UNIVERSIDADE ESTADUAL DE LONDRINA

ADRIANA OLIVEIRA DOS SANTOS

AVALIAÇÃO *IN VITRO* E *IN VIVO* DA ATIVIDADE DO ÓLEO DE COPAÍBA CONTRA *LEISHMANIA AMAZONENSIS*

Londrina
2011

ADRIANA OLIVEIRA DOS SANTOS

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

Orientação: Prof. Dr. Celso Vataru Nakamura

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Dedico este trabalho ao meu marido Eduardo, aos meus pais, Jorge e Maria Helena;

às minhas irmãs,

Andréia e Alexandra; com carinho.

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"Não vai demorar que passemos adiante uma grande e bela ciência, que faz arte em defesa da vida".

Carlos Chagas, 1928

SANTOS, Adriana Oliveira dos. **Avaliação *in vitro* e *in vivo* da atividade do óleo de copaíba contra *leishmania amazonensis***. 2011. 62f. Tese (Doutorado em Microbiologia) - Universidade Estadual de Londrina.

RESUMO

A leishmaniose é um problema grave de saúde pública, com altas taxas de morbidade e mortalidade. Esforços para encontrar novos fármacos de administração oral eficazes e seguros para o tratamento da leishmaniose estão em curso há várias décadas, a fim de evitar os problemas com o antimoniais usados atualmente. No presente estudo, verificou-se que o tratamento oral com óleo de copaíba ocasionou redução significativa no tamanho da lesão em camundongos infectados por *Leishmania amazonensis* quando comparados com camundongos não tratados. Para avaliar a segurança do óleo, a toxicidade e a genotoxicidade foram determinados. Avaliação histopatológica não revelou alterações nos animais tratados com óleo de copaíba em relação aos animais controle. Na avaliação de mutagenicidade (teste do micronúcleo), a dose testada (2000 mg/kg) não mostrou efeitos genotóxicos. Análises morfológicas e ultra-estruturais demonstraram mudanças notáveis em células de parasitas tratados com óleo de copaíba obtidos de *Copaifera. reticulata* e *Copaifera. martii.* O principal efeito ultraestrutural observado nas células dos parasitas tratados foi um acentuado inchaço mitocondrial. No presente estudo, também observou-se que o tratamento *in vitro* com óleo de copaíba ocasiona o aumento na permeabilidade da membrana plasmática e despolarização do potencial de membrana mitocondrial em células do parasita. Além disso, compostos isolados do óleo de copaíba obtidos de *Copaifera officinales* (ácido agático, ácido hidróxi-copálico, ácido caurenóico, copalato de metila, ácido pinifólico e ácido poliáltico) mostraram atividade frente a *L. amazonensis.* Ácido hidroxi-copálico e copalato de metila foram os mais efetivos contra formas promastigotas. Por outro lado, o ácido pinifólico e ácido caurenóico foram os mais efetivos na inibição do crescimento de formas amastigotas axênicas. O ácido agático, ácido caurenóico e ácido pinifólico induziram o aumento na permeabilidade da membrana plasmática e despolarização do potencial de membrana mitocondrial nas células dos parasitas. Embora o mecanismo de ação do óleo de copaíba ainda não esteja totalmete elucidado, estes resultados indicam que o óleo de copaíba pode ser uma opção no desenvolvimento de novos medicamentos para o tratamento da leishmaniose.

Palavras-chave: Microbiologia médica. Leishmaniose. Óleo de copaíba. Doenças parasitárias. Plantas – Uso terapêutico.

SANTOS, Adriana Oliveira dos. **Evaluation in vitro and in vivo activity of copaiba oil against Leishmania amazonensis**. 2011. 62p. Thesis (PhD in Microbiology) - Universidade Estadual de Londrina.

ABSTRACT

Leishmaniasis is a severe public-health problem, with high rates of morbidity and mortality. Efforts to find new, effective and safe oral agents for the treatment of leishmaniasis have been ongoing for several decades, in order to avoid the problems with the currently used antimonials. In the present study, we found that a copaiba oil oral treatment caused a significant reduction in the average lesion size against *Leishmania amazonensis* lesions compared with untreated mice. To prove the safety of the oil, the toxicity and genotoxicity were also determined. Histopathological evaluation did not reveal changes in the copaiba oil-treated animals compared to the control animals. In the mutagenicity evaluation, (micronucleus test) the dose tested (2,000 mg/kg) showed no genotoxic effects. Morphological and ultrastructural analyses demonstrated notable changes in parasite cells treated with copaiba oil from *Copaifera. reticulata and Copaifera martii.* The main ultrastructural effect was mitochondrial swelling. We also demonstrated that *in vitro* copaiba oil treatment of *L. amazonensis* led to an increase in plasma membrane permeability, and depolarization in the mitochondrial membrane potential in parasite cells. Additionally, isolated compounds from copaiba oil from *C. officinales* (agatic acid, hydroxy-copalic acid, kaurenoic acid, methyl copalate, pinifolic acid, polyaltic acid) showed some level of activity against *L. amazonensis.* Hydroxy-copalic acid and methyl copalate were the most activity against promastigote. On the other hand, pinifolic acid and kaurenoic acid were the most activity against axenic amastigote. The isolated compounds agatic acid, kaurenoic acid, and pinifolic acid showed remarkable increase in plasma-membrane permeability and mitochondrial membrane depolarization. Although the mechanism of action of the oleoresin is still unclear, these findings indicate that copaiba oil is a possible new drug, which would provide a safer, shorter, less-expensive, and more easily administered treatment for leishmaniasis.

Keywords: Medical Microbiology. Leishmaniasis. Copaiba oil. Parasitic diseases. Plants - Therapeutic use.

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CONCLUSÕES........

1 INTRODUÇÃO

Os protozoários do gênero *Leishmania* são responsáveis por patologias conhecidas coletivamente como leishmanioses (MCCONVILLE;HANDMAN, 2007). A prevalência mundial é de 12 milhões de casos. Enquanto a população em risco, por sua vez, gira em torno de 350 milhões em 88 países nos quatro continentes. Estima-se, atualmente, que a incidência mundial de novos casos é de cerca de 2 milhões por ano (ALVAR et al., 2006). Ressalte-se, porém, que os dados oficiais muitas vezes subestimam a realidade do problema, sobretudo devido ao fato de que a leishmaniose ainda não é uma doença de notificação obrigatória em todos os países onde é endêmica. Logo, um número substancial de casos nunca são registrados (DESJEUX et al., 2004; BUSTAMANTE et al., 2009).

As manifestações clínicas da infecção dependem da espécie de *Leishmania* e do sistema imunológico do hospedeirode maneira que a doença pode ser classificada como: leishmaniose cutânea, leishmaniose cutânea-difusa, leishmaniose mucocutânea e leishmaniose visceral, também conhecida como cala-zar (DESJEUX et al., 2004;. CLEM, 2010).

Leishmania amazonensis é uma espécie que causa a leishmaniose cutânea (LC), que pode evoluir de pequenos nódulos cutâneos para a destruição do tecido da mucosa (REITHINGER et al., 2007). Em pacientes anérgicos pode ocasionar a leishmaniose difusa, que produz lesões disseminadas (SINHA et al., 2008; SILVEIRA et al., 2009; MITROPOULOS et al., 2010).

Dentre os principais problemas e desafios para o controle da leishmaniose, destacam-se: o diagnóstico tardio, as dificuldades de controle de vetores, a epidemia de AIDS, a falta de vacinas eficazes, o desenvolvimento de resistência à quimioterapia e a migração populacional entre os continentes (ALVAR et al., 2006; MITROPOULOS et al., 2010).

Desde 1940, os compostos antimoniais pentavalente vêm sendo utilizados para tratar todas as formas de leishmaniose. Apesar de novos medicamentos ou formulações de fármacos estarem disponíveis para tratamento, tais como a anfotericina B lipossomal (AmBisome®), a Miltefosina® e a paromomicina, todos apresentam limitações de custo, toxicidade específica ou a necessidade de administração parenteral (LEE;HASBUN, 2003; CROFT, 2008; DUJARDIM et al., 2010, TIUMAN et al., 2011). Consequentemente, a necessidade de novos medicamentos eficazes contra a leishmaniose é urgente.

O emprego da fitoterapia em regiões endêmicas para leishmaniose renovou o interesse na avaliação de plantas usadas na medicina popular como fonte de novos fármacos com potencial antileishmania (IWU et al., 1994; MOREIRA et al., 2002;. GACHET et al.,

2010). Curiosamente, em vários estudos etnofarmacológicos com dados obtidos a partir do Peru, Estado do Maranhão, Norte da Amazônia no Brasil e Guiana Francesa, a utilização de óleos de copaíba vem sendo apontado como eficaz no tratamento da leishmaniose (GRENAND; MORETTI, 1987, FLEURY, 1997; MOREIRA et al., 2002; KVIST et al., 2006).

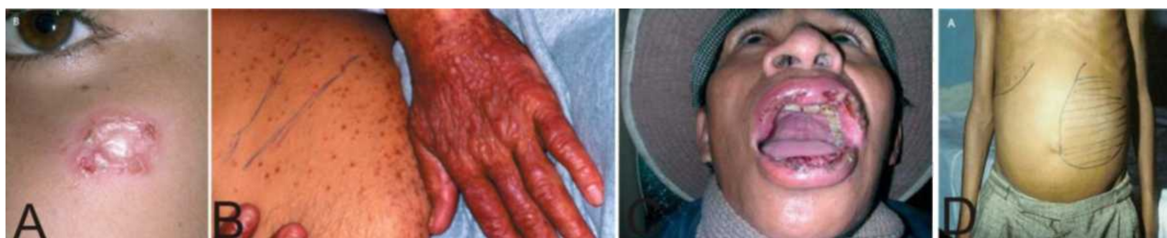
Recentemente, Santos et al. (2008) relataram que os óleos de copaíba de diferentes espécies de *Copaifera* têm atividade frente as formas promastigotas de *L. amazonensis.* Além disso, demonstraram a atividade do óleo obtido de *C. reticulata* contra formas amastigotas axênicas e amastigotas intracelulares de *L. amazonensis.* Essas descobertas levaram a investigação dos possíveis mecanismos de ação do óleo de copaíba em células de *L. amazonensis,* bem como avaliar a atividade *in vivo* do óleo de copaíba em modelos animais infectados com *L. amazonensis.*

2 REVISÃO BIBLIOGRÁFICA

As leishmanioses são doenças causadas por protozoários flagelados pertencentes à ordem Kinetoplastida, família *Trypanosomatidae,* gênero *Leishmania* (ROSS, 1903). As principais síndromes de leishmaniose humana, em ordem crescente de acometimento sistêmico e provável gravidade clínica, são as seguintes: forma cutânea, cutânea difusa, mucocutânea e visceral (MURRAY et al., 2005; MINODIER; PAROLA, 2007; CLEM, 2010) (Figura 1).

1) *Leishmaniose cutânea (LC):* apresenta exclusivamente lesões cutâneas, ulcerosas ou não. A leishmaniose cutânea afeta principalmente o rosto, o nariz, os braços e as pernas, que são os locais mais propícios para a picada do inseto. As úlceras têm como característica as bordas elevadas, em moldura (HEPBURN, 2000; ALMEIDA e SANTOS, 2011).
2) *Leishmaniose cutâneo-difusa (LCD):* caracteriza-se pela formação de lesões difusas não ulceradas por toda a pele. A disseminação do parasita no organismo se dá por meio dos vasos linfáticos, bem como pela migração dos macrófagos parasitados (SINHA et al., 2008; SILVEIRA et al., 2009).
3) *Leishmaniose mucocutânea ou cutâneo-mucosa (LCM):* a forma clínica mucocutânea é conhecida por espundia, nariz de tapir ou anta. As lesões ocorrem devido à disseminação do parasita por via hematogênica ou linfática. As regiões mais comumente afetadas são o nariz, a faringe, a boca e a laringe. Os processos ulcerativos causam graves mutilações nos pacientes, que nos estágios avançados da doença manifestam dificuldades de respirar, falar e de se alimentar. Nesta fase, complicações respiratórias por infecções secundárias podem ocasionar a morte do paciente (AMATO et al., 2008; DANESHBOD et al., 2010).
4) *Leishmaniose visceral ou calazar (LV):* a forma visceral da doença é crônica, progressiva e afeta vários órgãos, dentre os quais: o baço, o fígado, a medula óssea, os linfonodos e a pele. A leishmaniose visceral é a forma mais grave e, caso não seja tratada, pode ser fatal (GUERIN et al., 2002; SUNDAR; CHATTERJEE, 2006; CHAPPUIS et al., 2007; MURRAY et al., 2010).

Figura 1 – Síndromes de leishmaniose humana. (A) Leishmaniose cutânea (LC); (B) Leishmaniose Cutânea Difusa (LCD) (C) Leishmaniose Mucocutânea (LMC) e Leishmaniose Visceral (LV). Adaptado de MURRAY et al., 2005.

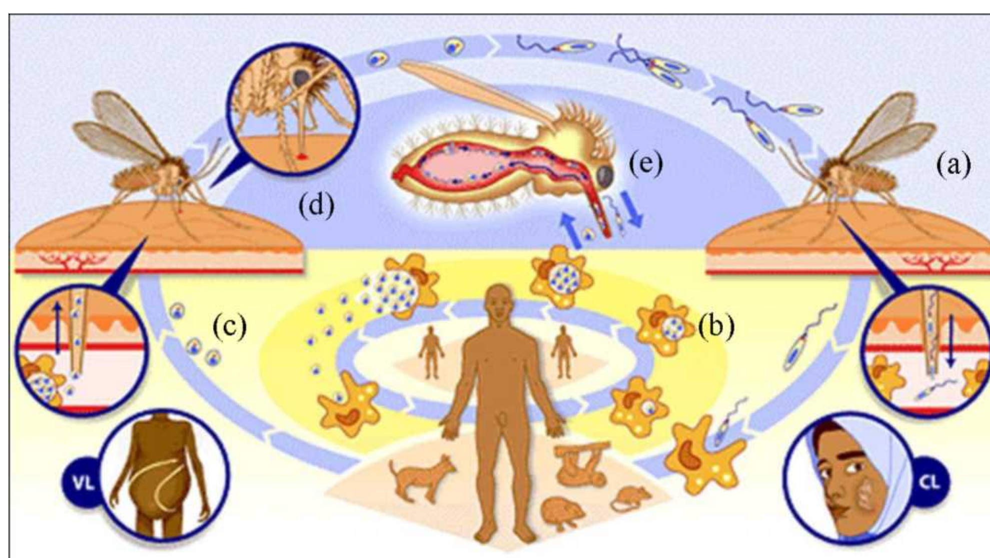


Os desenvolvimentos das diferentes formas clínicas da leishmaniose estão diretamente relacionados com as espécies ou subespécies do parasita infectante, a distribuição dos macrófagos infectados e, sobretudo, com a resposta imune do hospedeiro. Há mais de 20 espécies de *Leishmania* capazes de infectar o homem. Dividem-se em 2 sub-gêneros *(Leishmania* e *Viannia)* e 4 complexos (CUPOLILLO et al., 1998; COHENFREUE et al., 2007; SAKTHIANANDESWAREN et al., 2009; SEN; CHATTERJEE, 2011). Parasitas do gênero *Leishmania* apresentam um ciclo de vida heteroxênico, alternando entre formas promastigotas (extracelulares) e amastigotas (intracelulares). As amastigotas são formas ovais ou esféricas com núcleo grande e arredondado ocupando, aproximadamente, um terço do corpo do parasita. Multiplicam-se no citoplasma dos macrófagos localizados na pele, nas vísceras ou no sangue, onde produzem pouco ou nenhum efeito patológico nos hospedeiros naturais. Todavia, nos hospedeiros eventuais, entre os quais está o homem, pode-se verificar uma violenta reação celular ocasionada pelo parasita, da qual resulta lesões na pele e nas mucosas (leishmaniose tegumentar) ou severas alterações patológicas nos órgãos internos (leishmaniose visceral). As formas promastigotas são alongadas, com um flagelo livre e longo, emergindo da porção anterior do corpo do parasita. O núcleo é arredondado ou oval e está situado na região mediana do corpo do parasita. Estas formas do parasita são encontradas aderidas à parede intestinal em pontos diferentes ou livres no lúmen, em processo de multiplicação. As formas infectantes, promastigotas metacíclicas, são encontradas principalmente livres ou aderidas na porção anterior do aparelho bucal do inseto (CUNNINGHAM, 2002; McCONVILLE et al., 2007; BESTEIRO et al., 2007; CHAPPUIS et al., 2007).

