Jéssica Adriana de Jesus

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Tese apresentada à Faculdade de Medicina da Universidade de São Paulo, para obtenção do título de Doutor em Ciências.

Programa de Fisiopatologia Experimental
Orientador: Prof. Dr. Luiz Felipe Domingues Passero

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"Sobe mais alto aquele que ajuda o outro a subir"

George Adams

"O tempo muito me ensinou:

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Cora Coralina

Normalização adotada

Esta tese está de acordo com as seguintes normas, em vigor no momento desta publicação:

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Abreviaturas dos títulos dos periódicos de acordo com *List of Journals Indexed in Index Medicus*.

SUMÁRIO

LISTA DE ABREVIATURAS E SIGLAS

AgT	Antígeno total
AM	Ácido Maslínico
AmB	Anfotericina B
ALT	Alanina aminotransferase
AST	Aspartato aminotransferase
ATP	Adenosina trifosfato
AU	Ácido Ursólico
Be	Betulina
CE_{50}	Concentração Efetiva a 50%
CEUA	Comite de Ética no Uso de Animais
CLAE	Cromatografia Líquida de Alta Eficiência
Cmax	Concentração máxima
CP	Palmitato de Cetila
CTL	Controle
DLS	Dispersão dinâmica da luz (Dynamic Light Scattering)
DMSO	Dimetilsulfóxido ou sulfóxido de dimetilo
DMSO-d6	Dimetilsulfóxido deuterado
DSC	Calorimetria exploratória diferencial
EE	Eficácia de encapsulação
FFUP	Faculdade de Farmácia da Universidade do Porto
FICI	Concentração inibitória fracionada
FMUSP	Faculdade de Medicina da Universidade de São Paulo
FTIR	Espectroscopia de infravermelho com transformada de Fourier
FP	Bolsa flagelar
G	Glicossomo
GLU	Glucantime
HP	Alta Pressão de homogeneização
HE	Hematoxilina e eosina
IgG	Imunoglobulina G
IMT	Instituto de Medicina Tropical
iNOS	Óxido nítrico sintase induzível

IP	Índice de Polidispersão
K	Cinetoplasto
LD	Difração a laser (Laser Diffraction)
LF	Lignana lipossolúvel
Log P	Coeficiente de partição
Lu	Lupeol
LV	Leishmaniose Visceral
M	Mitocôndria
MF	Figuras mielínicas
Mil	Miltefosina
MTT	Brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio
NLC	Carreadores Lipídicos Nanoestruturados
NO	Óxido nítrico
NMR	Ressonância Magnética Nuclear
OMS	Organização Mundial da Saúde
PBS	Tampão salino fosfatado
PBST	Tampão salino fosfatado com Tween
PDI	Polidispersão
PLGA	poli-lactídeo-co-glicolídeo
PM	Sulfato de paromomicina
PS	Tamanho de partícula
RMN	Ressonância Magnética Nuclear
ROS	Espécies reativas de oxigênio
SLN	Nanopartículas Lipídicas Sólidas
SFB	Soro fetal bovino
Son	Ultrasonicação
t1/2	Meia vida
Th	T auxiliar (do inglês T helper)
Th1	T helper do tipo 1
Th2	T he**l**per do tipo 2
TNF-α	Fator de necrose tumoral alfa
USP	Universidade de São Paulo
ZP	Potencial zeta

LISTA DE SÍMBOLOS

%	por cento
nM	nanomolar
mV	milivolts
γ	gama
μg/mL	microgamas por mililitro
mL	mililitros
μL	microlitros
μM	micromolar
mg	miligramas
mg/dL	miligramas por decilitro
h	horas
α	alfa
H_2O_2	peróxido de hidrogênio
$CDCl_3$	deuterioclorofórmio
CD_3OD	metanol deuterado
MeOH	metanol
CO_2	dióxido de carbono

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Resumo

Jesus JA. *Nanopartículas lípidicas como carreadores de triterpenos para o tratamento da leishmaniose visceral experimental* [Tese]. São Paulo: Faculdade de Medicina, Universidade de São Paulo; 2021.

A leishmaniose é uma doença causada por protozoários flagelados, e que afeta milhões de pessoas em todo o mundo. Os tratamentos disponíveis possuem eficácia limitada e induzem efeitos colaterais variando de leve a grave, dificultando a aderência de pacientes. Assim, torna-se essencial o desenvolvimento de novos medicamentos e estratégias relacionadas ao tratamento desta doença tropical negligenciada. O presente estudo avaliou as atividades leishmanicidas *in vitro* e/ou *in vivo* dos triterpenos ácido maslínico (AM), ácido ursólico (AU), betulina (Be) e lupeol (Lu) carreados ou não em nanosistemas. Os triterpenos AM, AU, Be e Lu foram testados em formas promastigotas e amastigotas intracelulares de *Leishmania (Leishmania) infantum*. Todos os triterpenos, exceto Be, foram ativos contra as formas promastigotas e amastigotas de *L. (L.) infantum*. As principais alterações ultraestruturais dos parasitos tratados com AM, AU e Lu estiveram relacionadas à formação de compartimentos vesiculares com ou sem figuras de mielina ou restos de membranas, além de desestruturação da mitocôndria do parasito, que se mostrou com volume aumentado e fragmentada. Adicionalmente, formas promastigotas de *L. (L.) infantum* apresentaram alteração no potencial de membrana após 15 minutos de incubação com AU e Lu. A cromatina dos parasitos também se mostrou fragmentada, sugerindo que os triterpenos induzem morte celular programada. Para maximizar o potential leishmanicida dos triterpenos Au e Lu, nanopartículas lipídicas sólidas (SLN) e carreadores lipídicos nanoestruturados (NLC) contendo estes triterpenos foram produzidos pelas técnicas homogeneização à alta pressão (HP) a quente e por ultrasonicação (Son), com diferentes concentrações de lipídeo (5 e 10%), tensoativo (1 e 2%) e fármaco (0,10%). Análises físico-quimicas mostraram que SLN e NLC exibiram forma esférica com superfícies lisas e com tamanho médio abaixo dos 272 nm; o potencial zeta das nanopartículas apresentaram se negativos e elevados, variando entre -26,11 a -37,22 mV, sugerindo que as nanoformulações são estáveis. Além disso, se apresentaram com índice de polidispersão menor que 0,25. Estudos de Calorimetria exploratória diferencial (DSC) e espectroscopia no infravermelho com transformada de Fourier (FTIR) mostraram que o AU e Lu foi solubilizado na matriz lipídica, tanto nas SLN como nos NLC, uma vez que não houve quaisquer eventos de fusão de droga nessas técnicas. Estudos *in vitro* mostraram que NLC carreando AU ou Lu apresentaram elevada atividade leishmanicida quando comparado às SLNc carreando os triterpenos. De acordo com os dados físicos e de potencial leishmanicida, NLC carreando AU (NLC-AU) ou Lu (NLC-Lu) foram selecionadas para realização de estudos *in vivo*. Em hamsters saudáveis, foi visto que NLC-AU ou NLC-Lu não alteraram a estrutura histológica de baço, fígado, rim, pulmão e coração, entretanto foi observado leve infiltrado inflamatório no espaço porta nos grupos tratados com o Lu livre. Animais infectados e tratados com NLC-AU ou NLC-Lu tiveram diminuição significativa do parasitismo hepático e esplênico e a eficácia das nanoformulação foi superior àquela do AU, Lu e anfotericina B administrados livremente. A atividade terapêutica se correlacionou com elevação da expressão de IFN-γ e/ou iNOS nos animais infectados e tratados com NLC-AU ou NLC-Lu. Os dados sugerem que os NLCs são potenciais sistemas para a entrega de triterpenos com atividade anti-*Leishmania* aprimorada.

Descritores: Leishmania; Leishmaniose visceral; Triterpenos; Potencial terapêutico; Nanopartículas; Lipídeos; Hamsters.

Abstract

Jesus JA. *Lipid nanoparticles as triterpene carriers for the treatment of experimental visceral leishmaniasis* [Thesis]. São Paulo: Faculdade de Medicina, Universidade de São Paulo; 2021.

Leishmaniasis is a disease caused by flagellated protozoa and affects millions of people around the world. Available treatments have limited efficacy and induce side effects ranging from mild to severe, affecting the compliance of patients. Thus, it is essential to develop new drugs and strategies related to the treatment of this neglected tropical disease. The present study evaluated the *in vitro* and/or *in vivo* leishmanicidal activities of the triterpenes maslinic acid (MA), ursolic acid (UA), betulin (Be) and lupeol (Lu) carried or not in nanosystems. The triterpenes MA, UA, Be and Lu were tested in promastigote and intracellular amastigote forms of *Leishmania (Leishmania) infantum*. All of them, except Be, were active on the promastigote and amastigote forms of *L. (L.) infantum*. The main ultrastructural alterations of the parasites treated with MA, UA, and Lu were related to the formation of vesicular compartments with or without myelin figures or membrane debris; in the treated parasites, the mitochondria were enlarged and fragmented. Additionally, parasites treated with UA and Lu exhibited an altered mitochondria membrane potential after 15 minutes of incubation. Fragmentation of chromatin was observed in treated parasites, suggesting that triterpenes induce programmed cell death. To maximize the leishmanicidal potential of UA and Lu, solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) containing triterpenes were produced by hot homogenization (HP) and ultrasonication (Son) techniques with different lipid concentrations (5 and 10%), surfactant (1 and 2%) and drug (0.10%). Physicochemical analysis showed that SLN and NLC exhibited a spherical shape with smooth surfaces and an average size below 272 nm; the zeta potential of the nanoparticles was negative and elevated, ranging from -26.11 to -37.22 mV, suggesting that the nanoformulations are stable. Furthermore, they had a polydispersion index lower than 0.25. Differential Scanning Calorimetry (DSC) and Fourier Transform Infrared Spectroscopy (FTIR) studies showed that UA and Lu were solubilized in the lipid matrix, both in SLN and NLC, since there were no drug fusion events in these techniques. *In vitro* studies showed that NLC carrying UA or Lu showed elevated leishmanicidal activity compared to SLN carrying the same triterpenes. Thus, according to physical and leishmanicidal data, NLC carrying UA (UA-NLC) or Lu (Lu-NLC) were selected for *in vivo* studies. In healthy golden hamsters, it was observed that UA-NLC and Lu-NLC did not alter the morphology of the spleen, liver, kidney, lung, and heart, however, mild inflammatory infiltrate was observed in the portal space in the groups treated with Lu. Animals infected and treated with UA-NLC or Lu-NLC exhibited a significant decrease in the number of parasites in the liver and spleen and and the effectiveness of the nanoformulations was superior to that of freely administered AU, Lu and amphotericin B. The therapeutic activity correlated with increased expression of IFN-γ and/or iNOS in animals infected and treated with UA-NLC or Lu-NLC. The data suggest that NLCs are potential systems to deliver triterpenes with enhanced anti-Leishmania activity.

Descriptors: Leishmania; Visceral leishmaniasis; Triterpenes; Therapeutic potential; Nanoparticles; Lipids; Hamsters.

INTRODUÇÃO

1 INTRODUÇÃO

1.1 Aspectos gerais da leishmaniose

A leishmaniose é uma doença infecciosa transmitida por vetores e causada por múltiplas espécies de *Leishmania*, que inclui um espectro de doenças que variam de infecções cutâneas debilitantes a infecções viscerais fatais [1]. Mundialmente distribuída, a leishmaniose possui uma prevalência global de aproximadamente 12 milhões de casos e cerca de 1 bilhão de pessoas vivendo em área de risco de transmissão [2]. Estima-se que ocorram anualmente entre 50 a 90 mil novos casos de leishmaniose visceral e 0,6-1 milhão de casos de leishmaniose cutânea em todo o mundo [2].

Os protozoários que causam a leishmaniose pertencentem a família Tripanosomatidae, ordem Kinetoplastida e ao gênero *Leishmania*; é uma doença parasitária vetorial cuja importância no contexto da saúde pública no Brasil aumentou significativamente nos últimos anos. Esse fato deve-se principalmente à expansão dos processos de urbanização e à modificação dos habitats das espécies envolvidas no ciclo de transmissão [2, 3].

A leishmaniose é considerada uma das prioridades pela Organização Mundial de Saúde (OMS) entre as doenças tropicais, pois afeta pessoas vulneráveis em cerca de 100 países, incluindo o Brasil, cujas áreas de ocorrência aumentam progressivamente [2, 4].

Mais de 20 espécies de *Leishmania* são transmidos ao hospedeiro durante o repasto sanguíneo das fêmeas dos insetos vetores, denominados coletivamente como flebotomíneos (Diptera: Psychodidae, subfamília Phlebotominae), os quais são classificados taxonomicamente em dois gêneros *Lutzomyia* (Novo Mundo) e *Phlebotomus* (Velho Mundo) [5]. Os reservatórios naturais destes parasitos são gambás, raposas, roedores em geral, e os cães por serem sensíveis a doença também podem agir como um reservatório doméstico para *Leishmania* [6, 7].

A infecção do vetor ocorre quando as fêmeas de flebotomíneos realizam o repasto sanguíneo de mamíferos infectados e ingerem macrófagos parasitados por formas amastigotas da *Leishmania*. No trato digestivo anterior ocorre o rompimento dos macrófagos liberando essas formas que se reproduzem por divisão binária e diferenciam-se rapidamente em formas flageladas denominadas de promastigotas caracterizadas por morfologia fusiforme (Figura 1 A). As formas promastigotas transformam-se em

paramastigotas as quais colonizam o esôfago e a faringe do vetor, onde permanecem aderidas ao epitélio pelo flagelo, quando se diferenciam em formas infectantes - promastigotas metacíclicas e migram para a probóscide. No hospedeiro mamífero, formas promastigotas são fagocitadas por macrófagos, perdem seu flagelo e se diferenciam em formas amastigotas intracelulares obrigatórias, com aspecto arredondado (Figura 1 B) [8].

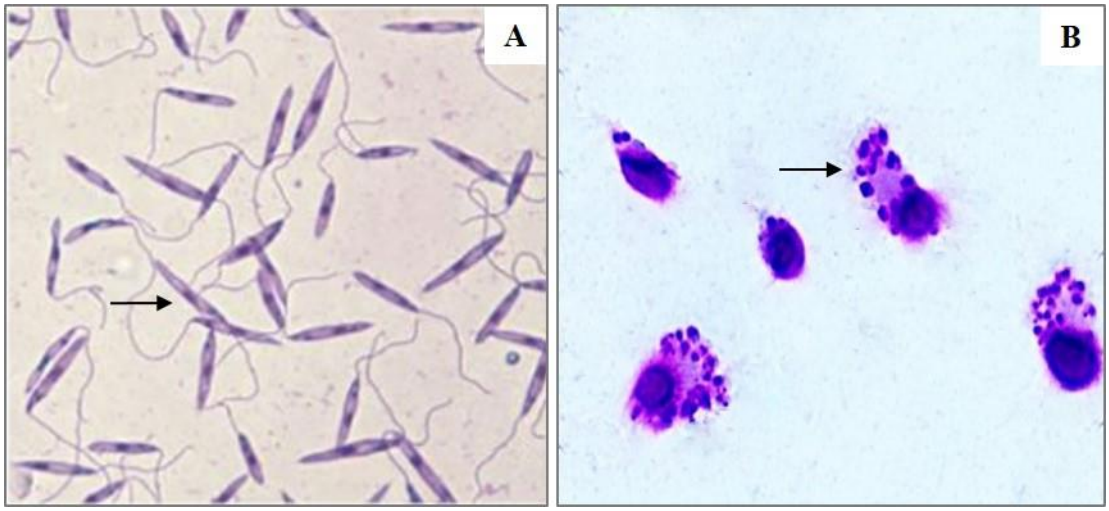

Figura 1 – A imagem mostra formas promastigotas de *Leishmania sp.* indicado pela seta (A) e formas amastigotas intracelulares, mostradas na seta (B). Coradas com Giemsa no aumento de 1000x. Barra da escala: 20 μm. Fonte: Figura A – Alynne Karen Mendonça de Santana; Figura B – arquivo pessoal.

Dentro dos fagolissomos dos macrófagos, formas promastigotas se diferenciam em amastigotas e se multiplicam por divisão binária. Assim, o número de amastigotas dentro dos macrófagos aumenta e a célula eventualmente se rompe, liberando parasitos para o meio extracelular, onde podem ser fagocitados por novos macrófagos [9]. Embora as células do sistema fagocítico mononuclear sejam capazes de eliminar grande parte dos parasitos por mecanismos leishmanicidas, alguns parasitos sobrevivem e se multiplicam causando os quadros clínicos típicos da leishmaniose, como a leishmaniose cutânea ou visceral [10]. O estabelecimento da infecção e o progresso da doença são dependentes da espécie do parasito e de fatores genéticos e imunológicos do hospedeiro [8, 11]. Dependendo da resposta imunológica inicial, o hospedeiro vertebrado pode apresentar resistência ou suscetibilidade à infecção, se a resposta for de susceptibilidade, resultará no avanço clínico e desenvolvimento da forma cutânea ou visceral da doença. Quando o

parasito se estabelece no hospedeiro vertebrado, e um inseto vetor saudável realiza o repasto sanguíneo, o flebotomíneo pode se infectar com *Leishmania* reiniciando o ciclo biológico (Figura 2).

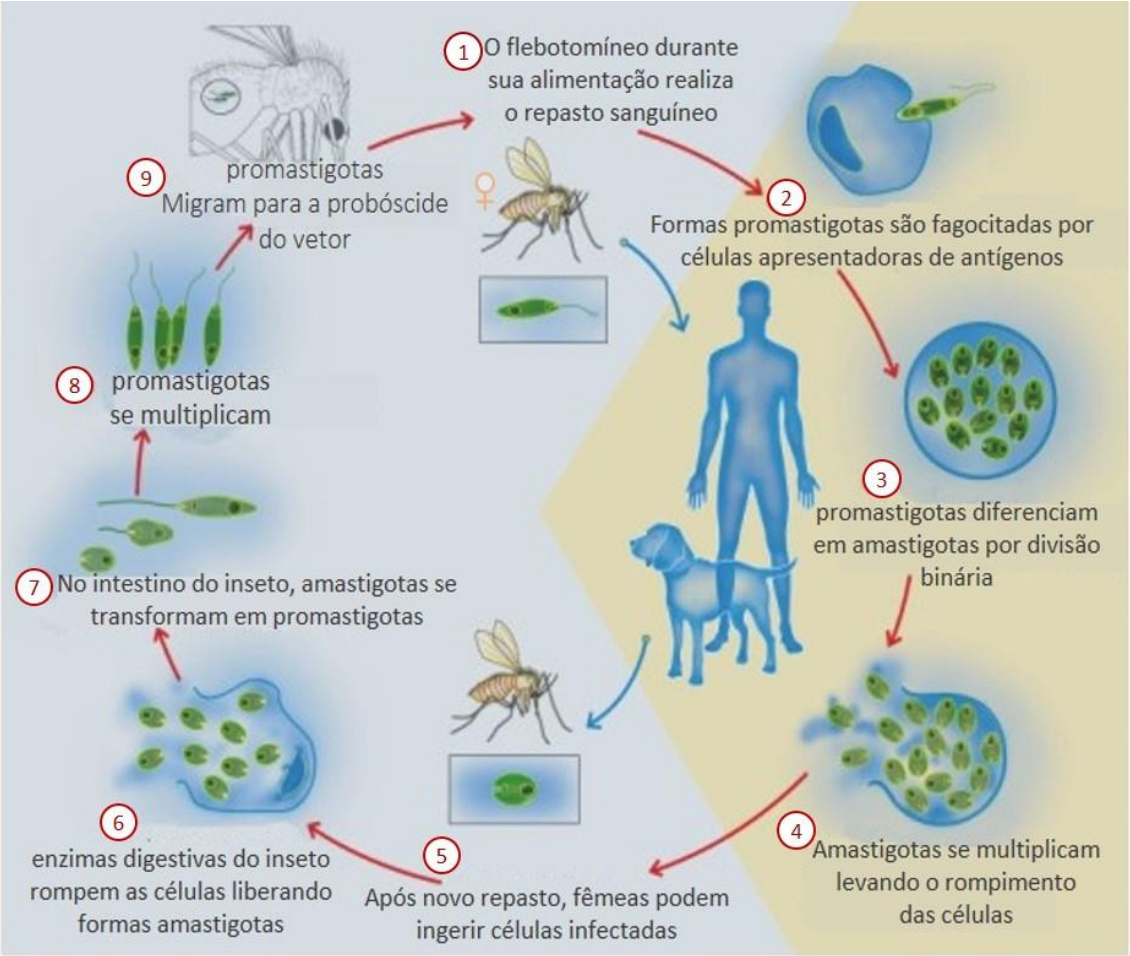

Figura 2 - Ciclo de vida dos parasitos pertencentes ao gênero *Leishmania*. (1) representa o repasto sanguíneo de flebotomíneos infectados e o início da infecção no hospedeiro mamifero; (2) após infecção, as formas promastigotas são fagocitadas por células apresentadoras de antígenos e se diferenciam em amastigotas dentro do vacúolo parasitóforo (3), que então se multiplicam ativamente nas células hospedeiras levando a ruptura destas células (4). Após novo repasto no hospedeiro vertebrado, as fêmeas de flebótomos podem ingerir células infectadas (5), que por ação de uma série de enzimas digestivas do inseto serão rompidas liberando as formas amastigotas, que novamente se diferenciarão em promastigotas (6), se ligarão à superfície intestinal destes insetos (7) e se multiplicarão (8), migrando para a probóscide do vetor, tornando capazes de infectar um novo hospedeiro vertebrado após novo repasto sanguíneo, reiniciando então o ciclo em (1). Fonte: Adaptado de Montalvo AM, Fraga J, Monzote L, García M, 2012 [12].

A leishmaniose visceral (LV) é a forma sistêmica e mais grave da doença, com a infecção bem estabelecida o parasito migra da pele para tecidos viscerais ricos em células

do sistema fagocítico mononuclear, como linfonodos, baço, fígado e medula óssea, alterando completamente a arquitetura desses órgãos, causando esplenomegalia, hepatomegalia e, levando à morte quando não tratada adequadamente [13, 14]. No Brasil, a manifestação visceral da leishmaniose ocorre por uma única espécie, a *Leishmania (Leishmania) infantum;* sinonímia: *Leishmania (Leishmania) chagasi* [15], e seu principal vetor é *Lutzomyia longipalpis*. A LV possui uma incidência de 1,6 casos por 100 mil habitantes [2].

1.2 Imunopatologia da leishmaniose visceral

A LV experimental é marcada pelo desenvolvimento de uma resposta imune órgão específica, sendo o baço e o fígado os principais órgãos alvos atingidos [10]. O baço é um local inicial para a produção da resposta imune mediada por células, mas também se torna um local para a persistência do parasito, levando ao quadro de esplenomegalia, dano tecidual e imunocomprometimento do hospedeiro [16]. Além do aumento do tamanho do órgão, ocorrem várias alterações na microarquitetura esplênica que incluem desorganização da polpa branca, hipertrofia da polpa vermelha e ruptura da zona marginal [17]. O baço é considerado um importante órgão para análise e compreensão das alterações imunológicas, que levam à disfunção da imunidade celular e à ativação de uma resposta humoral ineficaz, que são de extrema importância na patogênese da LV [18].

As células T iniciadas no baço possuem a capacidade de migrarem para o fígado gerando uma resposta granulomatosa, e ao mesmo tempo, durante a formação do granuloma, a fusão de macrófagos infectados ocorre formando células multinucleadas e contribuindo para a produção de citocinas inflamatórias [10, 19]. A inflamação granulomatosa é uma marca registrada da LV, caracterizada como uma resposta focal rica em células mononucleares, que favorece a concentração local de citocinas inflamatórias que, por sua vez, ativam de forma eficiente os mecanismos leishmanicidas das células de Kupffer, que são macrófagos especializados encontrados no fígado e os principais alvos para infecção por *Leishmania* [10, 20, 21]. Após infecção experimental, essas células secretam quimiocinas que leva ao recrutamento inicial de monócitos e neutrófilos, críticos no controle efetivo do crescimento do parasito [10]. Segundo Rodrigues e colaboradores, o ambiente inflamatório dos órgãos-alvo exerce influência na eliminação ou persistência

do parasito [18]. A falha em gerar uma resposta granulomatosa eficiente somado ao defeito no mecanismo de geração de moléculas anti-*Leishmania* acabam por contribuir para uma resposta imune deficiente na resolução da LV [19].

Uma resposta imune efetiva durante a LV requer a ação coordenada de várias células, tendo como a primeira etapa crítica a ativação de células do sistema imunológico inato, que incluem neutrófilos, macrófagos e células dendríticas [1]. A resposta imune contra LV se comporta de maneira distinta em diferentes organismos [22]. Em hamsters diferente da polarização observada em modelos murinos com LV, durante a fase ativa da doença os linfócitos TCD4+ exibem uma resposta imunológica mista Th1/Th2 [23]. A resolução da doença após a ativação de macrófagos é aumentada por respostas imunes do tipo T helper 1 (Th1) mediadas por meio da interação de células apresentadoras de antígeno, com secreção subsequente de citocinas pró-inflamatórias IFN-γ [9, 24]. No entanto, a inibição da ativação de macrófagos e desregulação imunológica pode ser mediada por fatores imunossupressores chaves, como a IL-10, que aumenta à medida que a infecção se estabelece e células T helper 2 (Th2) e Treg que secretam essa citocina, dando início ao desenvolvimento de uma condição imunossupressora grave [24]. O papel desempenhado pela IL-10 na imunopatologia e permanência de parasitos na LV tem sido demonstrado claramente em modelos experimentais, onde uma diminuição da razão IFN-γ/IL-10 está associada à suscetibilidade a LV [25, 26].

Comportamento semelhante tem sido descrito na leishmaniose visceral humana, em que uma baixa expressão de IFN-γ pode resultar em níveis relativamente mais elevados de IL-10, levando a desativação de macrófagos e consequentemente, aumento da replicação de amastigotas [27, 28]. Além disso, níveis elevados de IL-10 foram observados no plasma, soro e tecido lesionado de pacientes com LV progressiva, acompanhado do aumento da carga parasitária [27, 29–32]. Dados recentes sugerem que a produção de IL-10 por células T $CD4^+$ e dendríticas evita a ruptura da arquitetura esplênica, mesmo quando associada a um controle mais deficiente do crescimento parasitário [26].

A restauração da função dos macrófagos na LV, seja através do desenvolvimento de uma resposta imune apropriada ou por intervenção terapêutica, pode levar parasitos intracelulares à morte pela indução das funções microbicidas dos macrófagos, por meio da produção de óxido nítrico (NO) e espécies reativas de oxigênio (ROS) que são moléculas altamente eficazes em matar formas amastigotas [9]. Por outro lado, estudos

têm demonstrado que um dos mecanismos de sobrevivência de *Leishmania* ocorre através da inibição da produção de NO aumentando a expressão da arginase, que cliva competitivamente a L-arginina em ornitina favorecendo a proliferação de *Leishmania* dentro de macrófagos [33].

Embora respostas imunológicas e as vias imunorregulatórias tenham sido amplamente estudadas e definidas em camundongos como modelo experimental, esses animais não apresentam todas as características da doença humana [8, 34]. Em camundongos, a infecção por LV no fígado tem resolução automática, enquanto a infecção no baço é progressiva e a infecção geral é assintomática [10, 34]. Em contraste, a infecção por *L. (L.) infantum* em hamsters (*Mesocricetus auratus*) apresentam sinais clínicos semelhantes aos observados na LV clássica canina e humana, incluindo hipergamaglobulinemia, caquexia, grave perda de peso, anemia, leucopenia acompanhado do aumento do baço e do fígado, além da ausência de resposta de células T específicas após a infecção experimental [35, 36]. Dessa forma, o hamster por se assemelhar muito às características clínico-patológicas da leishmaniose visceral humana e canina e por apresentar uma resposta imunológica imparcial, tem sido reconhecido como o modelo mais adequado para estudos sobre a patogênese da doença, imunossupressão e a eficácia de novos métodos profiláticos e terapêuticos [37].

1.3 Tratamento das leishmanioses

O pequeno arsenal terapêutico disponível para o controle das leishmanioses é uma ilustração perfeita do quadro de negligência da doença e que apesar da diversidade de espécies de parasitos e formas clínicas da leishmaniose, o tratamento se restringe principalmente aos antimoniais pentavalentes e a anfotericina B [38].

O estibogluconato de sódio (Pentostam®) e o antimoniato de meglumina (Glucantime®), ambos compostos antimoniato pentavalente representados na figura 3, têm sido usados como agentes-chave na terapia desde 1940, como fármacos de primeira escolha no tratamento. Apesar do sucesso contra os parasitos, esses compostos são tóxicos, com efeitos colaterais graves, como hepatoxicidade e cardiotoxicidade, além de estarem associados ao aumento de casos de resistência parasitária, o que tem limitado seu uso [38–40].

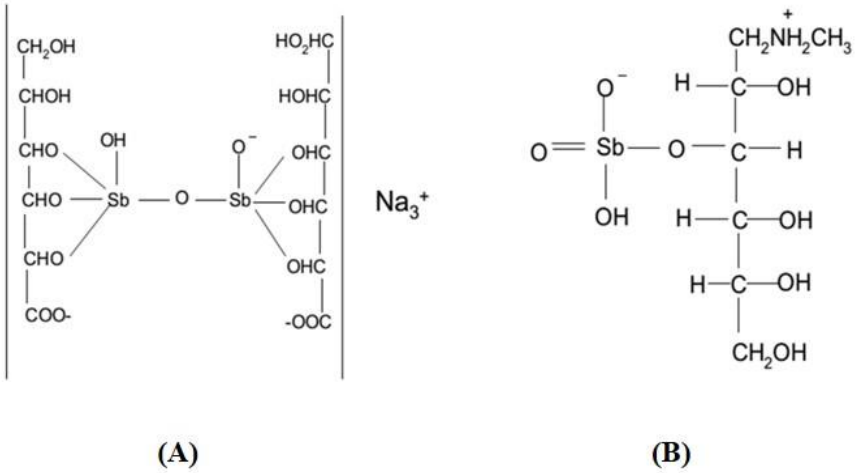

Figura 3 - Estrutura química do estibogluconato de sódio (A) e antimoniato de meglumina (B), ambos utilizados como fármaco de primeira escolha no tratamento da leishmaniose. Fonte: Rath et al., 2003 [41].

Algumas evidências sugerem que os antimoniais pentavalentes são pró-drogas que são convertidas no organismo em antimônios trivalentes, se tornando biologicamente ativas, apresentando interação com o metabolismo dos tióis e induzindo a fragmentação de DNA [42, 43]. Evidências sugerem que os antimoniais também eliminam parasitos através de mecanismos indiretos, pois estes ampliam a produção de citocinas inflamatórias (TNF-α) e ânion superóxido, elevando a capacidade fagocítica das células do sistema imune, como os macrófagos, ativando a ação microbicida dessas células através de espécies reativas de oxigênio [44].

Os antimoniais fazem parte do principal tratamento recomendado pela OMS, no entanto, a administração a pacientes por via parenteral diária requer um tratamento prolongado, por três semanas consecutivas com necessidade de um suporte hospitalar adequado para acompanhamento ao longo da terapia. Além do parasito, os antimoniais pentavalentes causam uma série de efeitos adversos locais e sistêmicos, como dor abdominal, náusea, problemas hepáticos e cardíacos, além da seleção de parasitos resistentes que acabam afetando gravemente a eficácia dessa classe de medicamentos [45].

Fármacos de segunda escolha, como anfotericina B e pentamidina (Figura 4), também são usados para tratar essa infecção, sendo geralmente administrados a pacientes que não respondem à terapia com antimonial ou têm alguma restrição quanto ao seu uso.

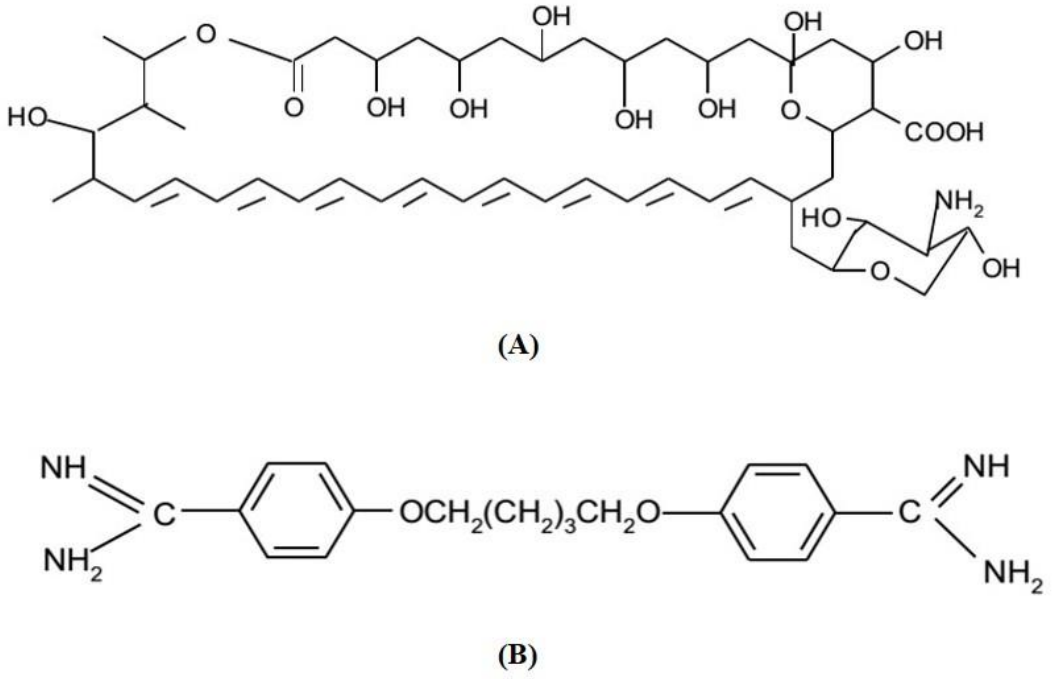

Figura 4 - Estrutura química da anfotericina B (A) e pentamidina (B), ambos utilizados como fármaco de segunda escolha no tratamento da leishmaniose. Fonte: Tempone; Martins De Oliveira; Berlinck, 2011 [46].

A anfotericina B é um fármaco anti-*Leishmania* conhecido, e foi desenvolvido a princípio como um medicamento antifúngico, isolado de uma cepa de *Streptomyces nodosus*, sendo bastante utilizada nos anos 90 no tratamento da leishmaniose [47]. Entretanto, o uso clínico desse composto tem sido prejudicado devido à sua alta toxicidade ao fígado, rim e coração [48–50]. A atividade terapêutica quanto os efeitos tóxicos derivam de sua interação com lipídeos, em particular com esteróis de membrana. Este fármaco se complexa com ergosterol (no caso de *Leishmania*) e com colesterol nas membranas das células de mamíferos, podendo gerar danos celulares extensivos, através da formação de poros na membrana [51], ainda devido a sua afinidade com lipídeos, pode haver sua incorporação em lipoproteínas plasmáticas, particularmente as de baixa densidade [52]. Assim, a endocitose dessas lipoproteínas carreando anfotericina B pelas células epiteliais renais pode ser um mecanismo associado à severa nefrotoxicidade frequentemente observada ao longo do tratamento [53]. As formulações lipossomais de

anfotericina (AmBisome®), apesar de melhorar seu efeito terapêutico e reduzir os efeitos adversos através de um maior direcionamento do fármaco para células hospedeiras parasitadas [54–56], possui um alto custo, restringindo sua aquisição e uso em muitas regiões endêmicas. A anfotericina B lipossomal tem sido usada com frequência crescente como primeira escolha em regiões endêmicas, particularmente nos países do norte da Europa do Mediterrâneo, em outros países como o Brasil, a OMS recomenda a forma lipossomal de anfotericina B apenas nos casos de LV graves, pacientes que apresentem comorbidades ou imunodeficiências, mulheres grávidas, e aqueles que desenvolveram toxicidade cardíaca ou insuficiência renal com o uso dos antimoniais [57–59].

A pentamidina foi sintetizada como um fármaco antiglicêmico, e posteriormente a sua atividade antiparasitária foi descoberta. Inicialmente, esta molécula foi empregada no tratamento da tripanossomíase africana e em seguida, foi demonstrada a sua atividade em *Leishmania*, e em 1940 foi utilizada no tratamento da LV [60]. Embora trabalhos indiquem que este fármaco interfere na síntese de poliamina e diminui o potencial de membrana mitocondrial, seu mecanismo de ação ainda não é bem compreendido [61]. O tratamento com a pentamidina apresenta diferentes efeitos colaterais, que incluem hipoglicemia e nefrotoxicidade [61]. Seu uso tem sido recomendado como profilaxia secundária em pacientes coinfectados por HIV e leishmaniose visceral na Etiópia, caso a contagem de linfócitos T CD4 esteja abaixo de 200 células/μL, com uso mensal da dose de 4 mg/kg por 12 meses [62, 63]. Estudos tem demonstrado que a eficácia do uso da pentamidina na leishmaniose tegumentar do Novo Mundo é baixa e varia de acordo com a espécie de *Leishmania*, duração, composição da terapia e região geográfica [64–66]. A resistência e a toxicidade emergentes acabam por limitar o uso da pentamidina [40, 67, 68].

A miltefosina (Figura 5) foi originalmente desenvolvida como medicamento antineoplásico e posteriormente reposicionada para o tratamento da leishmaniose visceral e cutânea tendo os primeiros relatos da sua atividade antiprotozoária na década de 80 [38]. Em 2002, a Índia concedeu o primeiro registro como medicamento oral para leishmaniose e até hoje é a única droga oral aprovada para o tratamento da leishmaniose [42, 69].

O mecanismo de ação desse fármaco ocorre pela interação com a membrana celular do parasito, modulando a composição lipídica, a permeabilidade e fluidez da membrana, assim como o metabolismo de fosfolipídios, induzindo morte celular por apoptose [70, 71]. Este medicamento é administrado oralmente e a eficácia pode ser

observada em um curto período de tratamento. Entretanto, a miltefosina oferece limitações ao tratamento da leishmaniose devido aos seus efeitos teratogênicos e longa vida útil, o que pode favorecer a resistência parasitária aos medicamentos [72, 73].

Na Índia, Bangladesh e Nepal a miltefosina foi utilizada no tratamento da LV devido à alta taxa de cura e fácil administração [74]. No entanto, após uma década de uso extensivo, a eficácia diminuiu e recidivas foram relatadas [75–77]. Isso levou a troca de miltefosina por anfotericina B lipossomal em dose única como estratégia terapêutica do programa de eliminação de LV no subcontinente indiano [42]. Atualmente, seu uso tem sido recomendado apenas em combinações com outros medicamentos [78, 79]. Em alguns países, os estudos com miltefosina mostraram desempenho variado no tratamento da leishmaniose, demonstrando a necessidade de doses mais elevadas do medicamento [80, 81]. Para a leishmaniose cutânea, a dose recomendada é 2,5 mg/ kg/dia por via oral durante 28 dias, no entanto, a miltefosina tem eficácia variada dependente da espécie infectante. Um ensaio clínico demonstrou que a miltefosina é útil contra *L. (V.) panamensis* na Colômbia, mas não contra *L. (V.) braziliensis* na Guatemala [82]. Em contrapartida, estudo conduzido por Machado e colaboradores, demonstrou que no Brasil, a terapia com miltefosina foi mais eficaz do que o tratamento com antimonial pentavalente para leishmaniose cutânea causada por *L. (V.) braziliensis* [83]. No entanto, o sucesso varia de 50 até 91% dependendo da cepa isolada em testes *in vitro* ou mesmo para testes clínicos, o que estaria longe do ideal para sua utilização como tratamento padrão [82, 84]. O principal efeito colateral da miltefosina está associado a eventos gastrointestinais, mas hepatotoxicidade e nefrotoxicidade podem ocorrer [42].

