µM), respectively. In addition, retusin, miltefosine and pentamidine exhibited antileishmanial activity against *L. amazonensis* promastigotes with the maximum effects of 82.6 ± 9.9% (IC_{50} = 69.7 ± 5.5 µM), 95.7 ± 1.8 (IC_{50} = 3.3 ± 0.2 µM), and 97.4 ± 4.4% (IC_{50} = 5.7 ± 1.6 µM), respectively (Table 2).

The results of the evaluation of leishmanicidal activity against intracellular forms of *L. chagasi* revealed that retusin induced growth inhibition of 65.8 ± 1.9% and IC_{50} of 5.6 ± 1.3 µM, miltefosine induced growth inhibition of 85.1 ± 0.6% and IC_{50}= 76.7 ± 2.9 µM, and pentamidine induced growth inhibition of 66.4 ± 3.7%, and IC_{50}= 65.1 ± 4.9 µM. Concerning inhibiting the growth of amastigotes *L.amazonensis,* retusin induced growth inhibition of 38.1 ± 8.0% against *L. amazonensis* amastigotes at 100 µM (Table 3).

Table 2. Leishmanicidal effect of pentamidine, miltefosine and retusin against the growth of promastigotes of *L. chagasi* and *L. amazonensis.*

Substances	*L. chagasi* Promastigotes		*L. amazonensis* Promastigotes	
	IC_{50} (µM)	Efficacy (%)	IC_{50} (µM)	Efficacy (%)
Pentamidine	4.0 ± 0.4	97.1 ± 5.0***	5.7 ± 1.6	97.4 ± 4.4***
Miltefosine	21.5 ± 1.3	96.7 ± 0.4***	3.3 ± 0.2	95.7 ± 1.8***
Retusin	65.8 ± 2.9	85.5 ± 5.0***	69.7 ± 5.5	82.6 ± 9.9***

Data are reported as the mean ± standard error of the mean, S.E.M. Differences with**p <0.01 and ***p <0.001 were considered significant in relation to the 0.1% DMSO group. IC_{50} is the concentration required to give 50% inhibition.

Table 3. Leishmanicidal effect of pentamidine, miltefosine and retusin against the growth of amastigotes of *L. amazonensis.*

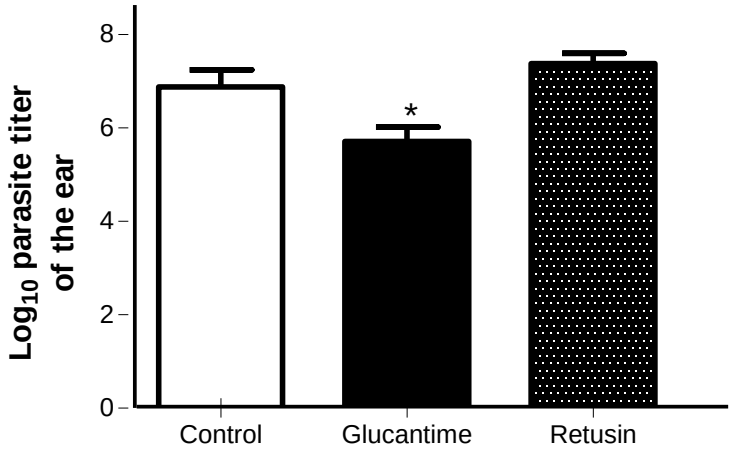
Substances	*L. chagasi* amastigotes		*L. amazonensis* P amastigotes	
	IC_{50} (µM)	Efficacy (%)	IC_{50} (µM)	Efficacy (%)
Pentamidine	65.1 ± 4.9	66.4 ± 3.7***	6.4 ± 0.1	99.91 ± 0.04***
Miltefosine	76.7 ± 2.9	85.1 ± 0.6***	22.0 ± 1.8	59.1 ± 5.6**
Retusin	5.6 ± 1.3	65.8 ± 1.9***	>100	38.1 ± 8.0*

Data are reported as the mean ± standard error of the mean, S.E.M. The values of efficacy were considered significant when * $p < 0.05$, ** $p < 0.01$, and*** $p < 0.01$ compared to the 0.1% DMSO group. IC_{50} is the concentration required to give 50% inhibition; NA: compound is not active.

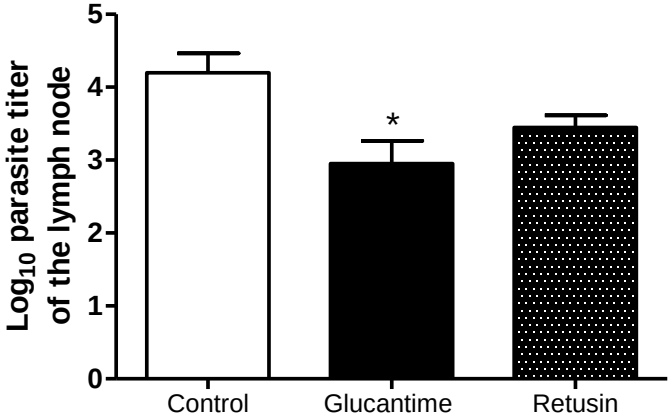
Once the *screening in vitro* of the leishmanicidal activity of retusin and pentamidine was performed, *in vivo* infection assays were conducted with *L. amazonensis* promastigotes in BALB/c mice and with *L.chagasi* promastigotes in hamsters. In the *in vivo* assay for leishmanicidal activity against *L. amazonensis*, the intraperitoneal treatment with retusin at 100 µmol/kg/day x 28 days did not decrease the parasite load in the infected ear, and draining lymph node. This result indicates that either the dose 100 µmol/kg/day was not sufficient to control the infection (Figure 1). Glucantime was able to reduce the parasite load on both the infected ear and draining lymph node (Figure 1). It was used as a standard drug for being the treatment of first choice recommended by the World Health Organization, despite literature showing that there are already strains resistant to this drug.

Figure 1. Parasite load in the ear and draining lymph node were determined at 4 weeks post-infection via a limiting dilution assay.

(A)



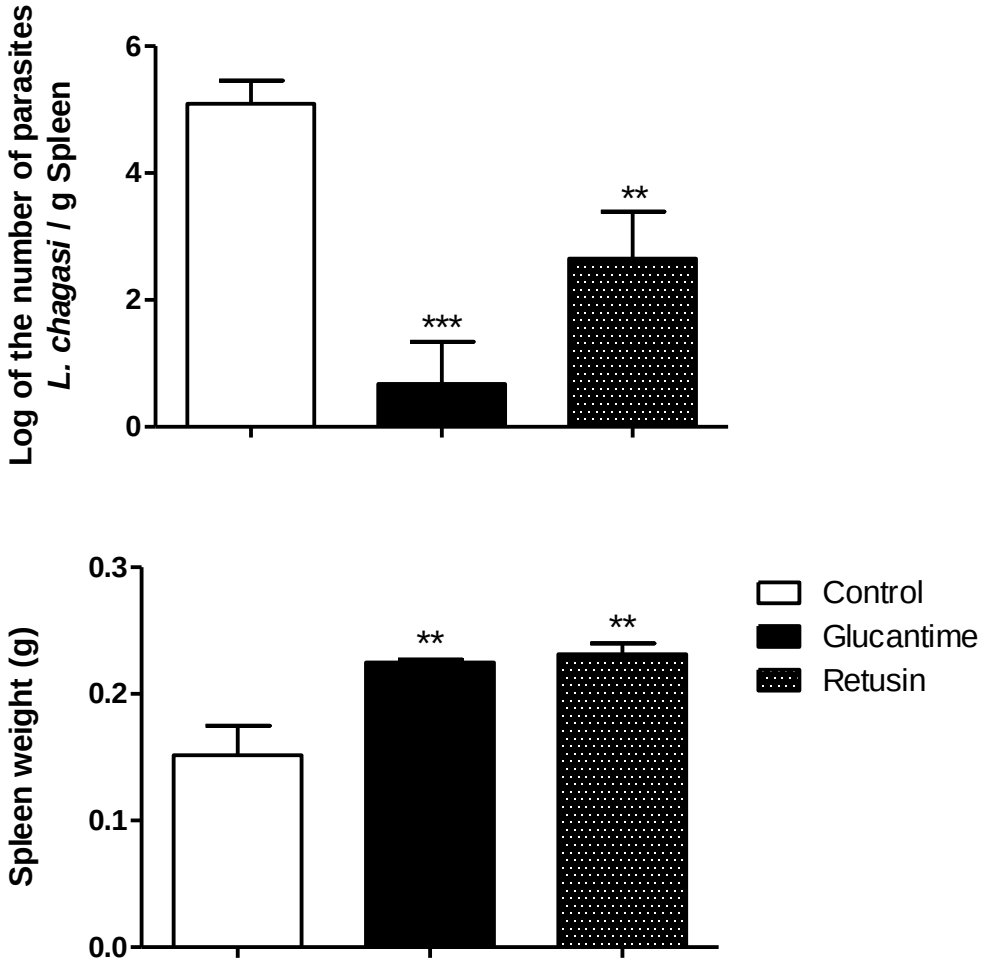
(B)