Todas as espécies de *Leishmania* são transmitidas por flebotomíneos (Diptera: Psychodidae) dos gêneros: *Lutzomyia,* vetores das leishmanioses no Novo Mundo, e *Phlebotomus,* correspondendo aos transmissores da doença no Velho Mundo (KAMHAWI, 2006; CARVALHO, 2011). Adicionalmente, há aproximadamente 30 espécies diferentes de

flebótomos que as podem transmitir ao homem (HERWALDT, 1999).A infecção se dá durante o repasto sanguíneo das fêmeas, sendo que a saliva do inseto desempenha um papel relevante na infecção. O ciclo evolutivo de *Leishmania* está representado na Figura 2 e inclui as formas promastigotas (a), alongadas, flageladas e móveis, que são inoculadas na derme do hospedeiro vertebrado e fagocitadas por macrófagos (b). No interior do vacúolo parasitóforo, o parasita se diferencia em amastigota, forma arredondada e aflagelada. Após a multiplicação por divisão binária os amastigotas rompem o macrófago, são liberados do meio intracelular e novamente fagocitados por outros macrófagos (c). Durante o repasto sanguíneo, a fêmea do flebotomíneo ingere os macrófagos infectados com as formas amastigotas (d) que ao longo do tubo digestivo do vetor se diferenciam em promastigotas. Os promastigotas multiplicam-se e migram para a probóscida do inseto, diferenciam-se em formas infectivas metacíclicas (e) que são inoculadas no hospedeiro vertebrado juntamente com a saliva durante a picada, reiniciando o ciclo (Figura 2) (HANDMAN; BULLEN, 2002; KIMA, 2007; McCONVILLE; HANDMAN, 2007; WHO, 2011a).

Figura 2 – Ciclo evolutivo de parasitas do gênero *Leishmania,* mostrando as formas promastigotas extracelulares existentes no hospedeiro invertebrado e as formas amastigotas intracelulares no hospedeiro vertebrado. VL, leishmaniose visceral; e CL, leishmaniose cutânea. Figura retirada do site http://www.who.int/tdr/diseases/.



Ambas as formas do agente etiológico da leishmaniose, pertencentes à ordem Kinetoplastidae, apresentam como característica da ordem a presença de um cinetoplasto mitocondrial conspícuo, situado próximo à base do flagelo. O cinetoplasto é um compartimento especializado da mitocôndria onde se concentra grande quantidade de DNA organizado em mini e maxi-círculos, denominado DNA do cinetoplasto (kDNA). Observados

ao microscópio eletrônico de transmissão, observa-se que esses parasitas são delimitados por uma membrana citoplasmática lipoprotéica, sob a qual estão dispostos microtúbulos subpeliculares em número variável.

Nas diferentes espécies de *Leishmania,* a membrana plasmática apresenta uma invaginação na região anterior do corpo do parasita formando a bolsa flagelar, onde se localiza o flagelo, e caracteriza-se por apresentar intensa atividade endocítica. Na matriz citoplasmática é possível observar a mitocôndria, que se estende por toda a célula, retículo endoplasmático, aparelho de Golgi, compartimentalização da via glicolítica em glicossomas e vacúolos de reserva, tais como os acidocalcissomas. Vale ressaltar que formas amastigotas do parasita *Leishmania* do complexo mexicana apresentam abundantes estruturas elétron-densas no citoplasma, as quais variam em tamanho e número, designadas como megasomas. Estudos citoquímicos demonstram que megasomas contém várias enzimas hidrolíticas, tais como as cisteínas proteinases, consideradas cruciais na diferenciação celular entre as formas morfológicas distintas do parasita (UEDA-NAKAMURA et al., 2001; DE SOUZA, 2002; BESTEIRO et al., 2007; UEDA-NAKAMURA et al., 2007).

Segundo a Organização Mundial de Saúde, 12 milhões de pessoas estão com leishmaniose, enquanto 350 milhões vivem sob o risco de infecção. Todo ano, são diagnosticados cerca de 2 milhões de novos casos. Ressalte-se, ainda, que as leishmanioses ocorrem em 88 países, mas somente em 30 sua notificação é compulsória. As áreas endêmicas, onde estão concentrados 90% dos casos de leishmaniose visceral, incluem o subcontinente indiano (Índia, Nepal e Bangladesh), a África (Sudão e Etiópia), o Brasil e a bacia mediterrânica. Quanto à leishmaniose cutânea, frise-se que 90% dos casos ocorrem no Afeganistão, Argélia, Irã, Arábia Saudita, Síria, Brasil, Colômbia, Peru e Bolívia (RATH et al., 2003; DESJEUX et al., 2004; ALVAR et al., 2006; BUSTAMANTE et al., 2009; CDC, 2010; WHO, 2011).

No Brasil, as leishmanioses apresentam-se como duas doenças distintas: a leishmaniose tegumentar, que pode ser causada por diferentes espécies, sendo as mais importantes, *Leishmania braziliensis, Leishmania guyanensis* e *Leishmania amazonensis,* e a leishmaniose visceral, também conhecida por calazar, causada pela *Leishmania chagasi.* A tegumentar, por sua vez, possui grande variedade de formase podem ser agrupadas da seguinte maneira: leishmaniose cutânea, cutânea difusa e mucocutâneas (FUNASA/MS, 2000; GONTIJO; CARVALHO, 2003; DESJEUX et al., 2004).

A incidência de leishmaniose tegumentar tem aumentado em praticamente todas as regiões do Brasil. No período de 2007 a 2010, o número de casos confirmados de

leishmaniose tegumentar e visceral foi de 91.320 e 15.387, respectivamente. Destes, 13 casos de leishmaniose visceral e 1774 casos de leishmaniose tegumentar foram diagnosticados no Estado do Paraná, onde a leishmaniose tegumentar é endêmica, ocorrendo em 289 dos seus 399 municípios (LONARDONI et al., 2006). Estatísticas preocupantes revelam que o calazar atinge 19 estados, especialmente da região Nordeste, onde se concentram mais de 90% dos casos humanos da doença. Por fim, vale ressaltar que há focos importantes também nas regiões Norte, Sudeste e Centro-Oeste. (MINISTÉRIO DA SAÚDE, 2011a ; 2011b).

As formas clássicas das leishmanioses (leishmaniose tegumentar e visceral) impõem dificuldades específicas em termos de diagnóstico e tratamento. Os protozoários, por serem eucariotos, apresentam processos metabólicos que se assemelham aos das células do hospedeiro, dessa forma, os medicamentos, causam sérios efeitos tóxicos para os hospedeiros humanos (SOTO; SOTO, 2006; KHATIMA AIT-OUDHIA et al., 2011). Os efeitos tóxicos dos medicamentos ocorrem, particularmente, em células com alta atividade metabólica, tais como: células neuronais, tubulares renais, intestinais e germinativas da medula óssea (HARVEY et al., 1998; DELORENZI et al., 2001; MONDAL et al., 2010; ALMEIDA; SANTOS, 2011).

Dentre os principais problemas e desafios para o controle da leishmaniose, destacam-se: o diagnóstico tardio, as dificuldades de controle de vetores, a epidemia de AIDS, a falta de vacinas eficazes, o desenvolvimento de resistência à quimioterapia e a migração populacional entre os continentes (HERWALDT, 1999; HEPBURN, 2000; DESJEUX et al., 2001; ALVAR et al., 2006; SUNDAR e CHATTERJEE, 2006; SHAW, 2007; MITROPOULOS et al., 2009).

O arsenal terapêutico para o tratamento da leishmaniose é ainda dependente de um limitado número de fármacos (MALTEZOU, 2009; ALMEIDA; SANTOS, 2011). O fármaco de primeira escolha é o antimonial pentavalente: antimoniato de VV-metil-glucamina (Glucantime®). Este antimoniato é indicado para todas as formas de leishmaniose tegumentar, embora as formas mucosas exijam maiores cuidados, podendo apresentar respostas mais lentas aos fármacos e possibilidades de recidiva (FUNASA/MS, 2000; AÏT-OUDHIA et al., 2011). Todavia, fatores como eficácia reduzida (altas taxas de recidiva dos pacientes e resistência do parasita), dificuldades de administração (via parenteral, intramuscular ou endovenosa), aumento da frequência e gravidade dos efeitos colaterais (artralgia, mialgia, inapetência, náuseas, vômitos, plenitude gástrica, epigastralgia, pirose, dor abdominal, prurido, febre, fraqueza, cefaléia, tontura, palpitação, insônia, nervosismo, choque pirogênico,

edema e insuficiência renal aguda) têm estimulado a busca de novos fármacos anti-leishmania (FUNASA/MS, 2000; ALMEIDA; SANTOS, 2011; TIUMAN et al., 2011).

Não havendo resposta satisfatória com Glucantime® ou na impossibilidade do seu uso, os fármacos de segunda escolha são a anfotericina B e a pentamidina. A anfotericina B é um antibiótico poliênico com atividade antifúngica que foi originalmente extraído de *Streptomyces nodosus* e está sendo utilizado frente leishmaniose desde 1960 (CROFT et al., 2006; MALTEZOU, 2009). Todavia, apesar de ser eficaz no tratamento de pacientes intolerantes aos antimoniais, apresenta um alto grau de toxicidade e efeitos colaterais como hipocalemia, febre, nefrotoxicidade e reações anafiláticas (FUNASA/MS, 2000). Por conseguinte, anfotericina B desoxicolato (Fungizone®), é uma formulação micelar eficaz contra as formas cutâneas e mucosas. Está sendo usado como primeira linha de tratamento em áreas com altos índices de insensibilidade aos antimoniais (ALVAR et al., 2006; AMATO et al., 2008). Contudo, também apresenta desvantagens, tais como a necessidade de hospitalização, duração prolongada do tratamento e efeitos colaterais como febre, calafrios e tromboflebite (CROFT; COOMBS, 2003; OLLIARO et al., 2005; SEIFERT, 2011).

Um importante avanço no tratamento das leishmanioses foi o advento da anfotericina B lipídicas e sua formulações. A anfotericina B lipossomal (AmBisome ®) foi aprovada para o tratamento da leishmaniose visceral pela *Food and Drug Administration* dos Estados Unidos da América (FDA) (MEYERHOFF, 1999).

Essa formulação apresenta maior segurança e menor duração do tratamento, uma vez que apresenta toxicidade reduzida, possibilita regimes terapêuticos mais curtos, redução do tempo de hospitalização e diminuição dos efeitos secundários, como a nefrotoxicidade. Destaque-se, ainda, que um tratamento de dose única com anfotericina B lipossomal cura mais de 90% por cento dos pacientes (SEIFERT, 2011). Murray, (2010) reportou em seu editorial a respeito do tratamento de uma paciente com leishmaniose tratada com AmBisome®: "...ela foi hospitalizada, mas apenas por 48 h para receber, uma vez por dia, durante dois dias, infusões intravenosas de 10 mg/kg de AmBisome®. O tratamento foi bem tolerado, a recuperação foi rápida e a infecção resolvida. Após seis meses, foi realizado os exames e constatou-se que a paciente estava totalmente saudável". Todavia, seu alto custo é o obstáculo que impede a sua acessibilidade generalizada em países endêmicos (CROFT; COOMBS, 2003; LIMA et al., 2007; MURRAY, 2010).

A pentamidina, comercializada sob o nome de Lomidina®, tem sido utilizada como fármaco de segunda escolha para o tratamento contra as leishmanioses cutânea

e visceral há mais de quatro décadas. No tratamento da leishmaniose visceral a pentamidina foi usada com sucesso, em séries de 12 a 15 doses. A segunda série, administrada após intervalo de 1 a 2 semanas, pode ser necessária em áreas onde se sabe que a infecção responde de modo insatisfatório ao tratamento (RATH et al., 2003). Contudo, também apresenta efeitos colaterais severos como diabetes, hipoglicemia, nefrotoxicidade, taquicardia e muita dor no local da injeção (MALTEZOU, 2009; TIUMAN et al., 2011).

A paramomicina é um antibiótico aminoglicosídico produzido por *Streptomyces* spp e pode ser utilizado para o tratamento da Leishmaniose Visceral (LV) e Leishmaniose Cutânea (LC). Entretanto, não é bem absorvido de forma oral. De maneira que é administrado de forma parenteral e tópica para as formas visceral e cutânea, respectivamente. Garnier e Croft, (2002) destacam a importância de formulações tópicas para o tratamento da LC, porém, as duas pomadas de paromomicina que estão comercialmente disponíveis têm seu uso limitado pela toxicidade ou ausência de eficácia (CROFT et al., 2006; MINODIER; PAROLA, 2007; SUNDAR et al., 2007; SEIFERT, 2011).

A Miltefosina® (hexadecilfosfocolina), um composto originalmente desenvolvido como um agente antitumoral, é considerada o primeiro fármaco de aplicação oral efetiva (MURRAY et al., 2005). Está registrado para o tratamento de leishmaniose visceral na Índia e Alemanha e para leishmaniose cutânea na Colômbia (ALVAR et al., 2006; SUNDAR; CHATTERJEE, 2006; LIMA et al., 2007; SEIFERT, 2011). Desde 1998, pesquisadores indianos realizam ensaios clínicos avaliando o fármaco Miltefosina® em pacientes com leishmaniose visceral. Em 1999, foram iniciados estudos clínicos na Colômbia, em pacientes com leishmaniose tegumentar. Estima-se que mais de 2.500 pacientes com leishmaniose foram tratados com Miltefosina® na Índia (LV por *L. donovani)* e Colômbia (LC causada por *L. panamensis)* (SOTO; SOTO, 2006). Obteve-se taxas de cura de mais de 90% desses pacientes quando tratados com a dose de 2,5 mg/kg/dia durante 28 dias. Na Guatemala, a taxa de cura para pacientes com leishmaniose cutânea foi de 53% (33% para *L. braziliensis* e 60% em *L. mexicana),* enquanto no Afeganistão a taxa de cura de pacientes com *L. tropica* foi de 63%. Pacientes com leishmaniose cutânea difusa, mucocutânea, e co-infectados com HIV têm sido tratados com sucesso inicialmente, porém, estas doenças têm recidivas freqüentes (SOTO et al., 2004; SOTO; SOTO, 2006).

Corroborando, Zerpa et al. (2006) demonstraram que pacientes com leishmaniose cutânea difusa apresentaram melhora clínica significativa e redução da carga parasitária nos primeiros 15 dias após o início do tratamento. Durante 45 dias, 15 pacientes

apresentaram 80-90% de melhora clínica. No entanto, a suspensão do tratamento foi seguido pelo desenvolvimento de novas lesões em todos, exceto um paciente.

Dentre as contra-indicações da Miltefosina®, deve ser enfatizado o seu potencial teratogênico, por isso não pode ser prescrito para mulheres grávidas. Ademais, a contracepção deve ser assegurada em mulheres em idade fértil. Achados de segurança em geral indicam o trato gastrointestinal como principal órgão alvo dos efeitos colaterais, além da elevação moderada e transitória de enzimas hepáticas (SOTO; SOTO, 2006; SUNDAR; CHATTERJEE, 2006; SEIFERT, 2011).

Todavia, ainda é muito pequeno o número de drogas efetivas, seguras e disponíveis (ALMEIDA; SANTOS, 2011; SEIFERT, 2011; TIUMAN et al., 2011). Espera-se que em um futuro próximo tenhamos os meios eficazes para prevenir e tratar todas as formas de leishmaniose sem desconforto aos pacientes.

Historicamente, os produtos naturais derivados de plantas têm-se revelado uma fecunda fonte de novos agentes terapêuticos (ROCHA et al., 2005; BEUTLER, 2009; SEN; CHATTERJEE, 2011; IZUMI et al., 2011; TIUMAN et al., 2011). O emprego das plantas no tratamento das doenças dava-se de maneira empírica, de tal forma que os conhecimentos adquiridos foram sendo passados ao longo do tempo, de geração para geração (CUNHA et al., 2003; SANTOS et al., 2008a, 2008b). Ante as limitações dos fármacos existentes, o emprego de conhecimentos calcados no método empírico para a síntese de drogas derivadas de plantas tornou-se uma alternativa bastante promissora. Com efeito, as plantas podem ser fontes potenciais de novas drogas seguras e efetivas de inestimável valor no tratamento de doenças infecciosas.