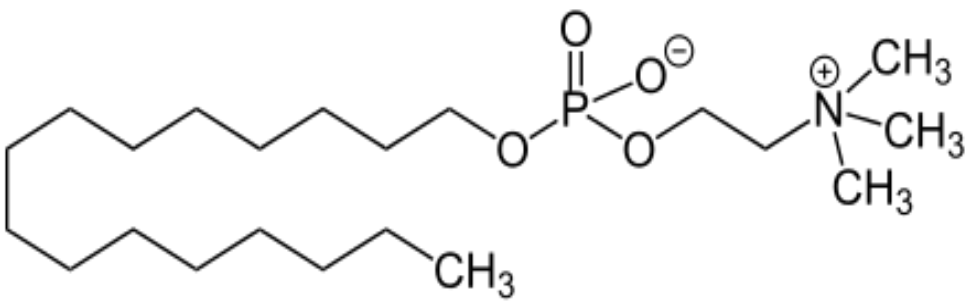

Figura 5 - Estrutura química da miltefosina, utilizado no tratamento da leishmaniose. Fonte: Macedo Bastos et al., 2016 [60].

1.4 Produtos naturais como fonte para o desenvolvimento de novos fármacos

Diante das muitas limitações devido aos efeitos colaterais tóxicos, alto custo e redução da eficácia, juntamente com o desenvolvimento da resistência a medicamentos utilizados no tratamento da leishmaniose [85, 86] a busca por novos candidatos a medicamentos menos tóxicos e de baixo custo se faz necessário. Nesse sentido, diferentes estudos tem demonstrado que moléculas extraídas de plantas medicinais possuem características interessantes para o desenvolvimento de novos agentes anti-*Leishmania* [6, 87, 88]. Esses produtos naturais têm numerosas funções biológicas contra diferentes patógenos e são de interesse para a saúde pública, tanto nas suas formas naturais quanto como modelos para modificação sintética [89–91].

Os compostos naturais atualmente utilizados na medicina exibem uma diversidade química muito ampla e, juntamente com seus análogos, demonstram a importância destes compostos na descoberta de novas drogas [92, 93]. Um exemplo bem sucedido de desenvolvimento de medicamentos a partir de produtos naturais é a artemisinina e seus análogos que são amplamente utilizados para o tratamento da malária. Isso mostra como a pesquisa usando produtos naturais fez uma contribuição significativa no desenvolvimento de medicamentos [94, 95]. Entre os medicamentos anticâncer de sucesso originalmente derivados de plantas estão os alcaloides da Vinca de *Catharanthus roseus* e o terpeno paclitaxel de *Taxus baccata* [96, 97].

No Brasil, o oléo essencial extraído de uma planta nativa da Mata Atlântica, a *Cordia verbenacea* conhecida pelo nome de erva-baleeira ou maria-milagrosa, é a base do analgésico e anti-inflamatório tópico comercializado como Acheflan® pela Aché Laboratórios [98, 99]. Um outro exemplo de medicamento originado de planta é Syntocalmy®, que é produzido a partir das folhas de *Passiflona incarnata* (planta europeia) e utilizado no tratamento de ansiedade e insônia comercializado pelo Aché Laboratórios e o Melagrião®, desenvolvido a partir das folhas de *Mikania glomerata* e utilizado no tratamento de tosse e asma, comercializado no Brasil pela Catarinense Pharma [98].

A aplicação de produtos naturais no desenvolvimento de novos medicamentos, especialmente na busca por novas estruturas químicas mostrou notável sucesso nos últimos anos, no entanto, estima-se que de 250.000 a 500.000 espécies de plantas

existentes no mundo, apenas uma pequena proporção foi cientificamente pesquisada para avaliação de suas atividades farmacológicas [98, 100]. Portanto, existe um grande potencial para descobertas futuras de plantas e outros produtos naturais com informações úteis sobre novas estruturas químicas e seus mecanismos de ação relacionados ao desenvolvimento de novos fármacos [94].

1.5 Triterpenos e sua ação leishmanicida

Uma classe de compostos de origem vegetal com interessante atividade biológica e farmacológica são os triterpenóides, que apresentam diversas funções nos vegetais como, na defesa contra patógenos e predadores [101]. Os triterpenos são formados estruturalmente por seis unidades de isopreno (C_5H_{10}), biosintetizados pela ciclização do esqualeno, tendo a sua diversidade altamente relacionada com a sua ampla gama de efeitos farmacológicos que permite que sirvam como candidatos ou protótipos de novos medicamentos [102]. Estudos mostram que os triterpenóides pentacíclicos com atividades biológicas bem caracterizadas incluem compostos da série ursano, oleanano e lupano (Figura 6), como o ácido ursólico (série ursano), ácido maslínico (série oleanano), betulina e lupeol (série lupano) [103].

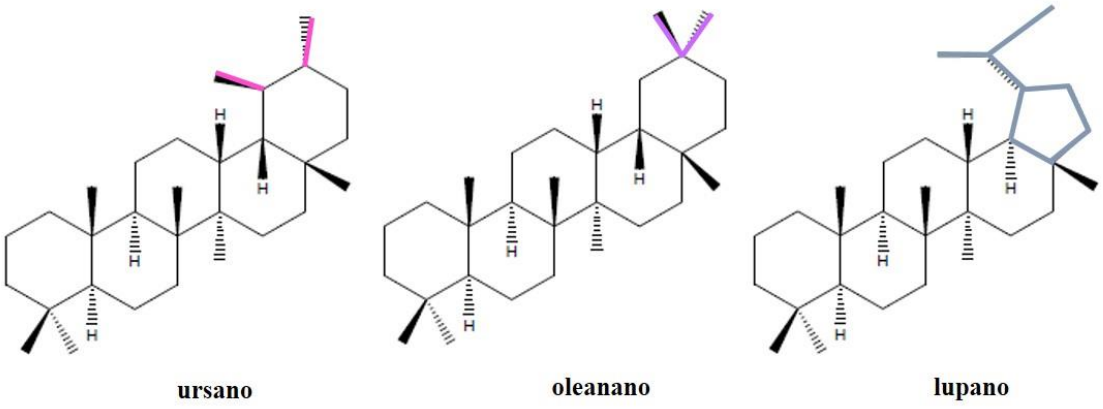

Figura 6 - Classificação das diferentes espécies de triterpenos pentacíclicos segundo sua estrutura, em destaque a diferença de cada estrutura. Fonte: James; Dubery, 2009 [104].

O ácido maslínico (ácido 2α,3β-dihidroxiolean-12-en-28-óico), o ácido ursólico (ácido 3β-hidroxiurs-12-en-28-oico), a betulina (lup-20(29)-eno-3β,28-diol) e o lupeol (lup-20(29)-en-3-ol, 3β) são triterpenóides amplamente distribuídos na natureza e que apresentam uma série de efeitos farmacológicos importantes já descritos na literatura, como propriedades antioxidantes, antialérgicas, antipruriginosas, antiangiogênicos, antifúngicas, antimalárica e antimicrobiana [105–113]. Além disso, alguns trabalhos já demonstraram que estes compostos têm um potencial antitumoral com uma elevada seletividade para as células tumorais, que na maioria dos casos induziu morte relacionada à apoptose [114–120].

Em meados de 2011, nosso grupo de pesquisa iniciou estudos com extratos produzidos com folhas de *Baccharis uncinella*, e através de purificações bioguiadas, verificou-se que uma fração contendo os triterpenos ácido oleanólico e ursólico eliminava formas promastigotas e amastigotas de *L. (L.) amazonensis* e *L. (V.) braziliensis* [121]. Posteriormente, verificou-se a ação terapêutica desta fração em camundongos infectados com *L. (L.) amazonensis* [87]. E em outro estudo, demonstrou-se que o AU era o composto ativo na fração triterpênica, e que *L. (L.) amazonensis* foi eliminada através da indução de morte programada. *In vivo*, o AU também foi capaz de eliminar formas amastigotas teciduais [122]. Estudos posteriores mostraram que na leishmaniose visceral experimental, ocorria uma diminuição significativa de parasitos no baço e fígado de hamsters quando tratado com AU, adicionalmente nenhum efeito tóxico foi observado. Animais tratados com anfotericina B também apresentaram diminuição do parasitismo tecidual, porém observou-se necrose de túbulos renais, que acompanhou o aumento dos níveis séricos de creatinina e ureia [123]. Este estudo mostrou que o triterpeno AU tem potencial farmacológico para o tratamento da leishmaniose visceral.

Objetivando caracterizar novos esquemas terapêuticos mais eficientes com o AU, estudos de combinação terapêutica entre este triterpeno e os fármacos convencionais utilizados na terapia, anfotericina B (AmB) e glucantime (Glu), foram efetuados e avaliados na leishmaniose visceral e tegumentar [124]. Na LV experimental, hamsters infectados com *L. (L.) infantum* e tratados por 15 dias consecutivos por via intraperitoneal, apresentaram redução significativa no parasitismo esplênico e hepático quando o AU foi adicionado a terapia, demonstrando que este triterpeno potencializou a atividade da AmB, principalmente nas doses de 0,2 e 1,0 mg/kg. No regime de monoterapia, realizado com AmB, a redução do parasitismo tanto no baço quanto no

fígado foi detectada apenas na maior dose estudada (5,0 mg/kg); isso significa que a AmB torna-se ativa *in vivo* quando os animais recebem uma dose total de 18,75 mg, que segundo trabalhos anteriores, essa quantidade de fármaco, assim como as menores, induz graves alterações morfológicas e bioquímicas no rim dos animais experimentais [53, 123]. Em contraste, doses não terapêuticas de AmB tornaram-se terapêuticas quando combinadas com o triterpeno AU, e isso pode ser considerado uma vantagem, uma vez que a combinação realizada com baixas doses de AmB e AU elimina um elevado número de parasitos viáveis e também pode induzir menos efeitos colaterais ao hospedeiro. Na leishmaniose cutânea, camundongos BALB/c infectados com *L. (L.) amazonensis* na base da cauda foram tratados via intralesional pelo mesmo período que os hamsters. O tratamento com glucantime (Glu) administrado em monoterapia foi inativo a 2,0 mg/kg; apresentou atividade moderada a 10,0 mg/kg, e a 50,0 mg/kg foi altamente ativo na eliminação de parasitos na pele. Quando os animais foram tratados com Glu mais AU, uma maior atividade leishmanicida foi observada em comparação a todos os grupos tratados com esquemas de monoterapia, e tal atividade foi relacionada à melhora da lesão e à elevação na expressão de IFN-γ. Estes dados sugerem que a associação de medicamentos para a terapia da leishmaniose pode aumentar a eficácia do tratamento e diminuir a toxicidade associada aos medicamentos convencionais [124].

Outros grupos de pesquisa também demonstraram o efeito antiparasitário do AU, como o estudo conduzido por Da Silva Filho e colaboradores, que mostrou que o AU possui atividade contra formas promastigotas de *L. (L.) donovani* [125], apresentando uma CE_{50} igual a 7,0 μg/mL. Em estudo mais recente, a eficácia *in vitro* e *in vivo* do AU foi investigada na leishmaniose cutânea e visceral [126]. A atividade anti-*Leishmania in vitro* contra as formas intracelulares foi seis e três vezes maior em comparação com as formas extracelulares de *L. (L.) amazonensis* e *L. (L.) infantum*, respectivamente. Em modelo experimental, o AU administrado por via parenteral na dose de 5 mg/kg por sete dias reduziu significativamente a carga parasitária no baço e no fígado de camundongos BALB/c, não apenas na infecção aguda murina, mas também em um modelo de infecção crônica contra *L. (L.) infantum*. Além disso, observou o potencial do AU formulado em creme (0,2%). A administrado tópica por quatro semanas levou a redução de 50% na progressão da lesão em um modelo de infecção cutânea causada por *L. (L.) amazonensis* [126].

Contrariamente, há escassez de estudos com o AM. Em formas promastigotas [127] foi demonstrado que o AM, isolado das folhas de oliveira (*Olea europaea* L.), foi ativo em formas promastigotas de *L. (L.) infantum* e *L. (L.) amazonensis*, apresentando CE_{50} de 9,32 ± 1,65 e 12,46 ± 1,25 μg/mL, respectivamente. Além disso, demonstrou-se que o AM age na membrana mitocondrial dos parasitos, através da depleção dos níveis de adenosina trifosfato (ATP) levando-o a morte celular. O mesmo grupo avaliou a atividade do AM em formas amastigotas, entretanto o método utilizado não é o ideal para mensurar a atividade de moléculas em formas amastigotas [128].

Em relação ao triterpeno Lu, um trabalho publicado por Das et al. (2017a), mostra que esta molécula, isolada a partir das cascas de *Sterculia villosa* Roxb. (Malvaceae), apresentou efeito leishmanicida significativo contra *L. (L.) donovani*, apresentando CE_{50} de 65,0 ± 0,41 μg/mL e 15 ± 0,45 μg/mL contra formas de promastigotas e amastigotas, respectivamente. Além disso, demonstrou-se que o possível alvo celular do Lu *L. (L.) donovani* é a membrana plasmática [129]. Estudo conduzido por Souza e colaboradores, mostrou que o Lu isolado obtido da casca do caule de *Platonia insignis* Mart. (Clusiaceae), apresentou efeitos leishmanicida contra promastigotas (CE_{50} = 39,06 μg/mL) e amastigotas *L. (L.) amazonensis* (CE_{50} de 44,10 μg/mL), com baixa atividade citotoxicidade e possíveis mecanismos de ação *in vitro* [130]. Além disso, foi observado aumento do volume lisossomal de macrófagos tratados com Lu e aumento da sua capacidade fagocítica, sugerindo que este triterpeno tem atividade imunomoduladora [130]. Mais recentemente, o potencial anti-*Leishmania in vitro* do Lu presente no extrato metanólico da casca de *Acacia nilótica* (Fabaceae) também foi descrito contra formas promastigotas (CE_{50} = 19,6 μg/mL), e amastigotas (CE_{50} = 77,52 μg/mL) de *L. (L.) donovani* [131]. O potencial sinérgico do Lu e AmB também foi descrito em estudos de combinação terapêutica durante a infecção de camundongos BALB/c por *L. (L.) donovani* [132]. O estudo mostrou que a combinação de Lu e AmB reduziu significativamente a carga parasitária esplênica e hepática quando os animais foram tratados com a dose (3 mg/kg de Lu + 0,1 mg/kg AmB) por via intraperitoneal. Além disso, verificou-se que o tratamento aumentou a produção de óxido nítrico, IL-12 e IFN-γ, e suprimiu a produção de IL-10, sugerindo que a associação do triterpeno Lu com AmB pode ser uma importante abordagem imunoterapêutica [132].

A variabilidade na atividade leishmanicida observada entre os triterpenos pode se correlacionar com as diferenças estruturais dessas moléculas. De estrutura semelhante ao

AU, o AM difere apenas na presença de uma hidroxila adicional em C-2 (representado na figura 7 por R1), além do grupo metila na posição C-20 (R4 na figura 7), enquanto no AU os grupos metila se encontram na posição C-19 e C-20 (R2 e R3 da figura 7 respectivamente), estes compostos têm sido referenciados na literatura como compostos biologicamente ativos e com importante potencial terapêutico [126, 133–135]. O mesmo pode ser observado para os triterpenos Be e Lu (Figura 7), que embora sejam membros do mesmo grupo estrutural de triterpenos conhecidos como lupanos e tenham suas estruturas bastante semelhantes, diferem pela presença do grupo OH presente em C-28 da Be (representado pela letra R na figura 7), e podem apresentar efeitos farmacológicos significantemente distintos.

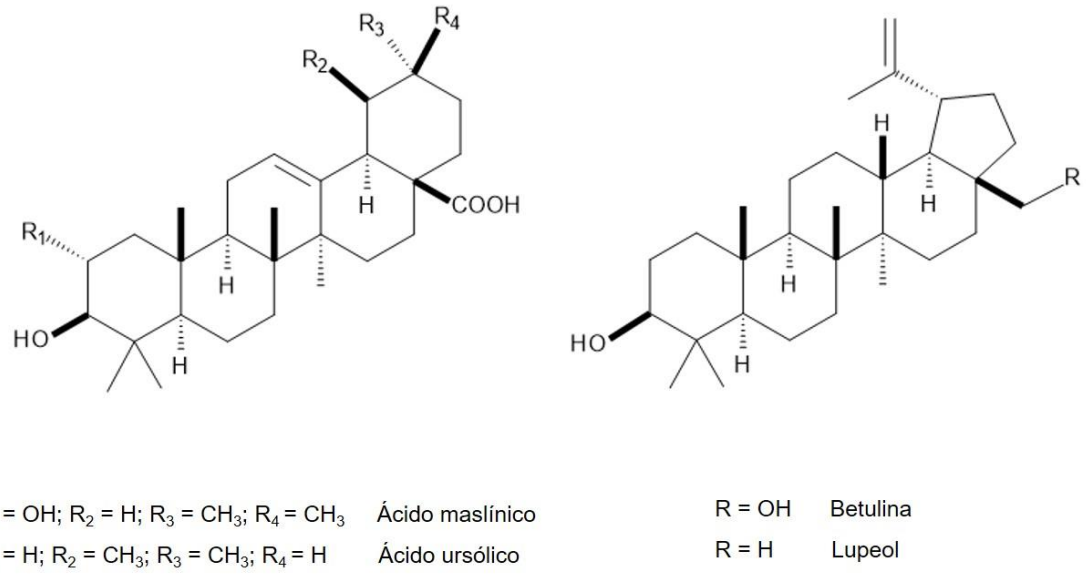

Figura 7 - Estrutura molecular dos triterpenos AM, AU, Be e Lu. Fonte: Adaptado De Jesus et al., 2021 [136].

1.6 Um breve histórico sobre o desenvolvimento de nanocarreadores de fármacos

A nanotecnologia tem fornecido soluções promissoras para o desenvolvimento de sistemas de entrega de medicamentos mais eficientes e seguros para o tratamento de diversas comorbidades [137]. As nanopartículas tem sido alvos de inúmeras e diversificada investigação em diferentes áreas da sáude, devido à sua capacidade de vetorizar uma grande variedade de substâncias com baixa biodisponibilidade e alta

toxicidade, possibilitando tratamentos mais adequados, efeitos colaterais baixos e até maior adesão do paciente à terapêutica [42, 138].

Os nanotransportadores são sistemas desenvolvidos à escala nanométrica (abaixo de 1μm) e que podem ser constituídos por diferentes materiais, incluindo os biodegradáveis tais como polímeros naturais ou sintéticos, lípideos e compostos organometálicos, que servem de veículo para fármacos ou outras substâncias ativas [139]. Atualmente, existem diferentes tipos de nanotransportadores tais como nanoemulsão, lipossomas, nanopartículas poliméricas, nanopartículas lipídicas sólidas, carreadores lipídicos nanoestruturados entre outros [140].

De uma maneira geral, os nanotransportadores são considerados como sendo excelentes veículos para o transporte e libertação de fármacos pelas inúmeras vantagens que oferecem. Entre estas destaca-se uma maior eficiência terapêutica alcançada através da libertação controlada de fármaco, a possibilidade de direcionamento a alvos específicos, a diminuição da toxicidade, a superação da resistência oferecida pelas barreiras biológicas do corpo devido ao reduzido tamanho que possuem, e a capacidade de incorporar substâncias hidrofílicas e lipofílicas aumentando consequentemente a biodisponibilidade de substâncias pouco solúveis [141]. Embora estas vantagens sejam significativas, existem desvantagens inerentes a cada sistema, que devem ser levadas em consideração, entre elas, uma possível toxicidade, ausência de biocompatibilidade dos materiais utilizados e o elevado custo de obtenção dos nanotransportadores [142].

O desenvolvimento de sistemas de transporte coloidal teve início com as microemulsões em 1943, alguns anos depois, em 1965 surgiram os lipossomas conforme demonstrado na figura 8, como um sistema potencial para transporte de fármacos e moléculas [140].

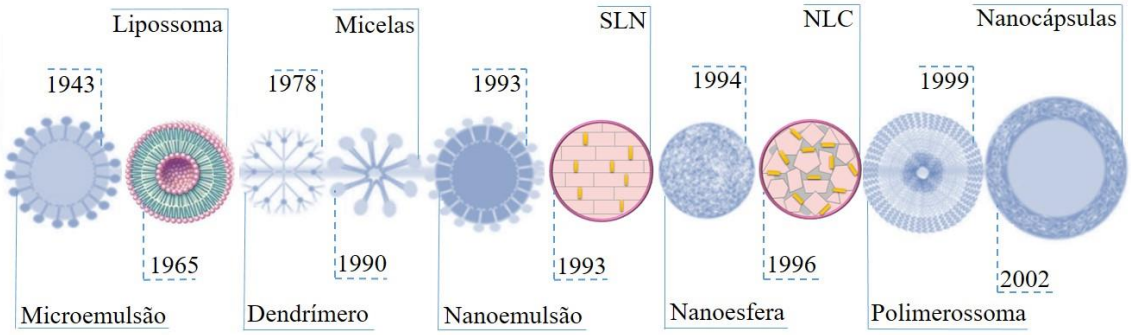

Figura 8 - Ordem cronológica de desenvolvimento dos principais nanocarreadores. Fonte: Adaptado de Apolinário et al., 2020 [140].

Os lipossomas são uma das estruturas vesiculares mais conhecidas e estudadas no meio científico. Estas partículas possuem um interior aquoso delimitado por uma (unilamelares) ou mais (multilamelares) bicamadas lipídicas concêntricas que podem conter colesterol para aumentar a rigidez [143]. Esse sistema foi amplamente explorado em estudos dérmicos, farmacêuticos e cosméticos por possuir vantagens importantes como transporte, proteção de fármacos contra a degradação de enzimas, baixa toxicidade e flexibilidade [144–146]. Os lipossomos são formados a partir de fosfolipídios estruturalmente semelhantes aos encontrados nas membranas celulares, os lipossomas são transportadores biocompatíveis, biodegradáveis e não imunogênicos capazes de encapsular substâncias lipofílicas como hidrofílicas na bicamada lipídica ou no núcleo aquoso, respectivamente [147].

A administração de produtos terapêuticos por lipossomas altera seu perfil de biodistribuição, aumentando o índice terapêutico de vários medicamentos [145]. Extensas pesquisas têm sido realizadas usando esses sistemas de entrega de nano drogas em diferentes áreas, incluindo a entrega de drogas para o tratamento do câncer, infecções fúngicas, virais entre outros. Aproximadamente 21 produtos lipossomais já foram aprovados pelo FDA e estão disponíveis para uso clínico, como o Doxil ®, Abelcet ®, AmBisome ®, DaunoXome ®, Depocyt ®, Inflexal V ®, Myocet ®, Visudyne ®, DepoDur ®, DepoCyt ®, Marqibo ®, Mepact ®, Exparel ®, Lipodox ®, Onivyde ®, doxorrubicina, Nocita ®, Vyxeos ®, Shingrix ®, Lipoplatin ™, e Arikayce ® [148–151]. Tais formulações são usadas em oncologia, infecções fúngicas e no controle da dor. Em resumo, 13 quimioterápicos e uma proteína formulada em

lipossomas convencionais foram aprovados pela União Europeia e EUA sendo comercializados com sucesso. Além disso, muitas outras formulações estão sendo testadas em diferentes fases dos ensaios clínicos [145, 152]

Apesar do fato dos lipossomas serem a marca registrada das nanopartículas para a entrega específica do fármaco no local de interesse, muitas das aplicações desse nanosistema são limitados por algumas de suas desvantagens, como curta meia-vida de circulação sanguínea, devido à sua interação com lipoproteínas de alta e baixa densidade que resulta na rápida liberação do fármaco, baixa estabilidade, eficácia de encapsulamento reduzida, rápida remoção pelo sistema reticulo endotelial e elevado custo de produção [147].

Além dos lipossomas, as microemulsões são um dos melhores sistemas de liberação de drogas devido à sua preparação simples, capacidade de solubilizar drogas hidrofóbicas, estabilidade físico-química e fácil aumento de escala [153]. São sistemas líquidos, translúcidos termodinamicamente estáveis, amplamente usados para administração sistêmica de medicamentos, formados por água (ou fase polar), óleo (ou fase apolar) e um ou mais tensoativos [154]. As nanopartículas poliméricas também são um sistema de nanopartículas amplamente utilizado, pois detêm propriedades para superar algumas das desvantagens dos lipossomas [155]. Essas nanopartículas possuem tamanho pequeno, baixa toxicidade e são econômicas, pois podem ser usados para administrar mais de um medicamento, sendo compostas de vários tipos de partículas coloidais biocompatíveis e biodegradáveis, sendo o poli-lactídeo-co-glicolídeo (PLGA) o mais empregado [156]. Esse nanosistema carregam drogas por diferentes abordagens, como adsorção, encapsulamento, dissolução, aprisionamento ou pela ligação química da droga na superfície de nanopartículas poliméricas. As nanopartículas polímericas estão presentes em duas formas diferentes: nanoesferas ou nanocápsulas. Nas nanocápsulas, a droga é encapsulada em uma cavidade cercada por uma membrana polimérica, enquanto na nanosfera, a droga não está confinada em uma cavidade, mas é dispersa uniformemente [157]. Uma diferença importante entre os lipossomas e nanopartículas poliméricas é sua estabilidade [158]. Ao contrário da natureza instável do lipossoma, as nanopartículas poliméricas não enfrentam a limitação do vazamento do medicamento para o sangue [159, 160].

Existem ainda alguns sistemas de nanocarreadores avançados, como nanopartículas metálicas, dendrímeros e nanomateriais como opções para transporte de

fármacos. Uma vantagem importante dos dendrímeros é sua capacidade de carregar mais de um medicamento devido à sua estrutura ramificada, aumentando a biodisponibilidade do medicamento [161]. Junto com as vantagens dos nanocarreadores como eficientes veículos de substâncias ativas, também existem alguns desafios a serem superados. Um dos principais obstáculos é o alto custo dessas nanoformulações, portanto, sua comercialização e produção em alta escala não são economicamente viáveis. Devido à sua viabilidade econômica, as nanopartículas lipídicas sólidas (SLN) e os carreadores lipídicos nanoestruturados (NLC) surgiram como opções potencialmente atraentes devido aos seus componentes naturais cuja escala de produção é menos dispendiosa [162].

As SLN e NLC são sistemas eficazes e alternativos aos lipossomas, emulsões e as nanopartículas poliméricas, uma vez que apresentam estabilidade físico-química melhorada, elevada biocompatibilidade, alta biodisponibilidade, proteção da degradação de fármacos incorporados, tanto hidrofílicos quanto lipofílicos, excelente tolerabilidade e também pode ser produzido em larga escala com menores custos [163, 164].

De acordo com a literatura a área superficial de uma partícula está diretamente relacionada com o seu tamanho e com a capacidade de dissolução em meio aquoso. Quanto menor for uma partícula, maior será a sua área superficial, como consequência maior será o contato com o meio [165]. As nanopartículas são partículas coloidais com diâmetro entre 1 nm a 1 μm (Figura 9) [166, 167] e podem ser constituídas por vários materiais como polímeros, lipídeos, metais, macromoléculas, entre outros [168].

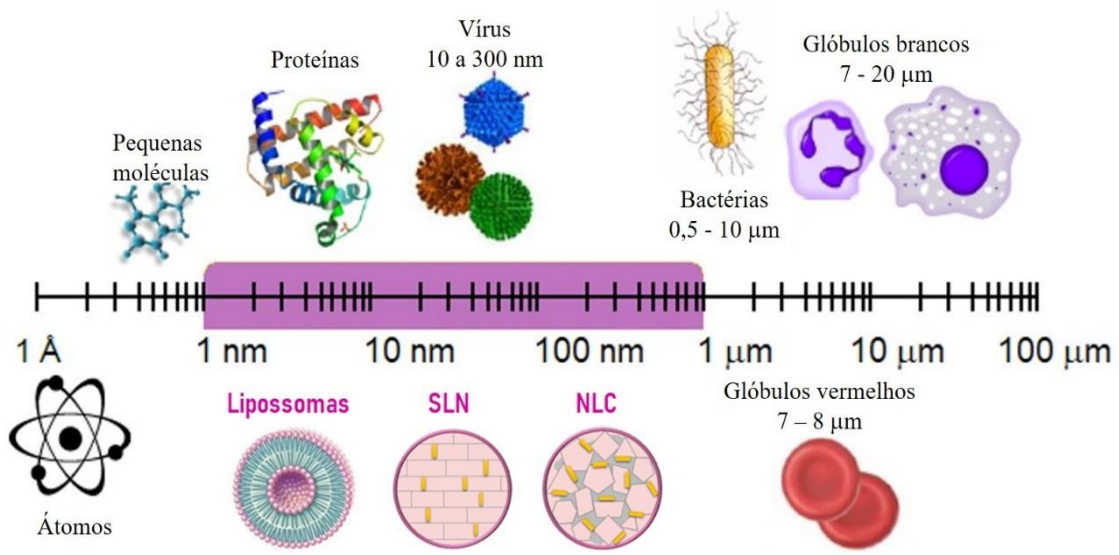

Figura 9 - Ilustração do tamanho de diferentes partículas, realçando o tamanho das nanopartículas lipídicas (roxo), abrangendo os lipossomas, nanopartículas lipídicas sólidas (SLN) e carreadores lipídicos nanoestruturados (NLC). Fonte: Adaptado de Apolinário et al., 2020 [140].

1.7 Nanopartículas lipídicas sólidas e carreadores lipídicos nanoestruturados

As SLN foram desenvolvidas no início dos anos 90 como uma alternativa aos lipossomas, às emulsões e às nanopartículas poliméricas como mencionado anteriormente, sendo definidas como um sistema de partículas lipídicas sólidas coloidais com um diâmetro entre 50 e 1000 nm [169].

Esse nanocarreador consiste de uma matriz composta de um lipídio sólido em temperatura ambiente e corporal, apresentando ponto de fusão ≥ 40°C. O lipídio sólido corresponde de 0,1-30% (massa de lipídio/massa da fase aquosa), disperso em uma solução aquosa contendo um agente tensoativo, que promove a estabilidade física do sistema, podendo variar entre 0,5-5% (massa de tensoativo/massa da fase aquosa) [164, 170].

Para a produção de SLN, ácidos graxos, misturas complexas de glicerídeos e triglicerídeos saturados ou ceras (palmitato de cetila), são normalmente usados como lipídeos sólidos; enquanto Pluronics e polissorbato 80 (Tween 80) estão entre os estabilizadores mais usados [171]. A natureza química e concentração dos lipídeos

utilizados influência na incorporação dos princípios ativos e tamanho das nanopartículas [163, 172, 173].

As SLN une as vantagens dos sistemas tradicionais de carreadores, como lipossomas, nanopartículas poliméricas e emulsões, mas sem desvantagens associados a eles, como a presença de solvente orgânico residual, instabilidade físico-química e problemas com ampliação de escala [174]. As SLN são versáteis, podendo ser ministrados por várias vias, utilizam excipientes seguros, biocompatíveis e com citotoxicidade reduzida [175], possuem um grande potencial para liberação sustentada de fármacos, são estáveis e possuem boa estabilidade física, principalmente, após o processo de liofilização que garante uma longa duração à temperatura ambiente de 12 a 24 meses, além de serem de baixo custo e facilmente escalonáveis, o que as tornam bastante atrativas para a indústria farmacêutica e cosmética [144, 146, 176].

Estudos de SLN carregados com drogas lipofílicas produziram resultados promissores, como o estudo conduzido por Carneiro e colaboradores, que demonstrou que as respostas antitripanossoma de 5-hidroxi-3-metil-5-fenil-pirazolina-1-(S-benzil ditiocarbazato) (H_2bdtc) foram aumentadas em 100x quando a droga foi carregada em SLN, reduzindo a parasitemia em camundongos infectados com *Tripanossoma cruzi e* diminuindo a inflamação e lesões do fígado e do coração [177]. Além disso, em estudo mais recente, pesquisadores observaram que o encapsulamento da sertralina (antidepressivo da classe dos inibidores seletivos de recaptação de serotonina) em SLN aumentou sua absorção gastrointestinal, conforme indicado por um aumento de 10x na biodisponibilidade do fármaco quando comparado com o fármaco livre [178]. Compostos citotóxicos, como topotecano e doxorrubicina, também foram carregados em SLN e ganharam ampla aceitação nesta formulação como tratamento de câncer devido ao aumento na eficácia da droga e diminuição dos efeitos colaterais [179, 180]. No entanto, tornou-se evidente que esse nanosistema apresenta algumas limitações, como uma baixa capacidade de encapsulamento e a possibilidade de ocorrer a expulsão de substâncias ativas durante o período de armazenamento [181]. Estas desvantagens ocorrem principalmente quando se utiliza um único tipo de lipídeo sólido, o que pode proporcionar o aparecimento de estruturas cristalinas perfeitas conforme estutura apresentada na figura 10 A [174]. Considerando que neste tipo de nanotransportador a substância ativa fica armazenada entre as cadeias lipídicas bem como nas imperfeições dos cristais, a alta organização dos cristais provoca uma diminuição da eficiência de encapsulamento [174].

No final dos anos 90 surgem os carreadores lipídicos nanoestruturados (NLC) para melhorar algumas das características das SLN, constituindo a segunda geração de veículos lipídicos [182]. Os NLC são um sistema carreador de fármaco mais inteligente de segunda geração com matriz sólida à temperatura ambiente e corporal, amplamente aceito pelas autoridades regulatórias para aplicação em diferentes sistemas de liberação de fármacos [183]. Esse sistema diferencia das SLN por apresentar uma matriz nanoestuturada imperfeita formada pela presença de pelo menos um lipídeo líquido à temperatura ambiente em sua composição, cujo objetivo é aumentar a capacidade de encapsulação de substâncias ativas e prevenir a expulsão do interior das nanopartículas durante o armazenamento, pela redução da cristalização da matriz lipídica, conferindo maior flexibilidade para modular a sua libertação [142]. As vantagens desse sistema consiste no aumento de carga e retenção de fármaco nas estruturas devido à redução de compressão e o alojamento da maior quantidade do princípio ativo nas imperfeições das nanopartículas, evitando a sua expulsão precoce e conferindo melhor estabilidade física e liberação prolongada das moléculas ativas encapsuladas [185], como ilustrado na Figura 10 B.

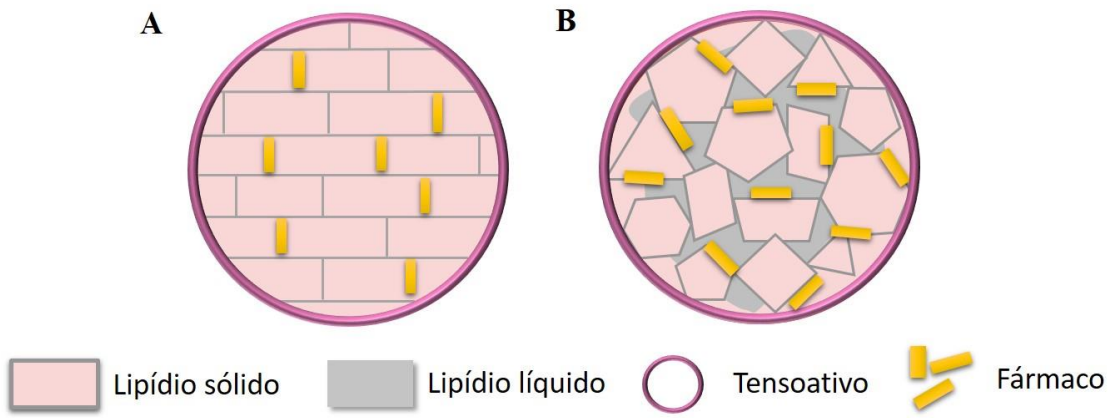

Figura 10 - Diferenças entre a estrutura lipídica cristalina "praticamente perfeita" das SLN (A) e a estrutura lipídica com bastantes imperfeições dos NLC (B). Adaptado de Laffleur; Keckeis, 2020 [142].

Assim como as SLN, os NLC podem ser produzidos utilizando triglicerídeos, ácidos graxos e ceras como matrizes, além de tensoativos como estabilizantes coloidais, que são capazes de reduzir a energia interfacial entre a fase lipídica e a fase aquosa durante

a preparação das partículas, devido à sua tendência a acumular na interface de ligação formando uma camada ao redor das partículas que favorece a estabilidade física da dispersão durante o armazenamento [175, 186]. Esses nanosistemas podem ser preparados por diversas técnicas que envolvem processos de fusão e cristalização, emulsificação, evaporação e homogeneização do tamanho das partículas. Atualmente, o método de fusão - emulsificação de solvente, seguido por homogeneização de alta pressão (HP), patenteado por Müller e Lucks destaca-se entre os demais [170]. Esta técnica consiste em solubilizar o fármaco no lipídeo previamente fundido, seguido de emulsificação em fase aquosa contendo o agente tensoativo, sob frequente agitação mecânica [187]. A homogeneização de alta pressão pode ser realizada a frio para princípios ativos termolábeis ou a quente para moléculas ativas mais estáveis, sendo esta última a mais comum [185].

Como novas gerações de nanopartículas, esses sistemas transportadores são capazes de desempenhar funções de entrega adicionais, permitindo o tratamento por meio de novas modalidades terapêuticas [171, 188, 189]. Durante as últimas décadas os estudos com nanopartículas à base de lipídeos aumentaram exponencialmente, e um dos fatores refere-se ao fato de que mais de 70% das novas moléculas com potencial terapêutico, apresentam baixa solubilidade em água e esta característica tem sido cada vez mais comum entre os candidatos, o que de fato limita o desenvolvimento de novos fármacos para o tratamento de doenças, a aplicação clínica e a comercialização devido à sua baixa dissolução e baixa biodisponibilidade [169]. Nesse sentido, o uso das SLN e NLC como sistemas de transporte de fármacos tem sido frequentemente utilizado como um método promissor para a superação desses problemas [162]. Além disso, como esses sistemas são a base de lipídeos, são considerados bastante seguros e alta biocompatibilidade é esperada [190].

O perfil de absorção e distribuição dos nanocarreadores depende muito das propriedades físico-químicas, como tamanho, hidrofobicidade, molécula de direcionamento e suas cargas [185]. Alguns processos, como a absorção e entrada de nanocarreadores na célula e sua posterior interação com o sistema imunológico, são dependentes do tamanho e da carga dos nanocarreadores [173]. Outra propriedade importante é a hidrofobicidade, pois controla a absorção e distribuição dos nanocarreadores, efetuando a interação das células imunológicas, interação com proteínas e a eliminação de partículas [137]. A carga dos nanocarreadores é usada na ligação de

proteínas plasmáticas, interações com proteínas, danos à membrana e na estimulação de células imunes [137]. Esses nanosistemas carregados com o fármaco entram na célula por fagocitose, uma forma de endocitose na qual a célula envolve partículas maiores que 0,75 µm de diâmetro [173].

O fato dos triterpenos deste estudo (AM, AU, Be e Lu) serem naturalmente hidrofóbicos e não solubilizarem facilmente, permite que sejam incorporados às SLN e NLC. A leishmaniose é uma doença particularmente interessante para ser tratada com nanocarreadores veiculando moléculas ativas, uma vez que os parasitos infectam exclusivamente as células do sistema fagocítico mononuclear, como macrófagos, que são as células hospedeiras finais de *Leishmania* [191]. Após administração as nanopartículas carregadas com o fármaco são preferencialmente fagocitadas por macrófagos, atuando diretamente sobre os parasitos (Figura 11).