(A) Ear parasite load data (normal distribution following log transformation) represent the mean ± SEM (n = 5). (B) Lymph node parasite load data were not normally distributed, even after log transformation, and are expressed as the median ± SEM (n = 5) (Dunnett's test, **p<0.01, *p<0.05).

In the *in vivo* model with *L. chagasi* using Golden Hamsters, which is a gold standard test to assess visceral leishmaniasis, it was possible to quantify the parasite load found in the spleen, as well as its weight. The flavonoid retusin at a dose of 100 µmol/kg/day x 15 days was able to reduce the *L. chagasi* parasitic load in the animal's spleen. Furthermore, it also increased the animals' spleen weight in a statistically significant manner after 15 days of treatment. (Figure 2).

Figure 2. Parasite burden at the spleen and spleen weight with intraperitoneal treatment using glucantime, and retusin (100 µmol/kg/day x 15 days).



It was analyzed the levels of AST, ALT, creatinine, and urea in the serum of infected animals treated with retusin and glucantime. The retusin flavonoid decreased serum ALT levels compared to infected control, as well as standard drug Glucantime, but did not alter the serum levels of AST. However, no statistically difference was observed compared to uninfected group. These enzymes are used as biomarkers of liver injury and can be altered by changes in liver metabolism caused for example by drug therapies or VL. Thus, it is suggested that retusin treatment did not cause damage to the liver during the study period (Figure 3). Furthermore, there was no change in creatinine and urea levels in treated with retusin and glucantime compared with infected group, leading us to suggest that the treatment did not cause kidney damage (Figure 4).

Figure 3. Dosages of alanine aminotransferase (ALT), and aspartate aminotransferase (AST) in the animals' plasma.

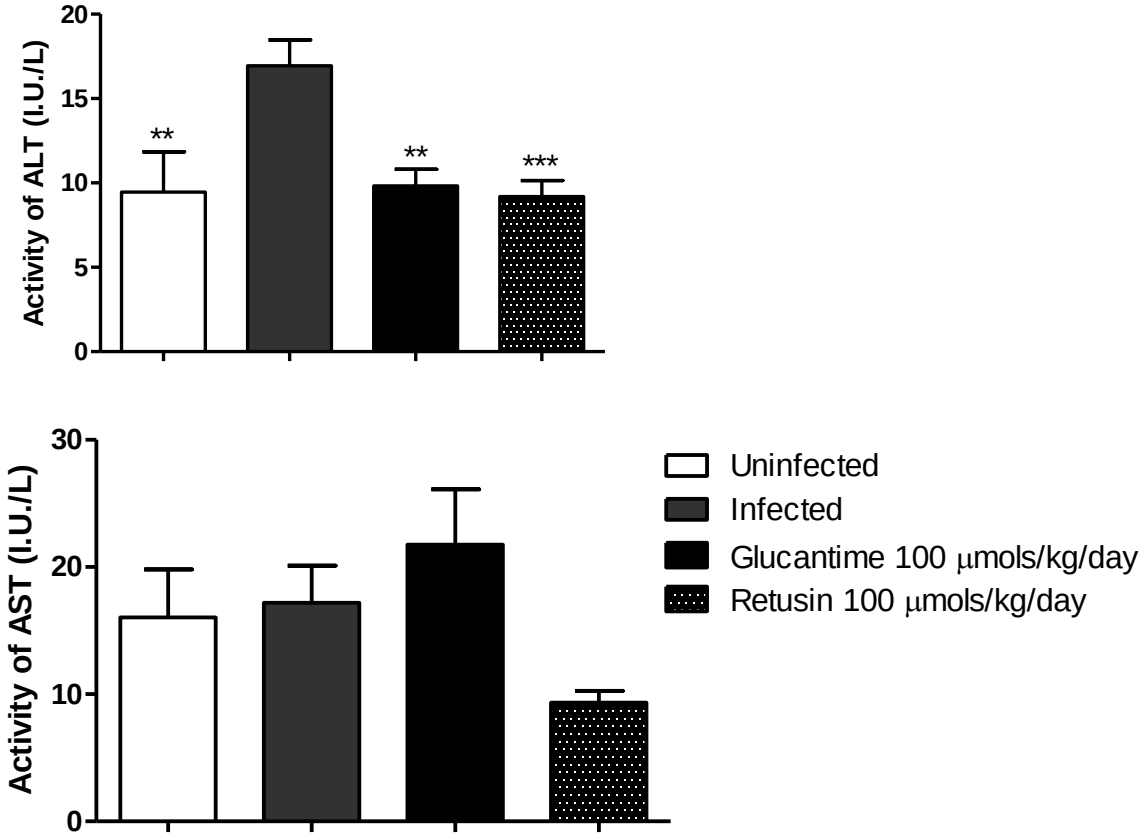
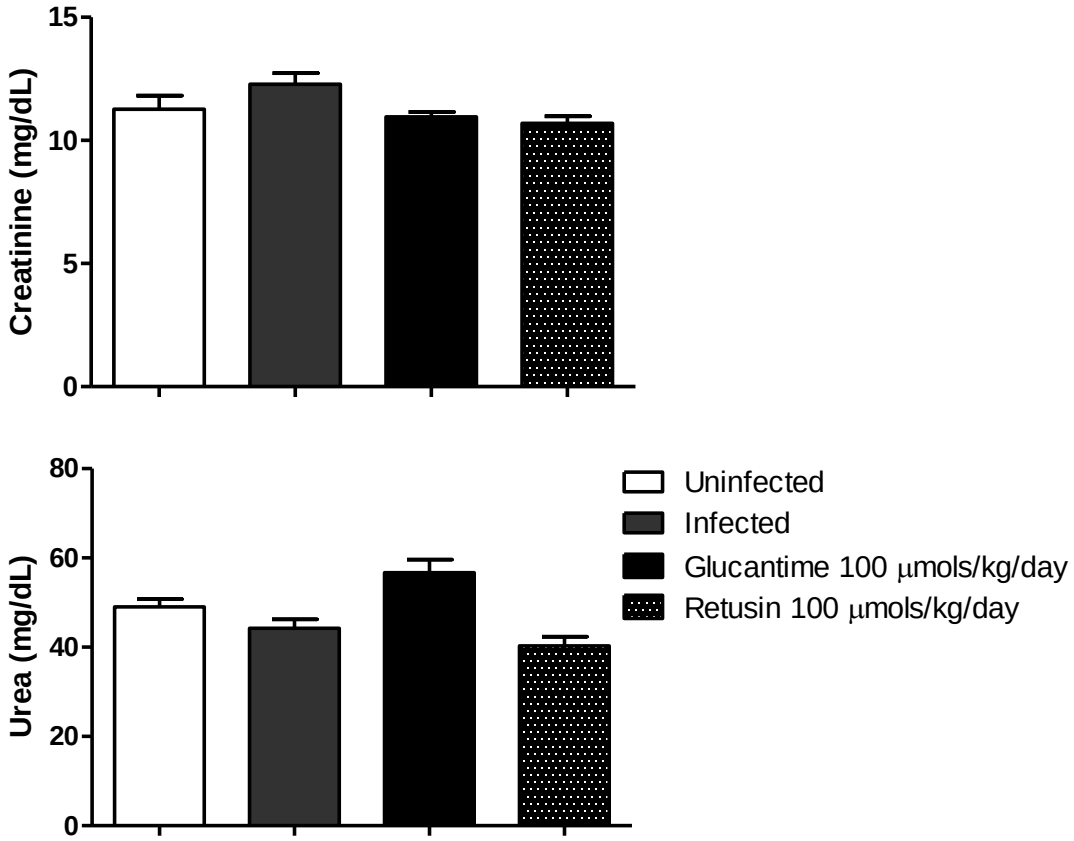
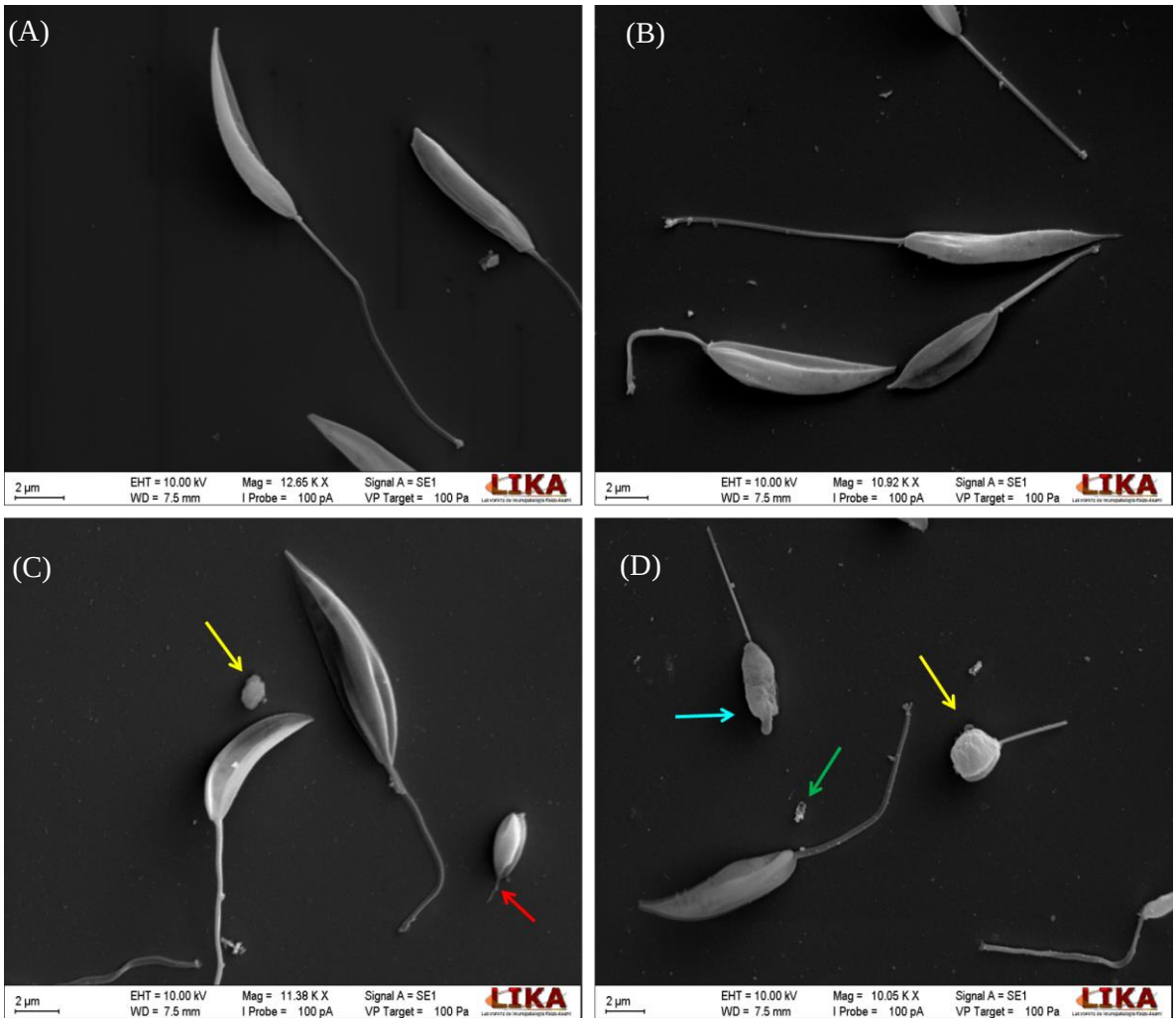