Nesse contexto, a busca por novos princípios ativos isolados de plantas, bem como protótipos moleculares para síntese de substâncias análogas mais potentes e seletivas, as quais podem ser obtidas mais facilmente e a custos menores, tem fomentado o investimento de muitas indústrias farmacêuticas em estudos envolvendo plantas (SIMÕES et al., 2004). Dentro desta perspectiva, pode-se afirmar que o Brasil é um país privilegiado, uma vez que detém extensa e diversificada flora, com destaque para as formas de vegetação encontradas por todo o território nacional - Floresta Amazônica, Mata Atlântica, Cerrado, Caatinga e Pantanal (SOUZA BRITO et al., 1993; CALIXTO, 2000; YUNES et al., 2001; WANG et al., 2008).

Inúmeros compostos encontrados em plantas superiores apresentam atividade seletiva em relação aos micro-organismos patogênicos, dentre os quais, destacam-se os alcalóides, terpenos, quinonas e flavonóides. Estes podem ser objetos de estudo para a

identificação de novas drogas e/ou para modificações semi-sintéticas para melhorar a atividade terapêutica e diminuir os efeitos tóxicos do composto (BRENZAN et al., 2008 SANTIN et al., 2009; SANTOS et al., 2010; VENDRAMETTO et al., 2010; SEN; CHATTERJEE, 2011).

Em todo o mundo, inúmeros grupos de pesquisa investigam as possíveis atividades biológicas das plantas, baseando-se em informações etnobotânicas. Os dados obtidos atestam cada vez mais, e de maneira científica, as propriedades terapêuticas das plantas, em detrimento dos resultados empíricos de outrora. O Quadro 1 apresenta estudos realizados nos últimos cinco anos que reportam a atividade antileishmania de diversas plantas.

Quadro 1 – Atividade antileishmania de extratos brutos, frações, compostos isolados e óleos essenciais obtidos de plantas.

FAMÍLIA/espécie da planta	Extratos/Frações ou Compostos	*Espécie Leishmania*	IC50 (ng/mL)		Ref
			PRO	AMA	
Aloeaceae					
Aloe nyeriensis	Fração metanólica	*L. major*	68.4	ND	56
	Fração aquosa	*L. major*	53.3	ND	56
Annonaceae					
Annona coriacea	Fração alcalóides totais	*L. chagasi*	41.6	ND	57
Annona crassiflora	Fração alcalóides totais	*L. chagasi*	24.9	ND	57
Annona muricata	Fração acetato de etila	*L. amazonensis*	25.0	NT	58
Guatteria australis	Fração alcalóides totais	*L. chagasi*	37.9	ND	57
Polyalthia suaveolens	Fração metanólica	*L. infantum*	1.8	8.6	59
Pseudomalmea boyacana	Fração acetato de etila	*L. amazonensis*	48.9	NT	58
Rollinia exsucca	Fração Hexano	*L. amazonensis*	20.8	NT	58
Rollinia pittieri	Fração Hexano	*L. amazonensis*	12.6	NT	58
Xylopia aromatica	Fração metanólica	*L. amazonensis*	20.8	NT	58
Apocynaceae					
Himatanthus sucuuba	Fração etanólica	*L. amazonensis*	20.0	5.0	60
Pagiantha cerifera	Fração diclorometano	*L. amazonensis*	25.0	12.5	61
Asteraceae					
Achillea millefolium	Oleo essencial	*L. amazonensis*	7.8	6.5	62
Anthemis auriculata	Anthecotulide	*L. donovani*	NT	8.18	63
	4-hydroxyanthecotulide	*L. donovani*	NT	3.27	63
	4-acetoxyanthecotulide	*L. donovani*	NT	12.5	63
Baccharis dracunculifolia	Extrato bruto	*L. donovani*	45.0	NT	64
	Hautriwaic acid lactone	*L. donovani*	7.0	NT	64
	Ursolic acid	*L. donovani*	3.7	NT	64
	Uvaol	*L. donovani*	15.0	NT	64
	2a-hydroxy-ursolic acid	*L. donovani*	19.9	NT	64
Calea montana	Fração etanólica	*L. amazonensis*	NT	10.0	65
Elephantopus mollis	Fração diclorometano	*L. donovani*	NT	0.6	66
Tanacetum parthenium	Extrato bruto	*L. amazonensis*	490	74.8	267
	Fração diclorometano	*L. amazonensis*	3.6	2.7	267
	Parthenolide	*L. amazonensis*	0.37	0.81	68
	Guaianolide	*L. amazonensis*	2.6	ND	69
Vernonia polyanthes	Fração metanólica	*L. amazonensis*	4.0	NT	70

Caricaceae					
Carica papaya	Fração etanólica	*L. amazonensis*	NT	11.0	65
Celastraceae					
Maytenus putterlickoides	Fração metanólica	*L. major*	60.0	ND	56
Clusiaceae					
Calophyllum brasiliense	(-) mammea A/BB	*L. amazonensis*	3.0	0.88	71
Crassulaceae					
Kalanchoe pinnata	Quercetin di-glycoside	*L. amazonensis*	NT	45.0	72
Fabaceae					
Acacia tortilis	Extrato aquoso	*L. major*	52.9	ND	56
Albizia coriara	Extrato aquoso	*L. major*	66.7	ND	56
Copaifera reticulata	Oleo resina	*L. amazonensis*	5.0	15.0	73
Flacourtiaceae					
Laetia procera	Casearlucine A	*L. amazonensis*	11.1	5.98	74
	Caseamembrol A	*L. amazonensis*	11.0	10.5	74
	Laetiaprocerine A	*L. amazonensis*	10.9	47.4	74
	Laetiaprocerine D	*L. amazonensis*	50.9	30.3	74
	Butanolide	*L. amazonensis*	111.0	129.0	74
Ginkgoaceae					
Ginkgo biloba	Isoginkgetin	*L. amazonensis*	NT	1.9	75
Goodneniaceae					
Scaevola balansae	Fração diclorometano	*L. amazonensis*	8.7	NT	76
Laminaceae					
Hyptis lacustris	Fração etanólica	*L. amazonensis*	NT	10.0	65
Ocimum gratissimum	Oleo essencial	*L. amazonensis*	135.0	100.0	77
	Eugenol	*L. amazonensis*	80.0	NT	77
	Fração metanólica	*L. chagasi*	71.0	NT	70
Premna serratifolia	Fração diclorometano	*L. amazonensis*	4.4	NT	76
Lecythidaceae					
Careya arborea	Arborenin	*L. donovani*	15.0	12.5	78
Liliaceae					
Asparagus racemosus	Fração metanólica	*L. major*	58.8	ND	56
	Extrato aquoso	*L. major*	56.8	ND	56
Malpighiaceae					
Lophanthera lactescens	LLD3	*L. amazonensis*	NT	0.41	79
Meliaceae					
Dysoxylum binectariferum	Fração clorofórmio	*L. donovani*	50.0	ND	80
	Rohitukine	*L. donovani*	100.0	ND	80
Menispermaceae					
Cissampelos ovalifolia	Fração de alcalóides totais	*L. chagasi*	63.9	ND	57
Olacaceae					
Minquartia guianensis	Fração diclorometano	*L. donovani*	NT	2.8	66
Papaveraceae					
Bocconia integrifolia	w-hexane extract	*L. donovani*	NT	1.8	66
	Fração diclorometano	*L. donovani*	NT	0.5	66
	Fração metanólica	*L. donovani*	NT	0.7	66
Piperaceae					
Piper auritum	Oleo essencial	*L. donovani*	12.8	22.3	81
Piper dennisii	Fração etanólica	*L. amazonensis*	NT	10.0	65
Piper hispidum	Fração etanólica	*L. amazonensis*	69.0	5.0	82
Piper regnellii	Eupomatenoid-5	*L. amazonensis*	9.0	5.0	83
Piper strigosum	Fração etanólica	*L. amazonensis*	>100	7.8	82
Piper sp.	Fração diclorometano	*L. donovani*	NT	2.2	66
Poaceae					
Cymbopogon citratus	Oleo essencial	*L. amazonensis*	1.7	3.2	84
	Citral	*L. amazonensis*	8.0	25	84

Rhamnacea					
Gouania lopuloides	Fração diclorometano	*L. donovani*	NT	1.9	66
	Fração metanólica	*L. donovani*	NT	2.9	66
Rutaceae					
Galipea panamensis	Coumarin 1	*L. panamensis*	NT	9.9	85
	Coumarin 2	*L. panamensis*	NT	10.5	85
	Phebalosin	*L. panamensis*	NT	14.1	85
	Artifact murralongin	*L. panamensis*	NT	>100	85
	Murrangatin acetonide	*L. panamensis*	NT	NT	85
Scrophulariaceae					
Scoparia dulcis	Fração diclorometano	*L. donovani*	NT	1.8	66
Scrophularia cryptophila	Crypthophilic acid A	*L. donovani*	NT	12.8	86
	Crypthophilic acid C	*L. donovani*	NT	5.8	86
	Harpagide	*L. donovani*	NT	2.0	86
	Acetylharpagide	*L. donovani*	NT	6.9	86
	Buddlejasaponin III	*L. donovani*	NT	6.2	86
Solanaceae					
Brugmansia sp.	Fração diclorometano	*L. donovani*	NT	3.0	66
Umbelliferae					
Ferula szowitsiana	Auraptene	*L. major*	5.1	NT	87
	Umbelliprenin	*L. major*	4.9	NT	87
Verbenaceae					
Lantana sp.	Fração etanólica	*L. amazonensis*	NT	10.0	65
Zingiberaceae					
Hedychium coronarium	Fração etanólica	*L. amazonensis*	NT	10.0	65

PRO: Promastigotas; AMA: Amastigotas; NT: não-testado; IC_{50} concentração em ug/mL que inibe 50% das células; ND: não determinado. Tabela adaptada de TIUMAN et al., 2011.

Ante um prospecto de sucesso no estudo de plantas com propriedades medicinais, no presente, resolvemos investigar os efeitos biológicos *in vitro* e *in vivo* do óleo de copaíba em *L. amazonensis.*

Plantas do gênero *Copaifera* (família Fabaceae, sub-família Caesalpinoideae) são árvores de grande porte (alcançam de 25 e 40 metros de altura), conhecidas popularmente como copaibeiras ou pau d'óleo. Tais árvores secretam um óleo-resina de odor forte e característico, com propriedades medicinais, chamado de óleo de copaíba, popularmente conhecido também como Panchimoiti, Palo de aceite, Cabimo, Copahyba, Copaibarana, Copaúba, Copaibo, Copal, Maram, Marimari e Bálsamo dos Jesuítas (VEIGA JR et al., 2001; VEIGA-JR; PINTO, 2002).

Contudo, a classificação química para o óleo de copaíba é óleo-resina, por ser um exsudato constituído por ácidos resinosos e compostos voláteis. Também é conhecido como "bálsamo" de copaíba, apesar de não ser um bálsamo verdadeiro, uma vez que não contém derivados do ácido benzóico ou cinâmico. Este óleo é encontrado em canais secretores formados pela dilatação de espaços intercelulares (meatos) que se intercomunicam no meristema, chamados de canais esquizógenos. Estes canais estão localizados em todas as

partes da árvore, no entanto, o caráter mais saliente deste aparelho secretor está no tronco (SIMÕES et al., 2004).

A coleta indicada do óleo da copaíba é a realizada através de uma incisão com trado a cerca de 1 metro de altura do tronco. Terminada a coleta é aconselhável vedar o orifício com argila para impedir a infestação da árvore por fungos ou cupins. Dessa forma, a argila pode ser facilmente retirada, permitindo que se façam outras coletas no mesmo tronco. Segundo relatos, uma única árvore pode gerar de 40 a 50 litros de óleo por ano, apesar de nem todas as espécies serem capazes de produzir essa quantidade (VEIGA-JR; PINTO, 2002; OLIVEIRA et al., 2006; GOMES et al., 2007). Fatores climáticos e a época do ano devem ser considerados no processo de extração do óleo resina. A composição química das espécies de *Copaifera* pode apresentar diferenças quantitativas e qualitativas. A presença de substâncias químicas em uma determinada espécie pode estar atrelada a sua sazonalidade, variando em função de alguns fatores, tais como: temperatura, radiação solar e precipitação pluviométrica (VEIGA JR et al., 1995; VEIGA JR et al., 1997; CASCON;GILBERT, 2000; OLIVEIRA et al., 2006; SANTOS et al., 2008b).

As copaibeiras são nativas da região tropical da América Latina e da África Ocidental. No Brasil, são encontradas nas Regiões Amazônicas, Nordeste e Centro-Oeste. Entre as espécies mais abundantes destacam-se: *C. officinalis* (norte do Amazonas, Roraima, Colombia, Venezuela e San Salvador), *C. guianensis* (Guianas), *C. reticulata, C. multijuga* (Amazônia), *C. confertiflora* (Piauí), *C. langsdorffii* (Brasil, Argentina e Paraguai), *C. coriacea* (Bahia) e *C. cearensis* (Ceará) (VEIGA-JR; PINTO, 2002).

Conforme relatado em documentos históricos, as propriedades medicinais do óleo de copaíba foram descritas por índios brasileiros. Tudo indica que o uso deste óleo veio da observação do comportamento de certos animais que, quando feridos, esfregavam-se nos troncos das copaibeiras. (VEIGA-JR; PINTO, 2002).

A utilização do óleo da copaíba na medicina popular ocorre de várias maneiras: anti-blenorrágico, antiinflamatório, anti-séptico, antiasmático, bronquite, espectorante, inflamações da garganta, pneumonia, dermatite, eczema, cicatrizante de feridas e úlceras, afrodisíaco, antitetânico, anti-reumático, anticancerígeno, leishmaniose, dores de cabeça, leucorréia e picadas de cobra. Com efeito, observa-se uma ampla gama de propriedades farmacológicas. Todavia, as principais atividades relatadas foram de antiinflamatório das vias superiores, inferiores e como cicatrizante (LIMA et al., 2003; CARVALHO et al., 2005; VEIGA-JR et al., 2006; FERNANDES et al., 2007; GOMES et al., 2007).

Hoje, o óleo da copaíba pode ser encontrado em qualquer farmácia natural e de manipulação do país. Estudos farmacológicos mostram que o uso do óleo pelos índios é plenamente justificado. Estudos *in vitro* e *in vivo* demonstram que o óleo de várias espécies de *Copaiferas* possui atividades biológicas. Dentre estas, destacam-se: antiinflamatória, antimicrobiana, antiparasitária, antitumoral, anti-blenorrágica, para cura da bronquite, , doenças da pele, úlcera, e também como cicatrizante de feridas (VEIGA-JR et al., 2001; LIMA et al., 2003; PAIVA et al., 2004; BARRETO-JR et al., 2005; BRITO et al., 2005; ARAÚJO-JR et al., 2005; CAVALCANTI et al., 2006; VEIGA-JR. et al., 2007; SANTOS et al., 2008a; SANTOS et al., 2008b; GOMES et al., 2010; IZUMI, 2011).

Estudo recente realizado por Santos et al. (2008a) reportou que óleo da copaíba obtido de diferentes espécies de *Copaifera* tem atividade contra formas promastigotas de *L. amazonensis.* Além disso, demonstrou a atividade do óleo obtido de *C. reticulata* contra formas amastigotas axênicas e amastigotas intracelulares de *L. amazonensis.*

3 OBJETIVOS

- Investigar a atividade *in vitro* do óleo de copaíba, bem como de seus compostos isolados, pela da determinação das concentrações inibitórias sobre formas promastigotas e amastigotas axênicas;
- Visualização das alterações morfológicas e ultraestruturais das células dos parasitas tratados;
- Avaliação da atividade do óleo de copaíba e compostos isolados na permeabilidade da membrana celular e potencial de membrana mitocondrial.
- Avaliar a atividade biológica *in vivo* do óleo de copaíba em modelos animais infectados com *L. amazonensis,* sua toxicidade e genotoxicidade.