A adsorção de proteínas séricas na superfície das nanopartículas (opsonização) é um fator importante, que permite aos macrófagos reconhecer e internalizar essas partículas, de forma mais eficiente do que o fármaco livre [137]. Após a fagocitose, o fármaco é liberado da nanoestrutura e pode interagir com o parasito, ocasionando danos celulares e morte [137, 191]. A internalização de nanopartículas por macrófagos aumenta a concentração intracelular do fármaco, especificamente no vacúolo parasitário [192]. Esse comportamento pode favorecer o aumento da eficácia terapêutica pela liberação do composto ativo diretamente em órgãos ricos em macrófagos, como medula óssea, baço e fígado [138]. Dessa forma, as nanopartículas à base de lipídeos representam uma oportunidade para maximizar a entrega do princípio ativo, melhorando a terapia das leishmanioses.

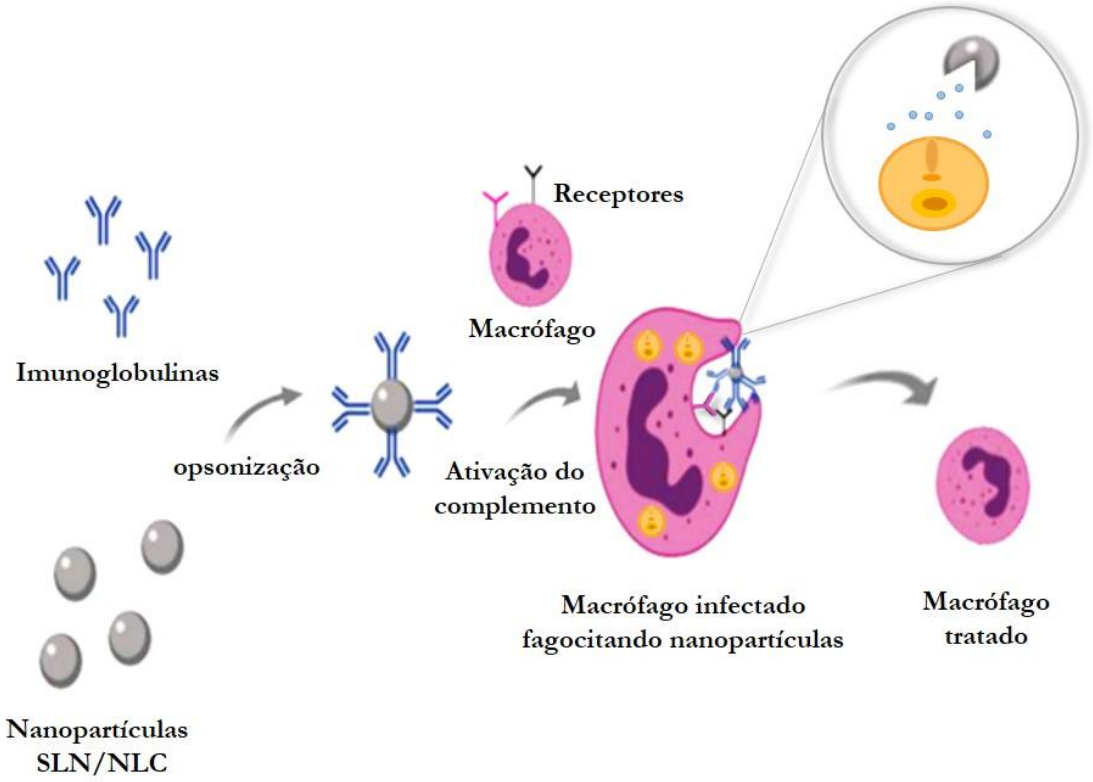

Figura 11 - Representação esquemática do processo de fagocitose para absorção de nanopartículas. Fonte: Adaptado de Saleem et al., 2019 [137].

O desenvolvimento de sistemas de entrega de medicamentos baseados em nanotecnologia para fármacos pouco solúveis, representa uma abordagem interessante para terapias menos tóxicas e igualmente ou mais eficazes do que o fármaco convencional [183]. As nanopartículas fornecem a oportunidade para o direcionamento seletivo dos triterpenos para células parasitadas e para a liberação sustentada e controlada da substância ativa, reduzindo os possíveis efeitos colaterais do fármaco e melhorando seu perfil terapêutico [162].

1.8 Nanopartículas lipídicas no tratamento da leishmaniose

O primeiro estudo a utilizar nanopartículas lipídicas na leishmaniose foi conduzido por Doroud et al., (2011) [194]. A cisteína proteinase tipo I (CPB) é uma candidata promissora à vacina; no entanto, requer um sistema de entrega para induzir uma resposta imunológica potente. Este estudo objetivava vacinar camundongos, e neste caso

SLN contendo o antígeno cisteína proteinase tipo I (CPB) com ou sem extensão C-terminal (CTE) foi injetada por via intraperitoneal em camundongos C57BL/6. Após desafio com *L. (L.) major*, verificou-se que camundongos imunizados com SLN-CTB exibiram menor carga parasitária e maior produção de IFN-γ e IgG2 assim como menor produção de IL-4, indicando que a resposta imune Th1 robusta foi induzida. Assim, foi demonstrado que o antígeno veiculado ao nanocarreador possui uma atividade imunogênica superior ao do antígeno administrado em nanocarreador com a extensão C-terminal.

Posteriormente, fármacos convencionais, como anfotericina B e miltefosina, utilizados no tratamento da leishmaniose também foram veiculados em nanocarreadores lipídicos na tentativa de aprimorar o potencial terapêutico e reduzir a toxicidade [195]. Nesse sentido um estudo mostrou que as SLN revestidas de quitosana carregadas com AmB apresentaram atividade anti-*Leishmania in vitro* aumentada em relação as formulações comerciais AmBisome e Fungizone. AmB veiculados em SLN revestidos com quitosana ativaram macrófagos e induziram uma resposta imune específica com produção aumentada de TNF-α e IL-12 em relação ao controle [196]. Além disso, estudos citotóxicos em macrófagos e estudos de toxicidade aguda em camundongos BALB/c evidenciaram o melhor perfil de segurança da formulação desenvolvida em comparação com as formulações convencionais comercializadas [196].

O fármaco sulfato de paromomicina (PM), utilizado como segunda escolha no tratamento da leishmaniose também foi encapsulado em SLN e sua eficácia avaliada *in vivo*. Verificou-se que em camundongos BALB/c infectados com *L. (L.) tropica* ou *L. (L.) major* a propagação do parasito foi inibida de forma mais eficaz com o tratamento de PM carregado com SLN em comparação com o sulfato de paromomicina livre; adicionalmente os animais tratados com PM carregado com SLN produziram forte resposta imune do tipo Th1 [197, 198].

Embora a AmB, seja um antibiótico padrão ouro usado no tratamento de infecções fúngicas sistêmicas e leishmaniose visceral, este fármaco é nefrotóxico quando administrado por via parenteral, por outro lado, sua administração oral, embora menos tóxica, apresenta biodisponibilidade baixa. Assim, para diminuir a toxicidade e melhorar a biodisponibilidade, SLN foram desenvolvidas carreando AmB para tratamento oral (AmbiOnp) [199]. Estudos farmacocinéticos e de biodistribuição *in vivo* foram conduzidos a fim de comparar a eficácia e a toxicidade desta nanoformulação com sua

versão comercial Fungizone®. Os estudos farmacocinéticos *in vivo* revelaram uma biodisponibilidade relativa de AmbiOnp de 1,05 vezes maior que a formulação comercializada (Fungizone®) administrado por via intravenosa. Além disso, os estudos de biodistribuição *in vivo* indicaram níveis reduzidos de anfotericina B nos rins quando administrado como AmbiOnp comparado com a formulação comercial, demonstrando que as SLN são sistemas seguros e estáveis [199]. A avaliação do potencial anti-*Leishmania* empregando SLN como veículos para AmB foi verificado em *L. (L.) major in vitro e in vivo* [200]. O estudo demonstrou que a AmB veiculada em SLN foi mais potente do que AmB na sua forma livre apresentando CE_{50} de 0,40; 0,26 e 0,14 μg/mL para formas promastigotas incubadas com SLN-AmB e CE_{50} de 38,18; 25,06 e 13,87 μg/mL para AmB livre, estimados após 24, 48 e 72 horas, respectivamente. Além disso, o diâmetro das lesões em camundongos BALB/c infectados com *L. (L.) major* foi menor no grupo tratado com AmB encapsulada do que no grupo tratado com o fármaco livre [200].

Além das SLN, outras pesquisas têm demonstrado o potencial dos NLC em veicular fármacos no tratamento da leishmaniose [201]. Tripathi e colaboradores desenvolveram NLC carreando AmB ancorados por quitosana e estabilizada com hexadecilfosfocolina (miltefosina). A miltefosina foi incorporada como um dos componentes das nanopartículas lipídicas utilizando sua propriedade surfactante como um agente tensoativo, isso porque a miltefosina apresenta uma estrutura anfifílica semelhante ao tensoativo [202]. Os NLC acumularam e liberaram AmB no baço e no fígado de hamsters infectados com *L. (L.) donovani*, culminando em uma significativa diminuição do parasitismo tecidual. Além disso, foi observado uma a atividade anti-*Leishmania* altamente significativa nos NLC estabilizados com miltefosina em comparação com as nanopartículas estabilizados com Tween 80 [202].

O potencial da AmB carreada em NLC também foi avaliado na infecção por *L. (V.) braziliensis in vitro* e *in vivo* [203]. Em ensaios biológicos, NLC-AmB apresentou um CE_{50} menor que AmB convencional (11,7± 1,73 e 13 ± 0,57 ng/mL, respectivamente), além de apresentar maior índice de seletividade e menor toxicidade para as células hospedeiras. Além disso, o potencial terapêutico da nanoformulação foi avaliado *in vivo* em camundongos BALB/c infectados com *L. (V.) braziliensis.* Neste caso, foram usados como fármacos convencionais o antimonial pentavalente e AmB lipossomal. Na sexta semana de infecção, verificou-se que o tratamento com NLC-AmB reduziu o tamanho

das lesões cutâneas de maneira superior ao dos fármacos convencionais, indicando a eficácia superior da nova formulação contendo AmB [203].

Outros pesquisadores desenvolveram NLC para entrega do medicamento veterinário denominado buparvaquona (BPQ) para o tratamento da leishmaniose [204]. A BPQ é tradicionalmente utilizada no tratamento de parasitos sanguíneos em bovinos, mas foi testada pela primeira vez em 1992 por Croft e colaboradores contra *L. (L.) donovani*. No entanto, devido à baixa solubilidade em água apresenta baixa atividade *in vivo* [205]. A citotoxicidade foi avaliada em macrófagos peritoneais de camundongos e a atividade leishmanicida em amastigotas de *L. (L.) infantum*. A formulação desenvolvida revelou atributos físico-químicos adequados ao tratamento, além disso, aumento da atividade leishmanicida (*in vitro*) de até 3,1 vezes foi observado quando comparadas ao fármaco livre [204].

Estudos relacionados a nanotecnologia também foram conduzidos com compostos naturais. Nesse sentido, Priyanka e colaboradores demonstraram que o Lu presente como principal constituinte no extrato de *Ficus religiosa* L. carreado em SLN teve sua biodisponibilidade melhorada em 9,2 vezes em relação a suspensão contendo Lu em ratos Wistar [206]. Além da biodisponibilidade, o estudo mostrou que as SLN melhoraram outros parâmetros farmacocinéticos do Lu, como a concentração máxima (Cmax), que representa o pico plasmático e a meia-vida (t1/2), que é o tempo que uma droga leva para reduzir sua concentração plasmática à metade, o que por sua vez, pode levar à redução da dose, ação prolongada e maior eficácia terapêutica do fármaco [206].

Mais recentemente, SLN foram desenvolvidas e carreadas com uma fração enriquecida em lignana lipossolúvel (LF) purificada de *Ocotea duckei* [207]. As nanopartículas produzidas apresentaram características físico-química adequadas, além disso, foi observado que tais nanopartículas apresentaram alta eficiência de encapsulação, e liberação sustentada. Nos modelos biológicos, verificou-se que SLN carregados com LF não apresentaram toxicidade *in vitro* a macrófagos no intervalo de 20-80 μg/mL e exerciam um efeito anti-*Leishmania* proeminente (20 μg/mL). O estudo demonstrou ainda, que o sistema de SLN melhorou a atividade leishmanicida da fração LF em relação ao fármaco livre [207].

Em outro estudo verificou-se que o NLC potencializou o efeito anti-*Leishmania* do sesquiterpeno cedrol em formas amastigotas de *L. (L.) donovani* resistentes ou não ao estibogluconato de sódio e paromomicina. A incorporação de cedrol em NLC resultou

em um aumento de 2 vezes nos índices de seletividade para cepas de tipo selvagem e resistentes a drogas. Além disso, estudos *in vivo* revelaram que a bioatividade do cedrol veiculado em NLC aumentou a atividade leishmanicida de 2,3 a 3,8 vezes na cepa selvagem, e de 3 a 4,9 vezes nas cepas resistentes aos medicamentos quando comparado ao cedrol livre administrado por via oral em camundongos infectados *L. (L.) donovani* [208].

Recentemente, um estudo mostrou o aumento do potencial do monoterpeno carvacrol carreado em NLC no tratamento da leishmaniose. Os NLC contendo o monoterpeno minimizaram a citotoxicidade da molécula livre, e o aumento da ação leishmanicida *in vitro* e *in vivo* também foi observada em comparado à molécula administrada livremente [209].

Esses resultados podem ser explicados com base em algumas características intrínsecas das nanopartículas lipídicas, incluindo fornecimento de liberação controlada de drogas, maior biodisponibilidade de drogas incorporadas por transporte melhorado para locais-alvo e potencial aprimorado de direcionamento aos macrófagos [210, 211]. Estes estudos reforçam o potencial promissor desses sistemas lipídicos em superar as desvantagens do tratamento atual da leishmaniose.

OBJETIVOS

2 OBJETIVOS

2.1 Objetivo geral

Avaliar a ação dos triterpenos ácido maslínico, ácido ursólico, betulina e lupeol em formas promastigotas e amastigotas de *L. (L.) infantum*, bem como avaliar o potencial terapêutico dos triterpenos ácido ursólico e lupeol encapsulados em carreadores lipídicos nanoestruturados na leishmaniose visceral experimental.

2.2 Objetivos específicos

1) Avaliar a atividade dos triterpenos AM, AU, Be e Lu contra formas promastigotas e amastigotas intracelulares de *L. (L.) infantum*;

2) Analisar alterações ultraestruturais e fisiológicas induzidas pelos triterpenos AM, AU, Be e Lu em formas promastigotas de *L. (L.) infantum*;

3) Produzir e caracterizar fisicamente e quimicamente as nanopartículas lipídicas (SLN e NLC) carregadas com AM, AU, Be e Lu;

4) Avaliar as alterações histológicas em baço, fígado, rim, coração e pulmão induzidos pelo AU e Lu encapsulados em NLC ou livres em hamsters saudáveis;

5) Avaliar o potencial terapêutico do AU e Lu nanoencapsulados em NLC ou livres em hamsters experimentalmente infectados com *L. (L.) infantum*;

6) Avaliar as respostas imune celular e humoral dos animais infectados e tratados com AU e Lu encapsulados em NLC ou livres;

METODOLOGIA

3 METODOLOGIA

Os resultados deste estudo foram publicados em quatro artigos. Portanto, esta tese está sendo apresentada como uma compilação de artigos científicos, reproduzida nas seções seguintes. O primeiro artigo foi publicado em *Pathogens*, em outubro de 2020 (fator de impacto 3.018, disponível em https://www.mdpi.com/2076-0817/9/10/855), e mostra a eficácia do AU em estudos de combinação de fármacos nas leishmanioses experimentais cutânea e visceral. Os dados revelaram que a adição do AU na terapia clássica, efetuada com antimonial pentavalente ou anfotericina B, potencializava o efeito da terapia.

O segundo estudo foi publicado na revista *Journal of Immunology Research* em fevereiro de 2021 (fator de impacto de 3.327, disponível em https://doi.org/10.1155/2021/6671287). O artigo apresenta os resultados comparativos *in vitro* da atividade anti-*Leishmania* dos triterpenos ácido ursólico (AU), betulina (Be) e lupeol (Lu), assim como a fisiologia e morfologia das organelas afetadas pelos triterpenos. Estudos *in vivo* abordam os resultados sobre a toxicidade dos triterpenos em hamsters, e também os dados sobre atividade terapêutica em hamsters infectados com *L.* (*L.*) *infantum*.

O terceiro artigo foi publicado em *Evidence-Based Complementary and Alternative Medicine* em maio de 2021 (fator de impacto de 1.813, disponível em https://doi.org/10.1155/2021/6671287). O estudo apresenta o potencial leishmanicida *in vitro* do ácido maslínico isolado das folhas de *Hyptidendron canum* (Lamiaceae) contra *L. (L.) infantum* assim como as alterações morfológicas e fisiológicas ocasionadas pelo triterpeno.

O quarto artigo foi publicado em *Pharmaceutics* em junho de 2021 (fator de impacto de 4.421, disponível em https://doi.org/10.3390/pharmaceutics13060908). O trabalho apresenta dados referente à produção de carreadores lipídicos nanoestruturados contendo o triterpeno ácido ursólico (NLC-AU). Parâmetros físico-químicos foram avaliados; e a toxicidade desse nanosistema foi analisada em hamsters saudáveis. Além disso, a eficácia do AU aprisionado em NLC foi avaliada na leishmaniose visceral experimental e sua eficácia comparada à anfotericina B.

4 ARTIGO PUBLICADO I

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Article

Ursolic Acid Potentializes Conventional Therapy in Experimental Leishmaniasis

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Abstract

Ursolic acid (UA) is a triterpene with a broad array of pharmacological activities. In leishmaniasis UA killed different species of parasites, and it was active in the experimental model of cutaneous and visceral leishmaniasis. Thus, the objective of this work was to study the therapeutic efficacy of the conventional drugs amphotericin B (AmB) or glucantime (Glu) combined with UA in experimental visceral and cutaneous leishmaniasis, respectively. *L. (L.) infantum*-infected hamsters were treated with AmB alone or combined with UA. *L. (L.) amazonensis*-infected BALB/c mice were treated with Glu alone or combined with UA. Animals were treated for 15 consecutive days by intraperitoneal or intralesional routes. Following one week after the last dose, the tissue parasitism and the cellular immune responses were analyzed. Hamsters treated with 0.2 and 1.0 mg/kg of AmB plus 1.0 mg/kg of UA showed low hepatic and splenic parasitisms, however AmB employed as monotherapy did not reduce the number of viable parasites in the spleen of treated animals. In cutaneous leishmaniasis, Glu given as monotherapy was inactive at 2.0 mg/kg, showed mild activity at 10.0 mg/kg and at 50.0 mg/kg was highly active at eliminating parasites in the skin. When animals were treated with Glu plus UA higher leishmanicidal activity was observed in comparison to all groups treated with monotherapy schemes, and such activity was related to lesion improvement and upregulation of IFN-γ production. Altogether data suggest that the association of drugs for the treatment of leishmaniasis can increase the efficiency of the treatment and decrease the toxicity associated to the conventional drugs.

Keywords: Leishmaniasis; Cutaneous leishmaniasis; Visceral leishmaniasis; Glucantime; Amphotericin B; Therapy

1. Introduction

Leishmaniasis is a parasitic disease that is found mainly in tropical and subtropical areas, and presents a prevalence of approximately 12 million cases, and over 350 million people live in areas at risk of transmission [1]. It is estimated that 0.7 to 1 million new cases and about 26,000 to 65,000 deaths occur annually [1]. Considering the main clinical forms, it has been officially reported approximately 58,000 cases of visceral and 220,000 cases of cutaneous leishmaniases per year [2].

Depending on the infecting species several clinical forms of leishmaniasis can be characterized, such as visceral or cutaneous forms. Visceral leishmaniasis (VL) or kala-azar is a severe and potentially fatal systemic disease [3]. Cutaneous leishmaniasis (CL), on the other hand, can manifest as a single skin lesion at the vector bite site to a less common clinical forms with multiple nodules throughout the body, such as anergic cutaneous diffuse leishmaniasis that can cause deformities and sequelae [3]. The evolution and occurrence of less common or recurrent clinical forms depends on the host general state of health, host genetics, parasite species, virulence as well as parasite-host interactions [4].

The therapeutic options for visceral and cutaneous leishmaniasis are focused on two major drugs, the pentavalent antimonials and amphotericin B [5]. In addition of these both medicines, other alternative drugs or formulations have been employed in the treatment of leishmaniasis as is the case of the high active drug Ambisome®, a liposomal drug containing amphotericin B, that has been used to minimize all side effects caused by amphotericin B, however the high costs related to the treatment is the major drawback in low income countries, such as East Africa and Brazil [6]. More recently, the antineoplasic miltefosine, the only available oral antileishmanial drug, has been used in some countries, but over a decade of use the effectiveness of miltefosine decreased significantly [7]. Of note, all conventional drugs induce local and systemic side effects in patients, and reports about the emergence of parasite resistance have been constantly published, limiting their use [8, 9]

In infectious diseases as well as leishmaniasis, monotherapy has been associated to the emergence of microbial drug resistance [10], and thus association of drugs represents a promising approach to eliminate intracellular parasites faster and at the same time avoid the emergence of resistance in Leishmania species. [10, 11]. Association between drugs has been considered a consensus among specialists for several reasons [6]. Combination of drugs from

different chemical classes may increase the effectiveness of therapy, reduce the dose and time of treatment that has a direct effect in the toxicity, and consequently the patient tolerance and compliance increases during the treatment [6]. Combined therapy can delay the onset of resistance and increase the shelf life of different drugs, as has been observed in the treatment of some infectious diseases, such as malaria, tuberculosis and HIV [10, 12, 13].

Clinical studies have investigated combination therapy, for example, using miltefosine (Mil) with other antileishmanial drugs in the treatment of VL [7, 14]. Two studies conducted in India showed that the association of Mil with a single injection of liposomal AmB (5.0 mg/kg) reduced the treatment time with Mil, which alone would take about 28 days to cure patients with visceral leishmaniasis, whereas patients under combined therapy got cure after seven days [15, 16]. In CL, previous studies have indicated that Glu associated with other drugs was more effective at eliminating parasites compared with Glu alone [17]. In this specific case, Glu combined with paromomycin or Mil synergistically reduced skin parasitism [17].

Due to the serious side effects of drugs commonly used in the chemotherapy of leishmaniasis and the emergence of parasitic resistance, it is necessary to look for new therapeutic targets, which require shorter administration cycles, are more effective and less toxic to patients [10]. Special metabolites from plants represents an interesting alternative in the search for new bioactive compounds, since different molecules have already been described with leishmanicidal activity [18], such as ursolic acid (UA).

UA is a natural pentacyclic triterpene isolated from several medicinal plants such as Baccharis uncinella, Rosemarinus officinalis and Radix actinidiae, and it shows anti-inflammatory [19], anti-cancer [20] and anti-microbial activities [20–25]. In *L. (L.) amazonensis* UA induced morphological, physiological and biochemical alterations that resembled programmed cell death, additionally BALB/c mice treated with UA by the intralesional route improved cutaneous lesion and showed lower tissue parasites than non-treated infected mice [26]. Golden hamsters infected with *L. (L.) infantum* under treatment with UA by intraperitoneal route showed fewer parasites in the spleen and liver, moreover, animals under UA treatment produced high amounts of inducible nitric oxide synthase in the spleen, suggesting enhancement of immune response in treated animals. Furthermore, UA was safe to be administered to BALB/c and golden hamsters since no histological and blood biochemical changes were detected in UA-treated animals [21, 26]. Thus, this compound has been proved to be safe and more importantly, effective in both cutaneous and visceral experimental

leishmaniasis, indicating that it may be considered an important target to develop new drugs directed to the treatment of leishmaniasis.

In order to avoid the emergence of parasites resistance, increase the efficacy and decrease the total amount of classical drugs used in the therapy, the aim of this study is to investigate if the association between UA with glucantime or amphotericin B can improve the efficacy of the treatment in leishmaniasis.

2. Results

2.1. - Chemical analysis of ursolic acid

^{1}H and ^{13}C NMR data were compared with those previously reported in the literature [27–29]. These results, in association with elemental analysis data, indicated that UA exhibited 100% of purity.

2.2. - Evaluation of the therapeutic potential of drug association in experimental visceral leishmaniasis

Hamsters infected with *L. (L.) infantum* and treated with 0.2 and 1.0 mg/kg AmB did not reduce the splenic parasitism in comparison to infected control but when 1.0 mg/kg of UA was added to these doses of AmB, significant reductions in the tissue parasitism (76 and 60.7, respectively, $p<0.05$) were observed (Figure 1A). Animals treated with 5.0 mg/kg AmB alone or associated to UA (5.0 mg/kg AmB plus UA) showed 99.2 and 97.0% of reduction in the splenic parasitism when compared to the infected control group, respectively (Figure 1A), however between these same groups no significative differences of therapeutic activity were observed ($p>0.05$). Additionally, animals treated with 1.0 mg/kg of UA alone showed significant reduction in the splenic parasitism (99.8%) compared to groups treated with monotherapy (0.2 or 1.0 mg/kg of AmB) and combined therapy (0.2 mg/kg AmB plus UA; 1.0 mg/kg of AmB plus UA) as well as infected control ($p<0.05$).

In the liver (Figure 1B), it was observed that animals treated with 0.2 mg/kg of AmB did not show significant reduction in the hepatic parasitism ($p>0.05$) in comparison to infected control, in contrast, animals treated with 1.0 and 5.0 mg/kg AmB alone or in association with UA (1.0 mg/kg) presented significantly less tissue parasites than infected control group ($p<0.05$). Animals treated with 1.0 mg/kg of UA presented significant reduction ($p<0.05$) of

the liver parasitism by 99.9% and comparatively it was more effective at eliminating parasites than 0.2 and 1mg/kg of AmB alone ($p < 0.05$) as showed in the Figure 1B.

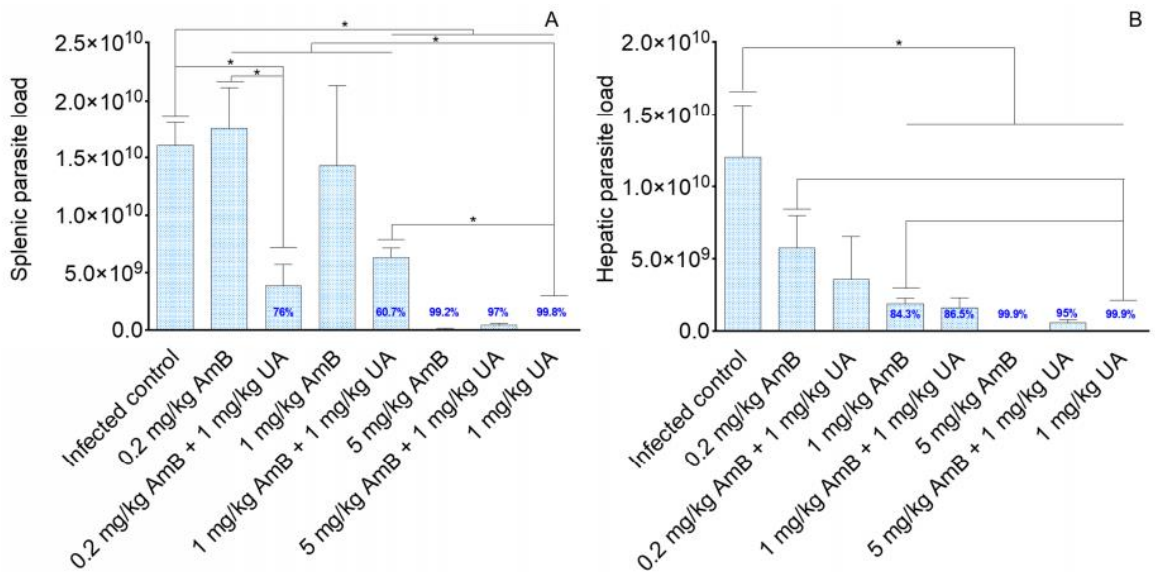

Figure 1 - Golden hamsters were infected intraperitoneally with $2x10^7$ promastigote forms of *L. (L.) infantum*. Four weeks after infection animals were intralesionally treated once, daily for 15 days with amphotericin B (AmB), ursolic acid (UA) or AmB plus UA. One week after the last injection, the number of viable parasites was estimated in the spleen (A) and liver (B) by limiting-dilution assay * $p<0.05$.

2.3. - Analysis of cellular immune response in visceral leishmaniasis

Analysis of cellular immune response in the spleen of *L. (L.) infantum*-infected hamsters showed that only animals treated with 1.0 mg/kg of UA, 5.0 mg/kg of AmB and the therapeutic combination of 5.0 mg/kg AmB + 1.0 mg/kg UA expressed higher levels of IFN-γ compared to the infected group (Figure 2A, $p<0.05$). Differences in the expression of IFN-γ were not observed between groups treated with mono and combined therapies.

The expression of IL-10 (Figure 2B) was similar among groups, except in animals treated with 1.0 mg/kg of UA that presented significant reduction in IL-10 expression in comparison to infected control ($p<0.05$).

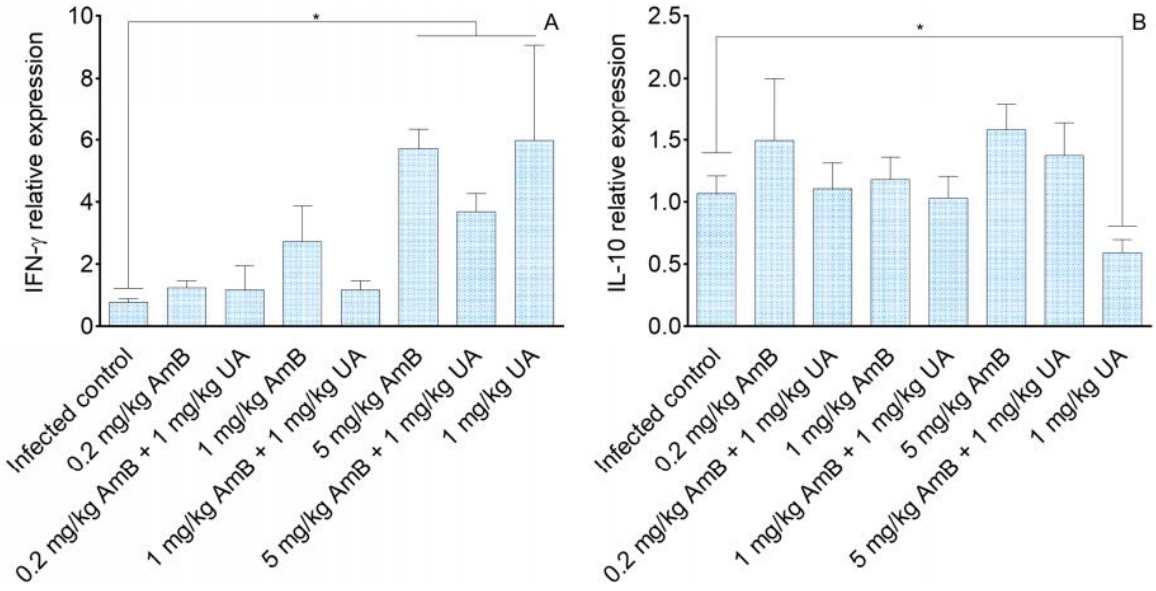

Figure 2 - IFN-γ (A) and IL-10 (B) relative gene expression in the spleen of infected golden hamsters treated with amphotericin B (AmB), ursolic acid (UA) or AmB plus UA. Relative gene expression was estimated by quantitative PCR. The expression levels of genes of interest were normalized to β-actin. * $p<0.05$ indicates statistical significance.

2.4. - Evaluation of the therapeutic potential of drug association in experimental cutaneous leishmaniasis

Infected BALB/c mice treated with 2.0 mg/kg of GLU showed no reduction in the skin parasitism in comparison to the infected control (Figure 3 A, $p>0.05$). In comparison to infected control, this treatment did not change the morphology of the skin lesions that were characterized by ulcerated and necrotic plates (Figure 3B, C and D, $p>0.05$). By the other side, when 2.0 mg/kg of Glu was associated with UA a significant decrease of the skin parasitism was observed compared to infected control and to animals treated with 2.0 mg/kg of Glu alone (figure 3A, $p<0.05$). Additionally, animals treated with 2.0 mg/kg of Glu plus UA presented skin lesions characterized by plaques of medium size in the base of tail, that was smaller in comparison to infected control and to animals treated with 2.0 mg/kg of Glu alone (Figures 3B and E, $p<0.05$). Animals treated with 10.0 mg/kg of Glu alone showed significant reduction in tissue parasitism (73.6%) compared to the infected control ($p < 0.05$), and a significative improvement of the skin lesions was verified, that were characterized as infiltrative plaques of medium size (Figure 3B and F). When 10.0 mg/kg of Glu was associated with UA significant reductions in the skin parasitism were observed in comparison to the skin parasitism from infected control and to animals treated with 10.0 mg/kg of Glu alone ($p<0.05$). Both

monotherapy and combined treatments improved the skin lesions morphology in comparison to infected control (Figures 3B, F and G; $p < 0.05$). Animals treated with 50.0 mg/kg of Glu showed significant reduction in tissue parasitism of 96.3% in comparison to the infected control (Figures 3A), and when UA was associated with 50.0 mg/kg of Glu the parasitism decreased by 99.4% compared to the infected control group ($p<0.05$). Although less parasites were observed in combined therapy (50.0 mg/kg of Glu plus UA), this difference was not significant ($p>0.05$). Similarly, animals treated with 50.0 mg/kg of Glu alone or combined with UA showed infiltrative plaques of small size compared to infected control (Figures 3B, H and I; $p<0.05$). Comparatively mono (50.0 mg/kg of Glu) or combined therapies (50.0 mg/kg of Glu plus UA) did not improve the overall aspect of the skin lesions ($p > 0.05$). Animals treated with UA alone showed reduced skin parasitism in comparison to infected control, and to animals treated with 2.0 and 10.0 mg of Glu ($p<0.05$). Morphologically small infiltrative plaques were identified in the base of tail from animals treated with UA as monotherapy (Figures 3B and J; $p<0.05$).

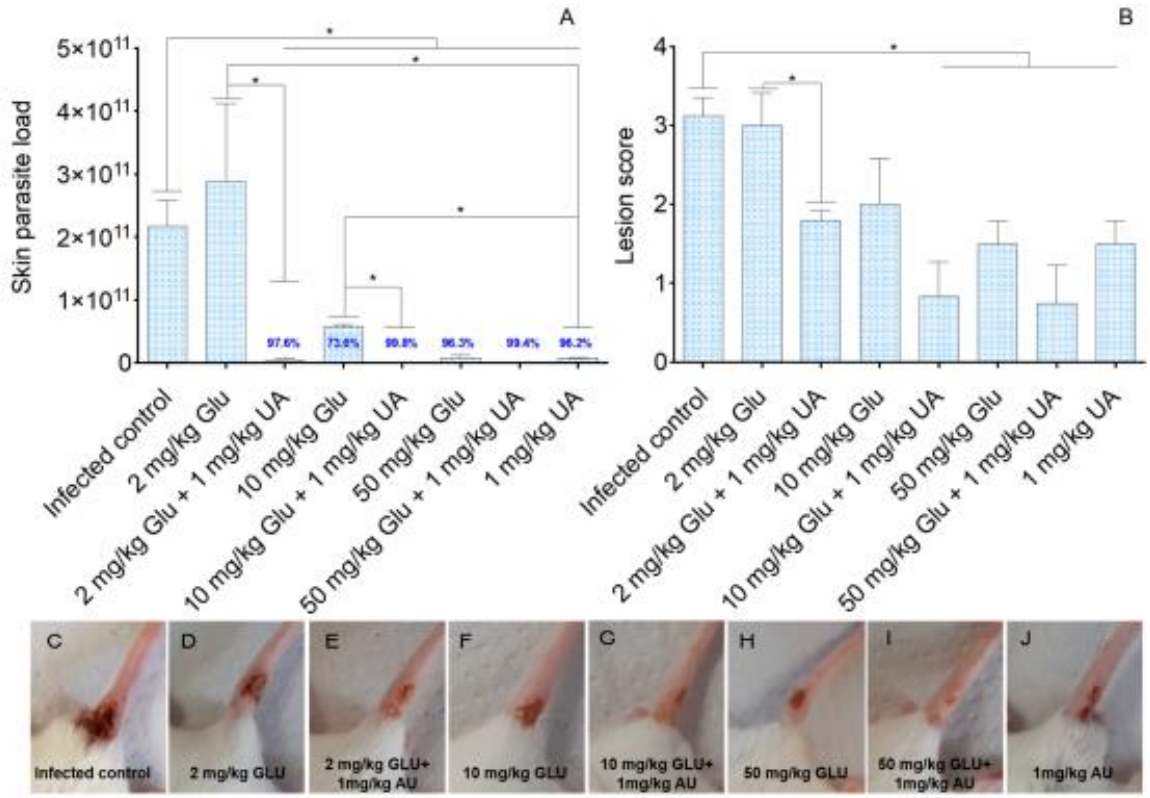

Figure 3 - BALB/c mice were infected into the base of the tail with 10^6 promastigote forms of *L. (L.) amazonensis*. Four weeks after infection animals were intralesionally treated once, daily for 15 days with Glucantime (Glu), ursolic acid (UA) or Glu associated with UA. Skin parasite load, quantified by limiting-dilution assay (A) was analyzed at the 8th weeks post-infection. Lesions were graded as 0 (no lesion), 1 (small infiltrative plaque), 2 (medium infiltrative plaques), 3 (large infiltrative plaques) and 4 (large ulcerated and necrotic plaques) according to the morphology of the lesions. Macroscopic images of the skin lesions from infected control and treated groups (C – J). * $p<0.05$ indicates statistical significance.

2.5. - Analysis of cellular immune response in experimental cutaneous leishmaniasis

Lymph nodes cells were stimulated with AgT from *L. (L.) amazonensis* and the amount of IFN-γ and IL-4 was quantified by ELISA. The treatments, performed as mono or combined therapies, enhanced the ability of lymph node cells to produce IFN-γ, except by the group treated with 2.0 and 50.0 mg/kg of Glu (Figure 4A). Additionally, it was verified that lymph node cells from animals treated with the combined therapy produced high levels of IFN-γ compared to lymph node cells from animals treated with Glu alone ($p<0.05$).

In comparison to the infected control (Figure 4B), IL-4 levels were increased in the group treated with the association between 50.0 mg/kg of Glu and UA ($p<0.05$) and low levels of IL-4 were detected in animals treated with the association between 10.0 mg/kg Glu and UA

($p<0.05$). Comparatively, it was observed that the association of 10.0 mg/kg of Glu with UA reduced the ability of lymph node cells to produce IL-4, and in contrast the association of 50.0 mg/kg of Glu enhanced the ability of lymph node cells to produce IL-4, as illustrated in the figure 4B ($p<0.05$).