Figure 4. Dosages of creatinine (CREA), and urea in the animals' plasma.



Then, the effects of retusin on the ultrastructural morphology of *L. chagasi* promastigotes were analyzed using scanning electron microscopy (SEM). This is a clinically important species because it is responsible for the most severe form of the disease. Control cells analyses revealed healthy promastigotes with the typical elongated shape, stable cell surface and long flagellum [Figure 5 (A- middle, B- 0.1% DMSO)]. Cells treated with Mitefosine [Figure 5 (c)] and retusin [Figure 5 (d)] exhibited different degrees of morphological alterations. Most of promastigotes presented a thin and tapered appearance, flagellum duplication, membrane folds, multi-septated or twisted body cell and atypical cell division. The main changes observed with the retusin treatment were shrinkage and overall rounding up with significant loss in body length and shortening of flagella compared to untreated cells [Figure 5 (A-B)]. These ultrastructural changes seen in the parasite promastigotes gives an indication of toxic effect, when compared to middle (Schneider's) and 0.1% DMSO controls.

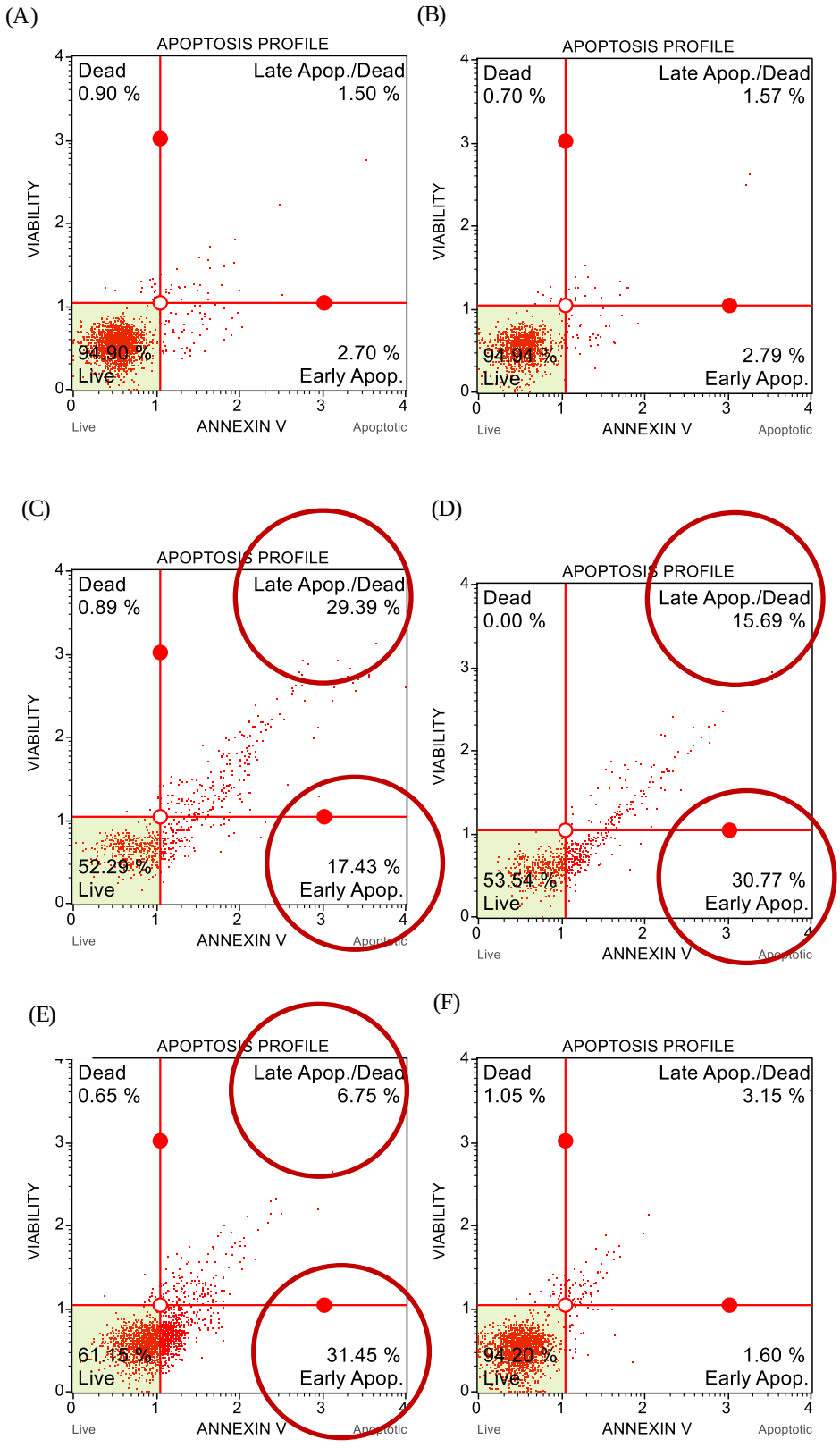
Figure 5. Scanning electron micrographs of *L. chagasi* promastigotes showing altered morphology after exposed to miltefosine (C) and retusin (D) (100 μM, 48 hours) compared to controls (A-Middle, B- 0.1% DMSO). Bars=2 μm; magnifications=11.000x.