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Research Article

Copaiba Oil: An Alternative to Development of New Drugs against Leishmaniasis

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Leishmaniasis is a neglected disease that is increasing globally at an alarming rate. Glucantime has been the therapy of choice for more than 50 years. A recent study reported the antileishmanial activity of copaiba oil against *Leishmania amazonensis*. These results led us to investigate morphological and ultrastructural changes in *L. amazonensis* treated with copaiba oil, using electron microscopy and flow cytometry to assess specific organelles as targets for copaiba oil. In the promastigote and axenic amastigote forms, this copaiba oil caused notable morphological and ultrastructural changes, including extensive mitochondrial damage and denaturation of the plasma membrane. Copaiba oil treatment also induced a decrease in Rh123 fluorescence, suggesting interference with the mitochondrial membrane potential and loss of cell viability with an increase in plasma membrane permeability, as observed by flow cytometry after staining with propidium iodide. In conclusion, copaiba oil could be exploited for the development of new antileishmanial drugs.

1. Introduction

Parasites of the genus *Leishmania* cause a wide spectrum of human infections, ranging from the disfiguring mucosal and cutaneous forms of the disease to the life-threatening visceral form [1–4]. The species and/or strains of the *Leishmania* parasites strongly influence the clinical features of leishmaniasis, including host immunity [5]. There are approximately 21 species of *Leishmania*, transmitted by about 30 phlebotomine sandfly species [6]. The number of cases of leishmaniasis is increasing globally at an alarming rate. According to the World Health Organization, this group of diseases is endemic in 88 countries in Africa, Asia, Europe, and North and South America, with a total of 350 million people at risk [7]. Despite its increasing worldwide incidence, leishmaniasis has become one of the so-called neglected diseases, with little interest by financial donors, public-health authorities, and professionals to implement activities to research, prevent, or control the disease [8–10]. During a complex digenetic life cycle, flagellated *Leishmania* parasites alternate between promastigote (living in the gut of their sandfly vector) and amastigote forms (living in their mammalian host), which differ significantly in cellular morphology and flagellum length [11]. The parasites have developed a variety of adaptive mechanisms to evade the vertebrate host immune responses, including survival within the host macrophage [12, 13]. Because leishmaniasis mainly affects poor countries, research and development of new drugs have been seriously neglected [6, 8, 10, 14]. The pentavalent antimonials (Glucantime) have been the therapy of choice for more than 50 years. Although the lipid formulations of amphotericin B are an important advance in therapy, their high cost precludes their use [15]. Consequently, there is an urgent need to discover new drugs that are effective against leishmaniasis. Plants are very promising subjects, since they have been important sources of substances with

many therapeutic uses [16–18]. In addition, the American flora is one of the world's wealthiest sources of material with pharmacological activity, due to its biodiversity [19]. Copaiba oil has been used in folk medicine since the 19th century [19, 20]. The use of copaiba oils to treat leishmaniasis is cited in several ethnopharmacological studies [21–24]. A recent study by Santos et al. [25] found that copaiba oils obtained from different species of *Copaifera* show activity against promastigote forms of *L. amazonensis*. Significant antileishmanial activity of copaiba oil from *C. reticulata* was demonstrated against axenic amastigote and intracellular amastigote forms of the parasite. These findings led us to investigate the morphological and ultrastructural changes in *L. amazonensis* treated with copaiba oil, using electron microscopy and flow cytometry to investigate specific organelles as targets for copaiba oil.

2. Materials and Methods

2.1. Plant Material. *Copaifera reticulata* Ducke was collected in Belém, state of Pará, and deposited in the Herbarium of the Instituto Nacional de Pesquisas da Amazônia (INPA, Manaus) under number INPA 61,212.

2.2. Parasites. *Leishmania amazonensis* MHOM/BR/75/Josefa strain was isolated from a patient with diffuse cutaneous leishmaniasis by Cesar Augusto Cuba-Cuba (Universidade de Brasília, Brazil). Promastigote forms were cultured at 25°C in Warren's medium (brain-heart infusion plus haemin and folic acid), pH 7.0, supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Gibco Invitrogen Corporation, New York, USA), in a tissue flask with weekly transfers. Axenic amastigote cultures obtained by *in vitro* transformations of infective promastigotes [26] were incubated at 32°C in Schneider's insect medium (Sigma Chemical Co., St. Louis, MO, USA), pH 4.6, with 20% foetal bovine serum.

2.3. Scanning Electron Microscopy. The parasites were treated with concentrations of copaiba oil from *C. reticulata* that inhibited 50% and 90% of growth (IC_{50} and IC_{90}, resp.). Promastigotes were treated with 5.0 and 80.0 μg/mL at 25°C, and axenic amastigote forms were treated with 15.0 and 70.0 μg/mL at 32°C. Next, the parasites were collected by centrifugation after 72 h incubation, washed in PBS buffer, fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 1.0 mM $CaCl_2$. After fixation, small drops of the sample were placed on a specimen support with poly-L-lysine. The samples were then dehydrated in graded ethanol, critical-point dried in CO_2, coated with gold, and observed in a Shimadzu SS-550 scanning electron microscope.

2.4. Transmission Electron Microscopy. Promastigote and axenic amastigote forms of *L. amazonensis* were treated with IC_{50} and IC_{90} of copaiba oil. After that, the samples were processed for transmission electron microscopy. Parasite cells were harvested and washed twice with PBS buffer, and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4°C, postfixed in a solution containing 1% OsO_4, 0.8% potassium ferrocyanide, and 10 mM $CaCl_2$ in 0.1 M cacodylate buffer, dehydrated in an increasing acetone gradient, and embedded in Epon resin. Next, ultrathin sections were stained with uranyl acetate and lead citrate, and images were obtained in a Zeiss 900 TEM.

2.5. Measurement of Mitochondrial Membrane Potential. The mitochondrial membrane potential was measured in *L. amazonensis* axenic amastigotes using Rhodamine 123 (Rh 123) reagent, following the manufacturer's protocol. Briefly, axenic amastigotes (5×10^6 parasites/mL) after treatment with copaiba oil from *C. reticulata* (100, 150, 200 μg/mL for 3 h at 32°C) or untreated cells were harvested and washed with PBS buffer. After that, the cells were incubated with Rh 123 (5 mg/mL for 30 min at 37°C) and washed twice with PBS buffer. The mean fluorescence intensity was analysed using FACSCalibur and CellQuest software. A total of 10,000 events were acquired in the region established as that corresponding to the parasites. The compound carbonyl cyanide m-chlorophenylhydrazone (CCCP) was used as a positive control.

2.6. Measurement of Cell Viability. Cell viability was checked by staining the cells with propidium iodide (PI). Axenic amastigotes treated with copaiba oil from *C. reticulata* (100 and 200 μg/mL for 3 h at 32°C) or untreated amastigotes were harvested and washed with PBS buffer. Then, the parasite cells were stained with PI (20 μg/mL for 5 min) as per instructions given by the manufacturer. The mean fluorescence intensity of the cells was analysed by flow cytometry, using FACSCalibur and CellQuest software. A total of 10,000 events were acquired in the region established as that corresponding to the parasites. Amphotericin B was used as a positive control.

2.7. Statistical Analysis. The comparison between the values of fluorescence for Rh123 and PI was performed with the program GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). Student's t-test was applied, and a P-value less than .05 was regarded as significant. The means and standard deviations were determined from at least three experiments.

3. Results and Discussion

Copaiba oils obtained from different species of *Copaifera* showed activity against promastigote forms of *L. amazonensis*. Significant antileishmanial activity of copaiba oil from *C. reticulata* was demonstrated against axenic amastigote and intracellular amastigote forms of the parasite with IC_{50} values of 15.0 μg/mL and 20.0 μg/mL, respectively. As reported recently, the major component of *C. reticulata* is β-caryophyllene (40.9%) [25]. Therefore, the antileishmanial activity of β-caryophyllene was tested against all forms of the parasite. However, β-caryophyllene did not show antileishmanial activity against amastigote-like forms, compared with copaiba oil (data not shown). Some investigators have attributed the biological activities of copaiba oil to the complex nature of the sesquiterpene and diterpene mixture,

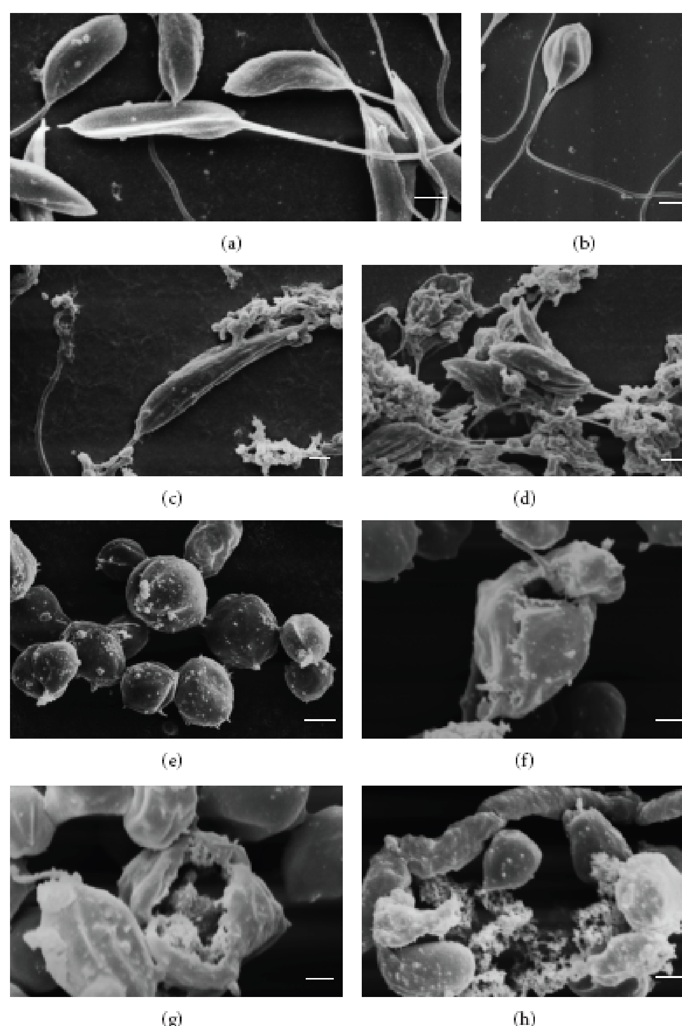

Figure 1: Scanning electron micrographs of promastigote and axenic amastigote forms of *L. amazonensis* treated with copaiba oil for 72 h. (a) promastigote, control; (b) promastigote after treatment with IC_{50} of copaiba oil; (c and d) promastigote after treatment with IC_{90} of copaiba oil; (e) amastigote, control; (f) amastigote after treatment with IC_{50} of copaiba oil; (g and h) amastigote after treatment with IC_{90} of copaiba oil. Bars = 1 μm.

which might affect the active component by a synergistic effect [27]. Additionally, Lima et al. [28] reported that fractionation of copaiba oils results in fractions that are less active than the crude oil itself.

These results led us to investigate the morphological and ultrastructural changes in *L. amazonensis* treated with copaiba oil from *C. reticulata,* using electron microscopy. As observed by scanning electron microscopy, promastigote and axenic amastigote forms treated with copaiba oil showed notable morphological changes (Figure 1). Untreated control promastigotes showed the typical elongated shape with a single flagellum (Figure 1(a)), and axenic amastigotes a rounded shape (Figure 1(e)). The typical morphology of promastigotes and axenic amastigotes changed drastically after treatment with copaiba oil. Promastigote forms treated with copaiba oil showed a rounded shape with two flagella (Figure 1(b)). In amastigote-like forms, treatment with copaiba oil led to rupture of the plasma membrane with loss of their contents (Figures 1(f) and 1(g)). Additionally, both forms showed protein denaturation of the cell surface (Figures 1(c), 1(d), 1(g), and 1(h)). Several studies have found that the cell surface (carbohydrates associated with lipids to form glycolipids) of parasitic protozoa plays an important role in various processes including cell recognition, cell adhesion, regulation of cell growth, expression of surface antigens, and receptors [29–31]. Consequently,

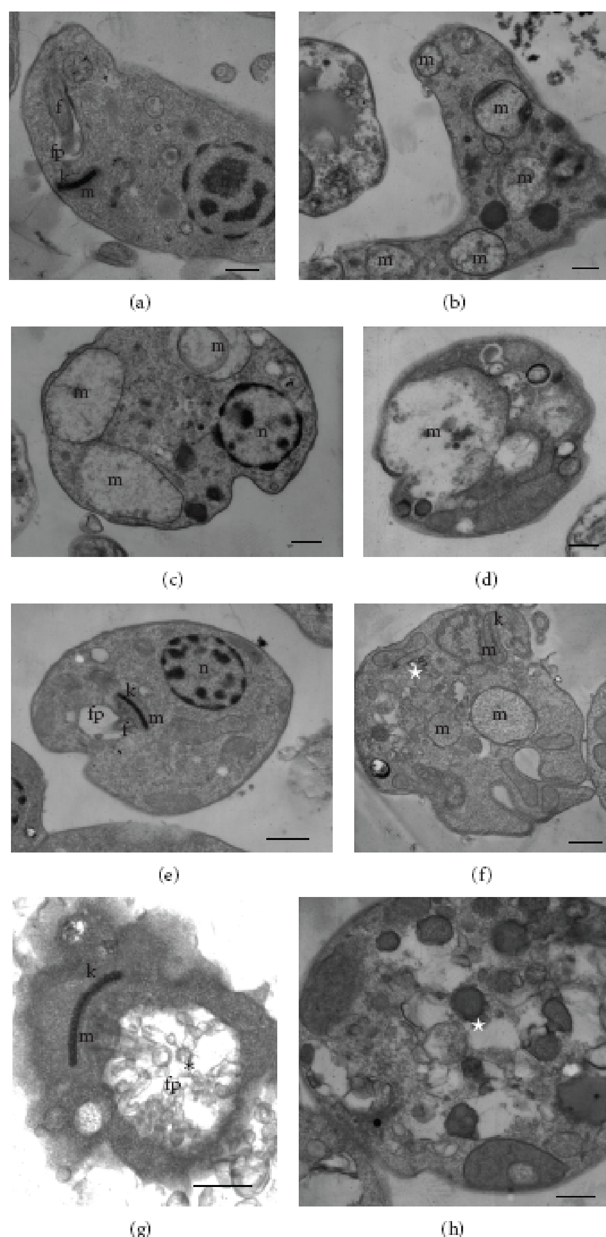

Figure 2: Ultrastructural effect of copaiba oil after incubation for 72 h on promastigote and axenic amastigote forms of *L. amazonensis*, observed by transmission electron microscopy. (a) promastigote control; (b) promastigote treated with IC_{50} of copaiba oil; (c and d) promastigote treated with IC_{90} of copaiba oil; (e) amastigote, control; (f) amastigote treated with IC_{50} of copaiba oil; (g and h) amastigote treated with IC_{90} of copaiba oil. Bars = 1 μm. The treatment with copaiba oil led to changes in the mitochondria (m), exocytic activity in the region of the flagellar pocket (asterisk), and cytoplasmic vacuolisation (white star). n, nucleus; f, flagellum; fp, flagellar pocket; k, kinetoplast; m, mitochondrion.

surface changes caused by copaiba oil treatment may affect the parasite-host interaction, decreasing the infectivity of the parasite. After treating the intracellular amastigotes with copaiba oil from *C. reticulata*, Santos et al. [25] observed a dose-dependent decrease of parasites in the host peritoneal macrophages. The cell viability of axenic amastigotes of *L. amazonensis* was checked by staining the cells with propidium iodide (PI), a fluorescent dye that binds specifically to DNA. In untreated axenic amastigotes, the degree of binding of PI was 1.86%. Following treatment of axenic amastigotes