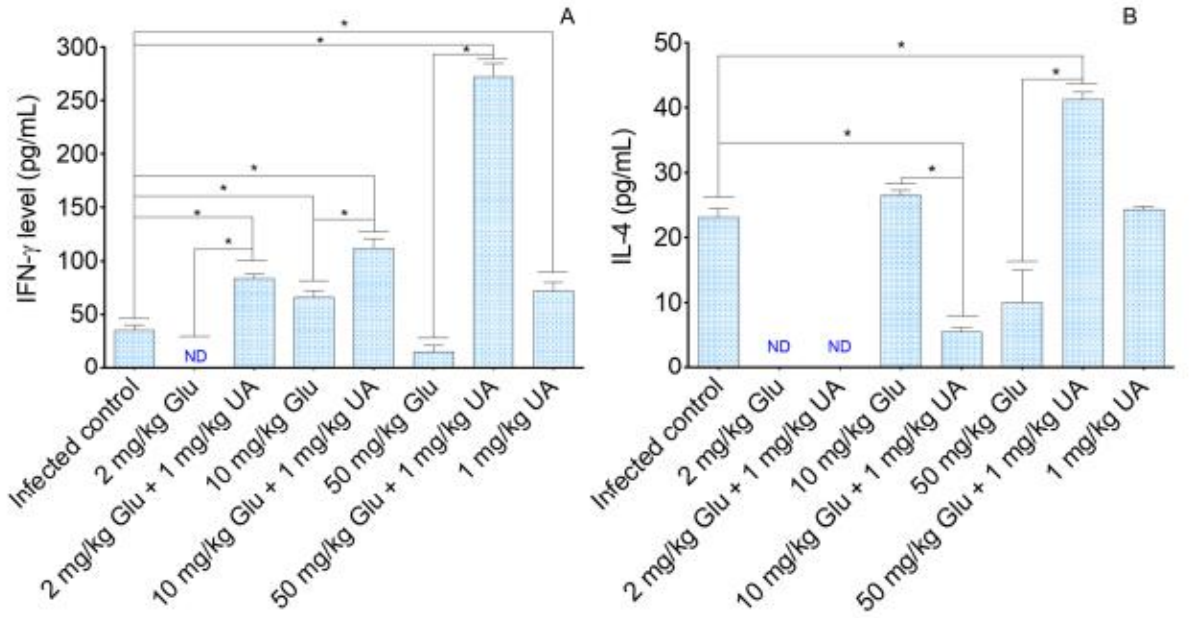

Figure 4 - Mononuclear cells from lymph nodes of treated and control BALB/c mice were isolated and cultured by 72 h under specific stimulation with the whole antigen of *L. (L.) amazonensis* (AgT), following the levels of IL-4 (A) and IFN-γ cytokines (B) were quantified by ELISA. *$p<0.05$ indicates statistical significance.

3. Discussion

The association of drugs brings relevant benefits in the treatment of infectious diseases and presents advantages over monotherapy. Clinical studies indicated that combined treatments can have three major consequences, such as 1) the clinical effects may be the sum of the effect of each drug alone (additive effect); 2) the effect of adding a second drug may exceed the individual effect of each drug alone (synergistic effect), or 3) the interaction between two drugs have opposite desired actions (antagonistic effect) [10]. As a consequence of additive or synergistic effects, patients can be treated with less amount of drugs in comparison to monotherapy [10], that in fact can increase tolerability of the treatment, reduce side effects of drugs as well as costs of hospitalization. Additionally, combined therapy has been considered

an important strategy to increase the efficacy of drugs in infectious diseases, and prevents microbial resistance [30]. In the present study it was verified that the association of UA with classical drugs used in the therapy of leishmaniasis was able to reduce parasite survival and the total amount of amphotericin B or glucantime injected in the experimental animals.

In experimental VL it was verified that 0.2 mg/kg of AmB presented any efficacy at reducing parasites in the spleen and liver of infected hamsters; and the dose of 1.0 mg/kg of AmB reduced the number of amastigote forms only in the liver. By adding UA in the therapy, significant reduction in the splenic and hepatic parasitism was observed, suggesting that this triterpene potentialized AmB activity, mainly the doses of 0.2 and 1.0 mg/kg. In the monotherapy regimen, performed with AmB, reduction in the parasitism in the both spleen and liver was detected only in the highest dose studied (5.0 mg/kg), and in general lines it means that AmB become active *in vivo* when animals receive a total dose of 18.75 mg, that according to previous works this amount of drug, as well as lower ones, induces severe morphological and biochemical changes in the kidney of experimental animals [21, 31]. In contrast, non-therapeutic doses of AmB become therapeutic when combined with the triterpene UA, and it can be considered as an advantage, since combination carried out with low doses of AmB and UA eliminated an elevated number of viable parasites and also may induce less side effects to the host. Additionally, it was verified that 1.0 mg/kg of UA administered alone reduced the number of viable parasites in the spleen and liver, as previously mentioned, in contrast when 5.0 mg/kg of AmB was combined with UA fewer parasites were eliminated in comparison to the respective monotherapy schemes, suggesting an antagonistic effect of this combination. However, it is still important to note that the association in VL eliminated tissue amastigotes in the spleen and liver, whereas low dose of AmB administered as monotherapy wasn't. These results are in agreement with those mentioned by Van Griensven [10], where the effect of two drugs administered in association may be smaller than the effect of each drug alone, suggesting that antagonistic effect in the *in vivo* association [10] may occur. In spite of that, it is still important to highlight that the treatment of animals with the combination between AmB and UA could be considered advantageous, since in this experimental situation only 0.2 mg/kg of AmB (total accumulated amount of AmB 0.75 mg) was needed to decrease the parasitism by 76% in the spleen, and thus, this dose of AmB may induce less side effects than 5.0 mg/kg (total accumulated amount of AmB 18.75 mg), it means that each animal will receive 25 times less AmB when UA is associated.

In experimental cutaneous leishmaniasis it was verified that Glu, given as monotherapy, had a dose-dependent therapeutic activity on infected BALB/c mice, in which 2.0 mg/kg had no impact in the parasitism, 10.0 mg/kg showed mild activity and 50.0 mg/kg drastically reduced the skin parasitism by 96.3% and improved the skin lesion morphology. Furthermore, UA administered alone also reduced parasitism by 96.2%. In addition to the monotherapy scheme, it was observed that UA given as a coadjutant drug became the non-therapeutic dose of Glu (2.0 mg/kg) into an active dose, decreasing the number of viable parasites in the skin by 97.6% and improving the morphology of lesions in the base of tail from infected BALB/c mice. Similar effect was observed in animals treated with 10.0 mg/kg of Glu, where monotherapy reduce parasitism by 73.6% while associated treatment decreased the skin parasitism by 99.8% and a trend to heal the skin lesions was observed. The treatment with 50.0 mg/kg of Glu alone or combined with UA significantly reduced the skin parasitism and although not statistically significant, the associated treatment tends to be more effective (99.4% of reduction) than monotherapy (96.3%). Altogether, these results strongly suggest an additive or synergistic effect of both drugs during the treatment. Previous studies also observed similar behavior when miltefosine, a drug used in the treatment of leishmaniasis, was combined with the experimental drug tamoxifen. In this case, combined drugs were highly effective at controlling parasites in the experimental cutaneous leishmaniasis than monotherapy [33], suggesting that combination of active drugs with different targets on *Leishmania* parasites can be more effective in the treatment than monotherapy.

In fact, Glu became more effective at treating experimental animals with cutaneous leishmaniasis when associated with UA. For example, animals almost reduced all parasites (97.5%) with a total dose of 0.75 mg Glu given along 15 days in association with UA. But in monotherapy scheme a similar reduction in the number of viable parasites in the skin was found only in animals treated with a total amount of 18.75 mg Glu; thus, by adding UA in the treatment 25 times less Glu was required to achieve similar rate of effectiveness. In fact, such reduction in the amount of drug injected in hosts can decrease all local and systemic side effects induced by antimonials [34–36].

In respect to the immunological response, it has been observed that in visceral leishmaniasis parasites downregulate IFN-γ, a Th1 related cytokine, and in contrast upregulate IL-10, a T regulatory associated cytokine [37, 38]. Thus, low or absent level of circulating IFN-γ won't activate macrophage to a leishmanicidal state, additionally, IL-10 is able to inhibit macrophage activation, and is associated to the parasite persistence in the host tissues [39]. In

the present study, it was observed that the spleen of infected hamsters treated with UA (1.0 mg/kg), AmB (5.0 mg/kg) and the combination (UA plus 5.0 mg/kg) expressed significantly more IFN-γ in comparison to infected control [40], suggesting that besides the leishmanicidal activity of both drugs [21, 41]) the therapeutic activity observed accounted by the immunomodulatory activity. By the other side, IL-10 levels did not reduce after treatment, and the maintenance of such cytokine during infection may limit or even inhibit the activity of macrophage [39, 42] in the spleen, explaining, at least in part, the persistence of parasites in animals treated with AmB alone or associated with UA.

In experimental cutaneous leishmaniasis resistance is associated to IFN-γ production and susceptibility to IL-4 [43, 44]. IL-4 cytokine is responsible to maturate B cells that in turn will produce antibodies, that in leishmaniasis are ineffective, since parasites are hidden in intracellular environment [45]. In monotherapy scheme, only the groups treated with 1.0 mg/kg of UA or 10.0 mg/kg of Glu increased IFN-γ production. In contrast, animals treated with all doses of Glu plus UA showed augmentation of this cytokine, suggesting that UA was able to modulate the immune system of treated animals, and such activity may be related to macrophage activation [22]. Previously, it was demonstrated that an UA-enriched fraction, purified from the leaves of *Baccharis uncinella*, was efficient at eliminating tissue amastigotes in experimental CL and these animals produced elevated amounts of IFN-γ [26], reinforcing that UA has modulatory activity on the immune system. Besides, it is still important to note that UA has a direct effect on *Leishmania* sp. [21, 22], and in *vivo* parasite elimination can be the sum of both leishmanicidal and immunomodulatory activities. Additionally, it is important to note that from the immunological point of view UA and Glu showed an additive or potentializing effect on the Th-1 immune response, since groups treated with combined drugs did not produce lower levels of IFN-γ than the respective monotherapy regimen, and importantly all doses in combined therapy improved the overall aspect of macroscopic lesions than the respective monotherapy control, as observed in figures 3E, G and I. In spite of IFN-γ increasing in the majority of treated groups, animals treated with Glu alone or combined still produced IL-4, that at least in part, may reduce the bioactivity of IFN-γ, avoiding getting sterile cure. In spite of that, in all experimental conditions IFN-γ levels were higher than IL-4 levels, reinforcing that the therapeutic activity of this combination may be related to direct effects of Glu, UA and immunomodulatory activity.

Taken together, the results presented herein suggest that UA combined with the conventional drugs AmB and Glu can potentialize their activity in leishmaniasis in comparison to monotherapy schemes, especially in experimental cutaneous leishmaniasis. Although in VL, AmB plus UA did not present additive effects, mainly in the highest dose, it may still be considered as a viable alternative for the treatment of VL, given that the association required lower amounts of AmB to reduce tissue parasitism, that in turn may create a less toxic treatment and consequently greater patient compliance and tolerance. In experimental cutaneous leishmaniasis UA potentialized the activity of Glu, and possibly an additive or synergistic effect was obtained, since a robust therapeutic effect compared to the monotherapy was observed, additionally Th1 immune response was higher in groups treated with combined therapy compared to monotherapy, that in fact, is an important factor to efficiently reduce the tissue parasitism. Altogether these data suggest that combination therapy may be an interesting strategy to increase efficacy of classical drugs in the treatment of leishmaniasis.

4. Materials and Methods

4.1. Drugs

UA was purchased from Cayman, USA, injectable glucatime and deoxycholate amphotericin B drugs were purchased from Sanofi-Aventis (Brazil) and Cristália (SP, Brazil), respectively.

4.2. Chemical analysis of ursolic acid

^{1}H (300 MHz) and ^{13}C (75 MHz) Nuclear Magnetic Resonance (NMR) spectra were recorded on deuterochloroform ($CDCl_3$), deuterated dimethyl sulfoxide (DMSO-d_6) or deuterated methanol (CD_3OD) (Merck) to UA using a Bruker DPX-300 spectrometer. Elemental analysis of UA was obtained in a Perkin-Elmer Elemental Analyzer model 2400 CHN.

4.3. Experimental animals and ethical considerations

Male Golden hamsters (*Mesocricetus auratus*), eight weeks old, were obtained in Anilab (São Paulo State, Brazil) and male BALB/c mice, five weeks old, in Medical School of the University of Sao Paulo, Brazil. This study was carried out in strict accordance with the recommendations detailed in the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation (http://www.cobea.org.br). The

protocol was approved by the Ethics Committee of Animal Experiments of the Institutional Committee of Animal Care and Use at the Medical School of São Paulo University (CEUA 056/16). Hamsters and mice were housed according to the standards of the Committee of Animal Welfare, and allowed access to food and water ad libitum throughout the study under a 12 h light cycle. The animals were anesthetized with intraperitoneal sodium thiopental.

4.4. - Parasites

L. (L.) infantum (MHOM/BR/72/46) and *L. (L.) amazonensis* (MHOM/BR/73/M2269) were kindly provided by Prof. Dr. Fernando Tobias Silveira from the cryobank of the "Leishmaniasis Laboratory Prof. Dr. Ralph Laison", Department of Parasitology, Ministry of Health, Evandro Chagas Institute (Belém, Pará - Brazil). They were identified using monoclonal antibodies and isoenzyme electrophoretic profiles at the Leishmaniasis Laboratory of the Evandro Chagas Institute. Parasites were maintained in Schneider's medium supplemented with 10% heat-inactivated fetal bovine serum, 10 μg/ml streptomycin and 10 IU/ml ampicillin (ThermoFisher) (S10). Parasites in late log stage were used for all experiments.

4.5. - Evaluation of the therapeutic potential of drug associations in visceral and cutaneous leishmaniasis

Forty golden hamsters (eight weeks old, weight ~ 250 g) were inoculated intraperitoneally with 100 μL of the parasite suspension (2×10^7 *L. (L.) infantum* promastigotes). Five healthy control group was injected with PBS alone. After 45 days of infection, groups were arranged as follows: groups 1; 2 and 3 were treated with 1.0 mg/kg UA plus 0.2, 1.0 or 5.0 mg/kg of AmB, respectively. Group 4; 5 and 6 were treated exclusively with AmB, at 0.2; 1.0 and 5.0 mg/kg respectively; group 7 was treated with 1.0 mg/kg of UA; group 8 was the infected control group injected with vehicle solution and group 9 was untreated and uninfected animals (healthy control), injected with vehicle solution. In experimental visceral leishmaniasis, amphotericin B was opted as the standard treatment, because in our model, glucantime showed low efficacy (data not show). A solution of associated UA + AmB was administered by the intraperitoneal route for 15 consecutive days. One week after the last injection, animals were sacrificed in CO_2 chamber, and the spleen and liver were collected to determine splenic and hepatic parasitism. Each group was composed of at least four hamsters and the experiment was repeated three times, independently. UA was solubilized in DMSO and

further PBS (never exceeding 1% DMSO); and AmB was solubilized in sterile water. The highest doses of 1.0 and 5.0 mg/kg from AmB and UA, respectively, were selected based on previously published works [21, 46, 47].

Forty BALB/c mice (four weeks old; weight ~ 25 g) were inoculated at the base of the tail with 2×10^7 promastigote forms of *L. amazonensis* in stationary phase of growth (final volume 25 µL) as described in previous published works [9, 47–50]. Five healthy control group was injected only with physiological solution. After 45 days of infection, groups were arranged as follows: groups 1; 2 and 3 were treated with 1.0 mg/kg of UA plus 2.0, 10.0 or 50.0 mg/kg of glucantime, respectively; groups 4; 5 and 6 were treated exclusively with Glu (2.0, 10.0 and 50.0 mg/kg); group 7 was treated with 1.0 mg/kg of UA, and group 8 was the infected control group injected with vehicle solution while group 9 was untreated and uninfected animals, injected with vehicle solution (healthy control). All treatments were given with a single daily dose of 20 µL, under intralesional route. The highest doses of 50.0 mg/kg from Glu was selected based on previously published works [48, 51, 52].Treatment outcome was determined by the skin parasitism that was analyzed one week after the end of treatment. Before, during or after experimental treatments deaths were not recorded.

4.6. - Determination of parasite load

The parasite load was determined by limiting dilution assay [21, 53]. In VL, parasitism was quantified in the spleen and liver, while in cutaneous leishmaniasis, it was analyzed in the skin at the point of parasite inoculation. Briefly, the skin, spleen and liver were individually weighed, homogenized in S10 and them diluted 1:2000 (skin) or 1:500 (spleen and liver). An initial homogenized suspension was placed into the first well (200 µL) and serial dilutions (1:4) were distributed in a 96-multiwell plate (Nunc, Germany) and subjected to 12 serial dilutions with four replicate wells. After 10 days at 25°C, each well was examined by optical microscopy and the final titer was set as the highest dilution for which the well contained at least one parasite. The viable parasitic load per gram of homogenized organ was calculated as follows: (reciprocal titer of the last positive well per total volume of homogenized tissue x dilution factor) divided by the weight (gram) of the homogenized tissue. The parasite load was expressed as the number of parasites per gram of homogenized organ.

4.7. - Analysis of cellular immune response in VL and CL

RNA from hamster spleen fragments (~10 mg) was extracted using the commercial RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. cDNA was synthesized with the SuperScript®VILO™ cDNA Synthesis Kit (Life Technologies). Amplification conditions consisted of an initial denaturation phase at 95°C for 10 min, followed by 40 amplification cycles consisting of 95°C for 15 s, 61°C for 90s, and 72°C for 30 s, using a thermocycler (Eppendorf, Hamburg, Germany). Prior to quantification, the efficiency of each qPCR reaction was verified using cDNA from spleens of healthy animal; it was always above 95%. Expression levels of genes of interest were normalized to β–actin (endogenous control). qPCR reaction was carried out using the GoTaq® 1-Step RT-qPCR System (Promega Corporation, Madison, WI, USA) and 75 nM of specific primer pairs. The primer sequences were as follows (5' to 3′): IFN- γ forward: GACAACCAGGCCATCC and reverse: CAAAACAGCACCGACT; IL-10 forward: TGGACAACATACTACTCACTG and reverse: GATGTCAAATTCATTCATGGC; b-actin forward: TCCTGTGGCATCCACGAAACTACA and reverse: ACAGCACTGTGTTGGCATAGAGGT [54]. The amplification conditions consisted of 95°C for 10 minutes followed by 40 cycles of 95°C for 15 s, 57°C for 30 s, and 72°C for 30 s. Each sample was analyzed at least in duplicate. Results were expressed as relative gene expression to samples from infected hamsters. PCR products were electrophoresed on 2% agarose gels to confirm amplification of products with the correct size.

The determination of IL-4 and IFN- γ cytokines in BALB/c was analyzed in the supernatants of lymph node cultures stimulated with the whole antigen produced with *L. (L.) amazonensis* promastigotes (AgT). Lymph node cells from treated animals (5×10^5 cells/well) were cultured in RPMI 1640 medium (R10 medium) supplemented with 10% fetal bovine serum, 1% L-glutamine, 10 μg/ml streptomycin and 10 IU/ml ampicillin (ThermoFisher) under stimulation with 50 μg of whole antigen of *L. amazonensis* or 10 μg of concanavalin A as a positive control; negative controls were incubated only with R10 medium. After 72 h, the supernatants of the different groups were collected, and the amounts of IL-4 and IFN-γ (BD, Franklin Lakes, NJ, USA) were quantified by sandwich enzyme-linked immunosorbent assay (ELISA) in accordance with the manufacturer's recommendations.

4.8. - Statistical analysis

All experiments were repeated at least three times, and the results were expressed by the arithmetic mean ± standard deviation. Statistical analyses were performed using GraphPad

Prism 5.0, and the statistical test Kolmogorov-Smirnov was used, followed by one-way ANOVA. Differences were considered statistically significant at a 5% significance level ($p<0.05$).

Author Contributions: "Conceptualization, J.A.J; L.F.D.P; methodology, J.A.J; J.H.G.L.; M.D.L; L.F.D.P; validation, J.A.J; J.H.G.L.; M.D.L; L.F.D.P.; formal analysis, J.A.J; J.H.G.L.; M.D.L; L.F.D.P.; investigation, J.A.J; T.N.F.S.; E.S.Y.; J.H.G.L.; M.D.L; L.F.D.P; resources, M.D.L; J.H.G.L.; L.F.D.P.; data curation, L.F.D.P.; writing-original draft preparation, J.A.J; J.H.G.L.; M.D.L; L.F.D.P.; writing—review and editing, J.A.J; J.H.G.L.; M.D.L; L.F.D.P; visualization, J.A.J; J.H.G.L.; M.D.L; L.F.D.P.; supervision, L.F.D.P.; project administration, L.F.D.P.; funding acquisition, L.F.D.P. All authors have read and agreed to the published version of the manuscript.", please turn to the CRediT taxonomy for the term explanation. Authorship must be limited to those who have contributed substantially to the work reported.

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Conflicts of Interest: The authors declare no conflict of interest

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Research Article

Related Pentacyclic Triterpenes Have Immunomodulatory Activity in Chronic Experimental Visceral Leishmaniasis

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Abstract

Leishmaniasis is a neglected tropical disease caused by the flagellated protozoa of the genus *Leishmania*, that affects millions of people around the World. Drugs employed in the treatment of leishmaniasis have limited efficacy and induce local and systemic side effects to the patients. Natural products are interesting alternative to treat leishmaniasis, because some purified molecules are selective toward parasites and not to the host cells. Thus, the aim of the present study was to compare the *in vitro* antileishmanial activity of the triterpenes betulin (Be), lupeol (Lu) and ursolic acid (UA); analyze the physiology and morphology of affected organelles; analyze the toxicity of selected triterpenes in golden hamsters; study the therapeutic activity of triterpenes in hamsters infected with *L. (L.) infantum* as well as the cellular immunity induced by studied molecules. The triterpenes Lu and UA were active on promastigote (IC_{50} = 4.0 ± 0.3 and 8.0 ± 0.2 μM, respectively) and amastigote forms (IC_{50} = 17.5 ± 0.4 and 3.0 ± 0.2 μM, respectively) of *L. (L.) infantum* and their selectivity indexes (SI) toward amastigote forms were higher (≥ 13.4 and 14, respectively) than SI of miltefosine (2.7). *L. (L.) infantum* promastigotes treated with Lu and UA showed cytoplasmic degradation, and in some of these areas cell debris were identified, resembling autophagic vacuoles, parasite mitochondria were swelled, fragmented and displayed membrane potential altered over time. Parasite cell membrane was not affected by studied triterpenes. Studies of toxicity in golden hamster showed that Lu did not altered blood biochemical parameters associated to liver and kidney functions, however it was detected a slight increase of aspartate aminotransferase level in animals treated with 2.5 mg/kg of UA. Lu and UA triterpenes eliminated amastigote forms in the spleen (87.5 and 95.9% of reduction, respectively) and liver of infected hamster (95.9 and 99.7% of reduction, respectively); and UA showed similar activity at eliminating amastigote forms in the spleen and liver than amphotericin B (99.2 and 99.8% of reduction). The therapeutic activity of both triterpenes was associated to the elevation of IFN-γ and/or iNOS expression in infected treated animals. This is the first comparative work showing the *in vitro* activity, toxicity and therapeutic activity of Lu and UA in the chronic model of visceral leishmaniasis caused by *L. (L.) infantum*, additionally both triterpenes activated cellular immune response in the hamster model of visceral leishmaniasis.

Keyswords Antileishmanial activity, triterpenes, cytotoxicity, mitochondria**,** visceral leishmaniasis

1 Introduction

Leishmaniasis is a neglected tropical disease and a public health problem worldwide, affecting vulnerable people in 98 countries, with 12 million cases detected worldwide per year, additionally 1 billion people live in areas at risk of transmission [1]. The most severe form of the disease is visceral leishmaniasis (VL), also known as kala-azar, and in Latin America it is caused by *L. (L.) chagasi* or *L. (L.) infantum* [2]. VL has an incidence of 1.6 cases per 100,000 inhabitants and is considered fatal if not properly treated. The main compromised organs are the spleen, liver and bone marrow. Hepatomegaly, splenomegaly, pancytopenia, prolonged fever and weight loss are the main clinical signs of manifested disease [3].

The therapeutic arsenal available for the treatment of leishmaniasis is scarce, and it is based on the use of the first-line drug, pentavalent antimonial [4]. Second-line drugs, such as amphotericin B, and its liposomal formulation Ambisome, paromomycin and more recently, miltefosine have also been used with different degrees of effectivity worldwide [4,5]. Additionally, all these second-line drugs have limitations, such as long duration of treatment, high costs, local and systemic side effects, that include pain in the local of application, nephrotoxicity, cardiotoxicity, gastrointestinal events and teratogenic effects [6,7].

Based on the aforementioned comments, it becomes clear the importance of identifying new leishmanicidal drugs. According to DNDi (Drugs for Neglected Diseases initiative) new prototype drugs need to be safe and affordable. In this sense, different studies have shown that natural molecules have these characteristics and can be considered prototype antileishmanial drugs [8]. Furthermore, these natural products are active and selective toward pathogens, additionally the selectivity can be improved after synthetic modifications [9,10].

Triterpenes are the most representative group of phytochemicals, comprising more than 20,000 recognized compounds and are biosynthesized in plants through the cyclization of squalene [11]. Due to the structural diversity associated with their pharmacological effects, these compounds are considered interesting candidates for the development of new drugs [12,13]. Moreover, in some Asian countries, triterpenes are used as anti-inflammatory, analgesic, hepatoprotectant, cardiotonic agents and sedatives [14,15].

Betulin (Be), lupeol (Lu) and ursolic acid (UA) are pentacyclic triterpenoids widely distributed in nature and exhibit important pharmacological effects such as antioxidant, antiallergic, antipruritic, antiangiogenic, and antimicrobial agents [16–18]. Additionally, these

pentacyclic triterpenes have immunomodulatory activity, and depending on the dose employed and route of administration Th1-associated cytokines as well as specific mediators of inflammation can be produced by innate or acquired immune cells [19]. In visceral leishmaniasis antigen-specific immune suppression is caused by *L. (L.) infantum* [20,21], and thus becomes essential to identify immunomodulatory-leishmanicidal prototype drugs, that are able to reverse the immunosuppression caused by parasites. Considering such inherent properties of pentacyclic triterpenes on the immunity, the scarcity of drugs available to treat leishmaniasis, the severe side effects of antimonial and amphotericin B and the emergence of resistance in Leishmania sp., the present study aimed at analyze the activity and selectivity of Be, Lu and UA on an American strain of *L. (L.) infantum*, as well as the possible target organelles. Additionally, the toxicity, therapeutic and immunomodulatory activities of these triterpenes were studied in the experimental model of chronic visceral leishmaniasis.

2 Material and methods

2.1 Chemical analysis of the triterpenes Be, Lu and UA

Be, Lu and UA triterpenes were purchased from Cayman Chemicals (USA). ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectra were recorded, respectively, at 300 and 75 MHz in a DPX-300 spectrometer (Bruker, USA) using deuterochloroform ($CDCl_3$), deuterated dimethyl sulfoxide (DMSO-d_6) or deuterated methanol (CD_3OD) as solvents and internal standard (Merck, Germany). Elemental analysis was obtained in an Elemental Analyzer 2400 CHN (Perkin-Elmer, USA).

2.2 Animals and ethical considerations

Golden hamsters (*Mesocricetus auratus*), 8 weeks old, were obtained from Anilab (Paulinia, São Paulo, Brazil). This study was carried out in strict accordance with the recommendations of the guide for the Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation (http://www.cobea.org.br). The protocol was approved by the Ethics Committee of Animal Experiments of the Institutional Committee of Animal Care and Use at the Medical School of São Paulo University (056/16). Hamsters were

housed in the Animal Experimental Institute of Tropical Medicine of São Paulo (IMT-USP), according to the standards of the Committee of Animal Welfare, and allowed access to food and water ad libitum throughout the study under a 12 h light cycle. The animals were anesthetized with intraperitoneal sodium thiopental at 1 mg/200 µL (Cristália, Brazil).

2.3 Cytotoxicity assay

Peritoneal macrophages from golden hamsters (*Mesocricetus auratus*) (10^6 macrophages / well) were cultured in 96-well plates in RPMI medium supplemented with 10% of fetal bovine serum (Thermo Fisher, USA), 2 mM L-glutamine (Sigma-Aldrich, USA), 10 mM Hepes (Sigma-Aldrich, USA), 1 mM sodium pyruvate, 1% v/v nonessential amino acid solution (Thermo Fisher, USA), 10 µg/mL of gentamicin (Thermo Fisher, USA) and 1000 U/mL of penicillin (Thermo Fisher, USA) (R10) along with triterpenes Be, Lu and UA or the standard drug miltefosine (2.0 to 240 µM). The plates were incubated at 37°C, 5% CO_2, for 24h and them centrifuged at 400 *g* for 10 min at 4°C and washed 3 times, followed by addition of 9.6 µM of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide - MTT (Sigma-Aldrich, USA). Four hours later, 50 µL of 10% sodium dodecyl sulfate (SDS) was added to each well. The plates were further incubated for 18 h and read in ELISA reader at 595 nm. Cytotoxic concentration 50% (CC_{50}) was estimated using the non-linear regression test with GraphPad Prism 5.0 software. The index of selectivity (SI) was estimated according to Passero and collaborators [22], and essentially it is the ratio between CC_{50} per IC_{50}.

2.4 Promastigote assay

Promastigote forms of *L. (L.) infantum* (MHOM/BR/72/46) were incubated in 96-well culture plate in Schneider's medium (Sigma-Aldrich, USA) supplemented with 10% heat inactivated fetal bovine serum and 50,000 IU/mL penicillin, 50 mg/mL streptomycin (S10), at $2x10^6$ promastigotes/well with the triterpenes Be, Lu and UA or standard drug miltefosine from 2.0 to 240 µM – (Sigma-Aldrich, USA). Negative control group was cultivated in medium and vehicle solution (PBS plus 1% DMSO). The parasites were incubated for 24 h at 25°C, then washed with 200 µL of PBS three times with centrifugation at 1200 g, 10 min at 4°C, followed

by addition of 9.6 µM of MTT. Four hours later, 50 µL of 10% sodium dodecyl sulfate (SDS) was added to each well [23]. The plates were further incubated for 18 h and read in ELISA reader at 595 nm. Inhibitory concentration 50% (IC_{50}) was estimated using Graph Pad Prism 5.0 software.

2.5 Activity of triterpenes in intracellular amastigotes, nitric oxide and hydrogen peroxide production

Peritoneal macrophages from golden hamsters (10^6 macrophages) were collected and cultured on round coverslips in 24-well plates during 4h in R10, and then cells were infected with *L. (L.) infantum* promastigotes (10 parasites per 1 peritoneal macrophage). The plates were incubated overnight at 5% CO_2 at 37 °C. Then the triterpenes Be (14 to 113 µM), Lu (12 to 96 µM), and UA (2 to 22 µM) were added to the infected macrophages. Of note, the highest concentration used was always below the values of the respective CC_{50} values. Miltefosine (18 to 74 µM) was used as the positive control. After 24 hours, macrophage supernatants were collected and stored at -80°C for quantifications of nitric oxide (NO) (Life Technologies, USA) and hydrogen peroxide (H_2O_2) (Life Technologies, USA) according to the manufacturer's instructions of the respective kits. The coverslips were dried at room temperature, fixed in methanol and stained by Giemsa (Sigma-Aldrich, USA). The number of infected macrophages and of parasites per macrophage was determined at least in 100 cells. The infection index (II) was expressed as the percentage of infected macrophages multiplied by the average number of amastigotes per macrophage according to Passero and collaborators [24], and the inhibitory concentration that inhibit 50% (IC_{50}) of the infection index was estimated using Graph Pad Prism 5.0. As a positive control for NO and H_2O_2 macrophages were incubated with 100 ng/mL LPS (Sigma-Aldrich, USA) according to Passero [22].

2.6 Ultrastructural alterations induced by triterpenes in *L. (L.) infantum*

Promastigote forms of *L. (L.) infantum* ($2x10^6$ promastigotes/well) were incubated in 24-well culture plate in S10 with Lu and UA at EC_{50} for 24h, 25°C. Control group was cultivated with medium and vehicle solution. The plate was centrifuged at 1200 g, 4°C, 10 min and washed three times with 200 µL of PBS. Then the pellets were resuspended in

glutaraldehyde 2% (Sigma-Aldrich, USA), pH 7.2 and incubated at 4°C, during 60 min. Parasites were post-fixed in 1% osmium tetroxide (Sigma-Aldrich, USA), were stained and block staining in 0.5% aqueous uranyl acetate overnight, dehydrated in a graded series of ethanol. Then, samples were embedded in a polyester resin, thin sectioned with Reichert ultramicrotome, double stained by uranyl acetate and lead citrate (Ladd Research Industries), and examined with a Jeol 1010 (Tokyo, Japan) transmission electron microscope (TEM).

2.7 Cell membrane integrity and mitochondrial membrane potential assays

Promastigote forms of *L. (L.) infantum* ($2x10^6$ promastigotes/well) were incubated in 96-well black culture plate (Corning Inc, USA) in S10 with the IC_{50} of triterpenes Lu and UA as well as miltefosine for 0, 10, 20, 30, 40, 50, 60, 120 and 1440 minutes. At each time-point the probes SYTOX Green at 0.5 μM/well (LifeTechonologies, USA) or Rhodamine 123 at 3 μM/well (Sigma-Aldrich, USA) were added in the parasite culture in order to evaluate cell membrane damage or mitochondrial membrane potential, respectively. Parasites were placed in the dark for 15 minutes at 25°C, then centrifuged at 1200 g, 5 min at 10°C and washed three times with 200 μL of PBS. Plates containing parasites stained with Sytox Green were read in fluorescence reader using 530 nm emission wavelength and 490 nm excitation, and parasites stained with Rhodamine 123 read with 520 nm emission wavelength and 485 nm excitation. Results were normalized in relation to the control, non-treated parasites. Triton X-100 (0.05 μL) (Sigma-Aldrich, USA) was used as a positive control for cell membrane damage and oligomycin at 0.1 μM/well (Cayman Chemicals, USA) as positive control of mitochondrial membrane potential inhibition.

2.8 Hepatic and renal biochemical parameters

Healthy golden hamsters (8 weeks old) were divided into four groups containing 5 animals/group. The experimental groups were arranged as follows: groups 1 and 2 were treated with 2.5 mg/kg of UA or Lu, respectively. Group 3 was treated with 5.0 mg/kg of amphotericin B [25] (Cristália, Brazil) and group 4 was constituted by animals that received only the vehicle solution (Control). Animals were treated by the intraperitoneal route, once a day, during 10

days. One week after the last injection, the animals were euthanized, sera collected and the following biochemical parameters quantified: serum alanine transaminase (ALT), aspartate aminotransferase (AST), urea and creatinine by colorimetric method on COBAS C111 equipment (Roche, USA).

2.9 Therapeutic activity of triterpenes in visceral leishmaniasis

Golden hamsters were infected intraperitoneally with $2x10^7$ *L. (L.) infantum* promastigotes (MHOM/BR/72/46). Non-infected control group was injected with PBS alone. Infected hamsters were divided into 4 groups, with 5 animals each. After 60 days of infection, animals were treated with UA, Lu or AmB and the last group was constituted by animals that received only the vehicle solution (Control). The protocol of the treatment is described in the item 2.8. One week after the last injection, animals were euthanized and the spleen and liver were collected to quantify the splenic and hepatic parasitism by limiting-dilution assay [26]. Additionally, amastigote forms in such organs were demonstrated by immunohistochemistry technique [27].

2.10 Cell immune response

RNA from hamster spleen fragments (~10 mg) was extracted using the commercial RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. cDNA was synthesized with the SuperScript®VILO™ cDNA synthesis kit (Life Technologies, USA). Amplification consisted of an initial denaturation phase at 95°C for 10 min, followed by 40 amplification cycles consisting of 95°C for 15 s, 61°C for 90s, and 72°C for 30 s, using a thermocycler (Eppendorf, Germany). Prior to quantification, the efficiency of each reaction was verified using cDNA from the spleens of a healthy animal; it was always above 95%. Expression levels of genes of interest were normalized to β-actin (endogenous control). Quantitative PCR (qPCR) reaction was carried out using the GoTaq® 1-Step RT-qPCR System (Promega Corporation, Madison, WI, USA) and 75 nM of primers. The primer sequences (Sigma-Aldrich, USA) were as follows (5´ to 3´): IFN-γ forward: GACAACCAGGCCATCC and reverse: CAAAACAGCACCGACT; IL-10 forward: TGGACAACATACTACTCACTG and reverse: GATGTCAAATTCATTCATGGC; iNOS forward:

CGACGGCACCATCAGAGG and reverse: AGGATCAGAGGCAGCACATC; β-actin forward: TCCTGTGGCATCCACGAAACTACA and reverse: ACAGCACTGTGTTGGCATAGAGGT. Quantification results are expressed in fold changes of $2^{-\Delta Ct}$ over the infected control group. PCR products were electrophoresed on 2% agarose gels to confirm amplification of products with the correct size; one single amplification product of predicted size, according to Lafuse [28], was always obtained for such reactions.

2.11 Statistical analysis

All data obtained have been reported as the mean of three independent assays. Values are expressed as mean ± standard error. Statistical analyses were performed using GraphPad Prism 5.0 software, and the ANOVA test was used to assess the differences between groups. Statistical significance was set at a p-value < 0.05.

3 Results

3.1 Chemical analysis of the triterpenes

NMR (^{1}H and ^{13}C) data triterpenes betulin (Be), lupeol (Lu) and ursolic acid (UA) were recorded and obtained data were compared with those reported in the literature [29–31]. These data, in association with elemental analysis, indicated that tested compounds exhibited more than 99.5% of purity (supplementary material 1).

3.2 Effect of triterpenes on promastigotes, amastigotes and in host cells

Promastigote forms of *L. (L.) infantum* were incubated with the triterpenes during 24h and the inhibitory 50% concentration (IC_{50}) for each compound was estimated. All triterpenes were active against promastigote forms of *L. (L.) infantum*, being Lu (IC_{50} = 4.0 ± 0.3 μM) more effective at inhibiting promastigote forms growth, followed by UA (IC_{50} = 8.0 ± 0.2 μM) and Be (IC_{50} = 133.0 ± 3.0 μM). Miltefosine, used as positive control, showed IC_{50} of 13.5 ± 0.9 μM (Table 1). Regarding the cytotoxic potential of triterpenes on peritoneal

macrophages of golden hamsters, it was verified that Lu presented CC_{50} above 200 μM. Triterpenes Be and UA displayed CC_{50} of 170.4 ± 0.5 μM and 42.1 ± 0.2 μM, respectively. Miltefosine eliminated 50% of the cell population with 89.3 ± 8.3 μM (Table 1).

Based on the ratio between IC_{50} and CC_{50}, it was possible to calculate the SI of the triterpenes towards *L. (L.) infantum*. In this regard, compound Lu was 58.5 times more selective to promastigote forms than the host macrophages, followed by triterpenes UA and Be (SI = 5.3 and 1.3, respectively). Miltefosine presented a SI of 6.6. These data are summarized in Table 1.

Macrophages infected with *L. (L.) infantum* and treated with triterpenes Lu and UA exhibited significant decrease in the parasitism, as demonstrated by the respective IC_{50} values. As shown in Table 1, the most active triterpene was UA followed by Lu, with IC_{50} values of 3.0 ± 0.2 μM and 17.5 ± 0.4 μM. Be was inactive on amastigote forms of *L. (L.) infantum*. Miltefosine killed amastigote forms with an IC_{50} of 33.4 ± 2.0 μM. Comparatively Lu and UA triterpenes were more effective at killing intracellular amastigote forms compared to the standard drug miltefosine. Selectivity indexes of analyzed triterpenes over amastigote forms of *L. (L.) infantum* were higher than that determined to miltefosine (SI = 2.7).