*Yellow: Rounded shapes/ multi chambered. Blue: Complete loss of morphology. Red: Double scourge / Plague loss. Green: Debris / content loss.

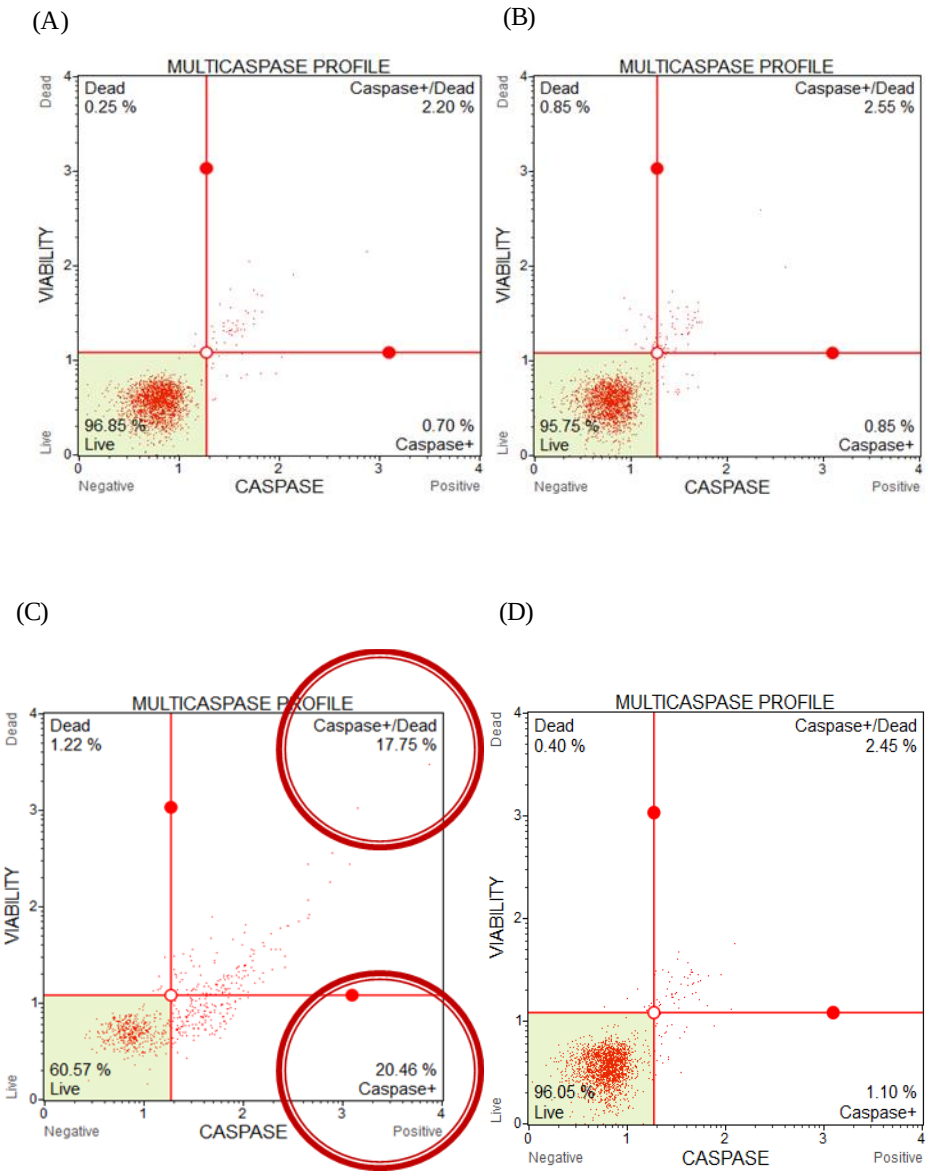
Other studies have been conducted to better understand the leishmanicidal mode of action. In order to determine the manner in which retusin exert their antiproliferative activity, it was performed a flow cytometric analysis using Annexin V-PE to investigate the externalization of phospholipids. As demonstrated in Figure 6, retusin at the concentration of 100 µM induced cell death through induction of apoptosis in *L. amazonensis* promastigotes after 48h of incubation (Figure 6). Retusin flavonoid induced 6.75% for late apoptosis and 31.45% of initial apoptosis. Furthermore, it was found that apoptosis triggered by the treatment with retusin is not dependent on caspase activation, as shown in Figure 7. Already, pentamidine (100µM) showed that apoptosis late apoptosis and early apoptosis is caspase-dependent (Figure 7).

Figure 6. Analysis of Externalization of phospholipids in *L. amazonensis* promastigotes death using flow cytometry after 48 h of treatment with pentamidine and retusin (100 µM and 10 µM)



***(A)** Middle **(B)**0.1% DMSO **(C)** 100 µM Pentamidine**(D)** 10 µM Pentamidine**(E)** 100 µM retusin**(F)** 10 µM retusin

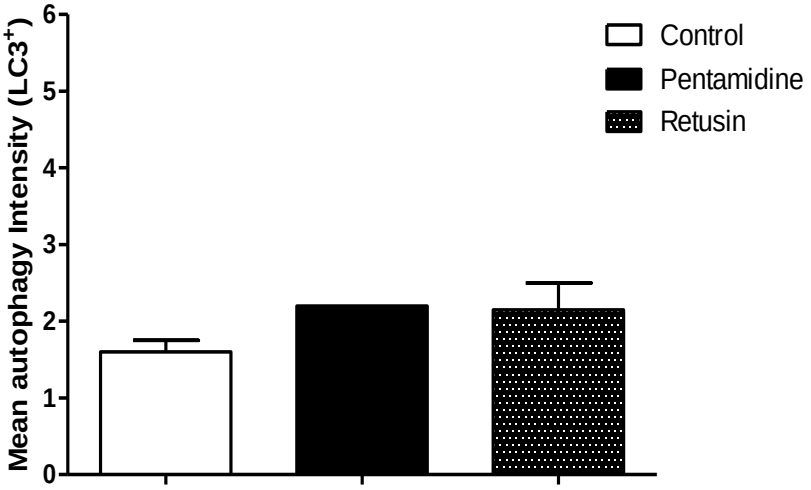
Figure 7. Caspase-like proteases in *L. amazonensis* promastigotes death using flow cytometry after treatment with pentamidine and retusin (100 µM) for 48 h.