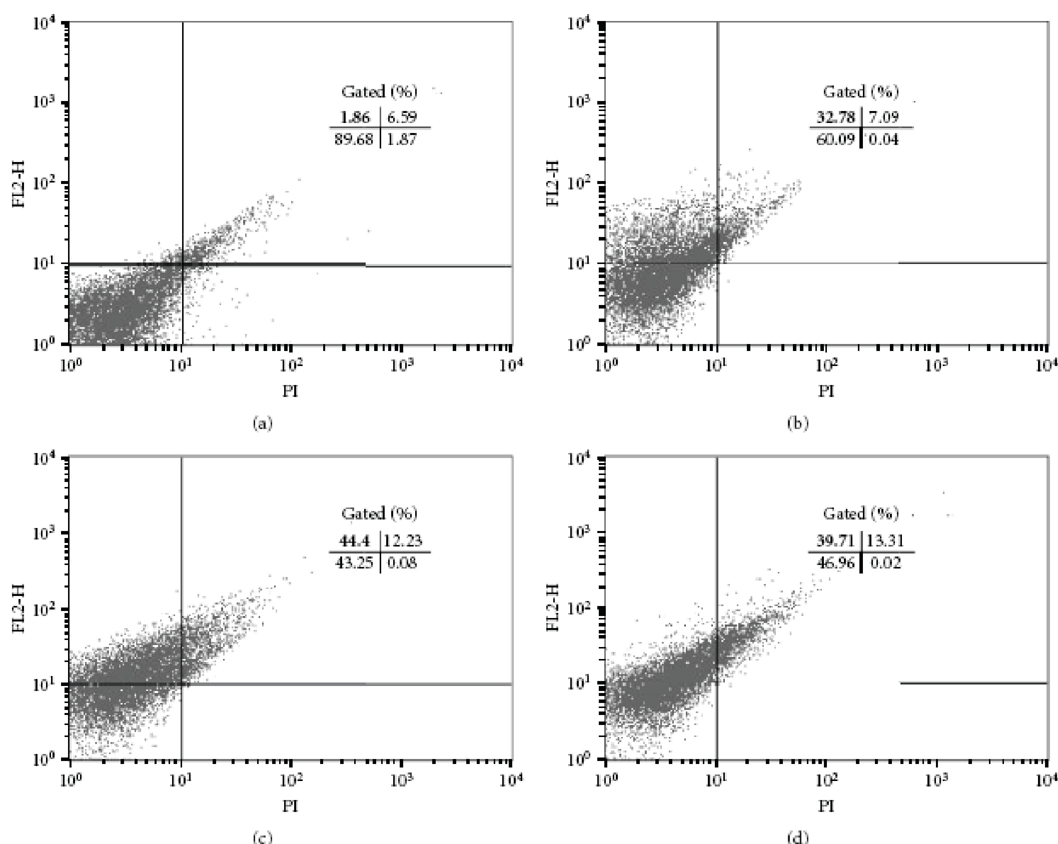

FIGURE 3: Flow cytometry analysis of *L. amazonensis* axenic amastigotes treated with copaiba oil and stained with propidium iodide (PI), showing a decrease of cell viability in treated cells, (a) Untreated cells (b) amphotericin-B; (c) amastigotes treated with 100 μg/mL; (d) amastigotes treated with 200 μg/mL. The numbers in bold show the percentage of PI-stained positive cells in the upper left quadrant.

with copaiba oil at 100 μg/mL and 200 μg/mL for 3 h, the gated percentage of PI-stained cells increased to 44.4% and 39.7%, respectively (Figure 3, upper-left quadrant). These results were similar to the positive control amphotericin B (32.78% treated with 5 μg/mL; Figure 3, upper-left quadrant). All treatments were applied for 3 h at 32°C. Student's t-test ($P < .05$) indicated significant differences between the group treated with copaiba oil compared to the negative control group. These data may indicate that in axenic amastigotes, copaiba oil induces a considerable increase in plasma-membrane permeability. Ultrastructural changes in promastigote and axenic amastigote forms of *L. amazonensis* treated with the copaiba oil from *C. reticulata* are illustrated in Figure 2. Control cells (Figures 2(a) and 2(e)) showed no plasma membrane alterations, a nucleus with a normally centred nucleolus, a kinetoplast, a mitochondrion with well-defined mitochondrial crypts, and a flagellar pocket with only one flagellum, or the short flagellum of the amastigote-like form is within a distended flagellar pocket. In both the promastigote and amastigote-like forms, the most prominent effect of the treatment was swollen mitochondria (Figures 2(b), 2(c), 2(d), and 2(f)). The copaiba oil treatment also induced intense exocytic activity in the region of the flagellar pocket (Figure 2(g)) and cytoplasmic vacuolisation (Figure 2(h)). Alterations in the mitochondrial membrane potential of *L. amazonensis* amastigotes were studied by treatment with copaiba oil for 3 h at 32°C and stained with Rh-123 as described in Methods. Mitochondrial energizing induces quenching of Rh-123 fluorescence, and the rate of fluorescence decay is proportional to the mitochondrial membrane potential. Data obtained from flow cytometry by using Rh123 showed a marked decrease in the percentage population of the upper right gated (49.11% and 38.76%), indicating depolarization of the mitochondrial membrane potential in the cells following treatment with copaiba oil at 100 μg/mL and 200 μg/mL, respectively (Figure 4). Similarly, a decrease in membrane potentials was also observed following treatment with the standard drug carbonyl cyanide m-chlorophenylhydrazone (CCCP) (76.4%) at 200 μM for 3 h at 32°C. In contrast, untreated cells maintained the

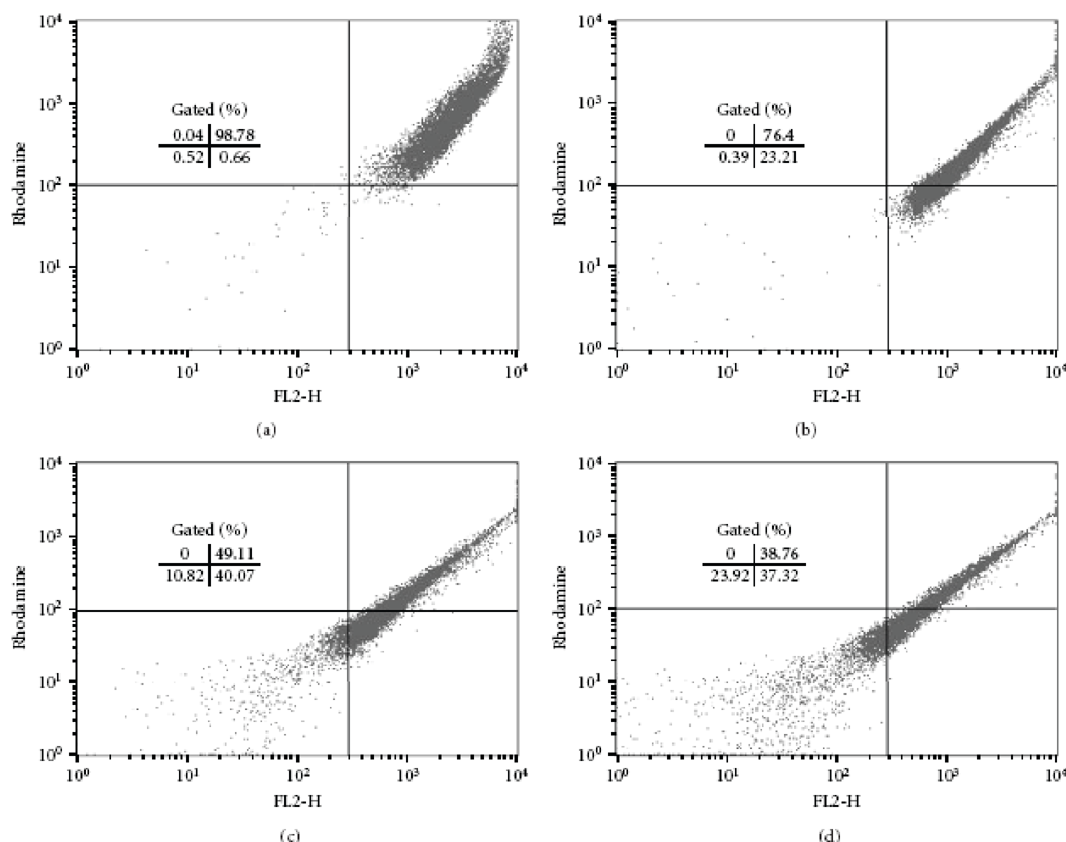

Figure 4: Flow cytometry analysis of Rh123-labeled axenic amastigotes of *L. amazonensis*. Copaiba oil collapsed the $\Delta\Psi_m$, leading to parasite death. (a) untreated cells; (b) CCCP 200 μM; (c) amastigotes treated with 100 μg/mL; (d) amastigotes treated with 200 μg/mL. The numbers in bold represent the percentage of collapsed $\Delta\Psi_m$ cells in the upper right quadrant.

membrane potential (98.78%) (Figure 4; upper right quadrant). Student's t-test ($P < .05$) indicated significant differences between cells treated copaiba oil compared to the negative control group. The maintenance of mitochondrion properties, including the mitochondrial membrane potential ($\Delta\Psi_m$), synthesis of ATP, and oxidative phosphorylation, is essential for the survival of *Leishmania*, because these parasites have a single mitochondrion [32–34]. Accordingly, the loss of the mitochondrial membrane potential is one indication of damage observed in the ultrastructural analyses and might lead to the death of the parasites.

In conclusion, the copaiba oil caused notable morphological and ultrastructural changes in the treated parasites compared with the untreated cells. In both forms of *L. amazonensis*, copaiba oil caused extensive mitochondrial damage and also led to plasma membrane denaturation. Since morphological and ultrastructural analysis demonstrated that treatment with copaiba oil induced alterations in the membrane and mitochondrion of the parasites, we incubated treated parasites with PI and Rh123. *L. amazonensis* treated with copaiba oil showed a decrease in Rh123 fluorescence, suggesting an effect on the mitochondrial membrane potential. Staining of the parasite cells with PI demonstrated that loss of cell viability occurred due to an increase in permeability of the plasma membrane. Therefore, it appears that copaiba oil could be exploited for the development of new antileishmanial drugs.

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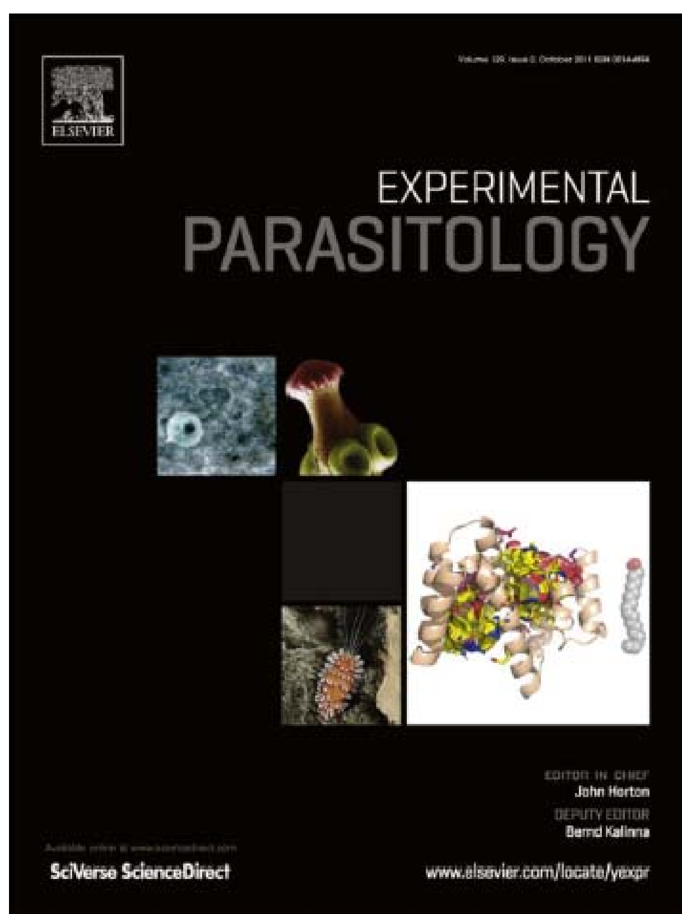

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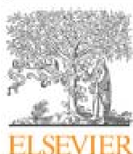

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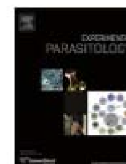

Leishmania amazonensis: Effects of oral treatment with copaiba oil in mice

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ABSTRACT

Leishmaniasis is a severe public-health problem, with high rates of morbidity and mortality. Efforts to find new, effective and safe oral agents for the treatment of leishmaniasis have been ongoing for several decades, in order to avoid the problems with the currently used antimonials. In the present study, we found that a copaiba oil oral treatment (Group IV) caused a significant reduction in the average lesion size (1.1 ± 0.4 mm) against *Leishmania amazonensis* lesions compared with untreated mice (Group I) (4.4 ± 1.3 mm). To prove the safety of the oil, the toxicity and genotoxicity were also determined. Histopathological evaluation did not reveal changes in the copaiba oil-treated animals compared to the control animals. In the mutagenicity evaluation, (micronucleus test) the dose tested (2000 mg/kg) showed no genotoxic effects. Morphological and ultrastructural analyses demonstrated notable changes in parasite cells treated with this oleoresin. The main ultrastructural effect was mitochondrial swelling. We also demonstrated that in vitro copaiba oil treatment of *L. amazonensis* led to an increase in plasma membrane permeability, and depolarization in the mitochondrial membrane potential in parasite cells. Although the mechanism of action of the oleoresin is still unclear, these findings indicate that copaiba oil is a possible new drug, which would provide a safer, shorter, less-expensive, and more easily administered treatment for leishmaniasis.

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1. Introduction

Leishmania is the protozoan parasite responsible for several pathologies known collectively as leishmaniasis (McConville and Handman, 2007). The worldwide prevalence is 12 million cases, and the estimated population at risk is about 350 million in 88 countries on four continents. Currently, the estimated global annual incidence of new cases is 2 million (Alvar et al., 2006). However, it is clear that official data frequently grossly underestimate the reality, because leishmaniasis is not a notifiable disease in all the countries where it is endemic. Therefore, a substantial number of cases are never recorded (Bustamante et al., 2009).

The clinical manifestations of this infection depend on the species of *Leishmania* and the immunological status of the host. The disease can be classified as cutaneous leishmaniasis, mucocutaneous leishmaniasis, and visceral leishmaniasis, also known as Kala-Azar (Clem, 2010). *Leishmania amazonensis* is a species that causes cutaneous leishmaniasis (CL), which ranges from small cutaneous nodules to gross mucosal tissue destruction (Reithinger et al., 2007).

The pentavalent antimonial compounds have been used since 1940 to treat visceral and cutaneous leishmaniasis. Although new drugs or drug formulations such as liposomal amphotericin B (AmBisome), miltefosine, and paromomycin should be available for treatment of leishmaniasis, they all have limitations of cost, specific toxicities, or the need for parenteral administration (Lee and Hasbun, 2003; Croft, 2008; Dujardin et al., 2010). Consequently there is an urgent need to discover new drugs effective against leishmaniasis.

The current use of herbal therapy in *Leishmania*-endemic regions has renewed interest in evaluation of plant remedies used in traditional medicine as sources of potential antileishmanials (Iwu et al., 1994; Gachet et al., 2010; Tiuman et al., 2011). Interestingly, the use of copaiba oils to treat leishmaniasis has been cited in several ethnopharmacological studies with data obtained from western Amazonia, in Peru (Kvist et al., 2006), eastern Amazonia, in the state of Maranhão, Brazil (Moreira et al., 2002), and northern Amazonia, in French Guiana (Fleury, 1997; Grenand and Moretti, 1987). Recently, Santos et al. (2008) reported that copaiba oils from different species of *Copaifera* show activity against promastigote forms of *L. amazonensis*. These results led us to investigate the

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in vivo antileishmanial activity of copaiba oil from *Copaifera martii*, together with in vitro studies by electron microscopy, biochemical analysis, and flow cytometry to determine the targets of copaiba oil in *L. amazonensis*.

2. Materials and methods

2.1. Copaiba oil

The copaiba oil was collected from the trunk of *Copaifera martii* tree at Tapará, Pará (DC 349), and the sample was deposited in the Herbarium Chico Mendes (Maricá, Rio de Janeiro). The chemical characterization was performed by high-resolution gas chromatography (HRGC) analyses with a Hewlett–Packard (HP) model 5890 instrument equipped with a flame ionization detector as published in Santos et al. (2008).