Table 1 - Leishmanicidal, cytotoxic activity and selective index of betulin (Be), lupeol (Lu) and ursolic acid (UA). Results are expressed by mean and standard error of triplicates from three different experiments.

Compound	IC_{50} (μM) promastigotes	IC_{50} (μM) amastigotes	CC_{50} (μM)	SI promastigotes	SI amastigotes
Be	133.0 ± 3.0	NA	170.4 ± 0.5	1.3	NA
Lu	4.0 ± 0.3	17.5 ± 0.4	≥ 240	≥ 58.5	≥ 13.4
UA	8.0 ± 0.2	3.0 ± 0.2	42.1 ± 0.2	5.3	14.0
Miltefosine	13.5 ± 0.9	33.4 ± 2.0	89.3 ± 8.3	6.6	2.7

3.3 Quantification of nitric oxide and hydrogen peroxide

Macrophages infected with *L. (L.) infantum* and treated with triterpenes Be, Lu and UA did not produce quantifiable levels of NO. By the other side, macrophages infected and treated with Lu increased the levels of H_2O_2 in a dose-dependent manner when compared to the control group ($p<0.05$), as indicated in figure 1. Infected macrophages treated with triterpenes Be, UA or miltefosine did not produce quantifiable H_2O_2. Non-infected macrophages did not produce measurable levels of H_2O_2. Macrophages treated with LPS produced high amounts of NO (17.1 ± 2.2 μM) and H_2O_2 (213.1 ± 24.4 μM).

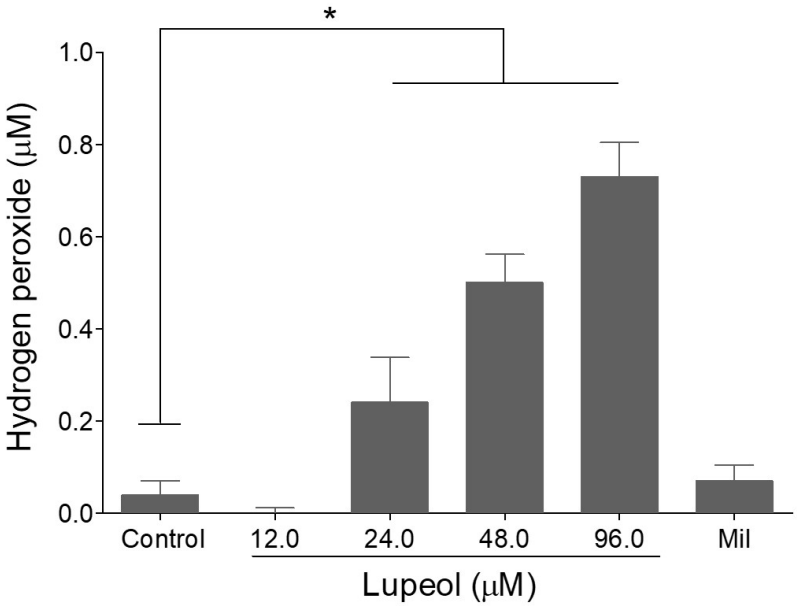

Figure 1 - Hydrogen peroxide production. Macrophages infected with *L. (L.) infantum* were incubated with different concentrations of betulin (Be), lupeol (Lu), ursolic acid (UA) and miltefosine. After 24h, the supernatants were collected and the NO and H_2O_2 levels were quantified. Only infected macrophages treated with Lu produced H_2O_2. * $p < 0.05$ compared to control.

3.4 Ultrastructural changes in promastigote forms treated with triterpenes Lu and UA

Control promastigote forms showed a well-preserved external structure, with fusiform shape and intact cell membrane (Figure 2 A). The cytoplasm, flagellum (F) and flagellar pocket (FP) exhibited regular morphology (Figure 2 A and B). The kinetoplast (K), presented in details in Figure 2 B, and the nucleus (N) (Figure 2 C) displayed normal morphology.

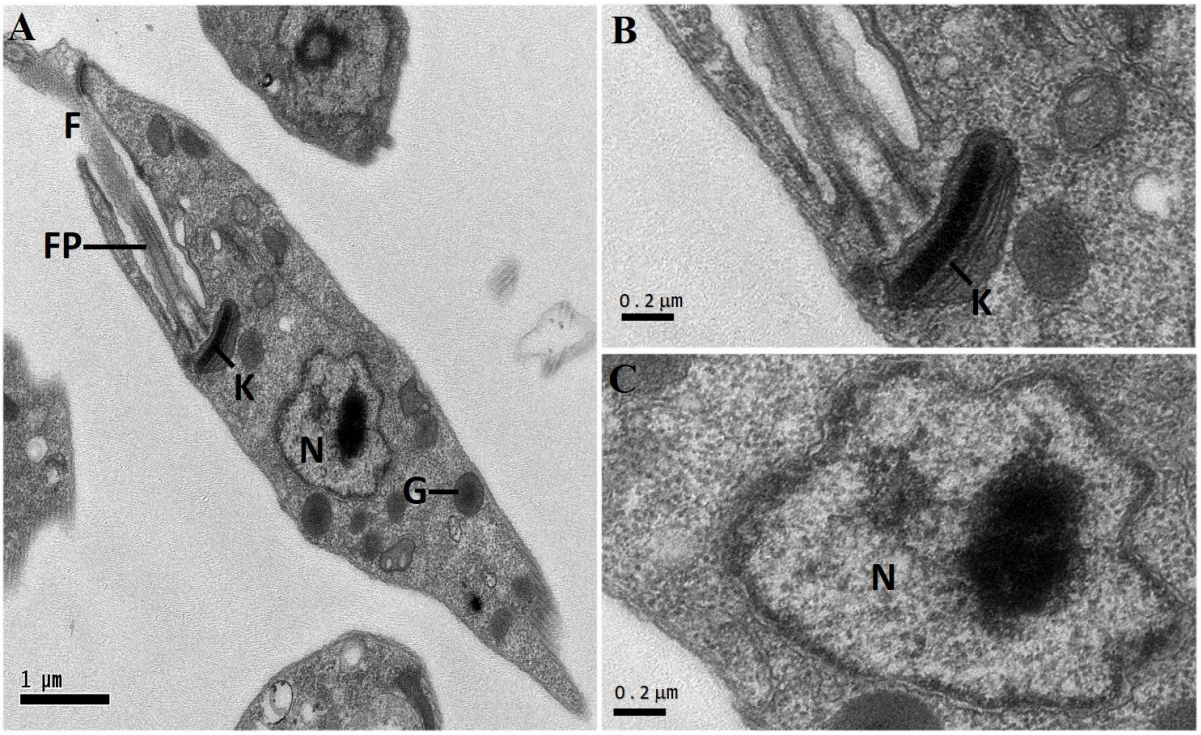

Figure 2 - Ultrastructure of promastigote forms of *L. (L.) infantum*. A - General morphology of *L. (L.) infantum* promastigote (x25k); B - Detail of flagellar pocket (FP) and kinetoplast (K) of control untreated (x80k); Glycosome (G) C – Nucleus (N), membrane and chromatin without changes in untreated control (x80k).

Promastigote forms treated with Lu at the IC_{50} for 24h showed major changes in the morphology (Figure 3 A). Cell membrane protrusions were detected (arrowhead), the cytoplasm presented areas of cytoplasm degradation (*), resembling autophagic vacuoles (Figure 3 A). The complex mitochondria (M) – kinetoplast (K) was swelled and disruption of the mitochondrial cristae (Figure 3 A and B) was observed. Parasite nucleus (N) seems to be degraded and fragmented (Figure 3 A and C).

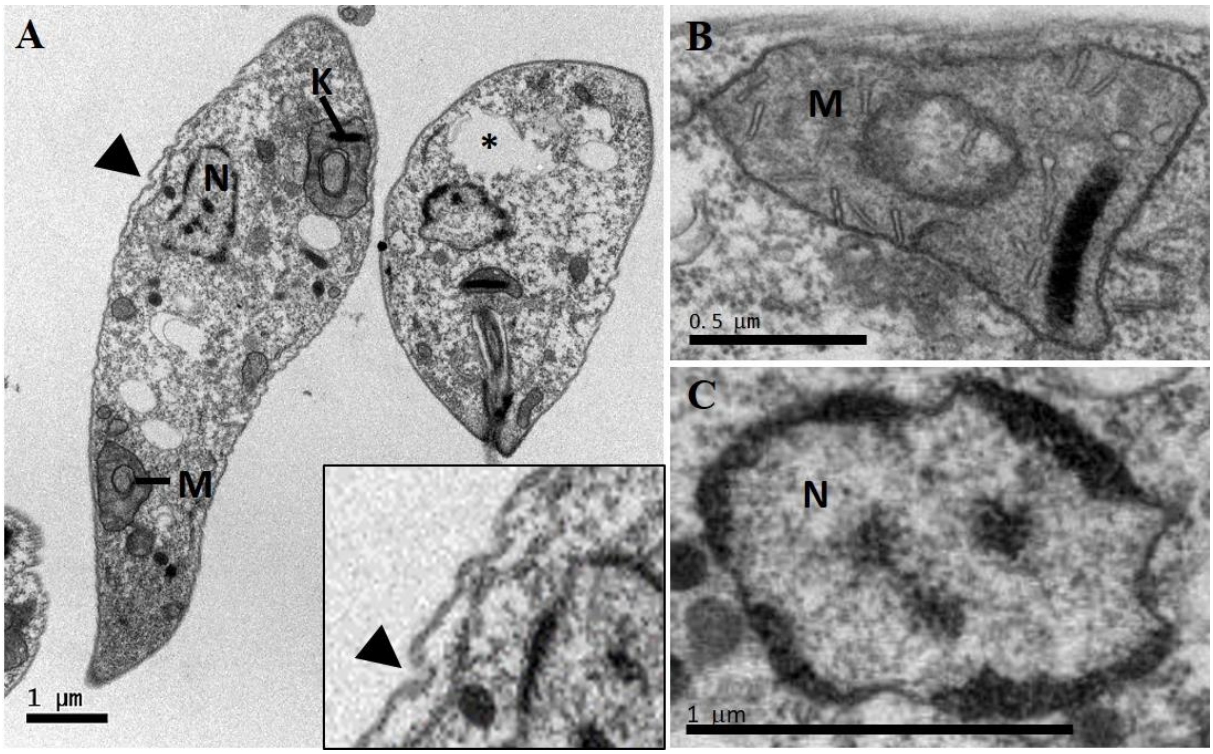

Figure 3 - Ultrastructure of promastigote forms of *L. (L.) infantum* treated with IC_{50} of lupeol (Lu) for 24h. A - Parasites treated with lupeol lost the fusiform shape, displayed cell membrane protrusions (arrowhead) and areas of cytoplasm degradation (*); B - Detail of the mitochondria and kinetoplast from parasites treated with Lu (x50k); C - Nucleus showing condensed and fragmented chromatin after Lu treatment (x50k).

Figure 4 A shows parasites treated with UA during 24h. Parasites lost the fusiform shape (Figure 4 A); the cytoplasm seems degraded showing changes resembling autophagic vacuoles (*) (Figure 4 A and B), myelin-like figures (MF) were detected (Figure 4 B). Blebs were identified in the outer cell membrane of promastigote forms treated with UA (Figure 4C - arrowhead). Moreover, mitochondrial bleb containing DNA was identified in the kDNA of parasites treated UA (Figure 4 A - arrowhead). The nucleus (N) of promastigote forms presented with dense and peripheral chromatin that appears to be fragmented (Figure 4 A).

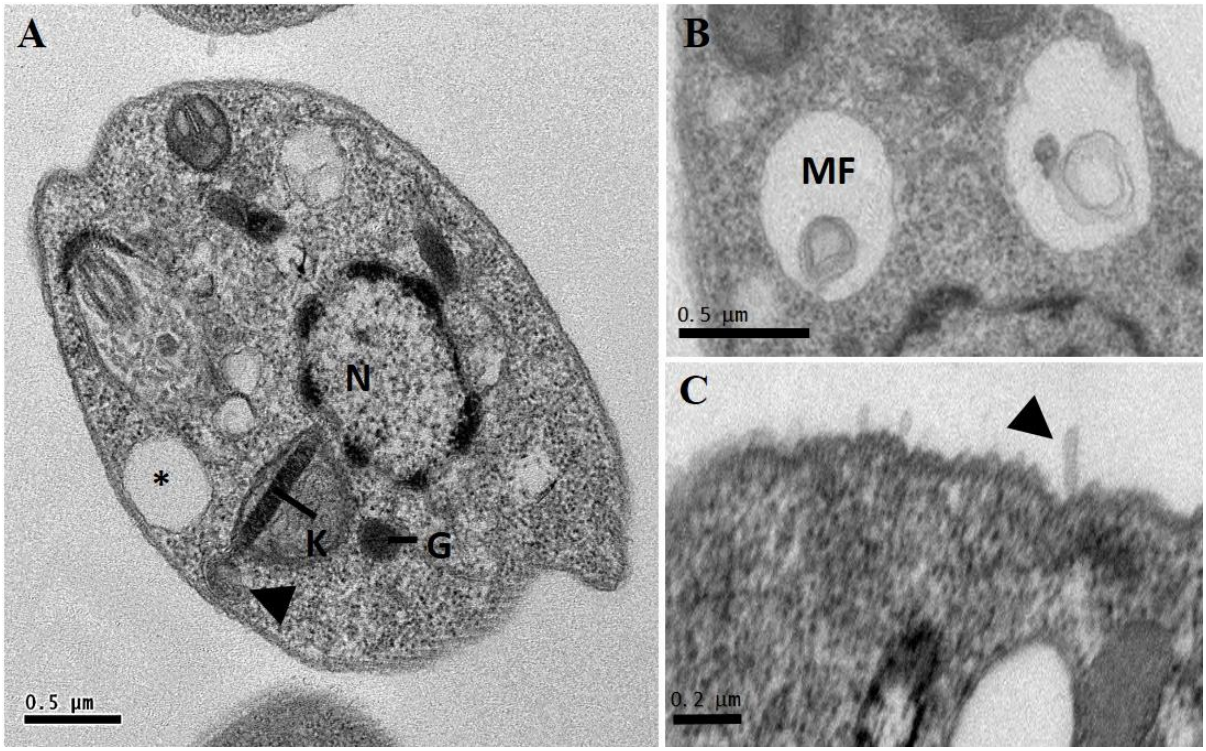

Figure 4 - Ultrastructure of promastigote forms of *L. (L.) infantum* treated with IC_{50} of ursolic acid (UA) for 24h. A - Parasite showing round shape morphology, with areas of cytoplasm degradation (*) and nucleus with condensed and peripheral chromatin; the kinetoplast was swollen, and a bleb was identified (arrowhead) (x 30k); B - Parasite treated with UA displayed myelin-like figures (MF) (x25k); C - Blebbing in the cell membrane (arrowhead) of promastigote forms treated with UA (x80k).

L. (L.) infantum treated with miltefosine lost the fusiform morphology, the cytoplasm was degraded, showing structures resembling autophagic vacuoles (*). Nuclear membrane detachment was observed (Figure 5 A, black arrow), additionally fragmentation of chromatin was detected in the nucleus (Figure 5 A and C). The complex kDNA-mitochondria (M) was swelled (Figure 5 A), fragmented, cristae were disrupted and blebs were observed (Figure 5B).

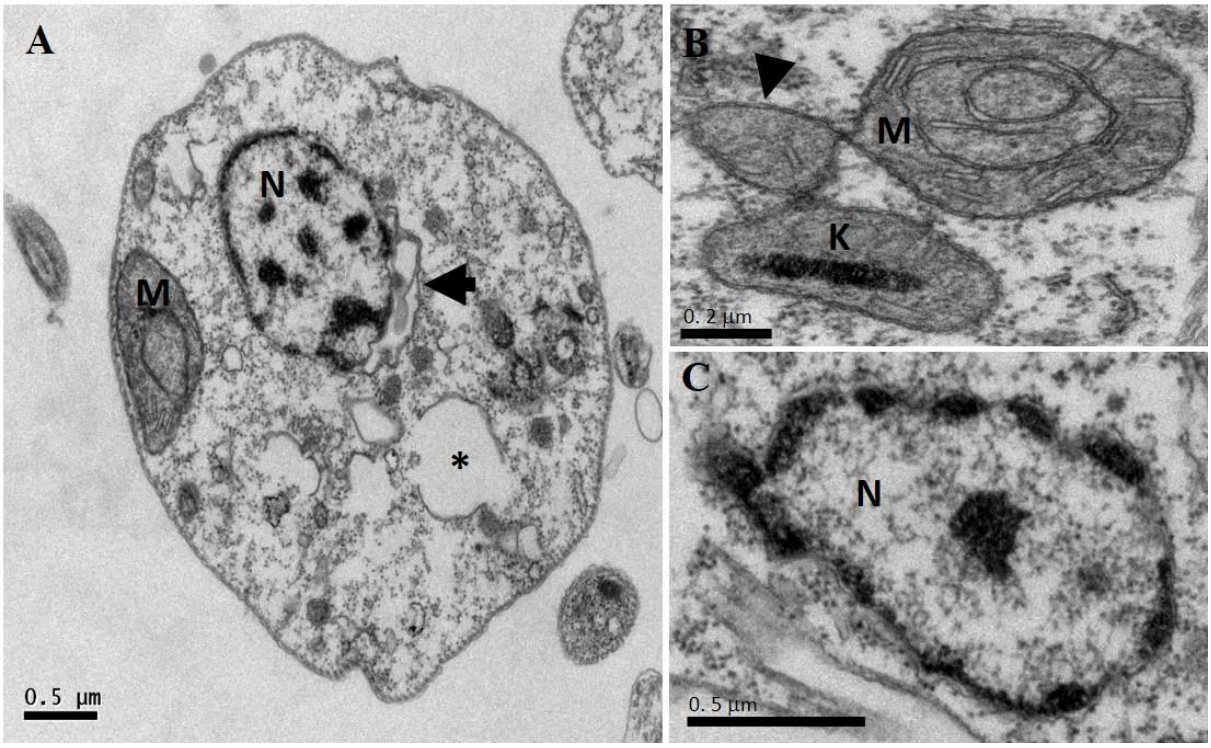

Figure 5 - Ultrastructure of promastigote forms of *L. (L.) infantum* treated with IC_{50} of miltefosine during 24h. A - Parasites treated with miltefosine lost its fusiform morphology, areas of cytoplasm degradation (*) and detachment of nuclear membrane were observed (black arrow) (x30k); B - Miltefosine altered the morphology of the kDNA (k) - mitochondria (M) complex, that presented fragmented and with blebs (arrowhead) (x80k); C - Nucleus (N) with condensed chromatin in parasites treated with miltefosine (x50k).

3.5 Cell membrane integrity and mitochondrial membrane potential assays

Corroborating the morphological changes, it was observed that the triterpenes Lu and UA as well as miltefosine did not damage the cell membrane of promastigote forms of *L. (L.) infantum* (Figure 6 A-C). In addition, parasites reduced the emission of fluorescence by 50% at 1440, and it is associated with the reduction of the parasite population by 50%. Parasites incubated with Triton X-100 emitted high levels of fluorescence (Figure 6 A-C).

As observed in the TEM images, treated parasites showed morphological changes in the mitochondrial-kDNA complex, and to validate such alterations, the mitochondrial membrane potential of treated parasites was analyzed. Triterpenes Lu and UA as well as positive control miltefosine (Figure 6 D-F) decreased the mitochondrial membrane potential ($\Delta\Psi m$) in a time-dependent manner at 30 minutes of incubation. Additionally, at 45 minutes a hyperpolarization was detected in parasites treated with UA and miltefosine. Parasites treated

with triterpenes Lu and UA displayed complete inhibition of the mitochondrial membrane potential at 60 and 1440 minutes of incubation (Figure 6 D and E, respectively). The mitochondria of parasites treated with miltefosine showed complete inhibition of the mitochondrial membrane potential at 1440 minutes (Figure 6 E). Oligomycin (positive control) inhibited the mitochondrial membrane potential over time.

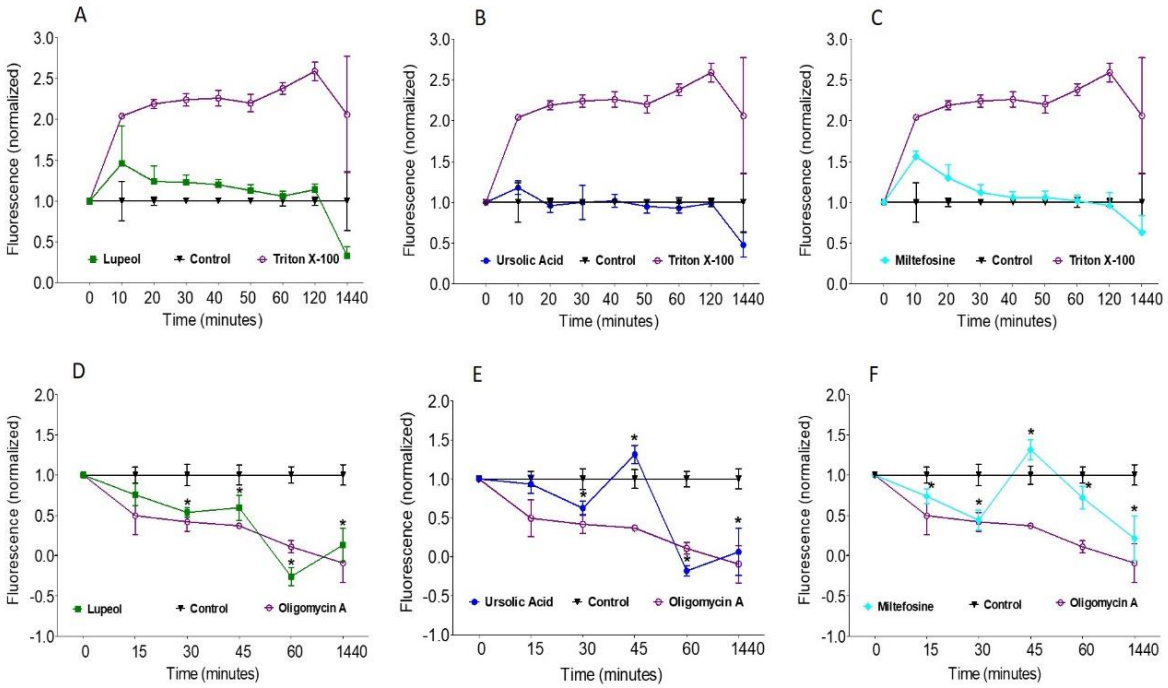

Figure 6 - Cell membrane integrity and mitochondrial membrane potential assays of *L. (L.) infantum* promastigotes. Parasites were treated with Lu (A), UA (B) and miltefosine (C) at the IC_{50} and at different time points, the probe SYTOX® Green (0.5 µM) was added, and the fluorescence intensity analyzed. Triton X-100 was used as a positive control. In figures D, E and F, promastigote forms were incubated with the IC_{50}s of the Lu, UA and miltefosine, respectively, and at different time-points Rhodamine 123 probe (3 µM) was added to the parasites and the fluorescence intensity analyzed. Oligomycin A was used as a positive control. Fluorescence was normalized relative to the control parasites.

3.6 Hepatic and renal biochemical parameters

Deficiencies in the metabolism and excretion of triterpenes were analyzed by hepatic and renal functions, respectively. In respect to hepatic function, it was verified that UA treatment caused a significant increase in the level of AST (Figure 7 A) but not ALT (Figure 7 B). Lu and AmB did not change the biochemical parameters of the liver. Animals treated with Lu and UA did not change renal functions, as the levels of urea and creatinine were similar to

the healthy group (Figures 7 C and D). By the other side, animals treated with AmB significantly increased the level of serum creatinine, as demonstrated in the figure 7 D.

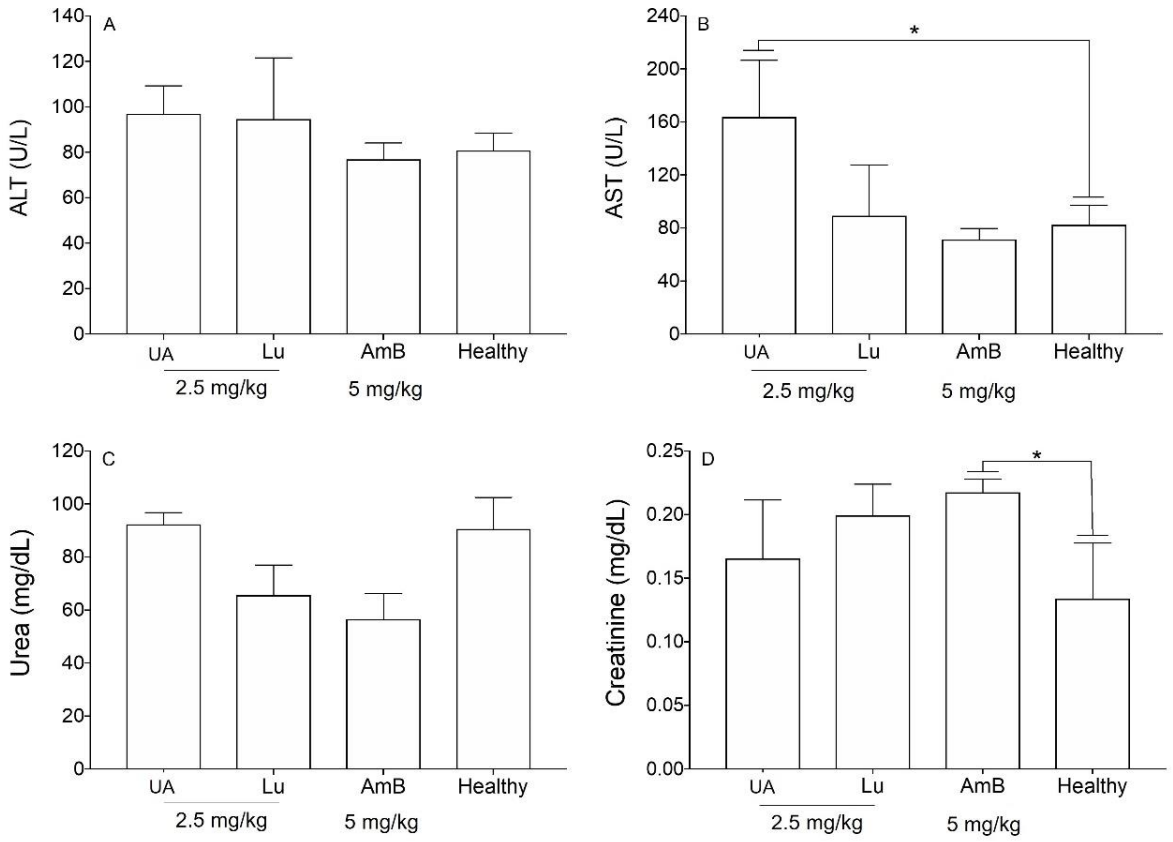

Figure 7 - Blood biochemical parameters of hamster treated with lupeol (Lu), ursolic acid (UA) and amphotericin B (AmB). Levels of ALT (A), AST (B), urea (C) and creatinine (D) were quantified in the serum of animals treated with 2.5 mg/kg of lupeol (Lu), ursolic acid (UA) or 5 mg/kg of amphotericin B (AmB) during 10 consecutive days by intraperitoneal route. * $p < 0.05$ indicates statistical significance.

3.7 Therapeutic activity of triterpenes in visceral leishmaniasis

Infected hamsters treated with 2.5 mg/kg of UA or Lu showed significant reduction in the splenic and hepatic parasitism (Figure 8 A and B, respectively) in comparison to infected control ($p < 0.05$), as demonstrated by limiting-dilution assay. In addition, tissue amastigote forms were stained by immunohistochemistry, and it is possible to observe that the treatment

carried out with UA or Lu drastically decreased the number of parasites in comparison to the spleen and liver of infected control.

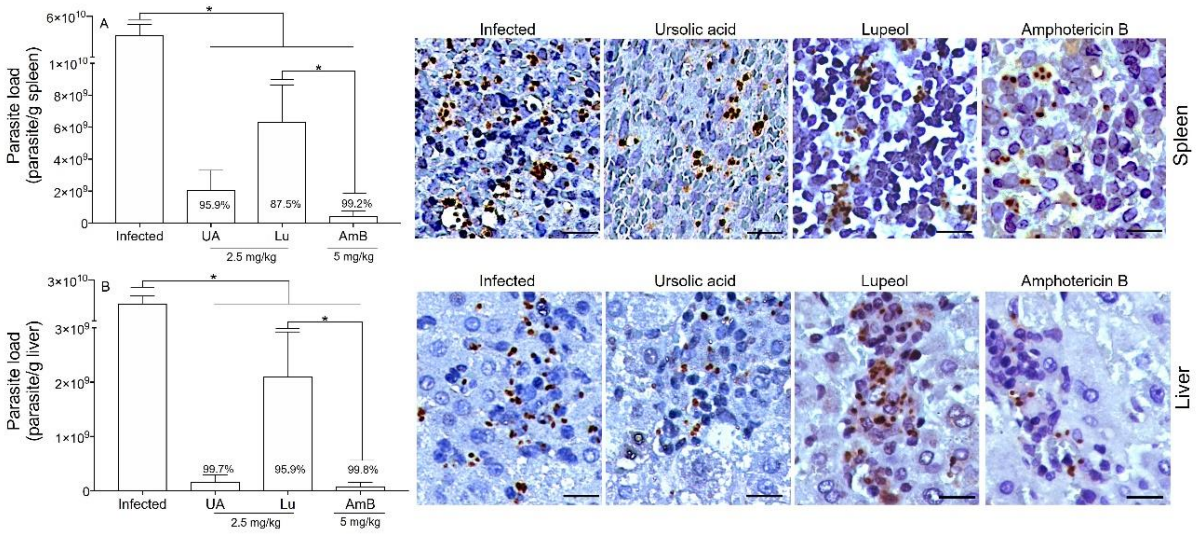

Figure 8 - Splenic and hepatic parasitism from hamsters infected with *L. (L.) infantum* and treated with triterpenes. Hamsters were infected with promastigote forms of *L. (L.) infantum*, and four weeks after infection, animals were intraperitoneally treated once daily during 10 days with 2.5 mg/kg of ursolic acid (UA), lupeol (Lu) or 5 mg/kg of amphotericin B (AmB). The splenic and hepatic parasitism was quantified by limiting-dilution assay, additionally, amastigote forms were stained by immunohistochemistry technique.

3.8 Cell immune response

Analysis of cellular immune response in the spleen of *L. (L.) infantum*-infected hamsters showed that animals treated with 2.5 mg/kg of UA or Lu expressed higher levels of IFN-γ compared to the infected control (Figure 9 A, $p < 0.05$). Animals treated with amphotericin B expressed lower levels of IFN-γ in comparison to the infected control, and it was similar to the healthy control. In respect to iNOS gene expression, only animals treated with lupeol showed significant elevation in the expression of this mediator of inflammation compared to the infected control (Figure 9 B, $p < 0.05$). Animals treated with 5 mg/kg of amphotericin B exhibited a significant decrease expression of IL-10 in comparison to the infected control (Figure 9 C, $p < 0.05$).

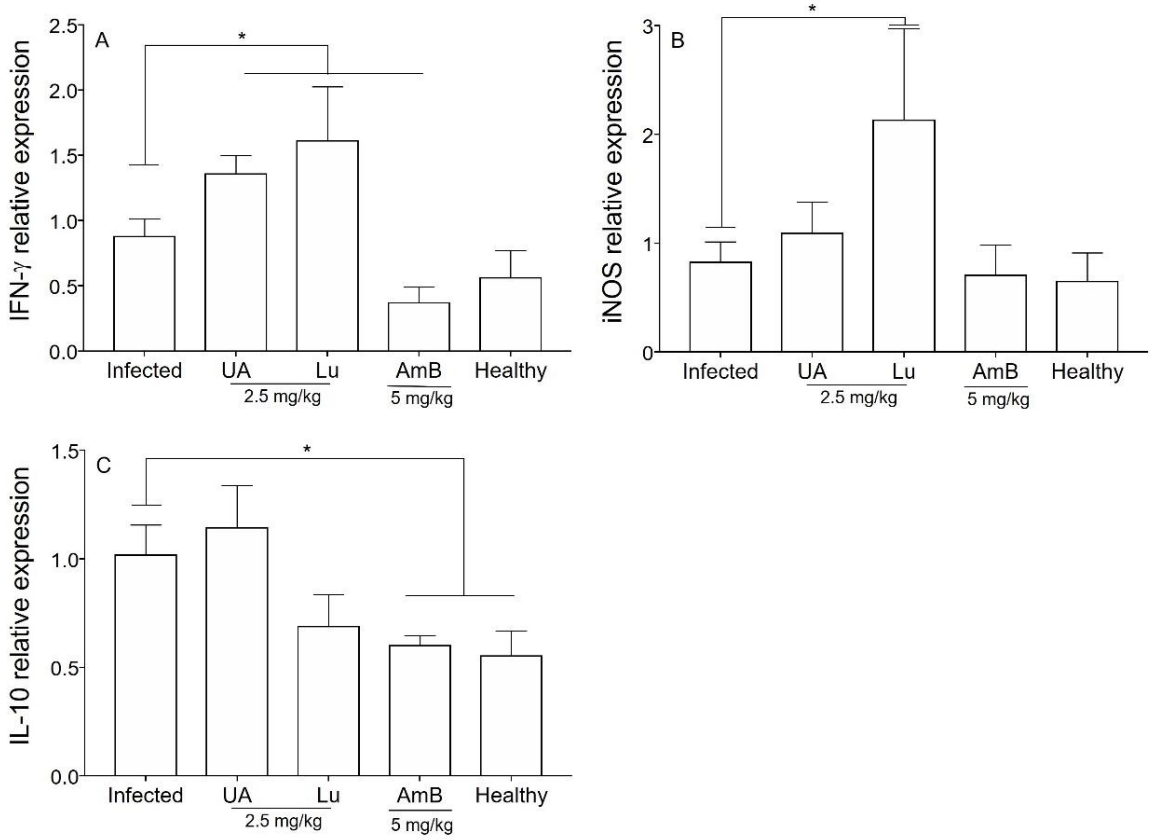

Figure 9 - Cell immune response of hamsters infected with *L. (L.) infantum* and treated with triterpenes and amphotericin B. IFN-γ (A), iNOS (B) and IL-10 (C) relative gene expression in the spleen of infected golden hamsters treated with 2.5 mg/kg of ursolic acid (UA) or lupeol (Lu) and 5 mg/kg of amphotericin B (AmB). Relative gene expression was estimated by quantitative PCR. The expression levels of genes of interest were normalized to β-actin. * $p < 0.05$ indicates statistical significance.

4 Discussion

The quality of drugs is a fundamental factor to ensure pharmacological activity and minimize the occurrence of unwanted effects resulting from the presence of impurities and/ or degradation products. Thus, according to the nuclear magnetic resonance, associated with the elemental analysis it was possible to confirm the purity degree of all studied triterpene (see supplementary material 1). Additionally, NMR spectral analysis was useful to confirm the identity of triterpenes as betulin (Be), lupeol (Lu) and ursolic acid (UA). These triterpenes showed in vitro leishmanicidal activity on promastigote forms of *L. (L.) infantum* that were more sensitive to Lu, followed by UA and Be. However, Lu and UA impacted the survival of intracellular amastigote forms, while Be was inactive. Although triterpenes presented similar

structures they also displayed different activities on promastigote and amastigote forms. Possibly the liposolubility [32] is one factor associated with the biological activities found herein, since to interact with the cell membrane, molecules need to present hydrophobic groups, allowing their uptake by the cell [33]. The lipophilicity of a compound can be characterized by its partition coefficient between octanol and water (log P), octanol being assumed to have a similar lipophilicity to cell membranes [32]. This coefficient may be used as one of the predictors of drug absorption by passive diffusion [34]. Lu is the triterpene with the highest lipophilicity, with log P of 7.67 [35], followed by the UA and Be with log P values determined, respectively, as 6.43 [36] and 6.17 [32], suggesting that these triterpenes have intermediate lipophilicity to interact with the cell membranes and access to the intracellular environment.

Additionally, minor structural differences among studies triterpenes can determine the potency of leishmanicidal effect. Considering Be structure, the presence of two hydroxyl groups at positions C-3 and C-28 should be crucial to the absence of the leishmanicidal activity of this triterpene. By the other side, Lu exhibits only one hydroxyl group at position C-3 and it is 33 times more effective at killing promastigote forms than Be. Additionally, on amastigote forms, this difference is higher, since Be was inactive, while Lu showed high activity and selectivity toward intracellular forms. UA was the most active at killing amastigote forms (5.8 times) in comparison to Lu, and such pharmacological activity has been related to the presence of the carboxylic acid at the position C-28 in the structure of compound UA, that was not observed in the structure of Lu. By comparing the structure of all studied triterpenes, the presence of hydroxyl group at the position C-28 found in the structure of Be may determine the low or absent leishmanicidal activity found herein. Additionally, it is important to note that Lu and UA showed higher activity and selectivity toward parasites than miltefosine, and thus these molecules should be considered to develop new leishmanicidal drugs. Additionally, previous studies have been verified that both Lu and UA are active on promastigote and amastigote forms of different *Leishmania* species [26,37–39], suggesting that besides high selectivity both triterpenes have multispectral activity, that needs to be considered for drug development.

Regarding cytotoxic potential of triterpenes on peritoneal macrophages of golden hamsters, it is important to note that Lu had CC_{50} above 240 μM, that was higher than that of miltefosine. UA showed the highest toxic potential towards macrophage and it was associated with the presence of carboxylic acid at C-28 and hydroxyl group at C-3 position. In spite of showing some toxicity to the host cell, the selectivity towards amastigote forms (SI ~ 14)

suggests that this triterpene is still an important leishmanicidal agent. Moreover, other studies have already shown that these triterpenes have absent or low cytotoxic effects on different cell lineages [40–43], suggesting the applicability of such molecules in therapeutic studies.

During infection, *Leishmania sp*. suppresses respiratory burst in the host macrophages, so compounds able to increase macrophage respiratory activity can aid the parasite elimination. In this regard, it was observed that infected macrophages treated with Lu produced elevated amounts of H_2O_2, that is able to trigger programmed cell death in *Leishmania* sp. [42,44,45]. Thus, Lu was able to activate infected host macrophages to a leishmanicidal state, and such activity, triggered by Lu, can be an additive mechanism to eliminate parasites [41].

In order to analyze major morphological changes induced by triterpenes, promastigote forms of *L. (L.) infantum* were analyzed by transmission electron microscopy, followed by physiological changes in the plasma and mitochondrial membranes. In general, parasites showed altered morphology, intracellular disorganization, blebs, chromatin condensation, nuclear fragmentation and different cytoplasmic alterations, mainly related to the cytoplasmic extraction. Additionally, changes in the parasite mitochondria and nucleus suggest that triterpenes act on the parasite's bioenergetics or even inducing programmed cell death [46–48]. In fact, Lu and UA were able to inhibit mitochondrial transmembrane potential after 30 minutes of incubation, suggesting that the mitochondria can be the target organelle of these triterpenes. Additionally, parasites treated with UA showed a transient recovery of the mitochondrial membrane potential, that may be associated with a hyperpolarization of this organelle as previously mentioned by Yamamoto and collaborators [38], suggesting that UA triggers programmed cell death in *L. (L.) infantum*. According to Vannier-Santos and Castro [49], pyknosis and nuclear fragmentation observed in parasites treated with drugs can be considered a morphological indicator of programmed cell death. In parasites incubated with miltefosine, shrinkage of the cell surface was observed, presence of myelin-like figures and fragmentation of mitochondria and nuclear DNA suggests the occurrence of programmed cell death, as previously demonstrated [50,51], suggesting that miltefosine can be a good control of programmed cell death in *Leishmania* sp. Previous studies have shown that programmed cell death may be a common mechanism of cell death in *Trypanosoma* sp. [52–54] and *Leishmania* [53,55] in response to chemotherapeutic agents such as miltefosine [50] and triterpenes [39,42,43]. Thus, the leishmanicidal activity of triterpenes can be associated with the major changes in the mitochondria, nucleus and in the intracellular compartments, that might be related to programmed cell death in *L. (L.) infantum*.