*(A) Middle (B) 0.1% DMSO (C) 100 µM Pentamidine (D) 100 µM Retusin.

It was evaluated the induction of autophagic cell death in *L.amazonensis* promastigotes treated with retusin using anti-LC3 Alexa Fluor® 555-conjugated antibody. In this study, retusin treatment for 48 h did not alter the rate of the promastigote autophagy induction, indicating that the antiproliferative activity of retusin is probably not the result of an exacerbated process of autophagy (Figure 8).

Figure 8. Determination of the presence of autophagic LC3 analysis of *L. amazonensis* promastigotes death using flow cytometry after treatment with pentamidine and retusin for 48h.



The analysis of the population of cells in different cell cycle phases was possible after staining with 7-AAD and subsequent analysis by flow cytometry. In Figure 9 it was observed that pentamidine (10µM) induced a marked decrease in the number of cells in S and G2/M phase compared with the control group. These phases are caracterized of mitotic catastrophe and topoisomerase inhibition, in which occurs disintegration of organelles and consequently cellular damage. Retusin was subjected to topoisomerase inhibition assay (LcTopI) *in vitro*. The results presented in Figure 10, show that retusin was able to inhibit relaxation activity of LcTopI at concentrations of 100 up 3.125 µM (IC_{50} = 2.23±0.8).

Figure 9. Analysis of Cell cycle of *L. amazonensis* promastigotes death using flow cytometry after treatment with pentamidine and retusin for 48h. The values represent the means ± SEMs of three samples.

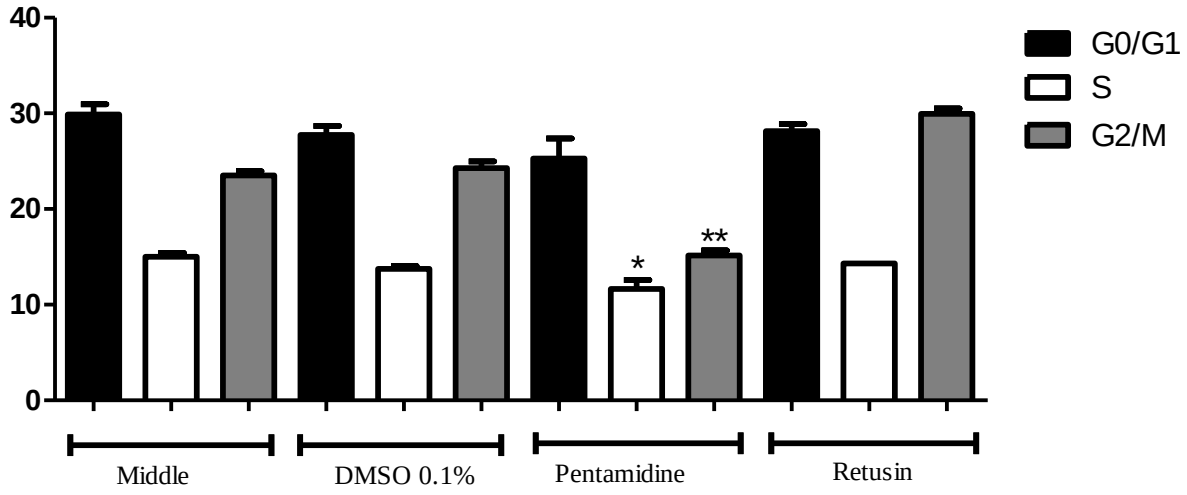
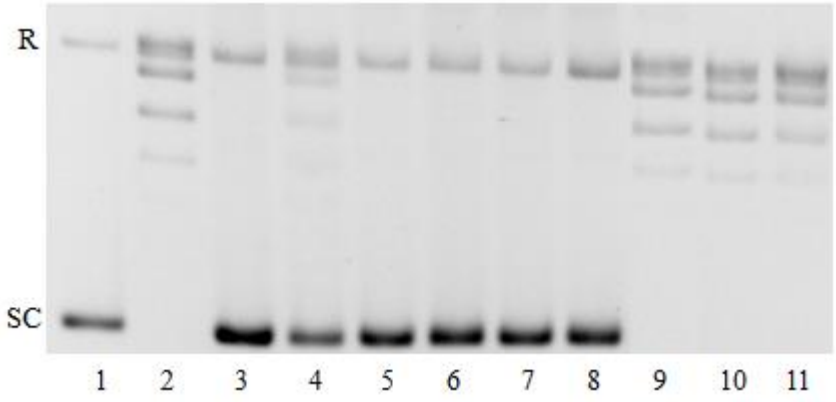


Figure 10. Inhibition of LcTopI-mediated DNA relaxation by different concentrations of retusin. Key to lanes: (1) Relaxed pBR332 plasmid DNA; (2) 1U LcTopI without inhibitor; (3-11) 1U LcTopI plus 100-0.39 µM retusin; (R) DNA relaxed; (SC) DNA supercoiled. The reaction was incubated in 30 min at 37 °C. (IC_{50} = 2.23±0.8).



4. Discussion

The therapeutic areas of infectious diseases have benefited from abundant scaffold diversity in natural products, able to interact with many specific targets within the cell and indeed for many years have been source of inspiration for the majority of FDA approved drugs (25).

Various studies have shown promising natural products against leishmaniasis (25, 26). In the natural products, *Solanum* species have shown activity against trypanosomes, such as those causing leishmaniasis and Chagas disease (27, 28). In the study of Silva et al. (2002) it was noted a higher yield of the retusin substance in the chromatogram (29). Hence, the purpose of this research was to study the antileishmanial effect and investigate the mechanism of action of the retusin flavonoid from the acetate fraction of *Solanum paludosum*.

In this study, retusin showed leishmanicidal activity against amastigotes of *L. amazonensis*, data that corroborates with the study of Wong et al. (2014) that tested flavonoid and found potent antiamastigote activity. In the same study, flavonoid dimers weretested against cutaneous leishmaniasis, where it was observed reduction in the size of the lesion of leishmaniasis (30). However, in our study, it was not observed parasite load reduction in the ear and lymph node of the animal group treated with retusin.

There is a general consensus that the T-cell and cell-mediated immunity contribute to the pathogenesis of the different forms of *Leishmania*. The cellular immune response to *Leishmania* parasites occurs with the production of cytokines that are important for the development and control of immune response (31). In leishmaniasis, control of infection is mediated by IFN-gamma and TNF-alpha, while disease progression is associated with production of interleukin4 (IL-4), IL-5, IL-10 and transforming growth factor-beta (TGF-beta) (31). TNF and IL-1 mediatethe development of inflammatory diseases, and since IL-1β is typically activated in situations in which TNF-α is produced, it was hypothesized that IL-1β alters the function of inflammatory epithelial cells induced by TNF-α by changing the TNF receptor (32).

IL-10 performs a fundamental role in the inhibition of macrophage activation and contributes to parasite growth in lesions, since BALB/cIL10$^{-/-}$ mice were shown to be able to control disease progression during *L. major* infection (33). IL-10 is a key cytokine in limiting the Th1 mediated response and preventing damage to the host, but in large quantities it inhibits the immune response mediated by cells, thus facilitating the development of chronic diseases

(34). It was identified as an important cytokine in susceptibility to VL and CL in humans, and it is associated with the persistence of parasites in the lesions (35, 36). In our study, IL-10 production increased after treatment,which may suggest that regulatory T cells uses this cytokine to limit the action of the immune system, favoring the persistence of the parasite after clinical cure (35).These data can be correlated with the permanence of the parasite load in the ear and lymph nodes of the groups treated with retusin.