2.2. Preparation of copaiba oil formulations

The available formulations were prepared as follows: The topical cream (TE) was prepared using glycerin monostearate 6% (w/w), stearic acid 2% (w/w), beeswax 1.5% (w/w), cetiol 11% (w/w), ethoxylated lanolin 1% (w/w), triethanolamine 1% (w/w), methyl paraben 0.18% (w/w), propyl paraben 0.2% (w/w) and distilled water 50% (w/w). Copaiba oil was added in tween 80 at the proportions of 1:1 and mixed until to uniformity. So, this mixture was added in the

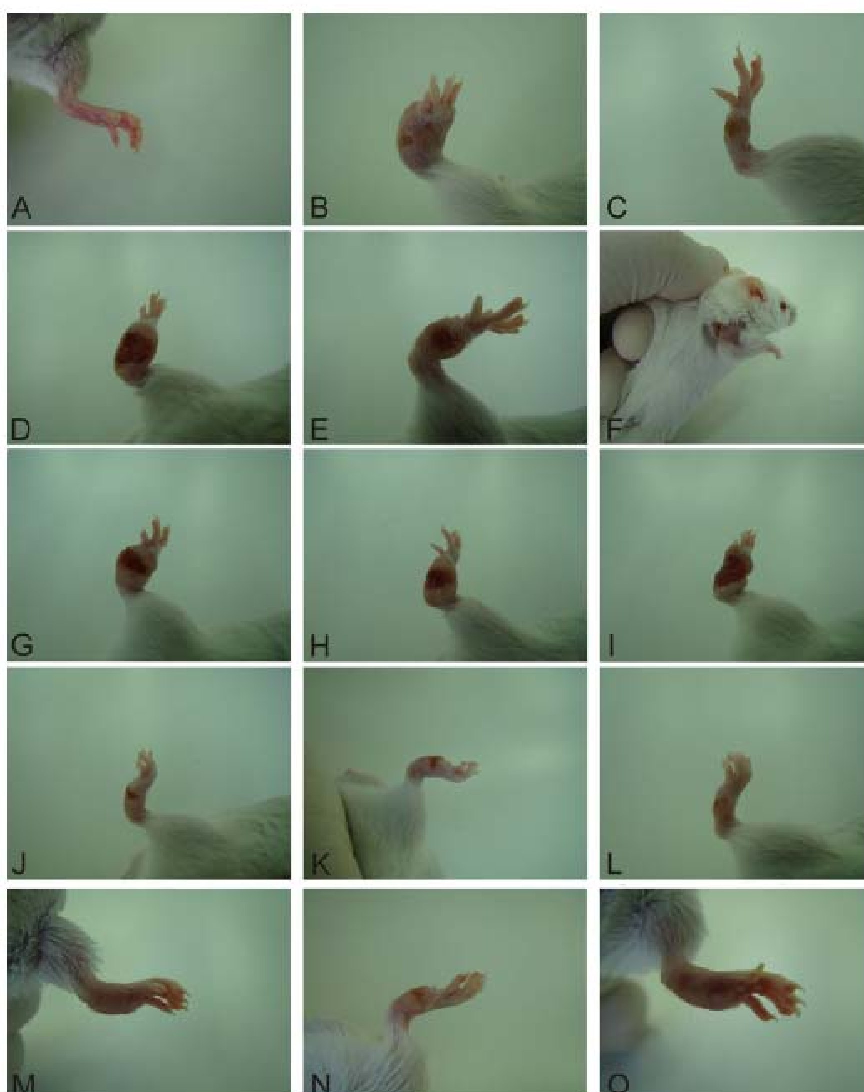

Fig. 1. Evaluation of cutaneous leishmaniasis development in mice treated with copaiba oil. (A) Group VII: Uninfected and untreated control; (B) Group I: infected and untreated control; (C) Group II: The reference drug Glucantime® (100 mg/kg/day) administered through intramuscular injection; (D–F) Group III: copaiba oil (100 mg/kg/day) by subcutaneous route; (G–I) Group V: Lesion treated topically with copaiba oil cream at a concentration of 4%, applied on the lesions in an amount of 1 mg/mm^2; (J–L) Group IV: copaiba oil emulsion at a dose of 100 mg/kg/day was administered orally by gavage. (M–O) Group VI: The animals received oral treatment by gavage (100 mg/kg/day) and also topical treatment with 4% copaiba oil cream applied on the lesions in an amount of 1 mg/mm^2.

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emulsion at concentration of 100 μg of copaiba oil for each g of the cream.

For the oral formulation, the guar gum at concentration of 5% (w/w) was dispersed in water under stirring for 4 h. After, the remaining water and 5% (w/w) of tween 80 were added in order to complete the homogeneous polymer dispersion. The copaiba oil was mixed in tween 80 at proportion of 1:1 and added in the preparation in order to obtain the oral emulsion (OE). The oral dose of copaiba oil was 100 mg for kg of the evaluated animal. The intramuscular preparations were obtained by the mixture of copaiba oil in tween and PBS at proportions of 1:1:1.

2.3. Parasites

The promastigote forms of *L. amazonensis* (MHOM/BR/75/Josefa) were isolated from a human case of diffuse cutaneous leishmaniasis. This strain has been maintained by weekly transfers in Warren's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 25 °C in a tissue flask. The infective promastigote was maintained by inoculation into the footpads of BALB/c mouse every 4–6 weeks. After development of lesion, we performed the excised of lesions and the isolation of *L. amazonensis*. The infective promastigotes were cultured in Warren's medium supplemented with 20% FBS, penicillin (100 U/ml), and streptomycin (0.1 mg/ml) at 28 °C. They were then used in the stationary phase of growth (day 6 of culture). Axenic amastigote forms were obtained as described by Ueda-Nakamura et al. (2001).

2.4. Animals

Male BALB/c mice between 4 and 6 weeks of age (20–25 g) and male and female (8–12 weeks) *Mus musculus* (Swiss) outbred mice, weighing 25–30 g were obtained from Universidade Estadual de Maringá (UEM, PR, Brazil). All animals were acclimatized to our laboratory conditions for 10 days before the beginning of the experiments. Animals were under standard laboratory conditions on a constant 12 h light/dark cycle with controlled temperature (22 ± 2 °C). Food (Nuvilab® Cr1) and water were given ad libitum. The Institutional Ethics Committee of Universidade Estadual de Maringá approved all procedures adopted in this study (Protocol 013/2010).

2.5. Infection of animals

BALB/c mice were divided into seven groups that consisted of at least 4 BALB/c mouse. Each mouse was inoculated subcutaneously with 1×10^7 metacyclic promastigotes using 27.5-gauge needle with a volume of 25 μl PBS into the left rear footpad of the mice. Lesion development was monitored weekly by measuring of the thickness of the infected footpad with a calliper (DIGIMESS® 150 mm) for a total period of 8 weeks. The treatments were started after development of lesions (30 days pos-infection) and continued for 4 weeks. Lesion size was expressed as the difference in thickness between parasite-inoculated footpad and the contralateral footpad of the same animal.

2.6. Treatments of infected animals and histological studies

The groups were distributed as follows: Group I: infected and untreated control; Group II: The reference drug Glucantime® (100 mg/kg/day) was dissolved in PBS and administered through intramuscular injection. Group III: copaiba oil (100 mg/kg/day) by subcutaneous route was made up in solution of Tween and PBS; Group IV: copaiba oil emulsion at a dose of 100 mg/kg/day was administered orally by gavage. The oil was suspended in Tween (Sigma Chemical Co. USA) and diluted in 5% Goma-Guar. Group V: Lesion received topical treatment. The copaiba oil cream at concentration of 4% was applied on the lesions in an amount of 1 mg/mm^2. Group VI: The animals received oral treatment by gavage (100 mg/kg/day) and also topical treatment with copaiba oil cream 4% being applied on the lesions in an amount of 1 mg/mm^2. Group VII: Uninfected and untreated control. The solutions were made up daily. All treatments were realized by 30 days after development of lesion. At the end of the 30-day-treatment period the animals were killed in a CO_2 chamber. Some selected organs (esophagus, stomach, duodenum, heart, kidney, liver, spleen, lung, and testiculum) were removed and processed for histological studies. Thus, the organs were fixed in Bouin' fluid, embedded in paraffin wax. Blocks were sectioned at 7 μm thickness in a microtome (Leica Microsystems Inc, Germany), mounted in a electrostatic slides and stained with hematoxylin-eosin.

2.7. Micronucleus test

Ten-animal groups (*Mus musculus* five males and five females) were treated orally by gavage with 2000 mg copaiba oil/kg body weight (bw), and were killed 24 h after the copaiba oil administration. The positive control (cyclophosphamide = 40 mg/kg bw) and negative control (distilled water) groups were also included (Costa et al., 2010). After the treatment periods, the femurs were exposed and sectioned, and the bone marrow was gently flushed out using fetal calf serum. After centrifugation (3000g, 5 min) the bone marrow cells were smeared on glass slides, coded for blind analysis, and air-dried. The smears were stained with May-Grunwald-Giemsa to detect micronucleated polychromatic erythrocytes (MNPCE) (Schmid, 1975). For each animal, three slides were prepared and 2000 polychromatic erythrocytes (PCE – polychromatic erythrocytes) were counted to determine the frequency of MNPCE. The slides were analyzed with the use of an Olympus BH-2 microscope (1000×).

Table 1
Effects of copaiba oil on lesion development in mice infected with *L. amazonensis* promastigotes. Results are shown as the mean ± SD for the four mice in each group.

Treatment	Average lesion size (mm) (mean ± SD)
Group I C(−): Infected and untreated	4.4 ± 1.3
Group II (C+): Glucantime®	0.9 ± 0.3[a]
Group III: C.O. Subcutaneous route	3.0 ± 1.0[b]
Group IV: C.O. Oral route	1.1 ± 0.4[a]
Group V: C.O. Topical	4.9 ± 0.3[b]
Group VI: C.O. Oral route and topical	1.2 ± 0.2[a]

C(−): negative control; C(+): positive control; C.O.: copaiba oil; SD: standard deviation.
[a] No significant difference when compared to positive control and significant difference when compared to negative control (ANOVA test; $p < 0.05$).
[b] Significant difference when compared to positive control (ANOVA test; $p < 0.05$).

Table 2
Micronucleus test – frequency of micronuclei in 2000 polychromatic erythrocytes (MNPCE) in bone marrow of mice after 24 h of oral treatment with copaiba oil (2000 mg copaiba oil/kg body weight – bw); positive control (cyclophosphamide = 40 mg/kg bw); and negative control (distilled water).

Treatment	MNPCE (mean ± SD)
Copaiba oil	9.0 ± 1.4[a]
Cyclophosphamide	27.0 ± 4.0[b]
Distilled water	7.0 ± 1.8

MNPCE: micronucleated polychromatic erythrocytes; SD: standard deviation.
[a] No significant difference when compared to negative control and significant difference when compared to positive control (ANOVA test; $p < 0.05$).
[b] Significant difference when compared to negative control (ANOVA test; $p < 0.05$).

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2.8. Study of parasite morphology and ultrastructure by electron microscopy

Promastigote forms were treated with copaiba oil obtained from *C. martii* and then processed for scanning and transmission electron microscopy as described previously in Santos et al. (2008).

2.9. Flow cytometry

L. amazonensis axenic amastigotes (5×10^6 parasites/mL) were treated with copaiba oil from *C. martii* (100 µg/mL for 3 h at 32 °C), or untreated. After that were harvested and washed with PBS. The integrity of the plasma membrane was assessed by the entrance of the propidium iodide (PI) (20 µg/mL for 5 min). To analyses of mitochondrial membrane potential (ΔΨm) parasites were stained with Rhodamine 123 (Rh 123) (5 mg/mL for 30 min at 37 °C) reagents. The compounds Amphotericin B (5 µM) and, Carbonyl Cyanide m-chlorophenylhydrazone (CCCP) (200 µM) were used as a positive control. The material was kept on ice until analysis. The mean of fluorescence intensity of the cells were analyzed by flow cytometry FACSCalibur and CellQuest software. A total of 10,000 events were acquired in the region previously established as that corresponding to the parasites.

2.10. Statistical analysis

Data are presented as mean ± SD. The statistical significance of differences in percentage between treated and untreated was ana-

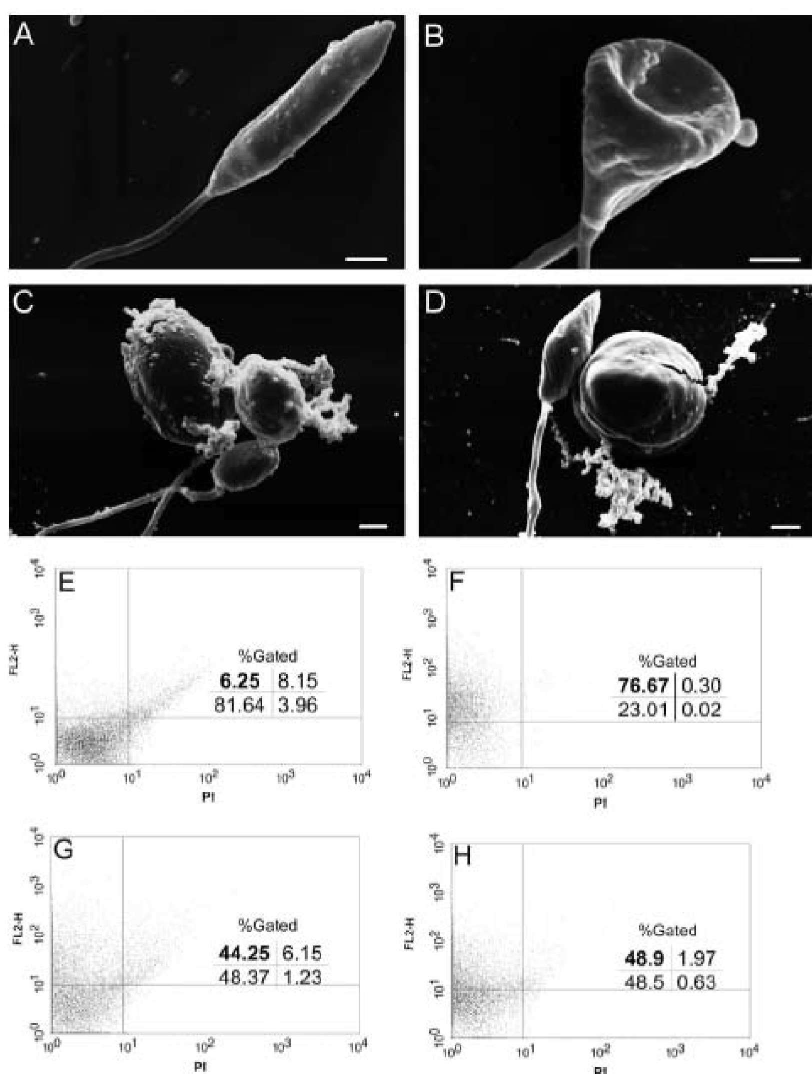

Fig. 2. Scanning electron micrographs of promastigote forms of *L. amazonensis* treated with copaiba oil (A) Promastigotes, control; (B) Promastigotes after treatment with IC_{50} of copaiba oil; (C and D) Promastigotes after treatment with IC_{90} of copaiba oil (Bars = 1 µm) and flow cytometry analysis of *L. amazonensis* axenic amastigotes treated with copaiba oil stained with propidium iodide (PI) (D) Untreated cells; (F) Amphotericin-B; (G) Amastigotes treated with 100 µg/mL; (H) Amastigotes treated with 200 µg/mL. The numbers in bold show the percentage of PI-stained positive cells in the upper left quadrant.

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lysed by Statistics 8.0®. The statistical analyses were performed by Anova one-way test followed by Dunnet test. Differences were considered significant at p value of less than 0.05.

3. Results and discussion

There is no single optimal treatment for cutaneous leishmaniasis (Hepburn, 2000; Markle and Makhoul, 2004; Palumbo, 2010). Natural products have made and are continuing to make important contributions to the search for new antileishmanial drugs (Tiuman et al., 2005; Khaliq et al., 2009; Singh et al., 2009; Vendrametto et al., 2010). Recently, Santos et al. (2008) reported that copaiba oils from different species of *Copaifera* show activity against promastigote forms of *L. amazonensis*. Taken together, these facts encouraged us to evaluate the treatment of experimentally infected animals with copaiba oil. For the in vivo tests, BALB/c mice were infected subcutaneously with *L. amazonensis* (1×10^7 cells/ml) in the left rear footpad. The treatment was started on the 4th week post-infection. Mice were treated subcutaneously (Group III), orally (Group IV), topically (Group V), or orally and topically (Group VI). Treatment with Glucantime® (Group II) was used as a positive control. The lesion size was measured with a caliper each week for one month of infection. Fig. 1 and Table 1 present the results of the treatments. Interestingly, the oral treatment (Fig. 1J, K, and L), and the oral and topical treatments (Fig. 1M, N, and O) caused significant ($p < 0.05$) reductions in the average lesion size (1.1 ± 0.4 mm), and (1.2 ± 0.2 mm) compared with untreated mice (Group I) (4.4 ± 1.3 mm) (Fig. 1B), and showed no significant difference compared to the positive control Glucantime® (Fig. 1C) (0.9 ± 1.3 mm). The topical (Fig. 1G, H, and I) (4.9 ± 0.3 mm) and subcutaneous (Fig. 1D and F) (3.0 ± 1.0 mm) treatments showed no significant reduction in the average lesion size ($p < 0.05$). Moreover, the subcutaneous treatment caused injuries in the animals during the applications, as shown in Fig. 1G.