Despite cell protrusions and blebs observed in the cell membrane of promastigote forms treated with Lu or UA, cell membrane lysis was not detected by morphology and physiology studies. Protrusions and blebs observed in the cell membrane of treated parasites can be the effect of the triterpenes on the cytoskeleton of promastigote forms [49]. Similar morphological changes were also observed in *Trypanosoma cruzi* treated with lysophospholipid analogues, such as edelfosine, ilmofosine, and miltefosine [56]. In addition to these changes, membrane debris were observed in areas resembling autophagic vacuoles. Previous works demonstrated that parasites recycle abnormal structures during an autophagic process [49,57,58]; besides targeting mitochondria, both triterpenes may also trigger autophagy in promastigote forms.

Both triterpenes showed promising *in vitro* activity and selectivity toward *L. (L.) infantum* amastigotes, and thus golden hamsters were injected with Lu or UA to evaluate hepatic and renal functions after treatment. Healthy golden hamsters treated with UA showed a significant increase of aspartate transaminase (AST) compared to the healthy animals. Both ALT and AST are produced by hepatocytes, therefore increased levels of ALT and AST indicate hepatocellular injury. In the present study UA-treated hamsters showed elevation only in AST, suggesting an initial hepatocellular injury. Previous studies already showed that low doses of UA are safe for BALB/c mice, and golden hamster treated with 2mg/kg showed no changes in serum ALT or AST, furthermore no morphological changes in the liver structure were observed; however higher doses of UA (150 mg/kg) administered with a diet over a 6-week can cause hepatic damage [59]. By the other side, UA can be found in fruits and vegetables present in the human diet, furthermore this triterpene also is used as a dietary supplement for humans [60]. Thus, UA hepatotoxicity may be related to the dosage, duration of treatment and route of administration. In contrast, Lu did not cause changes in the levels of ALT or AST.

Golden hamsters treated with Lu or UA did not change the levels of urea or creatinine, suggesting that both triterpenes are not nephrotoxic. By the other side, animals treated with 5mg/kg of amphotericin B showed an increase in the creatinine level, suggesting that AmB caused renal toxicity in golden hamsters. Previous study already showed that hamsters treated with 5.0 mg/kg/day of AmB during 16 days, displayed morphological changes compatible with acute renal toxicity [26]. AmB also induced nephrotoxicity in patients that presented reduced glomerular filtration rate and tubular dysfunction [61], reinforcing that one of the main side effects of AmB in vertebrates is renal failure.

Although the treatment with UA at 2.5mg/kg induced toxicity to the liver, its efficacy in the model of visceral leishmaniasis was assessed, since only the levels of AST marker increased after treatment. In fact, UA as well as Lu were able to eliminate amastigotes forms in the spleen and liver of *L. (L.) infantum* – infected hamsters, however UA treatment showed higher efficacy at eliminating hepatic parasites than Lu treatment; moreover, UA showed similar efficacy than AmB treatment. Lu, as well as UA, was poorly investigated concerning the leishmanicidal effect, and few available works showed the therapeutic activity of this triterpene only in the murine model of visceral leishmaniasis, that mimics the acute phase of infection, thus currently, this is the first work showing that Lu is able to decrease amastigote forms in the spleen and liver from hamsters in the chronic phase of visceral leishmaniasis.

In leishmaniasis, resistance to infection has been associated with the development of a Th1 immune response, with remarkable amounts of interferon gamma (IFN-γ) cytokine, that can be mainly produced by NK and T cells [62–64]. As phagocytes are exposed to IFN-γ, classical activation and induction of microbicidal mechanisms can occur, with the participation of inducible nitric oxide synthase enzyme (iNOS), as well as nitric oxide (NO), a mediator of inflammation, that along with other reactive oxygen and nitrogen species, can kill intracellular parasites [64–66]. In the present study it was observed an association between leishmanicidal activity *in vivo* and increased IFN-γ expression, suggesting that these both triterpenes may direct the immune response to a Th1 immune response, emphasizing that besides a direct activity on parasites, the therapeutic activity of triterpenes is mediated by immune response activation [26,39].

In contrast to resistance, the susceptibility in visceral leishmaniasis has been accompanied by elevation in the levels of IL-10, a cytokine with anti-inflammatory activity, and suppressive effects on the Th1 immune response. Thus, elevation in the level of IL-10 frequently results in parasite proliferation as well as chronification of leishmania infection [67,68]. UA or Lu treatment did not inhibit IL-10 expression. Possibly, in this chronic model of visceral leishmaniasis, where the disease is fully manifested, both Lu and UA lose the ability to restrain IL-10 expression and/or production, as mentioned previously [26], maintaining a small number of parasites in the spleen and liver of hamsters. Despite of that, it is still important to note that both triterpenes eliminated high amounts of amastigote forms in the spleen and liver of infected animals, and even Lu being less potent than UA and AmB, it was not toxic for hamsters, and should be viewed as an important target to develop new leishmanicidal drugs.

Previous studies already discussed the *in vitro* and *in vivo* activities of both Lu and UA [26,37,69], however this is the first comparative work showing the leishmanicidal activity of related pentacyclic triterpenes in association with their structures, as well as morphological and physiological changes that took place in *L. (L.) infantum*. In addition, this is the first work showing the comparative therapeutic activity of both Lu and UA in the experimental model of chronic visceral leishmaniasis, that is the most suitable model of natural infection. Taken together, data showed herein demonstrates that UA and Lu triterpenes were able to promote effective and selective antileishmanial activity on promastigote and amastigote forms of *L. (L.) infantum*, and possibly the parasite mitochondria may be the target organelle, since impairment of transmembrane potential was observed after 30 minutes of incubation, furthermore morphological studies showed a complete disorganization of such organelle. Both triterpenes increased the cellular immune response in hamsters with visceral leishmaniasis, and it was associated with amastigote elimination in the spleen and liver, suggesting that the leishmanicidal activity is mediated by immunomodulation of both innate and acquired immune system.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Supplementary material

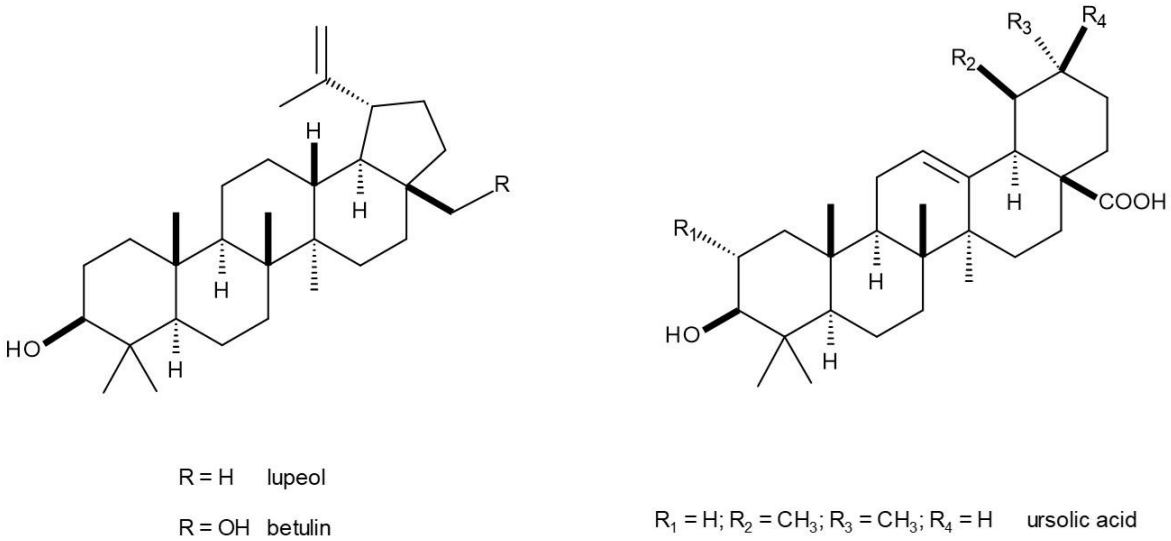

Figure 1 - Molecular structure of betulin, lupeol and ursolic acid.

Table 1 - ^{13}C NMR of botulin, lupeol and ursolic acid

	Betulin		**Lupeol**		**Ursolic Acid**	
Position	δC Observed [a]	δC Literature [a]	δC Observed [a]	δC Literature [a]	δC Observed [b]	δC Literature [a]
1	38.7	38.8	38.6	38.7	38.7	38.7
2	27.3	27.2	27.4	27.4	23.5	23.5
3	78.9	78.9	79.0	78.9	77,0	79.0
4	38.9	38.9	38.9	38.8	39.6	39.6
5	55.3	55.3	55.3	55.3	56.3	56.3
6	18.3	18.3	18.3	18.3	19.0	19.0
7	34.2	34.3	34.3	34.2	33.4	33.4
8	40.9	40.9	40.8	40.8	40.0	40.0
9	50.4	50.4	50.4	50.4	-	-
10	37.2	37.2	37.2	37.1	38.5	38.5
11	20.8	20.9	20.9	20.9	23.8	23.8
12	25.2	25.3	25.1	25.1	125.8	125.8
13	37.3	37.3	38.1	38.0	138.0	138.0
14	42.7	42.7	42.8	42.8	42.2	42.2
15	27.0	27.0	27.4	27.4	29.0	29.0
16	29.2	29.2	35.6	35.5	24.0	24.0
17	47.8	47.8	43.0	43.0	47.8	47.8
18	48.8	48.8	48.3	48.0	52.7	52.7
19	50.4	47.8	47.9	47.9	38.7	38.7
20	150.5	150.6	150.9	150.9	38.4	38.4
21	29.8	29.8	29.8	29.8	27,0	27.0
22	34.2	34,0	40.0	40.0	37,0	37.0
23	28.0	28.0	28.0	28.0	27.7	27.7
24	15.4	15.4	15.4	15.4	15.2	15.2
25	16.1	16.1	16.1	16.1	15.5	15.5
26	16.0	16.0	15.9	15.9	17.5	17.5
27	14.8	14.8	14.5	14.5	24.0	24.0
28	60.5	60.2	18.0	18.0	181.1	181.6
29	109.7	109.6	109.3	109.3	16.5	16.5
30	19.1	19.1	19.3	19.3	21.3	21.3

[a] $CDCL_{3;}$ [b] DMSO-$d_{6;}$

- data not identified in the experiment

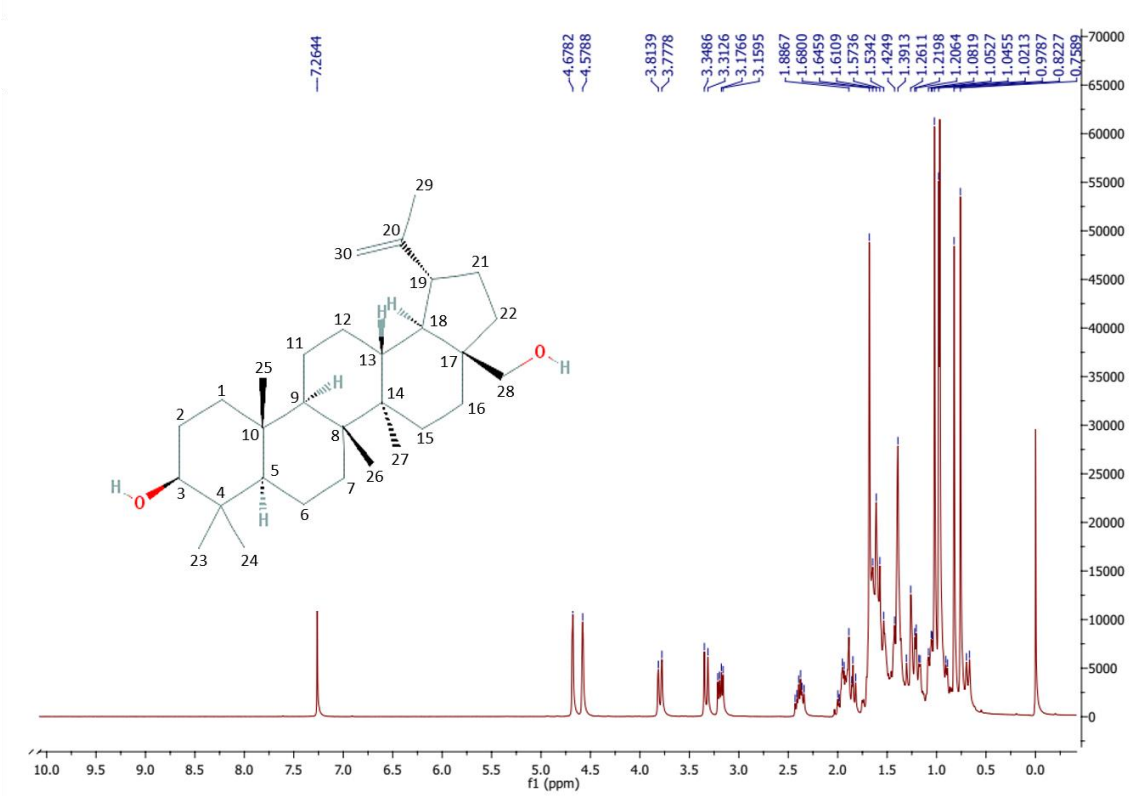

Figure 2 - ^{1}H NMR spectrum (δ, $CDCl_3$ + CD_3OD, 300 MHz) of betulin (Be).

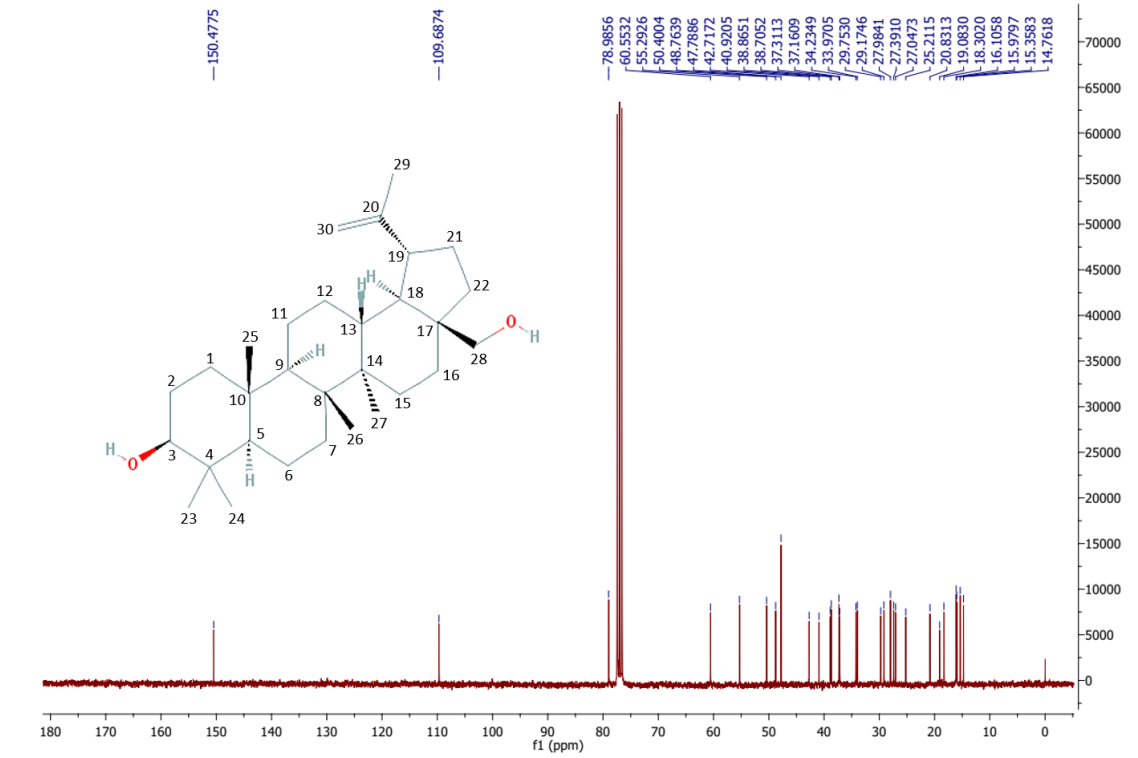

Figure 3 - ^{13}C NMR spectrum (δ, $CDCl_3$ + CD_3OD, 75 MHz) of betulin (Be).

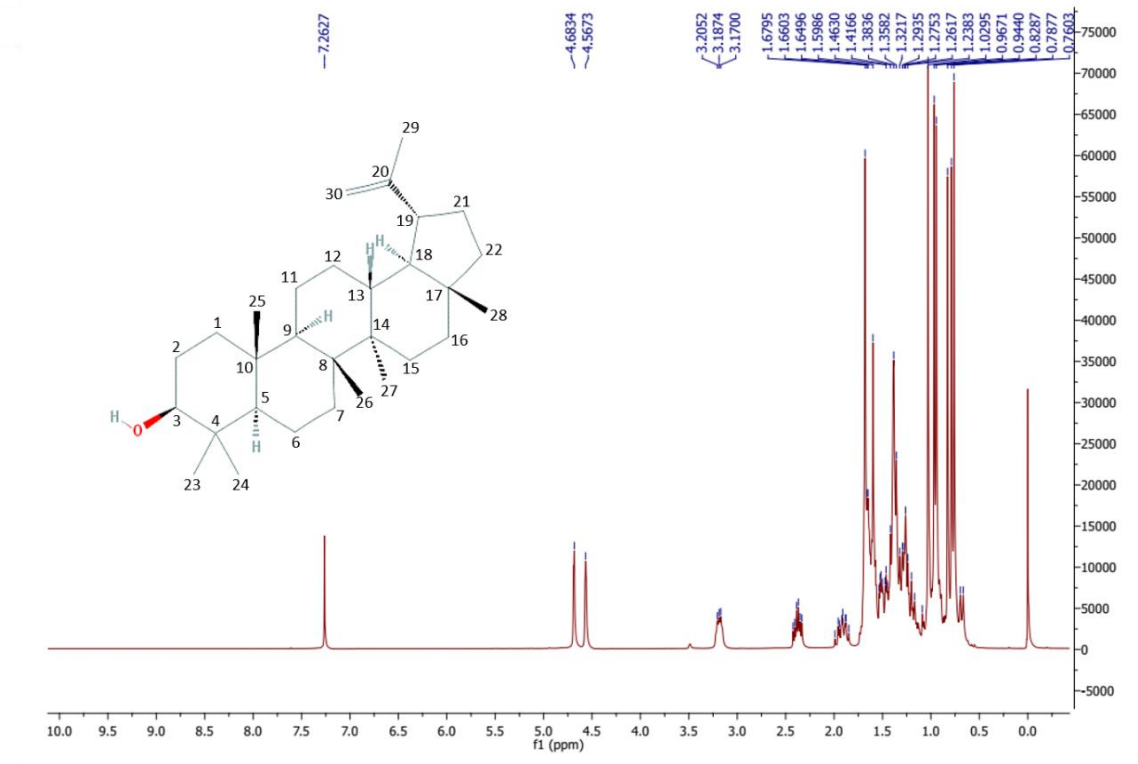

Figure 4 - ^{1}H NMR spectrum (δ, $CDCl_3$ + CD_3OD, 300 MHz) of lupeol (Lu).

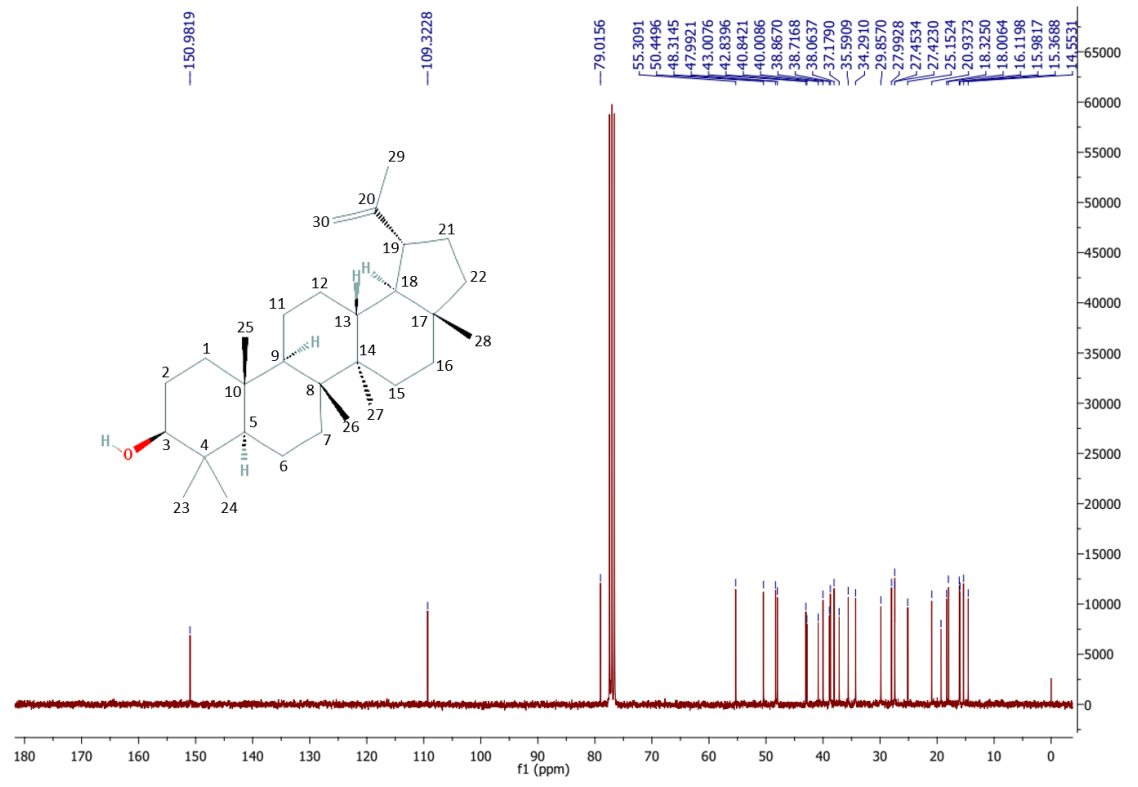

Figure 5 - ^{13}C NMR spectrum (δ, $CDCl_3$ + CD_3OD, 75 MHz) of lupeol (Lu).

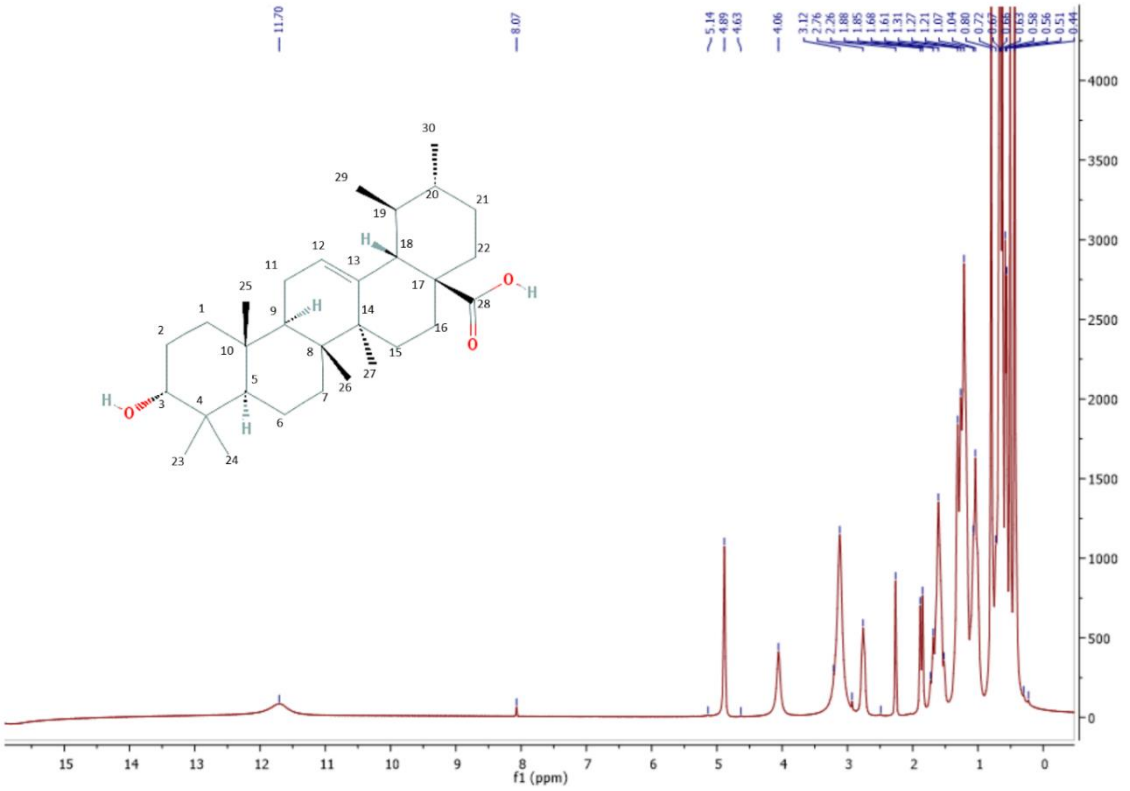

Figure 6 - ^{1}H NMR spectrum (δ, DMSO-d_6, 300 MHz) of ursolic acid (UA).

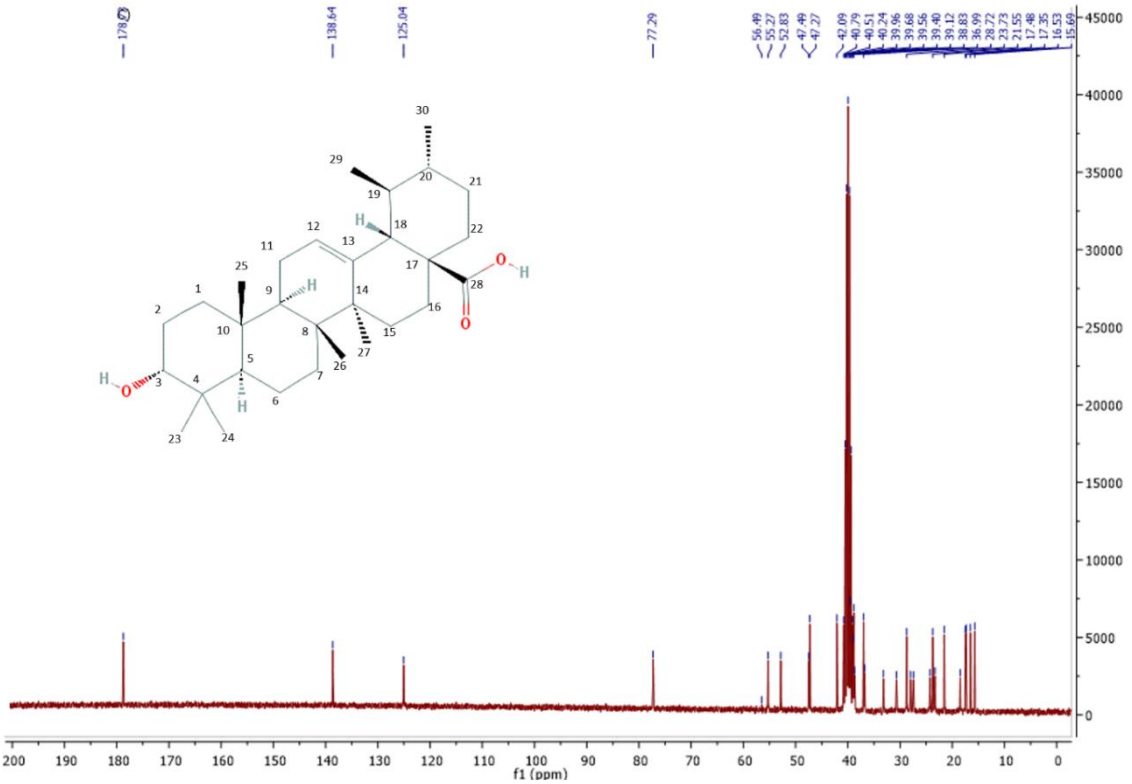

Figure 7 - ^{13}C NMR spectrum (δ, DMSO-d_6, 75 MHz) of ursolic acid (UA)..

Table 2 - Elemental analysis results

Triterpene	**Molecular formula**	**%C**		**%H**	
		Theorical	*Experimental*	*Theorical*	*Experimental*
Betulin	$C_{30}H_{50}O_2$	81.4	81.2	11.4	11.2
Lupeol	$C_{30}H_{50}O$	84.4	84.3	11.8	11.6
Ursolic acid	$C_{30}H_{48}O_3$	78.9	79.0	10.6	10.5

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6 ARTIGO PUBLICADO III

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Research Article

Leishmanicidal Activity and Ultrastructural Changes of Maslinic Acid Isolated from *Hyptidendron canum*

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Abstract

The therapeutic arsenal for the treatment of leishmaniasis is limited and has serious obstacles, such as variable activity, high toxicity, and costs. To overcome such limitations, it becomes urgent to characterize new bioactive molecules. Plants produce and accumulate different classes of bioactive compounds, and these molecules can be studied as a strategy to combat leishmaniasis. The study presented herein evaluated the leishmanicidal effect of maslinic acid isolated from the leaves of *Hyptidendron canum* (Lamiaceae) and investigated the morphological that occurred on *Leishmania (Leishmania) infantum* upon treatment. Maslinic acid was active and selective against promastigote and amastigote forms in a dose-dependent manner. Additionally, it was not toxic to peritoneal macrophages isolated from golden hamsters, while miltefosine and amphotericin B showed mild toxicity for macrophages. Morphological changes in promastigotes of *L. (L.) infantum* treated with maslinic acid were related to cytoplasmic degeneration, intense exocytic activity, and blebbing in the kDNA; disruption of mitochondrial cristae was observed in some parasites. The nucleus of promastigote forms seems to be degraded and the chromatin fragmented, suggesting that maslinic acid triggers programmed cell death. These results indicate that maslinic acid may be an interesting molecule to develop new classes of drugs against leishmaniasis.

Keywords: *Hyptidendron canum*; Maslinic acid; Triterpene; Antileishmanial activity; Cytotoxicity, Ultrastructural changes

1 Introduction

Leishmaniasis is an important neglected tropical disease caused by different species of the protozoan parasite that belong to the genus *Leishmania*. It affects vulnerable people in approximately 100 countries, with 12 million cases detected worldwide per year. Additionally, one billion people live in areas at risk of transmission [1]. The treatment of leishmaniasis relies on the use of the first-line drug pentavalent antimonial that is highly toxic for humans, limiting the use and adherence of patients with leishmaniasis [2]. Second-line drugs, such as amphotericin B, miltefosine, or paromomycin can be used in patients that do not tolerate treatment with antimonials, or in cases that it fails at eliminating parasites [3]. These drugs show different degrees of effectiveness in leishmaniasis, that depends on the infecting species and the clinical form of leishmaniasis, and similarly to antimonials, second-line drugs induce side effects that limit their use [4, 5]. As a consequence of the small number of available drugs currently used in therapy as well as different levels of therapeutic activity, it becomes urgent to implement leishmanial chemotherapy with new strategies. In this regard, it has been demonstrated that medicinal plants can be considered interesting scaffolds to isolate and characterize highly active molecules to be used in leishmaniasis [6–8].

Hyptidendron canum (syn. *Hyptis cana*) belongs to Lamiaceae and it is native to the Brazilian “cerrado” region (savannah like biome), especially in Goias, Mato Grosso, and Minas Gerais States [9]. Infusions and decoctions produced with the leaves and roots of *H. canun* have been used in folk medicine to treat inflammatory processes and infections caused by parasites and viruses [10–12]. All these pharmacological activities can be associated with the presence of active secondary metabolites that have been obtained from different organs of *H. canum* [13], such as the pentacyclic triterpenes betulinic, ursolic, and maslinic acids [14].

In leishmaniasis treatment, the most studied triterpene so far is ursolic acid, including in vitro and in vivo assays. The first report showed that this metabolite was active against promastigote and amastigote stages of *L. (L.) major* and *L. (L.) donovani* [15], further studies showed that ursolic acid triggered programmed cell death in *L. (L.) amazonensis* [16]. Besides, golden hamsters with visceral leishmaniasis and BALB/c mice with cutaneous leishmaniasis showed a significant reduction in tissue parasitism during the treatment with this triterpene [16, 17], and such activity was

reported to be linked with the immunomodulatory activity [7, 17]. Additionally, in experimental therapeutic approaches using combinatorial therapy, it was shown that ursolic acid potentialized the activity of glucantime in cutaneous leishmaniasis [18]. Although plenty of studies have shown the bioactivity of ursolic acid against leishmaniasis, few studies were performed to evaluate the antileishmanial activity of other related pentacyclic triterpenes, produced and accumulated by H. canum, as is the case of maslinic acid.

Maslinic acid is widely distributed among species, including edible plants such as spinach, eggplant, olive, and basil [19-23], suggesting that this triterpene has no toxicity for humans. In the present work, it has been demonstrated, for the first time, that maslinic acid isolated from the leaves of *H. canum* inhibited the growth of both stages of *L. (L.) infantum* and parasite death was associated with leishmanial organelle disorganization.

2 Material and methods

2.1 General

Column and thin layer chromatographic procedures were performed, respectively, using silica gel 60 (230−400 mesh, Merck) and silica gel 60 PF254 (Merck). Semipreparative HPLC procedure was performed on a Dionex Ultimate 3000 chromatography using a Luna Phenomenex C_{18} column (10 × 250 mm particle and pore size of 5 μm and 175 Å, respectively), and detector UVD-DAD – 170 V. NMR spectra were recorded on a Bruker Ultrashield 300 Avance III spectrometer (Billerica, MA, USA) operating at 300 and 75 MHz with ^{1}H and ^{13}C nuclei, respectively. All samples were dissolved in $CDCl_3$ and drops of CD_3OD (both solvents from Sigma-Aldrich) were added to make a homogeneous medium. ESI-HRMS spectra were measured using an ESI ion source in negative mode on a Bruker Daltonics MicroTOF QII (Billerica, MA, USA).

2.2 Plant material and preparation of crude extract

Fresh leaves of *Hyptidendron canum* (Pohl ex Benth.) Harley (Lamiaceae) were collected in Goiania, GO, Brazil, in December 2019. The plant material was compared

with a voucher specimen SP205332 deposited in the Herbarium of *Instituto de Botânica de São Paulo*, SP, Brazil. The leaves were dried at 35°C for 72h, milled, and the obtained material (312 g) was exhaustively extracted using MeOH at room temperature, to afford 11 g of MeOH extract after evaporation of the solvent under reduced pressure.

2.3 Isolation of maslinic acid

Part of MeOH extract from leaves of *H. canum* (8 g) was ressuspended in MeOH:H_2O 2:1 and sequentially partitioned with hexane and EtOAc to obtain, respectively, 3.4 g and 2.7 g of each organic phase. Part of the EtOAc phase (2.5 g) was subjected to column chromatography under silica gel eluted with increasing amounts of EtOAc in hexane to give six fractions (A – F). Fraction C (154 mg) was subjected to column chromatography over silica gel eluted with of hexane: EtOAc (8:2, 7:3, and 1:1) to give a mixture of triterpenes on fraction C-3 (34 mg). This subfraction was purified by semipreparative HPLC using MeOH: H_2O 9:1 as a mobile phase to afford pure maslinic acid (17.6 mg).

2.3.1 Maslinic acid

^{1}H NMR ($CDCl_3$ + drops of CD_3OD, 300 MHz): δ 5.32 (t, *J* = 3.7 Hz, H-12), 3.71 (m, H-2), 3.01 (d, *J* = 9.5 Hz), 1.15 (s, H-27), 1.03 (s, H-23), 1.00 (s, H-25), 0.93 (s, H-30), 0.91 (s, H-29), 0.84 (s, H-24), 0.78 (s, H-26). ^{13}C NMR ($CDCl_3$ + drops of CD_3OD, 75 MHz): δ 178.5 (C-28), 144.7 (C-13), 122.0 (C-12), 83.4 (C-3), 68.5 (C-2), 55.2 (C-5), 47.5 (C-1 and C-9), 46.3 (C-17), 46.1 (C-19), 41.6 (C-14), 41.1 (C-18), 39.2 (C-4), 39.1 (C-8), 38.1 (C-10), 33.9 (C-29), 33.8 (C-21), 32.9 (C-7 and C-22), 30.6 (C-20), 28.5 (C-23), 27.5 (C-15), 25.8 (C-27), 23.4 (C-11 and C-16), 23.3 (C-30), 18.3 (C-6), 17.4 (C-24), 17.1 (C-26), 16.4 (C-25). ESI-HRMS *m/z* 471.3471 $[M - H]^-$ (calcd. for $C_{30}H_{47}O_4$, 471.3474).

2.4 Parasites

L. (L.) infantum – synonymy *L. (L.) chagasi* - (MHOM/BR/72/46) was kindly provided by Prof. Dr. Fernando Tobias Silveira, from the cryobank of the Leishmaniasis Laboratory Prof. Dr. Ralph Laison, Department of Parasitology, Ministry of Health, Instituto Evandro Chagas (Belem, Para - Brazil). They were identified using monoclonal antibodies and isoenzyme electrophoretic profiles at the Leishmaniasis Laboratory of the Instituto Evandro Chagas. Stationary phase promastigotes were used throughout the entire study. Parasites were maintained in Schneider's Medium (Sigma Aldrich, Germany), supplemented with 10% heat-inactivated fetal bovine serum and 50,000 IU/mL penicillin, 50 μg/mL streptomycin (S10). Promastigote forms in the stationary phase of growth were used in all experiments.

2.5 Promastigote and cytotoxic assays

Promastigote forms of *L. (L.) infantum* ($2x10^6$ promastigotes/well) were incubated in a 96-well culture plate in S10 with maslinic acid and miltefosine (0.781 to 100 μg/mL) or amphotericin B (0.016 to 1 μg/mL). Control group was cultivated in medium and vehicle solution (PBS plus 1% DMSO). The parasites were incubated for 24 h at 25°C, then washed with 200 μL of PBS three times with centrifugation at 3000 rpm, 10 min at 4°C, followed by addition of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (10 mg/mL). Four hours later, 50 μL of 10% sodium dodecyl sulfate (SDS) was added to each well. The plates were further incubated for 18 h and read in an ELISA reader at 595 nm. Effective concentration 50% (EC_{50}) was estimated using the nonlinear regression test with GraphPad Prism 5.0 software.