The gold standard study of visceral leishmaniasis infection is the model in hamsters. In this study, it was observed a significant decrease in the number of spleen parasites. Furthermore, there was increased spleen size. Howerver, the histological analysis is need to confirm this effect. In order to supplement this data, hepatic enzymes were dosed because elevated ALT and AST activity can be indicative of disease or toxicity caused by the treatment.

ALT is a liver-specific enzyme which, when found in high quantities, can suggest a hepatobiliary disease. Moreover, increased AST may also be related damage to the heart, skeletal muscle, and liver parenchyma (37). Since these enzymes are use as biomarkers of liver injury and can be caused by changes in metabolism of the liver. It is suggested that treatment with retusin and glucantime did not cause damage to the liver during the study period.

The incidence and prevalence of chronic kidney disease (CKD) have rapidly increased worldwide, and CKD is recognized as a risk factor for all-cause mortality and cardiovascular mortality (38). High levels of creatinine and urea can cause kidney disease, which may lead the kidneys to stop functioning. Therefore, creatinine and urea levels of infected and treated groups were evaluated. The results demonstrated that retusin and glucantime treatment did not induce statistically significant changes in creatinine and urea levels, which suggests that it did not cause kidney damage.

The scanning electron microscope (SEM) is a powerful instrument which allows the observation and characterization of heterogeneous organic and inorganic materials and the determination of surface structures. In this study, as expected, control cells were with characteristic appearance, but promastigotes treated with retusin and miltefosine showed morphological change of promastigote with profound changes in the plasma membrane, loss of intracellular contents, and loss of nuclear envelope and plasma membrane integrity, which can lead to cell death by necrosis. This method may indicate the nature of cell death and some authors indicate how unique technique to identify the type of cell death (39, 40).

Morphological, biochemical or molecular changes lead to parasite death or prevent Parasite proliferation (41). Cell death pathways can be generally classified into apoptosis, necrosis, and exacerbation of autophagic processes (42). To determine the manner in which this flavonoid tested exerts their antiproliferative activity, flow cytometry analyzes were performed using annexin V-PE to investigate the externalization of phospholipids, typical characteristic of apoptosis in *Leishmania* (43). Lipids are essential for the structural and functional integrity of cells. Some changes in lipid distribution generally trigger apoptosis, so the externalization of phospholipids may be indicative of apoptotic death. In this study, the retusin flavonoid caused cell death through induction of apoptosis in *L. amazonensis* promastigotes after 48h of incubation. Death by apoptosis can be triggered by activation of cysteine proteases, known as caspases. Proteolytic cleavage of different cell substrates for caspases determines the main characteristics of the process of cell death by apoptosis (44). However, in this study, it was observed that death by apoptosis was not dependent on caspases, thus it could be a result of other mechanisms. Moreover, the tests also showed that there was not autophagy death induction, another important mechanism of cell death.

DNA topoisomerase are enzymes catalyzing charges in topological state of duplex DNA during replication, transcription, recombination and DNA repair process; there are three categories of such enzymes: DNA topoisomerase types IA, IB and II (45); particularly, type I topoisomerase are monomeric ATP-independent enzymes with relaxation activity for both positively and negatively supercoiled DNA (46). The effect of different concentrations of retusin (100 up 0.39 µM) on the relaxation activity of LcTopI has been monitored over 30 min of incubationg 5µL of LcTopI with 2 µL of compound in the presence of 0.5 µg supercoiled pBR322 plasmid DNA at 37°C. As show in figure 10, in absence of the compound (lane 2), the supercoiled substrate is fully processed after 30 min. The Retusin was able inhibit the relaxation of supercoiled DNA up 0.39 µM. The values of LcTopI relaxation activity inhibition by retusin are in agreement with values recorded by Prada et al. (2013) which showed inhibition in concentration 0.32 µM of compounds camptothecin and gimatecan (standard inhibitors of topoisomerase in tumor) in assays dose-dependent LiTopIB relaxation activity inhibition (47). Mittra et al. (2000) recorded relaxation activity inhibition of topoisomerase extracted of *L. donovani* to flavonoids quercetin, luteolin, rutin and isoorientin in concentrations 50, 12.5, 200 µM, respectively (48). The results presented by retusin suggest that this flavonoid can be active in inhibition of topoisomerase of *Leishmania*.

In conclusion, it was demonstrated in this work, that the flavonoid tested showed activity against *Leishmania* and showed excellent activity in the infection assay in hamsters because it reduced the parasite load in a statistically significant way. In the electron microscopy test, it was observed loss of parasite morphology, which led us to evaluate possible mechanisms of action. Retusin was able to inhibited topoisomerase *Leishmania*. Another mechanism of action suggested is death by apoptosis. The results confirm that retusin can become lead molecules for designing new prototypes of antileishmanial drugs.

Acknowledgments

The authors would also like to thank the CAPES, CNPq, MCT, FINEP, INCT-INOFAR (573.564/2008-6), CNPQ (479822/2013-1), CNPQ (404344/2012-7), FACEPE and FAPEAL (Pronem 20110722-006-0018-0010), FACEPE-PRONEM (APQ-1232-1.06/10) and CENAPESQ *for providing financial assistance for this research project. Moreover, the authors would like to thank several colleagues working at the UFAL for constructive criticism of and assistance with this project.*

Conflicts of Interest

The authors declare no conflict of interest.

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9 CONSIDERAÇÕES FINAIS E PERSPECTIVAS

Em resumo, a investigação de agentes anti-Leishmania resultou na identificação de novos derivados bioativos. Dentre estes, a série de derivados com substituintes bis-2-hidroxi-1,4-naftoquinona exibiram atividade leishmanicida significante contra promastigotas de *Leishmania amazonensis* e *Leishmania braziliensis* e o composto 3a reduziu o o tamanho da orelha infectada no tratamento no ensaio *in vivo* com *Leishmania amazonensis*, mostrando-se como substância promissora para estudos complementares.

A série com substituintes 2-N,N'-dialquillamino-1,4-naftoquinona apresentou atividade contra promastigotas de *L. chagasi* e *L. amazonensis,* com destaque para os derivados (1d, 1h e 1i) que exibiram pronunciada inibição do crescimento de promastigotas *L. amazonensis,* e os derivados (1d, 1e, 1f, 1h, 1k e 1n) que inibiram o crescimento de promastigotas de *L. chagasi.* No ensaio contra as formas intracelulares amastigotas, os derivados (1a, 1b, 1c, e 1m) apresentaram significante atividade contra amstigotas de *L. chagasi,* sem induzir efeito tóxico para célula hospedeira.

No geral, os flavonoides inibiram o crescimento de promastigotas de *L. amazonensis* e *L. chagasi.* No ensaio contra as formas intracelulares de *L. amazonensis* SP1, SP3 e o extrato bruto de *S. paludosum* exibiram siginificante inibição do crescimento de amastigotas. Além disso, no ensaio *in vivo* de infecção com *L. amazonensis* os compostos reduziram a carga parasitária da orelha infectada e induziram mudança na fase S e G2/M do ciclo celular, além disso o composto SP3 induziu morte por apoptose e autofagia na concentração de 100 µM.

O composto retusin apresentou significante atividade nos ensaios de infecção *in vitro* contra promastigotas e amastigotas de *L. amazonensis* e *L. chagasi.* No ensaio de infecção em hamsters com *L. chagasi,* o composto retusin diminuiu a carga parasitária no baço dos animais infectados e tratados com o composto. Entretanto, no ensaio *in vivo* de infecção com *L. amazonensis* não reduziu a carga parasitária da orelha e do linfonodo drenante. Além disso, o composto retusin induziu morte por apoptose na concentração de 100 µM, provavelmente não dependente de caspases. Outro mecanismo de ação sugerido do retusin é a inibição de topoisomerase de *Leishmania*. Os resultados indicam que os derivados 1,4-naftoquinonas e os compostos flavonoides derivados de *S. paladosum* como fortes candidatos a fármacos leishmanicidas.