The main chemical constituents of *C. martii* are sesquiterpenes (37.7%) and diterpenes (62.3%). The most common sesquiterpenes are β-bisabolene (10.7%) and α-zingiberene (7.2%). Kaurenoic (7.9%) and kovalenic (29.0%) acids are the main diterpenes (Santos et al., 2008). Several reports have demonstrated that the biological effect of copaiba oils may be explained by the complex nature of these sesquiterpene and diterpene mixtures, which might affect

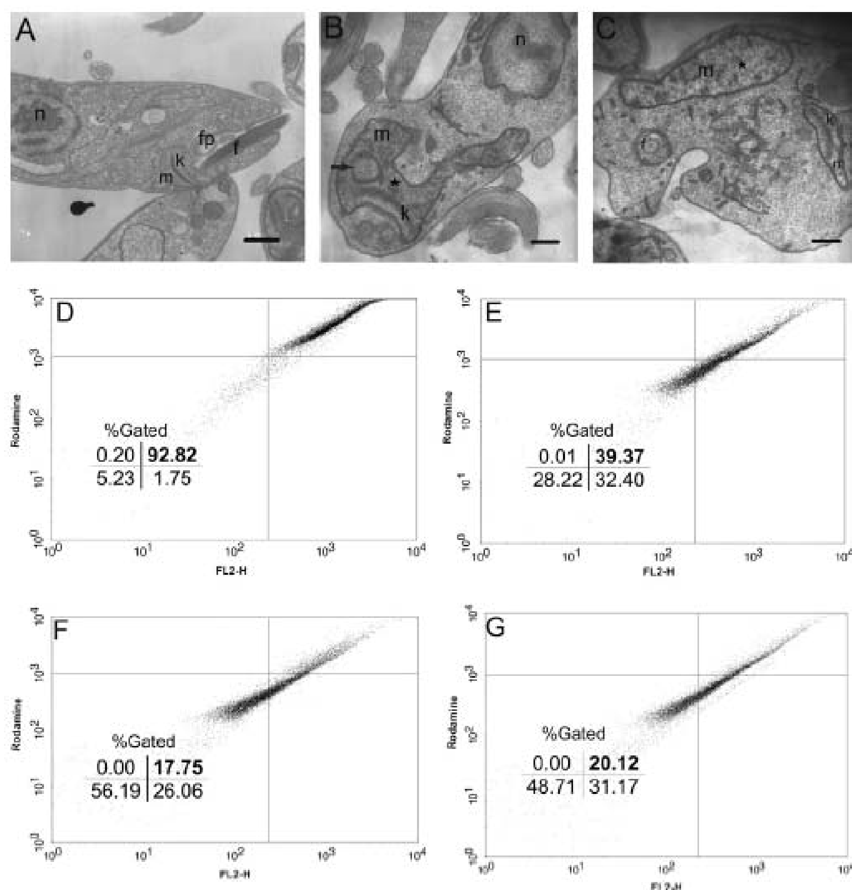

Fig. 3. Ultrastructural effect of copaiba oil on promastigote forms of *L. amazonensis*. (A) Promastigote control; (B) Promastigote treated with IC_{50} of copaiba oil; (C) Promastigote treated with IC_{90} of copaiba oil; The treatment with copaiba oil led to changes in the mitochondria (m) including mitochondrial swelling (stars) and the appearance of concentric membrane structures inside the organelle (arrow). n, nucleus; f, flagellum; fp, flagellar pocket; k, kinetoplast; m, mitochondrion. Bars = 1 μm. Flow Cytometry analysis of Rh123-labeled axenic amastigotes of *L. amazonensis*. (D) Untreated cells; (E) CCCP 200 μM; (F), Amastigotes treated with 100 μg/mL; (G) Amastigotes treated with 200 μg/mL. The numbers in bold represent the percentage of collapsed $\Delta\Psi m$ cells in the upper right quadrant.

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the active component by a synergistic effect (Fernandes and Freitas, 2007). Lima et al. (2003) reported that fractionation of copaiba oils results in fractions that are less active than the crude copaiba oil.

An important criterion in the search of active compounds with antiprotozoal activity is their toxicity to mammalian host cells. For this purpose, the toxicity and genotoxicity of the copaiba oil were determined. Histopathological evaluation did not reveal changes in the esophagus, stomach, duodenum, heart, kidney, liver, spleen, lung, or testicles of animals treated with copaiba oil compared to control animals. No clinical or behavioral changes were observed in the animals treated with the oleoresin. In the mutagenicity evaluation, the dose tested (Schmid, 1975) showed no genotoxic effects. The copaiba oil dose of 2000 mg/kg showed a frequency of MNPCE of 9.0 ± 1.4 (Mean ± SD) in 2000 polychromatic erythrocytes. These levels were significantly lower than the positive control (27.0 ± 4.0), and did not differ from the negative control (7.0 ± 1.8) (Table 2). However, are still further testing is needed to prove the total absence of genotoxicity.

To investigate which organelles might be the initial targets of copaiba oil, SEM and TEM techniques were employed. Figs. 2 and 3 show morphological alterations in promastigotes treated with concentrations corresponding to the IC_{50} (14.0 μg/mL) and IC_{90} (70.0 μg/mL) values of copaiba oil. Untreated control promastigotes (Fig. 2A) showed the typical elongated shape. In contrast, the parasites treated with copaiba oil showed notable morphological changes, such as the appearance of aberrant-shaped cells (Fig. 2B–D). Ultrastructural changes in promastigotes treated with copaiba are illustrated in Fig. 3. Untreated promastigotes showed no plasma membrane alterations, and organelles with normal morphology (Fig. 3A). The most prominent effects observed in treated parasites were swollen mitochondria in all the treated cells (Fig. 3B and C).

To improve drug therapies for leishmanial infections and for the development of new drugs, it is necessary to identify the targets of parasite cells that have the potential to be safe and effective treatments. The cell viability of axenic amastigotes of *L. amazonensis* was checked by staining the cells with propidium iodide (PI), a fluorescent dye that binds specifically to DNA. As shown in Fig. 2, the gated percentage (upper-left quadrant) of PI-stained cells after treatment with copaiba oil (44.25% in parasites treated with 100 μg/mL and 48.9% treated with 200 μg/mL) (Fig. 2G and H) was higher than the number in non-treated parasites (6.25% Fig. 2E) and similar to the positive control treated with Amphotericin B (76.67%, treated with 5 μg/mL) (Fig. 2F). All treatments were administered for 3 h at 32 °C. These data might indicate that copaiba oil induces in axenic amastigotes a considerable increase in plasma membrane permeability. This is in agreement with the observations of Santa-Rita et al. (2004) who demonstrated that treatment with edelfosine at concentrations above 1 mM in PBS for 10 min led to a gradual increase in membrane permeability.

The mitochondrial transmembrane potential was investigated by using the fluorescent probe Rh 123, which accumulates within energized mitochondria, in conjunction with flow cytometry analysis. Data obtained showed a marked decrease in the percentage population of the upper right gated (17.75% and 20.12%), indicating depolarization of the mitochondrial membrane potential in the cells following treatment with copaiba oil at 100 μg/mL and 200 μg/mL, respectively (Fig. 3F and G). Similarly, a decrease in membrane potentials was also observed following treatment with the standard drug Carbonyl Cyanide m-chlorophenylhydrazone (CCCP) (39.37%) at 200 μM for 3 h at 32 °C (Fig. 3E). In contrast, untreated cells maintained the membrane potential (92.82%) (Fig. 3D). Several antileishmanial drugs have produced a depolarization in the mitochondrial membrane potential. Investigations of the mitochondrial potential have reported that a main functional impact of mitochondrial alterations is programmed cell death by apoptosis (Singh et al., 2009; Santa-Rita et al., 2006; Sen et al., 2007; Misra et al., 2008; Kaur et al., 2010).

Copaiba oil from *C. martii* was identified as an orally active antileishmanial drug, through evaluation of BALB/c mice infected with *L. amazonensis*. Exposure of the parasites to copaiba oil induces an increase in plasma membrane permeability, and depolarization in the mitochondrial membrane potential. Although the mechanism of action of the oleoresin is still unclear, these findings indicate that copaiba oil might be a new drug, which would constitute a safer, shorter, less-expensive, and more easily administered treatment for leishmaniasis. Given the promising results obtained with copaiba oil (oral treatment) our next goals are to test new oral formulations containing copaiba oil, and to verify the effects of these oral formulations distribution (different sites of infection), providing an evidence about the pathologic lesion pre-and post-treatment, immuno-modulators studies, and tests of acute and chronic toxicity to provide information about the safety of the use of copaiba oil.

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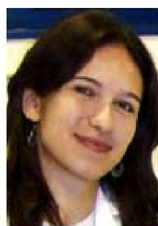

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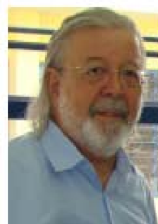

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IN VITRO ANTILEISHMANIAL ACTIVITIES OF ISOLATED COMPOUNDS FROM COPAIBA OIL AGAINST LEISHMANIA AMAZONENSIS

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ABSTRACT: Leishmaniasis are one neglected tropical diseases. According to the WHO there are about 1.5 - 2 million cases of cutaneous leishmaniasis each year worldwide. The current treatments suffer from several limitations. This study had as its goals investigated the antileishmanial activity and some possible targets of its action of isolated compounds from copaiba oil against promastigote and axenic amastigote forms of **Leishmania amazonensis.** Compounds utilized in this study include agatic acid, hydroxy-copalic acid, kaurenoic acid, methyl copalate, pinifolic acid, and polyaltic acid isolated from **Copaifera officinales** oleoresins. All compounds isolated from copaiba oil screened in this study had some level of activity against **L. amazonensis.** Hydroxy-copalic acid and methyl copalate were the most activity against promastigote. On the other hand, pinifolic acid and kaurenoic acid were the most activity against axenic amastigote. The isolated compounds agatic acid, kaurenoic acid, and pinifolic acid showed remarkable increase in plasma-membrane permeability and mitochondrial membrane depolarization.

Keywords: **Leishmania amazonensis.** Leshmaniasis. **Copaifera.** Isolated compounds.

Introduction

Leishmania is a genus of protozoan parasites that are transmitted by the bite of the sandfly and cause diseases collectively known as leishmaniasis (Kaye and Scott, 2011). The disease occurs in varying clinical manifestations, range from self-healing cutaneous and mucocutaneous skin ulcers to a fatal visceral form named visceral leishmaniasis or kala-azar (Murray et al., 2005; Murray et al., 2010; Ai't-Oudhia et al., 2011). Leishmaniasis is one of the most significant of the neglected tropical diseases (Kaye and Scott, 2011). According to the World Health Organization there are about 1.5 - 2 million cases of cutaneous leishmaniasis each year worldwide. It disease is endemic in 88 countries, with a total of 350 million people at risk. It is believed that worldwide 12 million people are affected by

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leishmaniasis (WHO, 2011). Despite the overwhelming impact of these parasites, there are still many aspects to be elucidated about the mechanisms of pathogenesis and how these organisms survive in the host.

There are no vaccines available for these diseases and current treatments with antimonials suffer from several limitations (Gelb and Hol, 2002; Cruz et al., 2009). The main alternative drugs include pentamidine, amphotericin B and amphotericin B encapsulated in liposomes. This encapsulation reduces the occurrence of side effects, but relapses still occur and the drug remains extremely expensive (Seifert, 2011; Tiuman et al., 2011). Natural products are a primary source of many pharmaceuticals used today. Several studies have shown that copaiba oil showing antileishmanial activity. Previously, our group observed that copaiba oils obtained from different species of **Copaifera** show activity against promastigote forms of **L. amazonensis** (Santos et al., 2008). Significant antileishmanial activity of copaiba oil from **C. reticulata** was demonstrated against axenic amastigote and intracellular amastigote forms of the parasite. Additionally, we demonstrated the morphological and ultrastructural changes in **L. amazonensis** treated with copaiba oil from **C. reticulata,** using electron microscopy and flow cytometry to investigate specific organelles as targets for copaiba oil (Santos et al., 2011a). More recently, Santos et al. (2011b) had shown that copaiba oil oral treatment caused a significant reduction in the average lesion size in mice. Therefore, this study had as its goals investigated the antileishmanial activity and some possible targets of its action of isolated compounds from copaiba oil against promastigote and axenic amastigote forms of **L. amazonensis.**

Materials and Methods

Compounds

Compounds utilized in this study include agatic acid, hydroxy-copalic acid, kaurenoic acid, methyl copalate, pinifolic acid, polyaltic acid. All were isolated from **Copaifera officinales** oleoresins (> 90% by NMR), utilizing potassium hydroxide impregnated silica gel chromatography (Izumi et al., 2011). Each compound was first dissolved in dimethylsulfoxide and then added to the appropriate medium so that its final concentration did not exceed 1%.

Parasite Culture

Promastigote forms of L. amazonensis (WHOM/BR/75/Josefa), originally isolated from a human case of diffuse cutaneous leishmaniasis, were cultured at 25 °C in Warren's medium (brain-heart infusion plus haemin and folic acid) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco Invitrogen Corporation, New York, U.S.A.) in a tissue flask. Axenic amastigote cultures, obtained by **in vitro** transformations of infective promastigotes (Ueda-Nakamura et al., 2001) were incubated at 32 °C in Schneider's insect medium (Sigma Chemical Co., St. Louis, Missouri, U.S.A.), pH 4.6, with 20% fetal bovine serum.

Antileishmanial activity

L. amazonensis promastigotes (1 x 10^6 parasites/mL) were inoculated in a 24-well plate containing Warren's medium supplemented with 10% inactivated fetal bovine serum with different concentrations of isolated compounds from copaiba oil, and incubated at 25 °C for 72 h. The cell density for each concentration was determined by counting in a hemocytometer (Improved Double Neubauer). The experiments with amastigote forms were performed as described above using Schneider's Insect Medium, pH 4.6 supplemented with 20% inactivated fetal bovine serum and incubated at 32 °C for 72 h. Amphotericin B was used as a positive control. Controls containing 0.5% dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, MO, USA) and medium alone were also included. The 50% inhibitory concentration (IC**50)** was determined by logarithm regression analysis of the data obtained.

Study of parasite morphology and ultrastructure by electron microscopy

Promastigote forms were treated with isolated compound hydroxy-copalic acid and then processed for electron microscopy. Parasite cells, were harvested and washed twice with PBS, and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4 °C. For scanning electron microscopy (SEM), promastigote forms were fixed as before. Thereafter, small drops of the sample were placed on a specimen support with poly-L-lysine. Subsequently, the samples were dehydrated in graded ethanol, critical point dried in CO**2,** coated with gold and observed on a Shimadzu SS-550 SEM. For transmission electron microscopy (TEM), cells were post-fixed in a solution containing 1% OsO_4, 0.8% potassium ferrocyanide and 10 mM $CaCl_2$ in 0.1 M cacodylate buffer, dehydrated in increasing acetone

gradient, and embedded in Epon® resin. Next, ultrathin sections were stained with uranyl acetate and lead citrate, and images were obtained on a Zeiss 900 TEM.

Studying of mitochondrial membrane potential

Axenic amastigote forms of **L. amazonensis** (5×10^6 cells/mL), treated or untreated with 100 ug/mL of isolated compounds from copaiba oil at 32 °C for 3 h, were washed and incubated with 5 mg/mL rhodamine 123 (Rh123) for 15 min at 37 °C to evaluate the mitochondrial membrane potential. The compound Carbonyl Cyanide m-chlorophenylhydrazone (CCCP) was used as a positive control. All the material was kept on ice until analysis. Data acquisition and analysis were performed using a FACS Calibur flow cytometer (Bectone Dickinson, Rutherford, NJ, USA) equipped with the CellQuest software (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA). A total of 10,000 events were acquired in the region previously established as that corresponding to the parasites. Alterations in the fluorescence of Rh123 were quantified as the percent of reduction of the fluorescence compared with the control (untreated parasites).