Peritoneal macrophages from golden hamsters (*Mesocricetus auratus*) at 10^6 macrophages/well were cultured in 96-well plates in RPMI 1640 medium supplemented with 10% of fetal bovine serum (Thermo Fisher, USA), 2 mM L-glutamine (Sigma-Aldrich, USA), 10 mM Hepes (Sigma-Aldrich, USA), 1 mM sodium pyruvate, 1% v/v nonessential amino acid solution (Thermo Fisher, USA), 10 μg/mL of gentamicin (Thermo Fisher, USA) and 1000 U/mL of penicillin (Thermo Fisher, USA) (R10 medium). Maslinic acid, miltefosine, or amphotericin B (0.781 to 100 μg/mL) were added

and macrophages were incubated at 37°C, 5% CO_2, during 24h. Cells were centrifuged at 1000 rpm for 10 min at 4°C and washed 3 times, then cell viability was assessed by the oxidation of MTT. Four hours later, 50 µL of 10% sodium dodecyl sulfate (SDS) was added to each well. The plates were further incubated for 18 h and read in an ELISA reader at 595 nm. Cytotoxic concentration 50% (CC_{50}) was estimated using the nonlinear regression test with GraphPad Prism 5.0 software. The index of selectivity (SI) was estimated according to Yamamoto and collaborators [16]. This study was carried out in strict accordance with the recommendations of the guide for Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation (http://www.cobea.org.br). The protocol was approved by the Ethics Committee of Animal Experiments of the Institutional Committee of Animal Care and Use at the Medical School of São Paulo University (056/16)

2.6 Activity of maslinic acid in intracellular amastigotes, nitric oxide, and hydrogen peroxide production

Peritoneal macrophages from golden hamsters (10^6 macrophages) were collected and cultured on round coverslips in 24-well plates for 4 h in R10 medium. After some washes with warm PBS, cells were infected with *L. (L.) infantum* promastigotes (10 parasites per peritoneal macrophage). The plates were incubated overnight at 5% CO_2 at 37°C. Maslinic acid and miltefosine were added to the infected macrophages at 7.5, 15, and 30 µg/mL; amphotericin B at 0.01, 0.1 and 1 µg/mL. Twenty-four hours later, macrophage supernatants were collected and stored at -80°C for quantification of nitric oxide (NO) (Life Technologies, USA) and hydrogen peroxide (H_2O_2) (Life Technologies, USA) according to the manufacturer's instructions. The coverslips were dried at room temperature, fixed in MeOH, and stained by Giemsa. The number of infected macrophages and parasites per macrophage was determined at least in 100 cells. The infection index (II) was expressed as the percentage of infected macrophages multiplied by the average number of amastigotes per macrophage according to Passero and collaborators [24]. As a positive control for NO and H_2O_2, macrophages were incubated with LPS (100 ng/mL) according to [25].

2.7 Ultrastructural alterations induced by maslinic acid in *L. (L.) infantum*

Promastigote forms of *L. (L.) infantum* ($2x10^6$ promastigotes/well) were incubated in a 24-well culture plate in S10 with maslinic acid, miltefosine or amphotericin B at the respective EC_{50} values. The control group was cultivated with medium and vehicle solution. Parasites were centrifuged at 3000 rpm, 4°C, 10 min, and washed three times with 200 μL of PBS. Then the pellets were resuspended in glutaraldehyde 2% in 0.1M sodium cacodylate buffer (pH7.4) and incubated at 4°C, for 60 min. Parasites were postfixed in 1% osmium tetroxide. The samples were washed, followed by dehydration in a graded series of acetone. Then, the samples were embedded in a polyester resin, thin sectioned in a Reichert ultramicrotome, double-stained with 7% uranyl acetate and lead citrate aqueous solution (Ladd Research Industries). Stained parasites were examined with a JEOL 1010 (Tokyo, Japan) transmission electron microscope (TEM).

2.8 Cell membrane damage and mitochondrial membrane potential assays

Promastigote forms of *L. (L.) infantum* ($2x10^6$ promastigotes/well) were incubated in 96-well black culture plates (Corning Inc, USA) in S10 at EC_{50} of maslinic acid, miltefosine, or amphotericin B for 24h, followed by the addition of Sytox Green at 0.5 μM/well (Life Techonologies, USA) or Rhodamine 123 at 3 μM/well (Sigma-Aldrich, USA) to evaluate cell membrane damage or mitochondrial membrane potential, respectively. Parasites were placed in the dark for 15 minutes at 25°C, centrifuged at 1200 g, 5 min at 10°C, and washed three times with 200 μL of PBS. Plates containing parasites stained with Sytox Green were read in a fluorescence reader using 530 nm emission wavelength and 490 nm excitation, and parasites stained with Rhodamine 123 read with 520 nm emission and 485 nm excitation wavelengths. Results were normalized concerning the respective control (non-treated parasites). Triton X-100 at 0.05 μL (Sigma Aldrich, USA) was used as a positive control for cell membrane damage and oligomycin at 0.1 μM/well (Cayman Chemicals, USA) as a positive control of mitochondrial membrane potential inhibition.

2.9 Statistical analysis

Data were reported as the mean of three independent assays. Values are expressed as means ± standard deviation. Statistical analyses were performed using GraphPad Prism 5.0 software, and the nonparametric Mann-Whitney was used to assess the differences between the treated groups and the control group (untreated). Statistical significance was set at a p-value < 0.05.

3 RESULTS

3.1 Chemical characterization of maslinic acid

ESI-HRMS spectrum of the isolated compound displayed the $[M - H]^-$ ion peak at *m/z* 471.3471, corresponding to the molecular formula $C_{30}H_{48}O_4$, suggesting the occurrence of a triterpene derivative. ^{1}H NMR spectrum displayed, besides other overlapped signals at range δ 1.07 – 2.05, seven singlets of methyl groups at δ 1.15 (H-27), 1.03 (H-23), 1.00 (H-25), 0.93 (H-30), 0.91 (H-29), 0.84 (H-24), and 0.78 (H-26) as well as two signals attributed to oximethyne hydrogens at δ 3.71 (m, H-2) and 3.01 (d, *J* = 9.5 Hz, H-3). These data, in association with a triplet at δ 5.32 (*J* = 3.7 Hz, H-12), indicated an oleanane triterpene derivative [26]. ^{13}C NMR spectrum exhibited 30 signals attributed to seven methyl, nine methylene, six methyne being two oxygenated at δ 83.4 (C-3) and 68.5 (C-2) as well as one sp^2 at δ 122.0 (C-12), and eight quaternary carbons, being two sp^2 at δ 144.7 (C-13) and 178.5 (C-28), this last characteristic of carboxylic acid [27]. The comparison of the obtained data with those reported in the literature [28], allowed the identification of maslinic acid (Figure 1).

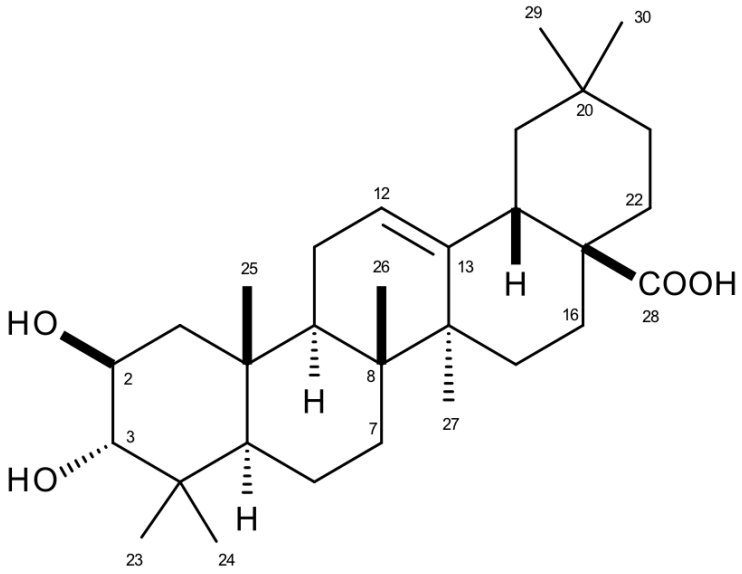

Figure 1 - Chemical structure of maslinic acid, isolated from the MeOH extract from the leaves of *H. canum.*

3.2 Antileishmanial and cytotoxic activities of maslinic acid

Maslinic acid, miltefosine and amphotericin B were active against promastigote forms of *L. (L.) infantum*, presenting dose-dependent antileishmanial activities (Figures 2A, B, and C, respectively). Comparatively, maslinic acid showed the lowest activity on promastigote forms (EC_{50} = 11.7 ± 0.4 µg/mL), in comparison with miltefosine (EC_{50} = 6.5 ± 0.3 µg/mL) and amphotericin B, that was highly active on promastigote forms (EC_{50} = 0.030 ± 0.006 µg/mL).

Maslinic acid was not toxic to peritoneal macrophages from golden hamsters (Figure 2 D), by contrast, miltefosine and amphotericin B killed macrophages in a dose-dependent manner, and in this case miltefosine displayed the highest cytotoxic potential (CC_{50} = 27.4 ± 0.2 µg/mL), followed by amphotericin B (CC_{50} = 87.7 ± 0.9 µg/mL), as shown in figure 2 E and F respectively.

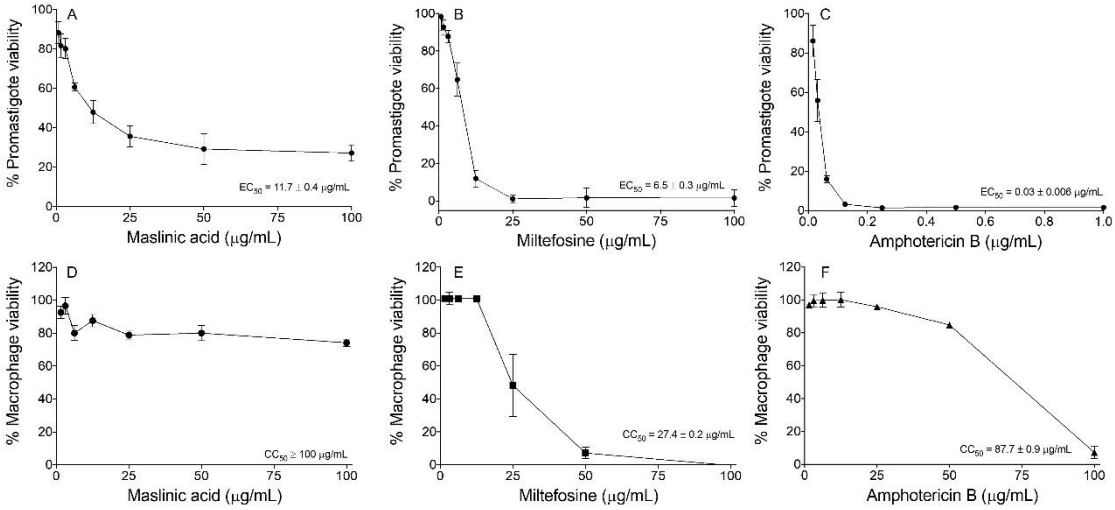

Figure 2 - Leishmanicidal and cytotoxic activity. Promastigote forms of *L. (L.) infantum* were incubated with different concentrations of maslinic acid (A), miltefosine (B), or amphotericin B (C) for 24h; in each of these treatments the effective concentrations 50% (EC_{50}) were estimated. In addition, peritoneal macrophages from golden hamsters were also incubated with maslinic acid (D), miltefosine (E), or amphotericin B (F) for 24h, and the the cytotoxic concentration 50% (CC_{50}) was calculated. These experiments were repeated three times, and the values are represented as mean ± standard deviation.

Based on these data, it was possible to estimate that against promastigote forms of *L. (L.) infantum,* amphotericin B displayed the highest selective index (SI = 2923.3), followed by maslinic acid (SI = 8.6) and miltefosine (SI = 4.2).

Maslinic acid (Figure 3 A) as well as miltefosine (Figure 3 B) and amphotericin B (Figure 3 C) exhibited anti-amastigote activity in a dose-dependent manner. Comparatively, maslinic acid presented intermediated antileishmanial activity (EC_{50} = 2.9 ± 0.2 μg/mL), being higher active than miltefosine (EC_{50} = 9.5 ± 0.5 μg/mL) and least active than amphotericin B (EC_{50} = 0.009 ± 0.001 μg/mL).

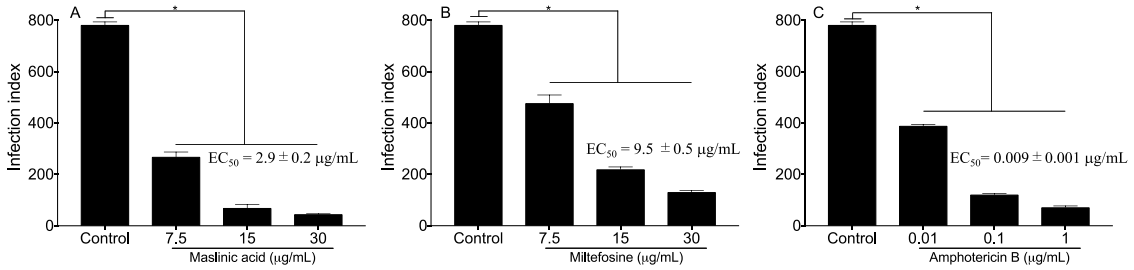

Figure 3 - Anti-amastigote activity of maslinic acid. Peritoneal macrophages from golden hamsters were isolated and infected with promastigote forms of *L. (L.) infantum* at 10:1 ratio. Twenty-four hours later, infected macrophages were treated with different concentrations of maslinic acid (A), miltefosine (B), and amphotericin B (C). Infected cells were incubated with the drugs during 24h and their infection indexes were estimated, as well as the effective concentration 50% for each treatment. * $p<0.05$ indicates a significant difference concerning the control group.

Considering data on the anti-amastigote action of these compounds, the estimated selective indexes for maslinic acid, miltefosine, and amphotericin were 34.5, 2.9, and 9744.4 respectively.

Infected macrophages treated with maslinic acid, miltefosine, or amphotericin B did not produce quantifiable levels of nitric oxide or hydrogen peroxide.

3.3 Ultrastructural changes in promastigote forms treated with maslinic acid

Promastigote forms treated with maslinic acid are shown in Figures 4A – C. Parasites did not display important changes in the cell membrane (Figure 4 A), but the cytoplasm presented signs of degeneration with areas containing membrane debris (Figure 4, A and B - black arrowhead) associated with an intense exocytic activity in the flagellar pocket (FP) zone (Figure 4 C). In the complex kinetoplast (K) - mitochondria (M), it was verified blebs in kDNA (Figure 4 B) and disruption of mitochondrial cristae (Figure 4 C) - however, some parasites showed preserved morphology of the kDNA and mitochondria (Figure 4 A). The nucleus of promastigote forms was affected by maslinic acid and the chromatin seems to be fragmented (Figure 4 A) and in some specimens, the nucleus was completely degraded (Figure 4 C).

The morphology of *L. (L.) infantum* was affected by miltefosine. The cytoplasm of the parasites was degraded, and blebbing was detected in the area of kDNA (K) (Figure 4D), mitochondrial cristae were disorganized (Figure 4 D). *L. (L.) infantum* treated with amphotericin B lost the fusiform morphology, small blebs were detected attached to the cell membrane (black arrow), and the cytoplasm seems to be degraded, presenting areas of cytoplasm extraction. Membrane debris (arrowhead) was detected, suggesting that autophagic vacuoles were triggered during the treatment with amphotericin B. Membrane debris was also detected in the flagellar pocket (FP), suggesting intense phagocytic activity (Figure 4 E).

Control promastigote forms showed a preserved external structure, with a fusiform shape and intact cell membrane (Figure 4 F). The cytoplasm, flagellum, flagellar pocket (FP), kinetoplast (K), and the nucleus (N) showed regular morphology (Figure 4 F).

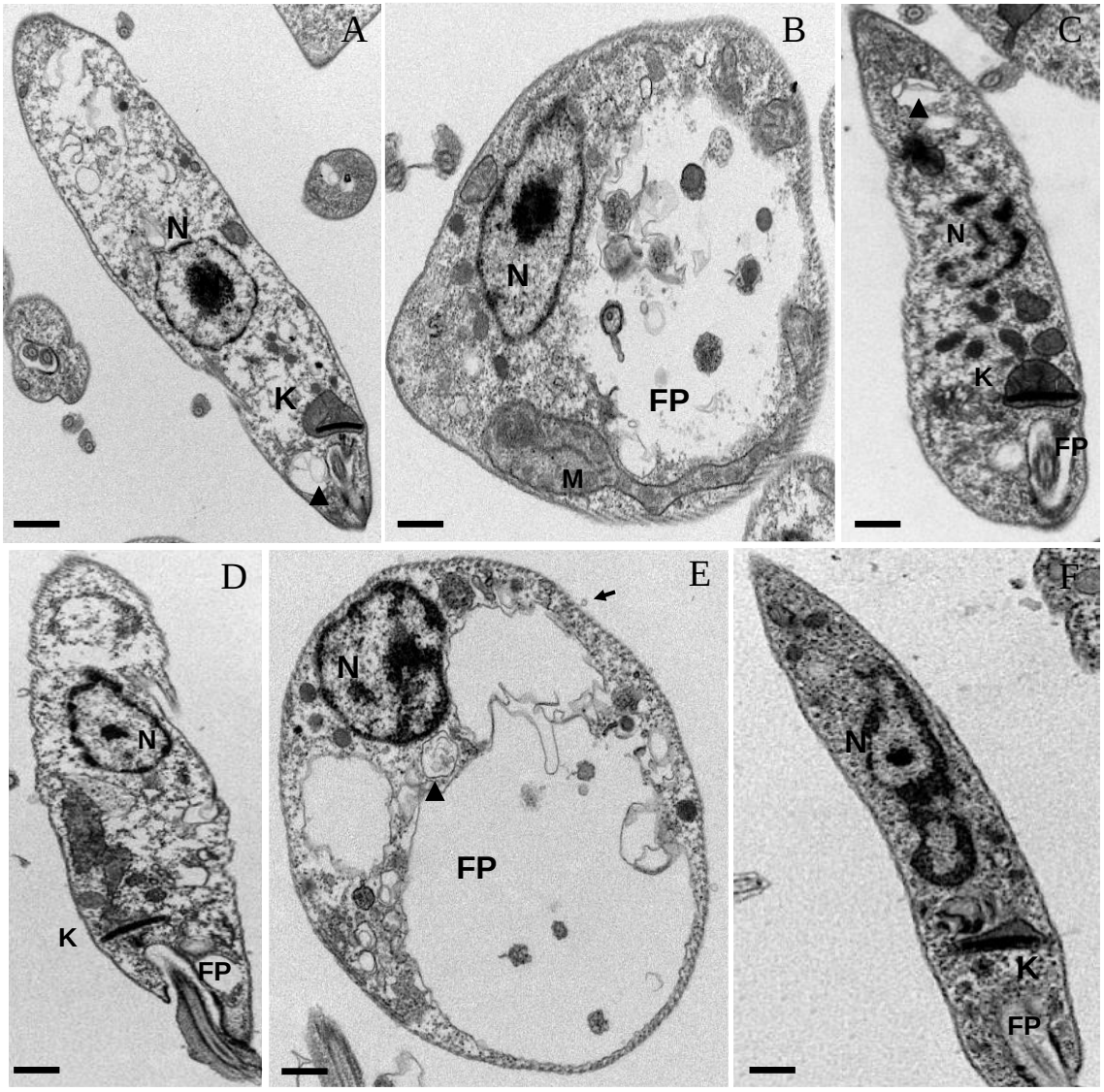

Figure 4 - Ultrastructural changes induced by maslinic acid (A, B, and C), miltefosine (D), or amphotericin B (E) in promastigote forms of *L. (L.) infantum*. Promastigote forms were incubated with EC_{50} of maslinic acid (A, B, and C), miltefosine (D), or amphotericin B (E) for 24h, then morphological changes were recorded. Control parasites are shown in F. N – nucleus; FP – flagellar pocket; K – kinetoplast. Black arrow indicates blebs in the cell membrane. Black arrow head indicates compartmentalized membrane debris.

3.4 Cell membrane integrity and mitochondrial membrane potential assays

Parasites treated with maslinic acid showed reduced fluorescence in comparison with the control group ($p < 0.05$), but parasites treated with amphotericin B displayed higher fluorescence intensity compared to the control ($p<0.05$), as shown in figure 5A,

suggesting that amphotericin B altered the integrity of the parasite cell membrane. On the other hand, this effect was not observed in parasites incubated with miltefosine.

Maslinic acid did not alter mitochondrial membrane potential in *L. (L.) infantum* promastigotes, in contrast, amphotericin B and miltefosine significantly ($p<0.05$) inhibited mitochondrial membrane potential in comparison with the control (Figure 5 B).

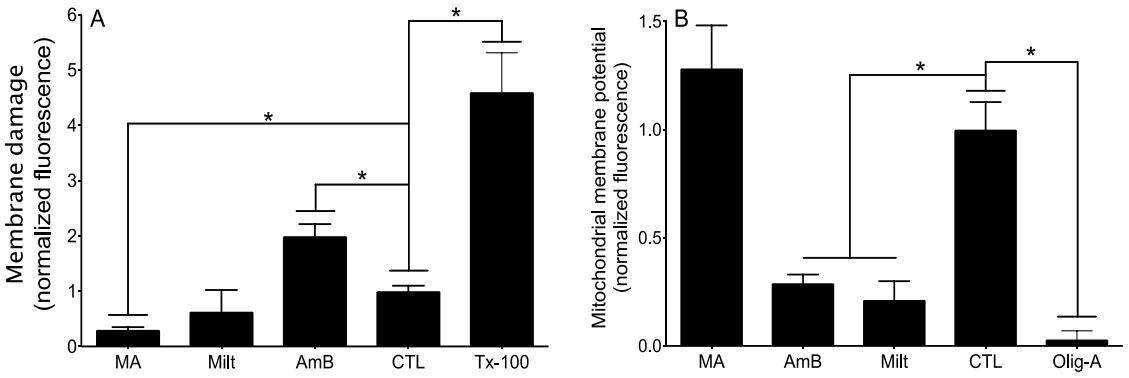

Figure 5 - Cell membrane integrity (A) and mitochondrial membrane potential assays (B). Promastigote forms of *L. (L.) infantum* were treated with EC_{50}s of maslinic acid, miltefosine, and amphotericin for 24h, then cell membrane integrity (A) and mitochondrial membrane potential were analyzed using the probes Sytox green and rhodamine 123, respectively. Triton X-100 was used as a positive control of membrane damage, and oligomycin A as an inhibitor of the mitochondrial membrane potential. * $p<0.05$ indicates a significant difference.

4 Discussion

In the present work, maslinic acid was isolated from the leaves of *H. canum*, a plant used to treat different medical conditions in traditional communities in Brazil. This compound was chemically identified by NMR analysis and ESI-HRMS data followed by comparison of the obtained data with those described in the literature [28]. Although several species of *Hyptidendron* accumulate bioactive molecules, currently, few works investigated the pharmacological activity of this genus, and despite the microbicide effect reported by the traditional populations, there are not available studies showing the leishmanicidal activity of bioactive molecules from *H. canum*.

Maslinic acid was active on promastigote forms of *L. (L.) infantum*, but it was less active than miltefosine and amphotericin B, both used in the treatment of leishmaniasis.

Although both miltefosine and amphotericin have had higher leishmanicidal activity than maslinic acid on promastigote forms, it was possible to observe that miltefosine and amphotericin B displayed high and moderate cytotoxic activity on peritoneal macrophages, respectively. In contrast, it was not observed cytotoxic events when peritoneal macrophages from golden hamsters were incubated with maslinic acid, and it positioned this triterpene as active and selective toward promastigote forms. A previous study reported that maslinic acid, isolated from the leaves of olive tree [29], displayed activity against an European strain of promastigote forms of *L. (L.) infantum* with EC_{50} values of 9.32 ± 1.65 μg/mL when parasites were incubated in the interval of 48 – 72h; comparatively, maslinic acid eliminated the Brazilian strain of *L. (L.) infantum* at 24h with EC_{50} of 11.7 ± 0.4 μg/mL. These data indicate that this triterpene has multispecies activity and the Brazilian strain of *L. (L.) infantum* may be more susceptible than the European species. Although maslinic acid exhibited absent or reduced cytotoxic activity on peritoneal macrophages from golden hamsters, an experimental model able to mimic natural infection [30], a previous study showed that this triterpene had a significant cytotoxic activity towards J774 macrophage [31], that is a tumor cell lineage, and thus the differences in the cytotoxicity data may be associated with the cell type employed. Taken together, our data suggest that maslinic acid could be considered an important molecule to develop new prototypes for the treatment of leishmaniasis.

Maslinic acid also impacted the survival of intracellular amastigote forms, and the EC_{50} (2.9 ± 0.2 μg/mL) of this triterpene was 3.3 times higher at eliminating amastigote forms than miltefosine. In the study conducted by Sifaoui and collaborators, maslinic acid also displayed an EC_{50} of 2.90 ± 0.06 μg/mL on intracellular forms of *L. (L.) infantum* isolated in the European continent, and although the EC_{50} value was the same that we found herein, the method to analyze the activity of maslinic acid was different, since herein we analyzed the infection index, that considers the number and morphology of intracellular amastigote forms, while Sifaoui and collaborators used the Alamar blue method. By using this method, intracellular amastigote forms are placed at 25^{o}C, and after differentiation of amastigote to promastigote forms the activity of mitochondria is recorded by Alamar blue reagent. Thus, based on the number and mitochondria activity the potency of maslinic acid was determined. The infection index is the most used method to analyze the activity of drugs on amastigote forms, and although Alamar blue is an innovative technique to analyze anti-amastigote activity of drugs it is not widely accepted.

Taking into consideration that maslinic acid showed superior anti-amastigote activity than miltefosine and in the present work it showed a selective index of 34.5, it becomes possible to emphasize that this triterpene is an interesting molecule to develop a new drug to treat visceral leishmaniasis in both New and Old Worlds.

In addition, it was observed that maslinic acid showed a higher selective index (SI = 34.5) over intracellular amastigote forms than miltefosine (SI = 4.2); however amphotericin B still displayed the highest SI on amastigote forms. Despite the highest activity and selectivity of amphotericin B on amastigotes, a mild toxicity of this drug was observed on macrophages from golden hamsters; additionally experimental [17, 31] and clinical [32, 33] studies already demonstrated severe side effects induced by amphotericin B during the treatment, that in turn have been associated with the low adherence to the treatment. In contrast, maslinic acid is present in different edible vegetables, commonly used in the human diet; thus it does not seem toxic for humans [20, 22, 34]. Furthermore, it was demonstrated that daily oral doses of maslinic acid did not alter the body weight, biochemical, or hematological parameters of experimental animals [35], and in diabetic rats this triterpene exhibited hepatic and renal protective properties [36, 37], suggesting that this triterpene is safe to be used in different medical conditions, including leishmaniasis.

Ultraestructural changes observed in promastigote forms of *L. (L.) infantum* incubated with maslinic acid and miltefosine were related with cytoplasm degradation and disruption of intracellular organelles, especially the mitochondria, which showed an increased volume, associated with disorganization of cristae. Furthermore, blebs were detected in the kDNA-mitochondria complex, suggesting that maslinic acid may target the mitochondria. In addition, the nucleus seems to be affected by this triterpene, since chromatin fragmentation has been detected, and in some individuals the nucleus seems to be completely fragmented, as observed in figure 4C, suggesting that maslinic acid also may affect the nucleus of *L. (L.) infantum* promastigote forms. In addition to these morphological changes, it was observed membrane debris in the flagellar pocket of parasites treated with maslinic acid. Possibly, similar structural changes observed in parasites treated with maslinic acid or miltefosine may be associated with a correlated mechanism of death.

It was demonstrated that *Leishmania* sp. treated with miltefosine displayed morphological changes compatible with programmed cell death [38-39], and biochemical

changes attested that the parasites underwent apoptosis upon treatment [40-42]. Similarly, a chemically related triterpene – ursolic acid – also induced morphological and molecular changes in treated parasites, resembling apoptosis [7, 16]. Thus, considering the structural similarity between both triterpenes and the similar morphological changes observed in parasites after treatment, it may be possible that maslinic acid triggers programmed cell death in *L. (L.) infantum*. Previously, it was demonstrated that maslinic acid may induce apoptosis in *L. (L.) infantum*, since parasites incubated with IC90 exposed phosphatidyl serine on the cell membrane, decreased the mitochondrial membrane potential as well as ATP levels [29]; however, no morphological evidence of programmed cell death was previously demonstrated. In addition, the apoptotic process triggered by maslinic acid was identified in tumor cell strains that displayed plasma membrane disintegration and nuclear fragmentation [43]. In contrast, it has been observed that the leishmanicidal activity of amphotericin B is related to the interaction of the drug with the cell membrane of parasites, causing changes in the integrity of the cell membrane [44, 45]. The main change in the cell membrane of parasites treated with amphotericin B was associated with the presence of small blebs, however pores in the membrane were not observed. In addition, it was verified areas with cytoplasm extraction and autophagic vacuoles, suggesting that amphotericin B degraded lipids [46, 47].

Despite the intracellular disorganization, maslinic acid did not change the integrity of the cell membrane of promastigote forms, as evaluated by morphology and SYTOX Green probe. By contrast, it was observed a significant decrease in the fluorescence units in parasites incubated with maslinic acid than in control. A possible explanation may be related to the morphology of the parasite nucleus. Upon treatment with maslinic acid (at EC_{50} value), it was observed that the nucleus was altered and, in some individuals, it was completely fragmented, that may have affected the potential of SYTOX green probe to bind to the nucleus and emit fluorescence. In contrast, Sifaoui and collaborators [29] observed that maslinic acid was able to damage the cell membrane of *L. (L.) infantum* promastigotes, however the highest time point recorded was 100 minutes, suggesting that cell damage is an early event triggered by maslinic acid. Unlike maslinic acid, the parasites treated with amphotericin B and incubated with Sytox displayed high levels of fluorescence, suggesting that this drug altered the integrity of the cell membrane, allowing the probe to access the nucleus of the parasites, which in this specific situation was more preserved than the nucleus of parasites treated with maslinic acid.

In this study, amphotericin B and miltefosine were able to inhibit the mitochondrial membrane potential, which is crucial for the generation of ATP in the respiratory chain, and as a consequence cells become depleted of energy with subsequent death [29, 48]. In contrast, maslinic acid did not change mitochondrial membrane potential, that was not expected, because it was observed that promastigote forms displayed morphological changes in the mitochondria. This can be explained in some ways - firstly, the morphological changes induced by maslinic acid at the EC_{50} were not enough to alter the mitochondrial membrane potential; secondly, the morphological changes in the mitochondria were observed only in some parasites, and thus the normal parameters observed could be associated with the parasites presenting preserved mitochondria; third, maslinic acid may not target mitochondria at the EC_{50}, and the morphological changes observed in the mitochondria of some parasites may be associated with a late process of death, in which all organelles are affected.

Taken together, in vitro data demonstrate that maslinic acid was able to eliminate promastigote forms and it exerted an important activity in the elimination of amastigote forms of *L. (L.) infantum* in a selective manner, with an action superior to that of miltefosine. Additionally, morphological data suggest that this triterpene would be acting by inducing programmed cell death in *L. (L.) infantum*. These results highlight that maslinic acid may be an interesting therapeutic alternative in the treatment of leishmaniasis.

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Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

Investigation, formal analysis, writing, and editing: JAJ, LFDP, MLS; Resources, review: LFDP, MDL, and JHGL; Conceptualization, validation, resources, writing, review, editing: LFDP, JAJ. All authors read and approved the final manuscript.

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7 ARTIGO PUBLICADO IV

pharmaceutics

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Article

Preclinical Assessment of Ursolic Acid Loaded into Nanostructured Lipid Carriers in Experimental Visceral Leishmaniasis

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Abstract

Ursolic acid, a triterpene produced by plants, displayed leishmanicidal activity *in vitro* and *in vivo*; however, the low solubility of this triterpene limits its efficacy. To increase the activity of ursolic acid (UA), this triterpene was entrapped in nanostructured lipid carriers (UA-NLC), physical-chemical parameters were estimated, the toxicity was assayed in healthy golden hamsters, and the efficacy of UA-NLC was studied in experimental visceral leishmanisis. UA-NLC exhibited a spherical shape with a smooth surface with a size of 266nm. UA-NLC displayed low polydispersity (PDI = 0.18) and good colloidal stability (−29.26mV). Hamsters treated with UA-NLC did not present morphological changes in visceral organs, and the levels of AST, ALT, urea and creatinine were normal. Animals infected with *Leishmania (Leishmania) infantum* and treated with UA-NLC showed lower parasitism than the infected controls, animals treated with UA or Amphotericin B (AmB). The therapeutic activity of UA-NLC was associated with the increase in a protective immune response, and it was associated with a high degree of spleen and liver preservation, and the normalization of hepatic and renal functions. These data indicate that the use of lipid nanoparticles as UA carriers can be an interesting strategy for the treatment of leishmaniasis.

Keywords: nanoparticles; nanostructured lipid carriers; ursolic acid; toxicity; visceral leishmaniasis

1 Introduction

Leishmaniasis is a neglected tropical disease, caused by a parasite of the genus *Leishmania* that is transmitted to mammalian hosts during a phlebotomine vector blood meal. Leishmaniasis has been considered a serious public health problem, given the wide geographical distribution, the number of pathogenic species to humans and other vertebrates, diversity of clinical forms, and the scarcity of drugs available for therapy [1,2].

The effectiveness of leishmaniasis treatment depends on several factors, such as drug choice, host immune response, parasite strain, treatment regimen as well as patient compliance [3]. The treatment of leishmaniasis is not specific to each species nor to the clinical forms and it is performed mainly with pentavalent antimonials. Miltefosine, pentamidine, paromomycin, and amphotericin B are considered second-choice drugs and complete the arsenal to treat all clinical forms of leishmaniasis [4] , however, these drugs have limitations due to side effects, high costs, and in some cases reduced efficacy, sometimes caused by resistant parasites [5,6]. Thus, it is essential to characterize new, efficient, affordable, and less toxic drugs or approaches to combat this neglected disease.

Studies have shown that medicinal plants and their purified molecules, present attractive features to develop new antileishmanial agents, such as selectivity, low toxicity to the experimental hosts, and importantly, high leishmanicidal potential [7]. In this regard, it has been demonstrated that the triterpene ursolic acid (UA) is an interesting antiprotozoal agent, since it is active towards *Leishmania* sp., *Trypanosoma cruzi*, and *Toxoplasma gondii* [8–10]. In leishmaniasis UA eliminated the promastigote and amastigote forms of *L. (L.) amazonensis, L. (L.) braziliensis, L. (L.) donovani, L. (L.) guyanensis,* and *L. (L.) infantum* [11–13], suggesting a multispectral activity. Furthermore, it displayed therapeutic activity in experimental cutaneous and visceral leishmaniasis [10,14]. Altogether, these studies show that UA is an interesting molecule to design rational methodologies to improve the treatment of leishmaniasis.

Although the activity of this triterpene has been recorded in experimental leishmaniasis, the main drawback of UA is related to the low solubility in common physiological diluents, which indeed may impact its efficacy in the treatment of leishmaniasis. To overcome this disadvantage, UA can be encapsulated into different types of nanocarriers, which would increase the solubility and availability of this

molecule *in vivo*. Additionally, it is well recognized that drug delivery using nanocarriers can overcome physiological barriers, such as the lipidic membrane of cells, and thus a high concentration of drug will be accumulated in the cytosol of cells as well as the phago-lysosomal compartment. This strategy of treatment may improve the efficacy of the therapy and reduce the toxicity, as the drug release happens at specific locations [15,16]. Among the different drug delivery systems, nanostructured lipid carriers (NLCs) are one of the most studied and show attractive attributes meeting the requirements of an ideal carrier system for lipophilic molecules, such as UA triterpene. The low cytotoxicity, high drug payload, and ability to passively target and release bioactive substances at the site of action are some of the advantages that promote NLC as carriers of interesting drugs for the treatment of leishmaniasis [17].

Considering the scarcity of safe and effective approved drugs for the treatment of leishmaniasis and the activity of UA towards *Leishmania* species, this study aimed to develop a nanocarrier loaded with UA to enhance the effectiveness of this triterpene in experimental visceral leishmaniasis caused by *L. (L.) infantum.*

2 Materials and Methods

2.1 Materials

Solid lipid cetyl palmitate (CP) was provided by Gattefossé SAS (St Priest, France), the liquid lipid miglyol 812, and polysorbate 80 (Tween® 80) were purchased from Acofarma® (Madrid, Spain). UA (purity ≥ 98%) and AmB (purity ≥ 99%) were purchased from Cayman chemical company (Ann Arbor, MI, USA) and Cristalia Laboratory (São Paulo, Brazil), respectively. For the HPLC assay, acetonitrile was obtained from VWR (Radnor, PAA, EUA), ultrapure water (type 1, Milli-Q®) was obtained from EMD Millipore (Billerica, MA, USA). Cell culture media were bought from Sigma-Aldrich (Darmstadt, Germany).

2.2 Preparation method of NLC

NLC and UA-NLC nanocarriers were prepared by high-pressure homogenization

technique. CP (solid lipid - 2%) with the mygliol-812 (liquid lipid - 3%) and an aqueous solution of polysorbate 80 were heated to 70°C separately. Then, UA (0.1%) was dissolved in the lipid phase and the aqueous phase was added. The emulsion was submitted to a homogenization process in Ultra-Turrax T25, with S25N - 18G dispersing element (IKA®-Labortechnik, Staufen, Germany) at a stirring speed of 10,400 rpm for 5 min. For the NLC formation, the prepared emulsion was quickly transferred to the high-pressure hot homogenizing equipment (High-Pressure Homogenizer SPCH-10, Stansted Fluid Power), being homogenized during five cycles at 600 bar, and then cooled to 25°C in an ice bath. The samples were stored in glass bottles at 4°C. Empty NLC was prepared similarly, without adding UA.

In all experiments, UA was quantified using the UltiMate 3000 HPLC apparatus (Dionex Corporation, Sunnyvale, USA), with a UV-VIS detector, and automatic injector. A reverse-phase C18 column (BDS-Hypesil-C18®, Thermo Scientific, Waltham, MA, USA), was used with acetonitrile: water (88:12, v: v) mixture as the mobile phase, with a flow rate of 1.0 mL.min-1; UA was detected at 210 nm wavelength [18].

2.3 Physical-chemical characterization of nanoparticles

2.3.1 Determination of Encapsulation Efficiency

The freshly prepared formulation was diluted in milli-Q water (1:5), filtered through a 5 µm nitrocellulose membrane filter (Millipore, Ireland), and diluted 1:10 in ethanol to extract UA from NLC. The mixture was centrifuged at 4620 xg at 25°C for 15 min (Thermo Scientific Heraeus Multifuge X1R Refrigerated Benchtop Centrifuge, Indianapolis, IN, USA), the supernatant collected, and filtered through a 0.45 µm PTFE syringe filter (Millipore, Ireland). The supernatant was diluted (1:6) in eluent solution and applied to the HPLC column and the amount of UA released quantified. The encapsulation efficiency (EE) was calculated according to the following equation:

$$EE\ \% = \frac{Amount\ of\ UA\ in\ the\ filtered\ formulation}{Total\ amount\ of\ UA}\ x\ 100$$

2.3.2 Determination of size, polydispersity, and zeta potential

Particle size and distribution (polydispersity index) were analyzed by Dynamic Light Scattering (DLS, ZetaPALS, Brookhaven Instruments, Holtsville, USA). The zeta potential (ZP) of NLC dispersions was measured by Electrophoretic Light Scattering in a zeta potential analyzer (ZetaPALS, Brookhaven Instruments, Holtsville, USA). Samples were diluted (1:200) in milli-Q water, yielding a suitable scattering intensity. The samples were analyzed at room temperature, with a fixed light incidence angle of 90º; and the mean hydrodynamic diameter (Z-average), PDI, and ZP were obtained by calculating the mean value of six measurements, performed in three samples.