Entre as perspectivas deste trabalho, pretende-se determinar as alterações ultraestruturais nas estruturas celulares de promastigotas dessa espécie de *Leishmania*, através de análise em microscópio eletrônico de transmissão, além de realizar a análise histológica dos órgãos coletados dos animais nos ensaios *in vivo* já realizados. Além disso, faz-se necessário continuar investigando o mecanismo destas substâncias, que poderão ser realizados através de ensaios bioquímicos e de biologia molecular, visando determinar o efeito destes derivados, principalmente, em cisteína proteases, serina proteases, tubulina, tripanotiona redutase e topoisomerases do parasito. Por fim, pretende-se ainda investigar uma possível interferência da transcrição de genes e tradução de transcritos de *Leishmania* spp, através de ensaios de microarray e western blot.

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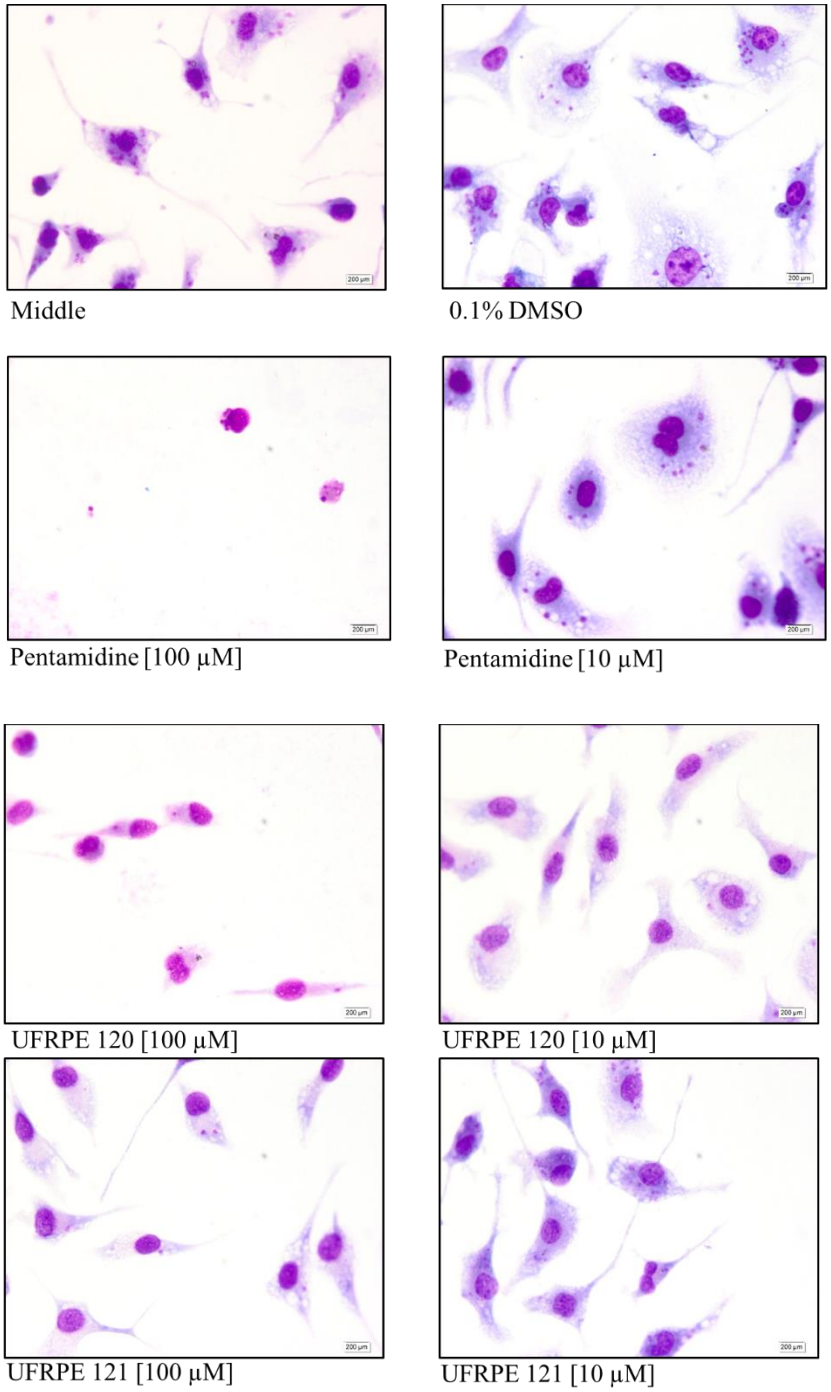
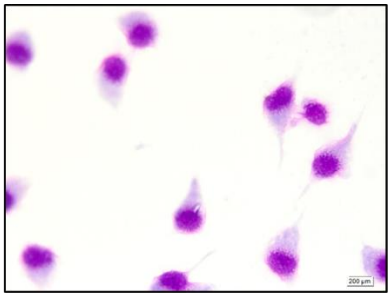
ANEXOS

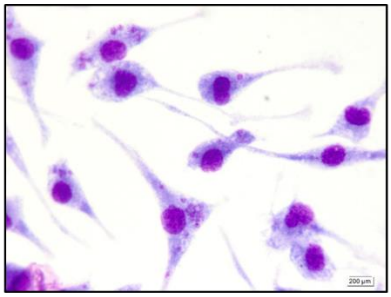
Anexo A: Fotografias do artigo 2

Figure 1. Photographs of the Leishmanicidal assay against the growth of amastigotes of *L. chagasi*. Viewing through optical microscope with objective of 100X. Staining May-Grunwald / Giemsa.

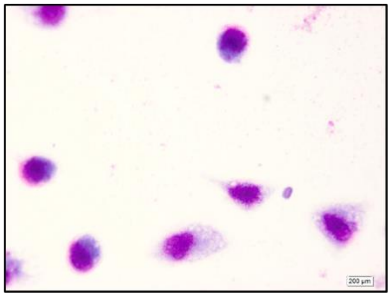




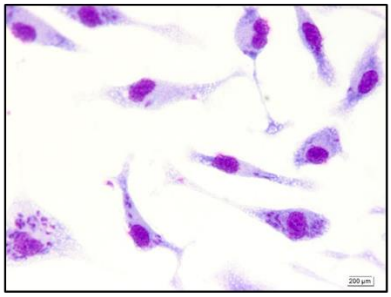
UFRPE 122 [100 µM]



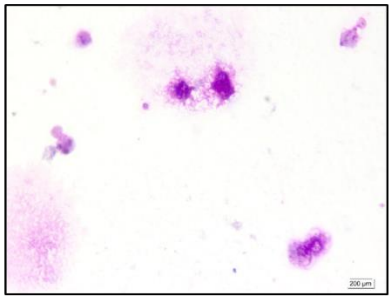
UFRPE 122 [10 µM]



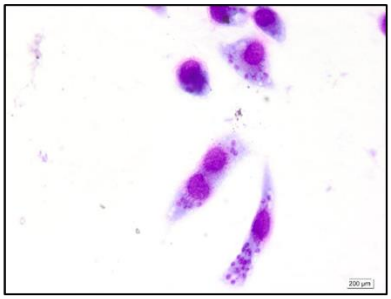
UFRPE 123 [100 µM]



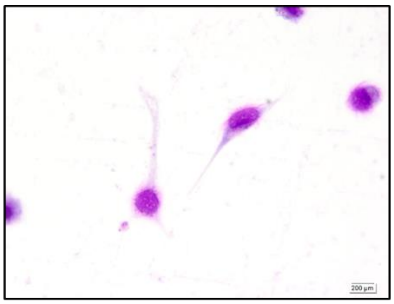
UFRPE 123 [10 µM]



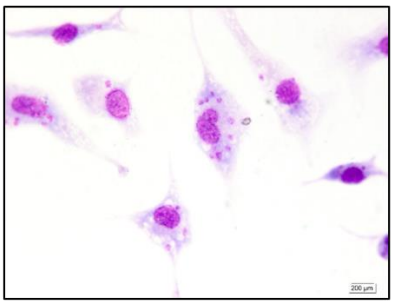
UFRPE 133 [100 µM]