Studing cell viability

Cell viability was checked by staining the cells with propidium iodide (PI). Axenic amastigotes (5×10^6 cells/mL), treated or untreated with 100 ug/mL of isolated compounds from copaiba oil at 32 °C for 3 h were harvested and washed with PBS buffer. Then, the parasite cells were stained with PI (20 //g/mL for 5 min) as per instructions given by the manufacturer. Amphotericin B was used as a positive control. The mean fluorescence intensity was analysed using FACSCalibur and CellQuest software. A total of 10,000 events were acquired in the region established as that corresponding to the parasites.

Statistical Analysis

Statistical analysis was performed with the program GraphPad Prism 4 (GraphPad Software, San Diego, California, U.S.A.). Student's **t** test was applied, and a p-value less than 0.05 was regarded as significant.

Results and Discussion

We have tested the in vitro antileishmanial activity of the isolated compounds from copaiba oil against promastigote and axenic amastigote forms of Leishmania amazonensis showing promising results. The compounds tested were: agatic acid, hydroxy-copalic acid, kaurenoic acid, methyl copalate, pinifolic acid, polyaltic acid, isolated from Copaifera officinales**.** Hydroxy-copalic acid and methyl copalate were the most active, with IC**50** of 2.5 and 6.0 ug/mL, respectively against promastigote forms of **L. amazonensis** (Table 1). Amphotericin B showed IC_{50} of 0.06 ug/mL against promastigote forms, after 72 h of treatment. In Student's **t** test (**p** < 0.05) indicated significant differences between isolated compound and the control group. According to data, the morphological and ultrastructural changes of the parasites were investigated after treatment with hydroxy-copalic acid as show in Figure 1a-d. By SEM is possible to note in untreated cells the typical elongated shape (Fig. 1a). However, when the parasites were treated with hydroxy-copalic acid, we observed notable morphological changes, such as the appearance of a rounded shape cells (Fig. 1d), rupture of the plasma membrane with loss of their contents (Fig. 1b), and the flagellar membrane also showed significant alterations (Fig. 1c). Additionally, TEM analyses of treated and untreated **L.** amazonensis with hydroxy-copalic acid are illustrated in Figure 1e-h. Untreated promastigotes, showed no plasma membrane alterations and organelles with normal morphology (Fig. 1e). In parasites treated with hydroxy-copalic acid, the most prominent effect of the treatment was swollen mitochondrial with appearance of concentric membrane structures inside the organelle (Fig. If, g and h). Others ultrastructural alterations induced by isolated compound were the abnormal chromatin condensation (Fig. 1g), and intense exocytic activity in the region of the flagellar pocket (Fig. 1g). Others studies with copaiba oils have often been associated with morphological changes and alteration in mitochondrial ultrastructure. Santos et al. (2011a) showed that **C.** reticulata treatment led to rupture of the plasma membrane with loss of their contents in amastigote-like forms of **L.** amazonensis**.** Additionally, have been demonstrated that **C.** martii caused the appearance of aberrant-shaped cells (Santos et al., 2011b). In both Copaifera species the most notable ultrastructural effect following the treatment was swollen mitochondrial. Several studies have demonstrated changes in mitochondria of Leishmania species treated with several antileishmanial agents (Delorenzi et al., 2001; Rosa et al., 2003; Santa-Rita et al., 2004; Ueda-Nakamura et al., 2006; Rodrigues et al., 2007; Santos et al., 2010). These compounds reported that significant

alterations in mitochondrial led to the loss of cell viability, confirming the importance of this organelle for the parasite's viability.

Isolated compounds from copaiba oil were also evaluated against axenic amastigote forms of L. amazonensis. The isolated compounds most active in reductions in the proliferation of axenic amastigotes, the vertebrate stage of the parasite, were pinifolic acid, and kaurenoic acid with IC_{50} of 4.0 and 3.5 ug/mL, respectively (Table 1). Amphotericin B showed IC_{50} of 0.23 ug/mL against amastigote forms. The statistical analysis by Student's **t** test (**p** < 0.05) showed significant differences between isolated compound and the control group.

As previously described (Santos et al., 2011a), PI labelling was employed to evaluate the cell viability of the parasite. PI is a well-known DNA marker, a fluorescent dye that binds specifically to DNA. As shown in Figure 2, column I, the relative numbers of PI-positive events was measured and compared with the upper-left quadrant gated percentage of positive control (75.9%) (Fig. 2h, column I) and negative control (11.4%) (Fig. 2g, column I). Cell membrane integrity was affected mainly by the compounds agatic acid (63.4%), kaurenoic acid (60.8%), and pinifolic acid (68.4%) (Figure 2a, c and e, column I, upper-left quadrant gated percentage). These isolated compounds from copaiba oil induce a considerable increase in plasma membrane permeability in axenic amastigote forms. Hydroxy-copalic acid, methyl copalate, polyaltic acid (Figure 2b, d and f column I) the percentage of gated cells was similar to that in the negative control (Figure 2g column I). All treatments were applied for 3 h at 32 °C.

Flow cytometry by using Rh123 was used for membrane potential measurements. Data obtained related a marked decrease in the percentage population of the upper right-gated percentage after treatments mainly with agatic acid (27.5%), kaurenoic acid (24.4%), and pinifolic acid (32.4%) (Figure 2a, c and e column II) indicating depolarization of the mitochondrial membrane potential in the cells after treatment at 100 [jg/mL for 3 h at 32 °C. Similarly, a decrease in membrane potentials was also observed following treatment with the standard drug CCCP (53.1%) (Figure 2h, column II upper right gated percentage) at 200 jM for 3 h at 32 °C. In contrast, untreated cells maintained the membrane potential (93.7%) (Figure 2g, column II, upper right quadrant).

However, not all the isolated compounds had similar effects (Figure 2). This suggests that different isolated compounds have different mechanisms of action to kill leishmania cells. Despite the isolated compounds have different levels of activity against **L.** amazonensis is interesting to use also them in the form of oleoresin or combinations of

compounds obtained from the same plant that could give better results than the standard drug evaluation method. Izumi et al. (2011) demonstrated in recent study that copalic acid and P-caryophyllene show synergistic activity against T. cruzi**,** and considering that many species of the genus Copaifera contain both of these compounds. This synergism could occur in almost all copaiba oils and may be one of the reasons for their medicinal properties.

Conclusions

All compounds isolated from copaiba oil screened in this study had some level of activity against **L. amazonensis.** Hydroxy-copalic acid and methyl copalate were the most activity against promastigote. On the other hand, pinifolic acid and kaurenoic acid were the most activity against axenic amastigote. The isolated compounds agatic acid, kaurenoic acid, and pinifolic acid showed remarkable increase in plasma-membrane permeability and mitochondrial membrane depolarization.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Table 1 – Antileishmanial activity against promastigote and axenic amastigote forms of *L. amazonensis* treated during 72 h with isolated compounds from copaiba oil

Compounds	IC_{50}	
	Promastigote	Amastigote
Agathic acid	28.0 ± 1.5	17.0 ± 2.0
Hydroxy-copalic acid	2.5 ± 0.4	18.0 ± 1.5
Kaurenoic acid	28.0 ± 0.7	3.5 ± 0.5
Methyl copalate	6.0 ± 0.9	14.0 ± 1.0
Pinifolic acid	70.0 ± 8.0	4.0 ± 0.4
Polyaltic acid	35.0 ± 2.0	15.0 ± 1.0

Values represent the mean ± S.D. of at least three experiments performed in duplicate.

Legends for figures

Figure 1 – Scanning and Transmission electron micrographs of promastigote forms of *L. amazonensis* **treated with hydroxy-copalic acid.** (a) Promastigotes, control; (bd) Promastigotes after treatment with IC_{50} of hydroxy-copalic acid; (e) Promastigotes control; (f-g) Promastigotes after treatment with IC**50** of hydroxy-copalic acid. The treatment with hydroxy-copalic acid led to changes in the mitochondria (stars), exocytic activity in the region of the flagellar pocket (asterisk), and abnormal chromatin condensation nuclear alterations (n*). n, nucleus; f, flagellum; fp, flagellar pocket; k, kinetoplast; m, mitochondrion. Bars = 1 um.

Figure 2 – Flow cytometry analysis of axenic amastigotes of *L. amazonensis*. Columns: (I) Studying cell viability, (II) Studying of mitochondrial membrane potential. Lines: (a) agathic acid, (b) hydroxy-copalic acid, (c) kaurenoic acid (d) methyl copalate, (e) pinifolic acid, (f) polyaltic acid, (g) Control cells without treatment (h) Positive control. Treatment with 100 [g/mL for 3 h. Analysis of 10,000 events with the percentage of gated cells indicated in the quadrants.

Figure 1

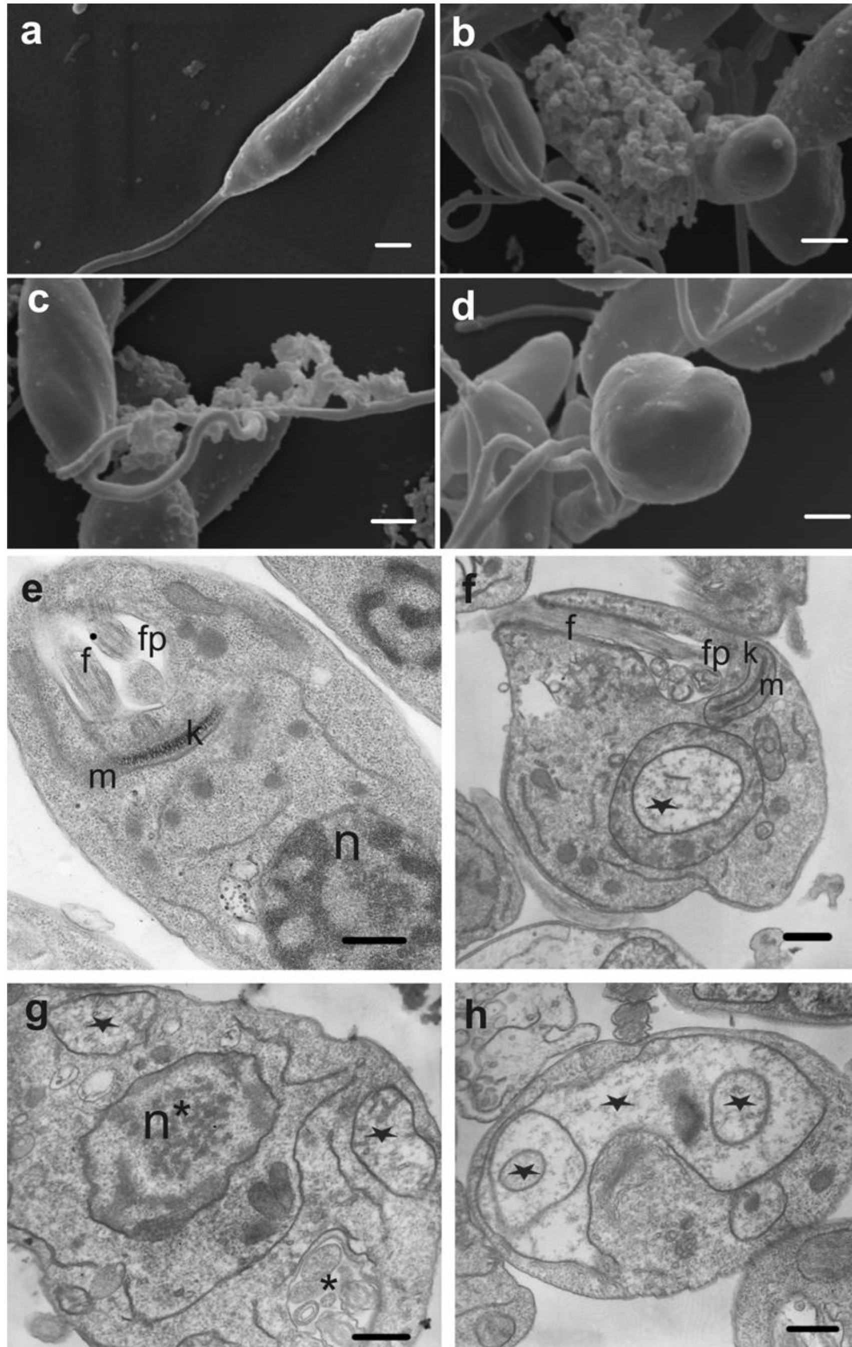
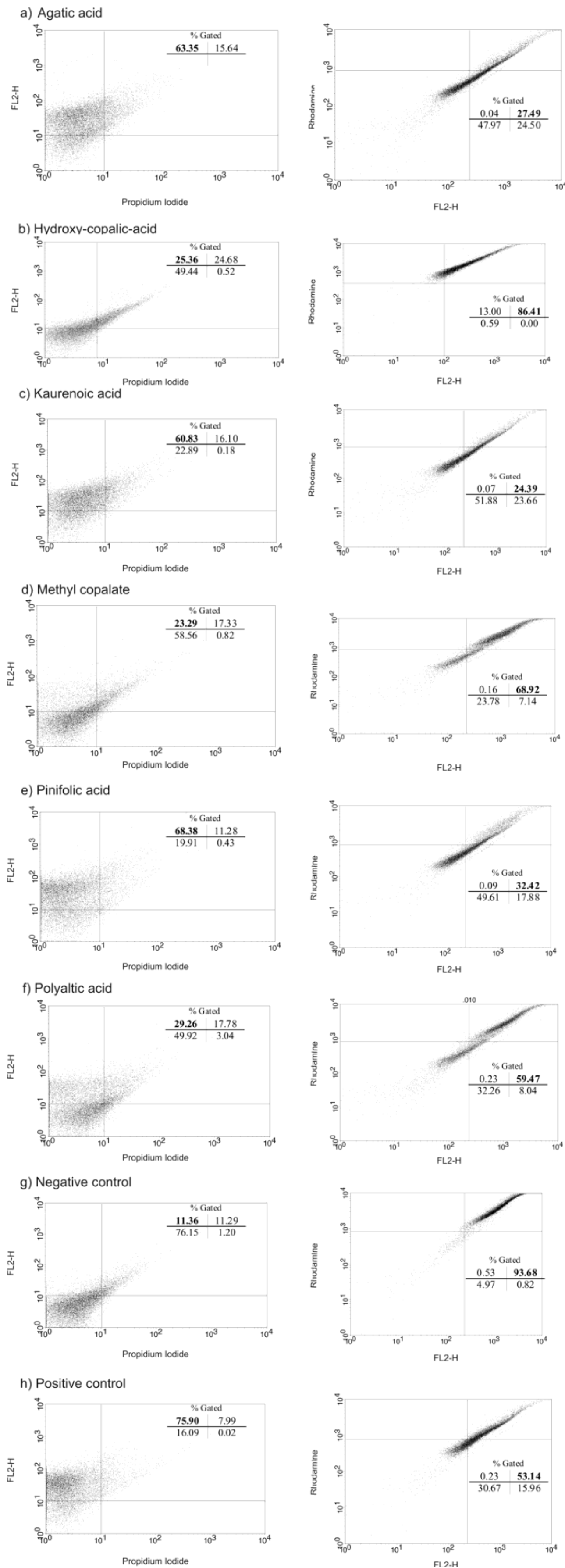


Figure 2



6 CONCLUSÕES

- No presente estudo foi demonstrado que o óleo de copaíba bem como os compostos isolados inibiu a multiplicação de formas promastigotas e amastigotas axênicas de *L. amazonensis.*
- As alterações morfológicas e ultraestruturais das células dos parasitas tratados foram visualizadas por microscopia eletrônica.
- Dentre os efeitos observados após os tratamentos, e mostrados pela técnica de citometria de fluxo estão o aumento na permeabilidade da membrana plasmática e a despolarização do potencial de membrana mitocondrial.
- Adicionalmente, o óleo de copaíba da espécie *C. martii* administrado por via oral reduziu de forma significativa as lesões no modelo animal infectado por *L. amazonensis.*
- Destaca-se que não apresentou genotoxicidade na concentração testada, nem mesmo toxicidade nos tecidos avaliados.
- Embora o mecanismo de ação do óleo de copaíba nos parasitas ainda não esteja elucidado, os resultados obtidos no presente estudo indicam que o óleo de copaíba pode ser uma opção para o desenvolvimento de novos fármacos, mais efetivos e seguros para o tratamento das leishmanioses.