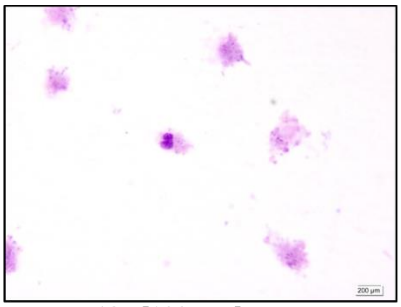
UFRPE 133 [10 µM]



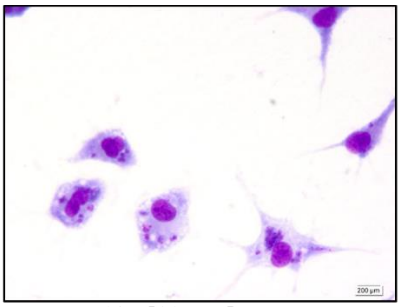
UFRPE 135 [100 µM]



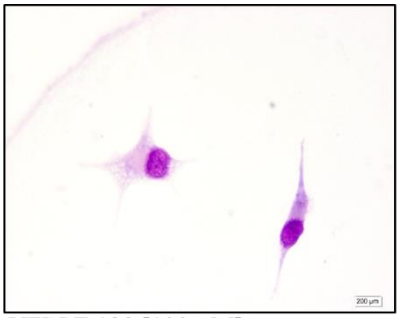
UFRPE 135 [10 µM]



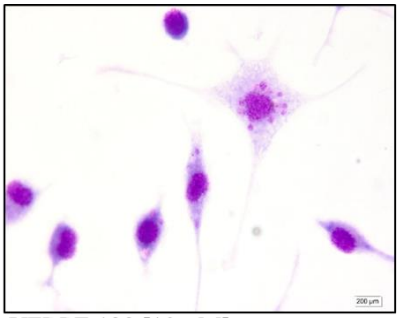
UFRPE 137 [100 µM]



UFRPE 137 [10 µM]



UFRPE 139 [100 µM]



UFRPE 139 [10 µM]

Anexo B: Aprovação do Comitê de Ética da UFAL para uso de camundongos Swiss

UNIVERSIDADE FEDERAL DE ALAGOAS
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

PARECER CONSUBSTANCIADO

PROJETO Nº 01/2015

TÍTULO: Avaliação de protótipos de fármacos leishmanicidas.

RESPONSÁVEL: Magna Suzana Alexandre Moreira

OBJETIVO: Este projeto visa à obtenção de protótipos que representem uma nova estratégia terapêutica contra doenças negligenciadas com características multifatoriais, com comprometimento imune e/ou infeccioso.

SITUAÇÃO: Aprovado

PERÍODO DE VIGÊNCIA: 22.06.2015 a 22.06.2016

DADOS DO ANIMAL:

ESPÉCIE	LINHAGEM	QUANTIDADE
Camundongo	Swiss	140

Maceió, 16 de junho de 2015.

Silvana Ayres Martins
Coordenadora da CEUA/UFAL

Profa. Dra. Silvana Ayres Martins
Coordenadora da Comissão de
Ética no uso de Animais
SIAPE 1120858

Anexo C: Aprovação do Comitê de Ética da UFAL para uso de camundongos Balb/C

UNIVERSIDADE FEDERAL DE ALAGOAS
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

ROTEIRO DE PARECER CONSUBSTANCIADO

O projeto de pesquisa Desenvolvimento de protótipos de fármacos leishmanicidas a partir de produtos naturais, derivados semi-sintéticos e/ou sintéticos
(2/2013) coordenado pelo Professora Magna Suzana Alexandre Moreira do Instituto de Ciências Biológicas e da Saúde / UFAL
Esse projeto visa determinar o efeito antiparasitário em modelos in vivo (murino), contra Leishmania amazonensis. O presente estudo visa à avaliação farmacológica de novos agentes candidatos a protótipos de fármacos imunomoduladores e leishmanicidas, capazes de atuar como estratégias terapêuticas em doenças negligenciáveis, especialmente a leishmaniose.
Não existe método substitutivo para evitar o uso de animais. Está linhagem de camundongo é utilizada no modelo clássico de indução de leishmaniose, por desenvolver um perfil de resposta imune Th2 com produção de IL-4, suscetível ao desenvolvendo lesões com riqueza parasitária.Será utilizada apenas para a(s) molécula(s) mais ativa para minimizar o número de animais.
Existe planejamento estatístico para determinar o tamanho das amostras. Cada grupo experimental será constituído por cinco animais (n=5 animais). Os 14 grupos presentes no estudo serão: Grupo Controle infectado (Balb/c infectado com tratamento apenas com veículo), Grupo Controle não-infectado (Balb/c não-infectado com tratamento apenas com veículo), Grupo Antimoniato de Meglumina (Balb/c infectado com tratamento utilizando fármaco de referência), Grupo Combretastatina A4 (Balb/c infectado com tratamento utilizando fármaco antitumoral), Grupo LASSBio 1483 (Balb/c infectado com tratamento utilizando derivado LASSBio 1483), Grupo LASSBio 1586 (Balb/c infectado com tratamento utilizando derivado LASSBio 1586), Grupo JB-42 (Balb/c infectado com tratamento utilizando derivado JB-42), Grupo Quercetina-permetilada (Balb/c infectado com tratamento utilizando Quercetina-permetilada), Grupo VR-67 (Balb/c infectado com tratamento utilizando derivado VR-67), Grupo L1-SbPh3 (Balb/c infectado com tratamento utilizando derivado L1-SbPh3), Grupo HDL-Sb (Balb/c infectado com tratamento utilizando HDL-Sb), Grupo HDL (Balb/c infectado com tratamento utilizando HDL), Grupo espectalina (Balb/c infectado com tratamento utilizando espectalina), Grupo WE-01 (Balb/c infectado com tratamento utilizando derivado WE01) e HB-04 (Balb/c infectado com tratamento utilizando derivado HB-04).

Devido à solicitação do CONCEA na Orientação técnica 2.2013 será necessário o envio de:
XIII - plantas baixas das áreas e das instalações utilizadas para criação, manutenção, manuseio e manejo de animais para fins de ensino ou pesquisa científica, contendo o detalhamento/dimensionamento das áreas e o registro do Responsável Técnico pela planta (engenheiro ou arquiteto), devidamente assinado.

Situação: Aprovado

Robinson Sabino

Robinson Sabino da Silva
Coordenador CEUA-UFAL

Robinson Sabino da Silva
Coordenador da CEUA-UFAL
Comissão de Ética no Uso de Animais

Anexo D: Aprovação do Comitê de Ética da UFAL para uso de hamsters Syrian Golden

UNIVERSIDADE FEDERAL DE ALAGOAS

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

PARECER CONSUBSTANCIADO

PROJETO Nº 56/2014

TÍTULO: Desenvolvimento de protótipos de fármacos leishmanicidas a partir de produtos naturais, derivados semi-sintéticos e/ou sintéticos.

RESPONSÁVEL: Magna Suzana Alexandre Moreira

OBJETIVO: Visa à obtenção de protótipos que representem uma nova estratégia terapêutica contra doenças negligenciadas com características multifatoriais, com comprometimento imune e/ou infeccioso.

JUSTIFICATIVA (APROVAÇÃO, PENDÊNCIA, NEGAÇÃO):

A pesquisa proposta é de relevada importância científica apresentando justificativas plausíveis para a utilização de animais. O projeto ressubmetido atendeu a todas as solicitações do parecer consubstanciado.

SITUAÇÃO: Aprovado

PERÍODO DE VIGÊNCIA: 15-03-2015 a 30-05-2015

DADOS DO ANIMAL:

ESPÉCIE	LINHAGEM	QUANTIDADE
Hamster	Syrian Golden	72

Maceió, 12 de fevereiro de 2015.

Silvana Ayres Martins
Coordenadora da CEUA/UFAL

Profa. Dra. Silvana Ayres Martins
Coordenadora da Comissão de Ética no uso de Animais
SIAPE 1120868