2.3.3 Morphological analysis of UA-NLC

The morphology of the NLC was analyzed by transmission electron microscopy JEOL JEM 1400 (Tokyo, Japan). An aliquot of nanoparticles (10 μL) was placed on nickel gratings with Formvar mesh / carbon film (Electron Microscopy Sciences, Hatfield, PA, USA). Samples were contrasted with 1% uranyl acetate solution. Samples were analyzed under a microscope at a voltage of 120 kV. Images were recorded using a CCD digital camera Orious 1100W Tokyo, Japan.

2.4. Animal and ethical considerations

Golden hamsters (Mesocricetus auratus), 8 weeks old, were obtained from the Anilab (Paulinia, São Paulo, Brazil). This study was performed in accordance with the recommendations of the guide for Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation. The Ethics Committee of Animal Experiments of the Institutional Committee of Animal Care and Use at the Faculdade de Medicina da Universidade de São Paulo (FMUSP) approved the following protocol 056/16, from 22 July 2016. Hamsters were housed in the Animal Experimental Instituto de Medicina Tropical da Universidade de São Paulo (IMTUSP), according to the

standards of the Committee of Animal Welfare.

2.5 Histological and biochemical changes of healthy hamsters treated with UA-NLC

Healthy golden hamsters were divided into 7 groups containing 5 animals/group. The experimental groups were arranged as follows: groups 1 and 2 were treated with NLC containing 1.25 and 5.0 mg/kg UA, respectively. Groups 3 and 4 were treated with 1.25 and 5.0 mg/kg of UA, respectively; Group 5 was treated with empty NLC (122.5 mg - the equivalent amount of NLC present in group 2); Group 6 was treated with 5.0 mg/kg of AmB [19] and group 7 consisted of healthy animals that received only the vehicle solution (PBS control plus 1% DMSO). Animals were treated by the intraperitoneal route, once a day, for 10 consecutive days. One week after the last injection, animals were anesthetized with intraperitoneal sodium thiopental (1 mg/200 μL) and euthanized after total blood collection. The blood was collected in 2 mL tubes without anticoagulants and centrifuged at 3000 rpm, 10 minutes, 4oC. The serum was collected and placed in eppendorffs; aliquots of 5 μL were used to analyze the following biochemical parameters: serum alanine transaminase (ALT), aspartate aminotransferase (AST), creatinine, and urea by colorimetric method on COBAS C111 equipment (ROCHE, Indianápolis, USA), as recommended previously by Spada and collaborators [20]. Fragments of the spleen, liver, kidney, lung, and heart were collected, fixed in buffered formalin 10%, processed using usual histological techniques, and 3 □m thick sections were stained with Hematoxylin and Eosin (HE).

2.6 Analysis of the therapeutic potential of UA-NLC

L. (L.) infantum (MHOM/BR/72/46) was provided by Prof. Dr. Fernando Tobias Silveira, from the cryobank of the Leishmaniasis Laboratory Prof. Dr. Ralph Laison, Department of Parasitology, Instituto Evandro Chagas (Para State, Brazil). Parasite was identified using monoclonal antibodies and isoenzyme electrophoretic profiles. *L. (L.) infantum* parasites were maintained in Schneider's Medium (Sigma Aldrich, Darmstadt, Germany), supplemented with 10% heat-inactivated fetal bovine serum and

50,000 IU/mL penicillin, 50 μg/mL streptomycin (S10). Stationary phase promastigotes were used throughout the entire study.

Golden hamsters (8 weeks old) were infected intraperitoneally with $2x10^7$ *L. (L.) infantum* promastigote forms. The noninfected control group was injected with PBS alone. Infected hamsters were divided into 8 groups, with 5 animals each. After 60 days of infection, the treatment was initiated with UA, UA-NLC, AmB or empty NLC. The experimental groups were arranged as follows: Groups 1 and 2 were treated with UA-NLC, containing 1.25 and 5.0 mg/kg of UA, respectively; Groups 3 and 4 were treated with 1.25 and 5.0 mg/kg of UA, respectively; Group 5 was treated with NLC (122.5 mg - the equivalent amount given to animals from group 2); Group 6 was treated with 5.0 mg/kg of AmB [19]; Group 7 was injected with vehicle solution (PBS plus 1% DMSO - Infected control group) and Group 8 consisted of the non-infected control group and received only vehicle solution. Animals were treated by the intraperitoneal route, once a day, for 10 consecutive days. One week after the last injection, the animals were euthanized and the serum was collected to quantify *Leishmania*-specific IgG and IgG2 by Enzyme-Linked Immunosorbent Assay (ELISA) (Southern Biotech, Birmingham, AL, USA); AST, ALT, creatinine, and urea were quantified by a colorimetric method on COBAS C111 equipment (ROCHE, Indianápolis, USA), as detailed in the section 2.5. Fragments of the spleen and liver were collected to determine splenic and hepatic parasitism as well as histological changes.

2.6.1 Determination of parasite load

The splenic and hepatic parasitism was quantified by limiting dilution assay [10]. Briefly, the spleen and liver were collected, weighted and aseptically homogenized in S10. Spleen and liver suspensions were subjected to 12 serial dilutions in sterile 96-well plates with four replicate wells. The number of parasites was determined based on the highest dilution that promastigote forms could grow after ten days of cultivation at 25°C. Additionally, parasitism in both organs was evidenced by the immunohistochemistry technique [21].

2.6.2 Analysis of cellular and humoral immune responses

RNA from hamster spleen fragments (~10 mg) was purified using the commercial RNeasy Mini Kit (Qiagen, Hilden, Germany). Isolated RNA was used to synthesize cDNA with the SuperScript®VILO™ cDNA Synthesis Kit (Life Technologies Carlsbad, CA, USA). Amplification conditions consisted of an initial denaturation phase at 95°C for 10 min, followed by 40 amplification cycles consisting of 95 °C for 15 s (s); 61°C for the 90s, and 72°C for 30s. All reactions were performed in a Mastercycler Nexus GSX1 (Eppendorff, Framingham, MA, USA). Before quantification, the efficiency of each reaction was verified using cDNA from the spleen of healthy animals; that was always above 95%. β-actin (endogenous control) was used to normalize the expression level of the genes. qPCR reactions were performed using the GoTaq® 1-Step RT-qPCR System (Promega Corporation, Madison, WI, USA) and 75 nM of primers. The primer sequences were as follows (5´ to 3´): IFN-γ forward: GACAACCAGGCCATCC and reverse: CAAAACAGCACCGACT; interleukin -10 (IL-10) forward: TGGACAACATACTACTCACTG and reverse: GATGTCAAATTCATTCATGGC; enzyme inducible nitric oxide synthase (iNOS) forward: CGACGGCACCATCAGAGG and reverse: AGGATCAGAGGCAGCACATC; β-actin forward: TCCTGTGGCATCCACGAAACTACA and reverse: ACAGCACTGTGTTGGCATAGAGGT [22–24]. Quantification results were expressed in fold changes of $2^{-\Delta\Delta Ct}$ over the infected control group [25]. PCR products were electrophoresed on 2% agarose to confirm the amplification of products. For each reaction, one single product of predicted size [22] was always obtained.

Soluble antigen of *L. (L.) infantum* was used to analyze the humoral immune response by ELISA. Briefly, promastigote forms in stationary phase of growth were collected by centrifugation at 3000 rpm, 10 min, 4ºC, and the pellet was washed three times with PBS. The pellet was resuspended in 100 μL of PBS and immediately frozen in liquid nitrogen. Following this step, the pellet was thawed at room temperature. This cycle of freeze and thaw was repeated three times, allowing parasite lysis. The lysate was centrifuged at 10000 rpm, 30min, 4ºC; the supernatant was collected and the concentration of protein was determined using the Bradford method (Biorad, Hercules, CA, USA). Ninety-six-well high-binding ELISA plates (Costar, USA) were coated with

the soluble antigen of the promastigote forms of *L. (L.) infantum* (1.0 μg of protein/well) in the carbonate-bicarbonate buffer, pH 9.6 (100 mM $NaHCO_3$; 6 mM Na_2CO_3), for 18h at 4°C. After this period, the wells were washed three times with PBS plus 0.05% Tween 20 (PBST), and nonspecific bindings were blocked with 10% skimmed milk diluted in PBS for 120 min at 37°C. The wells were washed three times with PBST, and 100 μL of animal serum (diluted 1: 1000 in PBT) were added to each well and the plate was incubated for 60 min at 37°C. After this period, the wells were washed three times with PBST, and the secondary antibodies goat anti-hamster IgG (1:10000) or IgG2 (1:16000), both conjugated with horseradish peroxidase - HPR - Southernbiotech, Birmingham, AL, USA), were added to the wells for 60 min at 37°C. After this step, the wells were washed five times with PBST, and the substrate 3.3', 5.5' tetramethylbenzidine – TMB (B&D, USA) was added to the wells for 15 min. The reaction was blocked by the addition of 50 μL / well of sulfuric acid (2N) and the absorbances were read in an ELISA reader at 450 nm wavelength. Serum from animals chronically infected with *L. (L.) infantum* and healthy animals were used as positive and negative controls of the reactions, respectively.

2.7 Statistical Analyses

Statistical analyzes were performed using the GraphPad Prism 5.0 software (San Diego, California, USA) and the nonparametric test Kruskal-Wallis, followed by Dunn's multiple comparison test, was used. Differences between two groups were analyzed by an unpaired t-test. Differences were considered statistically significant at the 5% level ($p<0.05$). Values were expressed as mean ± standard deviation from a minimum of three independent experiments.

3. Results

3.1. Physical characterization of NLC

The size of nanoparticles (PS), index of polydispersity (PDI), zeta potential (ZP) and efficacy of encapsulation (EE) are shown in Table 1. In general, the mean size of UA-NLC and NLC was below 267 nm. The PDI of UA-NLC was 0.18 while NLC was 0.16

and the value of ZP for UA-NLC was -29.26 mV and NLC was -26.12 mV, the EE of UA was 59.71%.

Table 1 - Particle size (PS), polydispersity (PDI), zeta potential (ZP), and efficacy of encapsulation (EE) of nanostructured lipid carriers (NLC) or UA-NLC. Data are presented as mean ± standard deviation (n = 3).

Nanoparticle	PS (nm)	PDI	ZP (mV)	EE (%)
NLC	261.1 ± 3.9	0.16 ± 0.013	-26.12 ± 1.18	
UA-NLC	266.3 ± 5.4	0.18 ± 0.022	-29.26 ± 1.16	59.71 ± 0.2

The morphology of the lipid nanoparticles, observed by TEM, revealed that both NLC (Figure 1 A) and UA-NLC (Figure 1 B) were spherical and uniform in shape with smooth surfaces, corroborating the results shown in Table 1. Additionally, it was possible to observe that by loading UA into NLC the morphology of the nanocarrier was not altered in comparison to NLC (Figure 1 A and B).

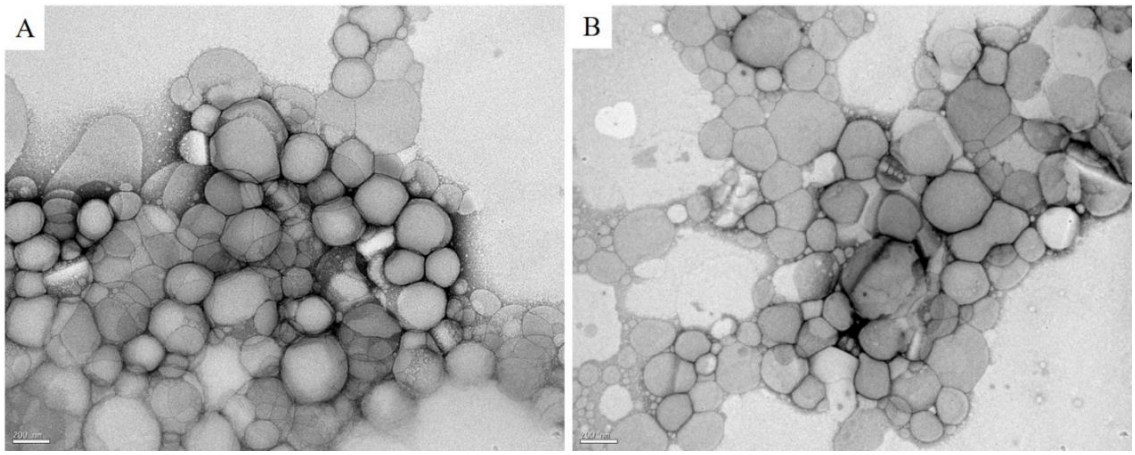

Figure 1 - TEM images of the Nanostructured Lipid Carriers (NLC) (A) and UA-NLC (B). Magnification of 50,000x.

3.2 - Biochemical and histological changes of healthy hamsters treated with UA-NLC

Golden hamsters treated with UA-NLC did not show significant changes in the levels of AST (Figure 2 A) and ALT (Figure 2 B) in comparison with the control group.

ALT levels were not altered in animals treated with UA, however a significant elevation in the levels of AST were observed in animals treated with 5.0 mg/kg of UA ($p<0.05$), as observed in Figure 2 A. In the liver, no histological changes were observed in animals treated with NLC, UA, or UA-NLC (Figure 2 C-G). However, inflammatory nodules were observed in the portal areas of the liver from animals treated with AmB (arrowhead in Figure 2 H).

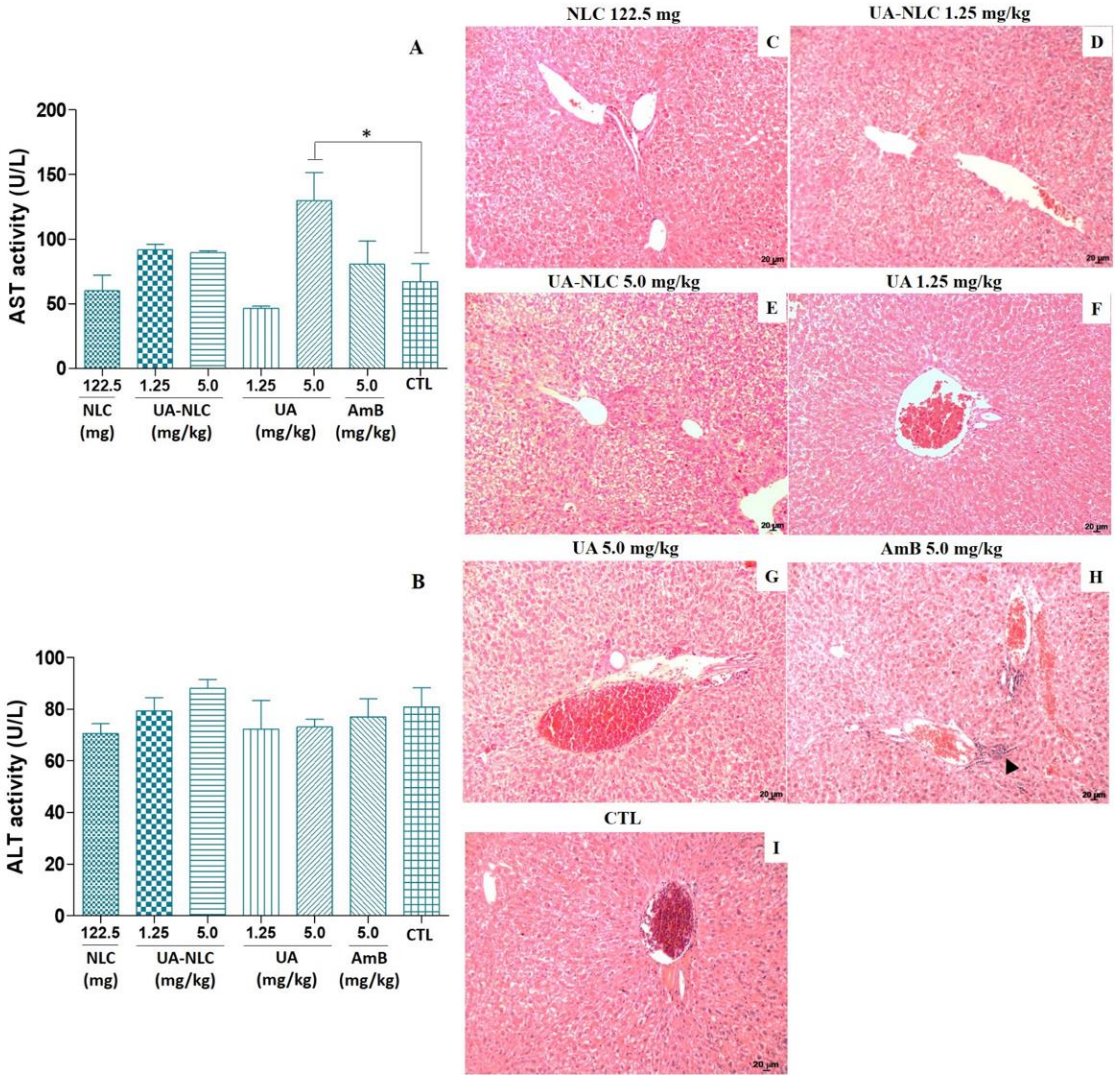

Figure 2 - Biochemical and histological changes were analyzed in golden hamsters treated with UA or UA-NLC. Seric levels of AST (A) and ALT (B); histological sections of the liver from *Mesocricetus auratus* treated with NLC (C) or UA-NLC at 1.25 or 5.0 mg/kg (D,E, respectively) were monitored; UA at 1.25 and 5.0 mg/kg (F,G, respectively); 5.0 mg/kg of AmB (H), and control animals untreated – CTL - (I). Hematoxylin-Eosin. 100× magnification. * $p < 0.05$ indicates statistical significance.

Experimental animals treated with 1.25 and 5.0 mg/kg of UA or UA-NLCs did

not alter the levels of creatinine (Figure 3 A) and urea (Figure 3 B) in comparison with the control. In contrast, a significant increase in the level of creatinine was detected in animals treated with 5.0 mg/kg of AmB ($p<0.05$) compared with the control group (Figure 3 A). Levels of urea were similar in all experimental groups (Figure 3 B). The treatment of animals with UA-NLC or UA did not alter the morphology of the cortical and medullary regions of the kidney (Figures 3 D-G). However, in animals treated with AmB, a vacuolization of epithelial cells of the proximal and distal tubules of the medullary region of the kidney was observed (black arrow in Figure 3 H).

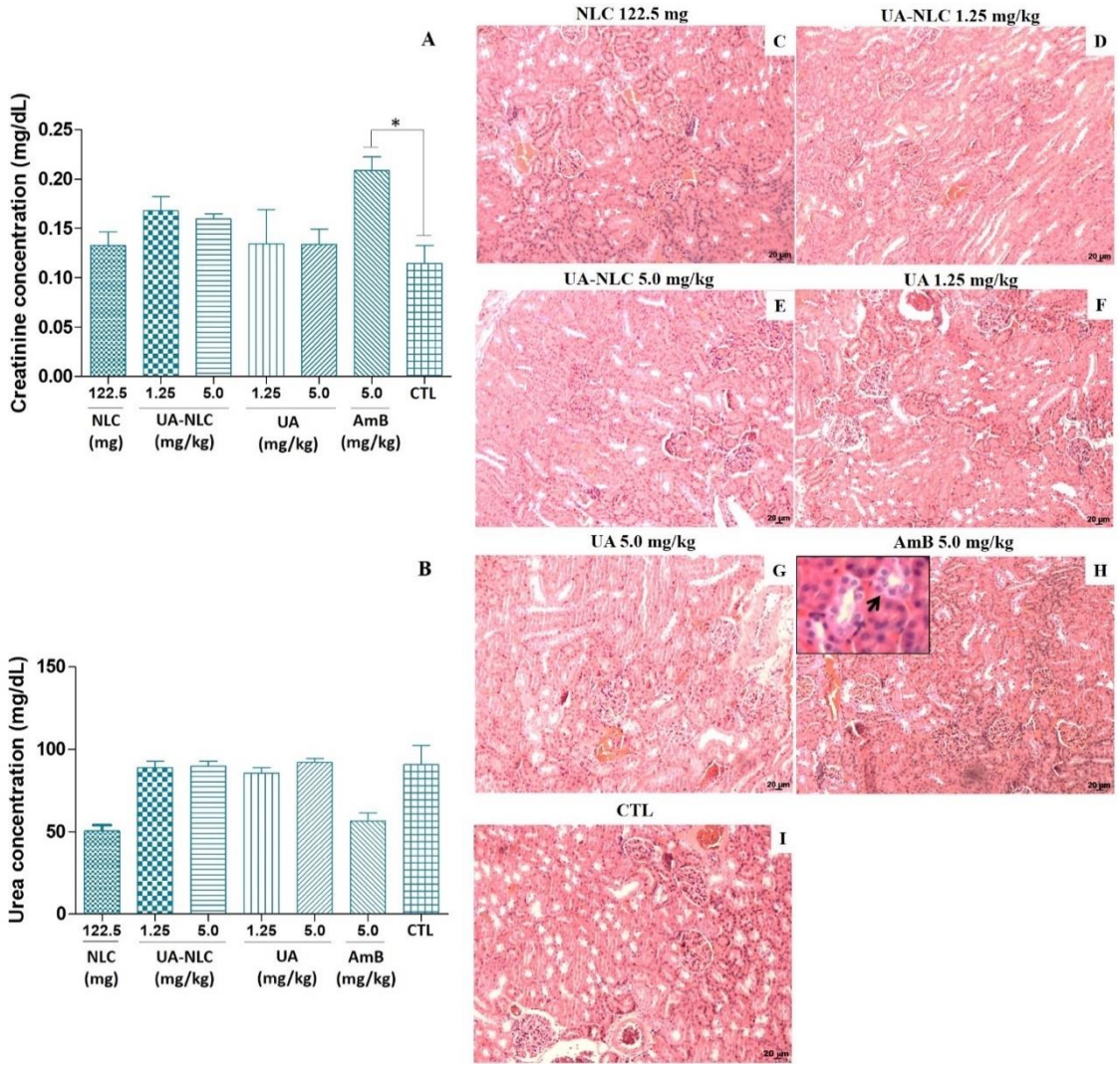

Figure 3 - Biochemical and histological changes were analyzed in golden hamsters treated with UA or UA-NLC. Levels of serum creatinine (A) and urea (B) were estimated, and histological sections of the kidney from healthy *Mesocricetus auratus* treated with NLC (C) or UA-NLC at 1.25 or 5.0 mg/kg (D,E, respectively); UA at 1.25 and 5.0 mg/kg (F,G, respectively); 5.0 mg/kg of AmB (H), and control animals untreated - CTL - (I). Hematoxylin-Eosin. 100x magnification. *$p<0.05$ indicates statistical significance

Hamsters treated with 1.25 or 5.0 mg/kg of UA-NLC, 1.25 or 5.0 mg/kg of UA; 5.0 mg/kg of AmB or NLC did not present significant histological changes in the spleen, lung, and heart (data not shown).

3.3 Analysis of the therapeutic potential of UA-NLC

In the spleen (Figure 4 A) and liver (Figure 4 B) of animals infected with *L. (L.) infantum* and treated with 1.25 or 5.0 mg/kg of UA-NLC or UA was observed a significant reduction in the number of parasites in comparison with the infected controls ($p<0.05$). Furthermore, it was observed that UA-NLC (1.25 mg/kg) showed superior therapeutic activity than UA (1.25 mg/kg) at eliminating splenic and hepatic amastigotes ($p<0.05$). AmB also reduced splenic and hepatic parasites in comparison with the infected control ($p<0.05$), however, animals treated with 5.0 mg/kg of UA-NLC displayed superior antileishmanial properties than AmB in both organs ($p<0.05$).

Histological sections of the spleen and liver were immunolabelled to observe amastigote forms (Figure 4 C-I and K-Q, respectively). In the spleen (Figure 4 C and 4D) and liver (Figure 4 K and 4L) of infected controls an elevated number of amastigote forms were detected. In comparison with the controls, a low number of splenic and hepatic amastigote forms were observed in animals treated with 1.25 and 5.0 mg/kg UA-NLC, UA (Figure 4 E-H and 4 M-P), or AmB (Figure 4 I and Q).

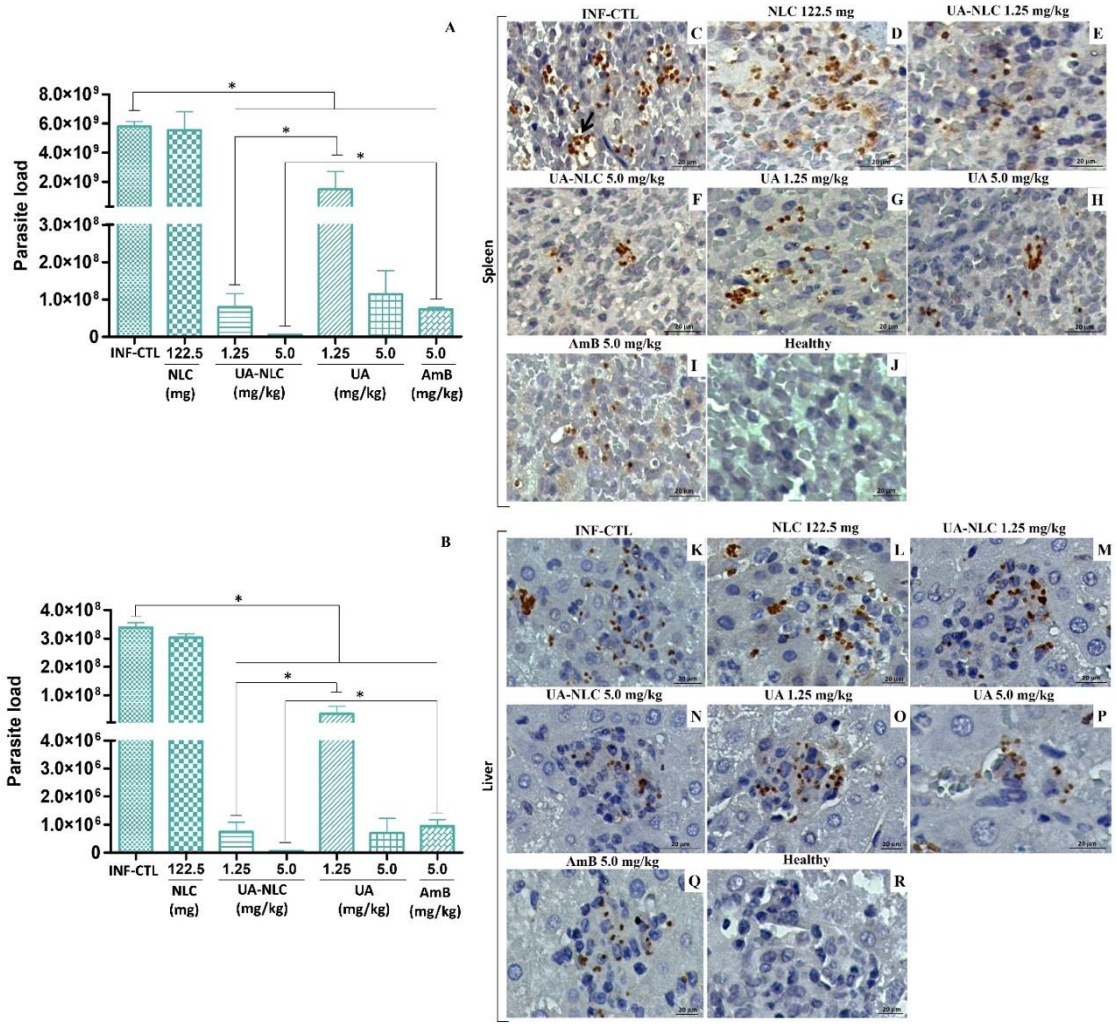

Figure 4 - Golden hamsters infected with *L. (L.) infantum* were treated with UA-NLC or UA and the tissue parasitism was analyzed by limiting dilution assay. Parasitic load on the spleen (A) and liver (B) of animals infected with *L. (L.) infantum* and treated with UA-NLC or UA at concentrations 1.25 and 5.0 mg/kg; AmB was given at 5.0 mg/kg. Photomicrographs of histological sections stained by immunohistochemistry show amastigote forms (stained in dark brown) in the spleen (C–J) and liver (K–R) of the infected control group (C,K), as well as empty NLC (D,L), UA-NLC (E,F,M,N), UA (G,H,O,P) or treated hamsters with AmB (I,Q) (magnification 400; scale bar: 20 μm). * $p < 0.05$ indicates statistical significance.

3.4 Histopathological changes in the spleen and liver of animals treated with UA-NLC or UA

Histological sections from the spleen of infected control groups (infected nontreated or treated with empty NLC), shown in Figures 5 A and B, respectively, displayed replacement of lymphoid follicles by infected macrophages (inset 5 A and B).

Furthermore, polymorphonuclear cells were observed in both control groups, indicating high disease severity. Comparatively, histological sections of the spleen of animals treated with 1.25 and 5.0 mg/kg of UA-NLC showed a low number of parasites and polymorphonuclear cells compared to the infected controls (inset 5 C and D), and preservation of the white pulp, suggesting a better host response after treatment (Figure 5 C and D, respectively). Animals treated with 1.25 and 5.0 mg/kg of UA (Figure 5 E and F, respectively) or AmB (Figure 5 G), also showed preservation of the white pulp, expansion of the red pulp marked by the presence of few parasitized macrophages, lymphocytes and moderate presence of neutrophils (details in the inset of the respective figures). The histological sections of the spleen of healthy animals, on the other hand, had a normal histological aspect, with well-preserved white and red pulp, as shown in Figure 5 H.

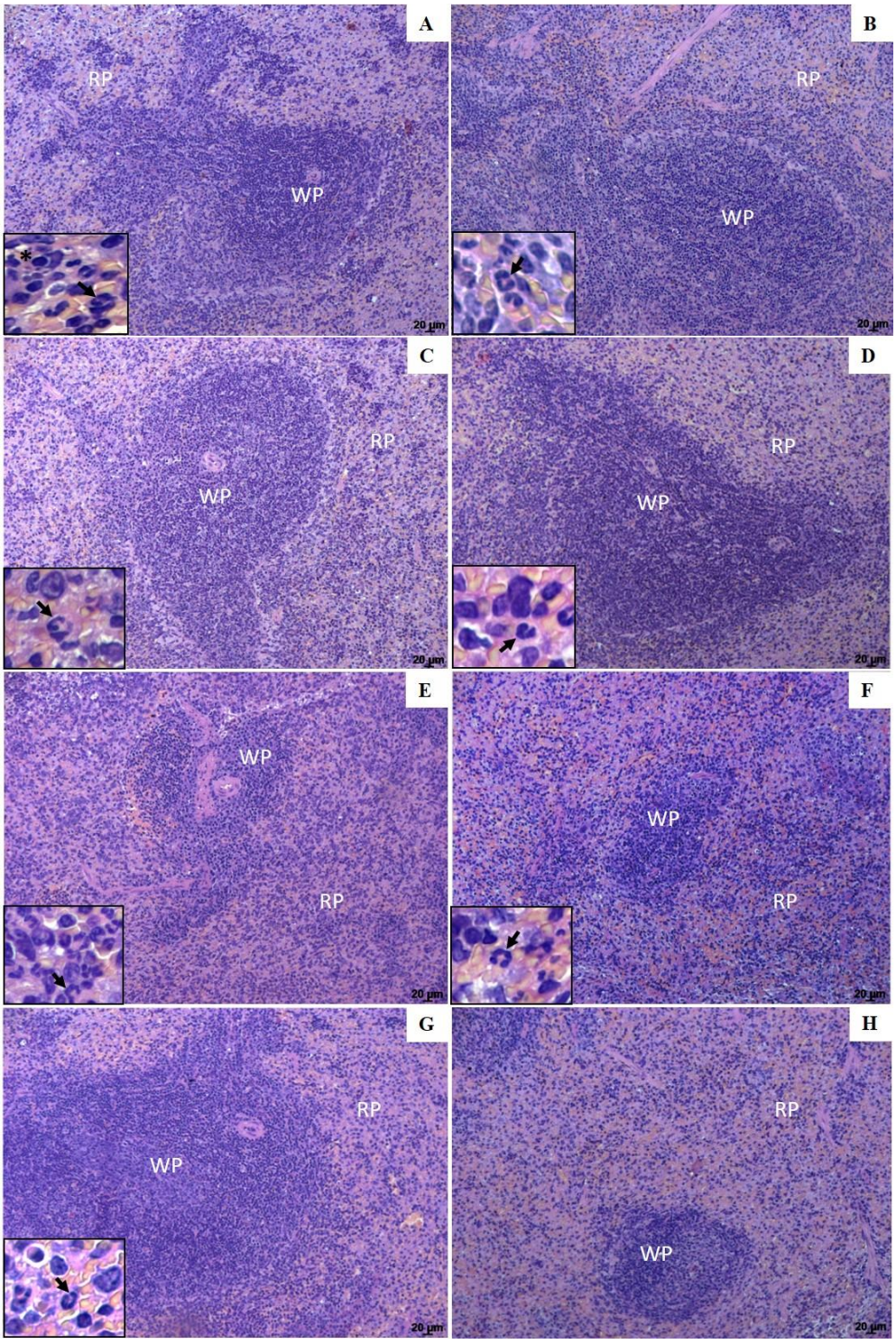

Figure 5 - Photomicrographs of histological sections of the white pulp (WP) and red pulp (RP) areas of the spleen from golden hamsters. Infected control (A); Infected and treated with empty NLC (B); Infected and treated with 1.25 and 5.0 mg/kg UA loaded in NLC (C,D, respectively), Infected and treated with 1.25 and 5.0 mg/kg of UA (E,F, respectively) or AmB (G). Spleen histological section from healthy animals is shown in image H. Insets show in detail amastigotes forms (*) and polymorphonuclear cells (arrows) of the spleen histological sections. Magnification of 100×; scale bars: 20 µm (A–H).

In the animals from the infected control group, NLC, and treated with 1.25 and 5.0 mg/kg of UA-NLC or UA, the main histopathological finding in the liver was related to periportal inflammation with the presence of parasitized macrophages, and granulomas in the portal space and parenchyma (Figure 6 A-H). Additionally, all infected animals showed hyperplasia and hypertrophy of Kupffer cells which were sometimes parasitized; however, periportal inflammation, parasitism, and granuloma formation in the parenchyma was less frequent in animals treated with UA-NLC (Figure 6 C and D), followed by UA (Figure 6 E and F) and AmB (Figure 6 G) than infected controls (Figures 6A and B). Healthy animals displayed normal hepatic morphology (Figure 6 H).

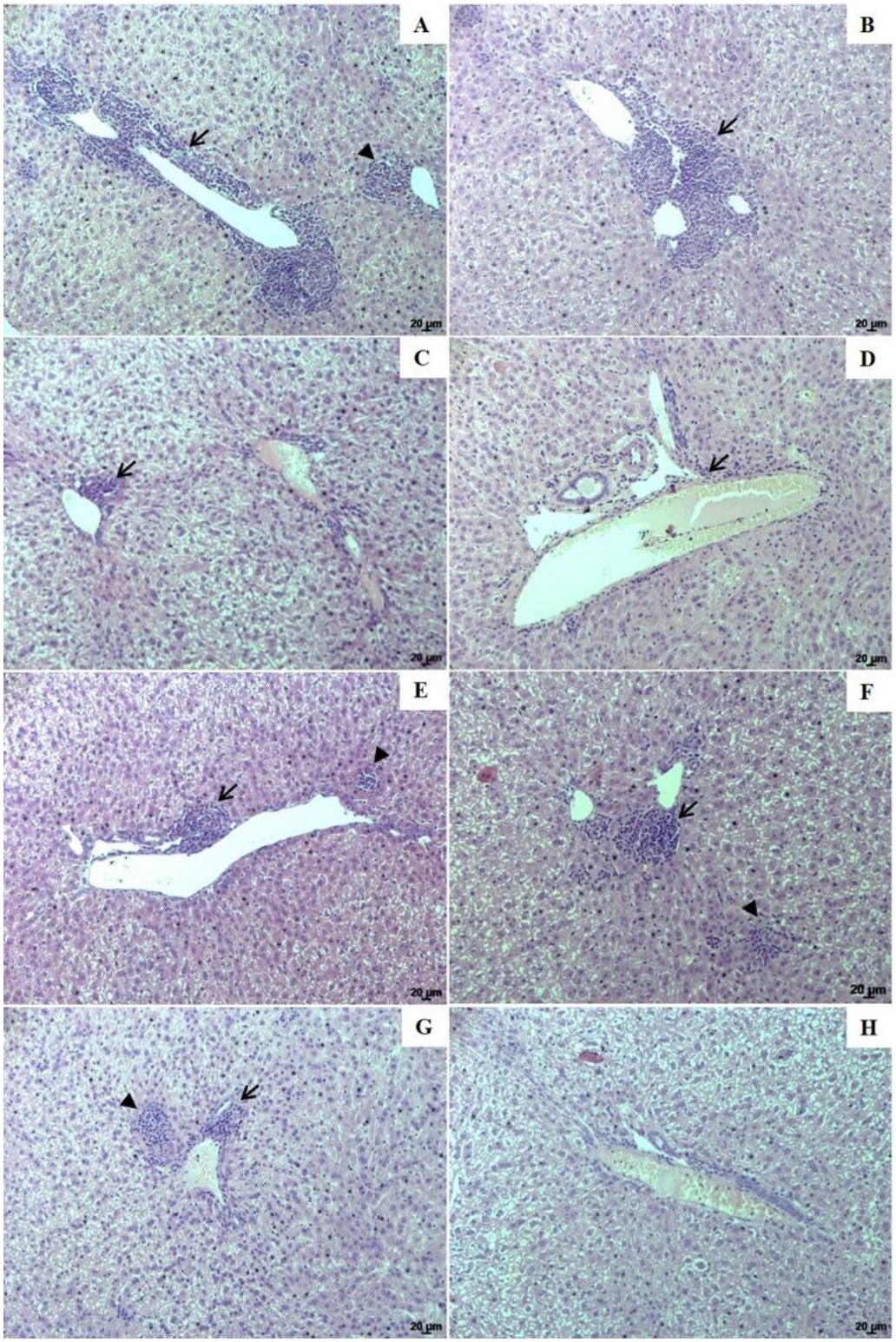

Figure 6 - Histological changes of the liver from golden hamsters infected with *L. (L.) infantum*. Liver histological sections from A – infected control; B – infected and treated with empty NLC; animals treated with 1.25 and 5.0 mg/kg UA loaded in NLC (C,D, respectively), animals treated with 1.25 and 5.0 mg/kg free UA (E, F, respectively) or AmB (G). Liver histological sections from healthy animals are shown in image H. Inflammation foci (arrows) and granulomas (arrowhead). Magnification of 100x; scale bars: 20 µm (A-H).

3.5 Analysis of cellular and humoral immune responses

Animals treated with UA-NLC (1.25 or 5.0 mg/kg) or UA (5.0 mg/kg) expressed higher levels of IFN-γ (Figure 7 A) than the infected control group ($p<0.05$). Additionally, it was found that the groups treated with UA-NLC (1.25 or 5.0 mg/kg) expressed higher levels of IFN-γ than animals treated with UA at the same doses (Figure 7A). The expression of IL-10 in the spleen was similar among all analyzed groups (Figure 7 B). A significant expression of iNOS gene in the spleen of animals treated with UA-NLC (1.25 or 5.0 mg/kg) or UA (5.0 mg/kg) was verified in comparison to the infected control group (Figure 7C). A significant increase in iNOS expression was observed in the group treated with UA-NLC (1.25 mg/kg) compared to UA at the same dose (Figure 7C).

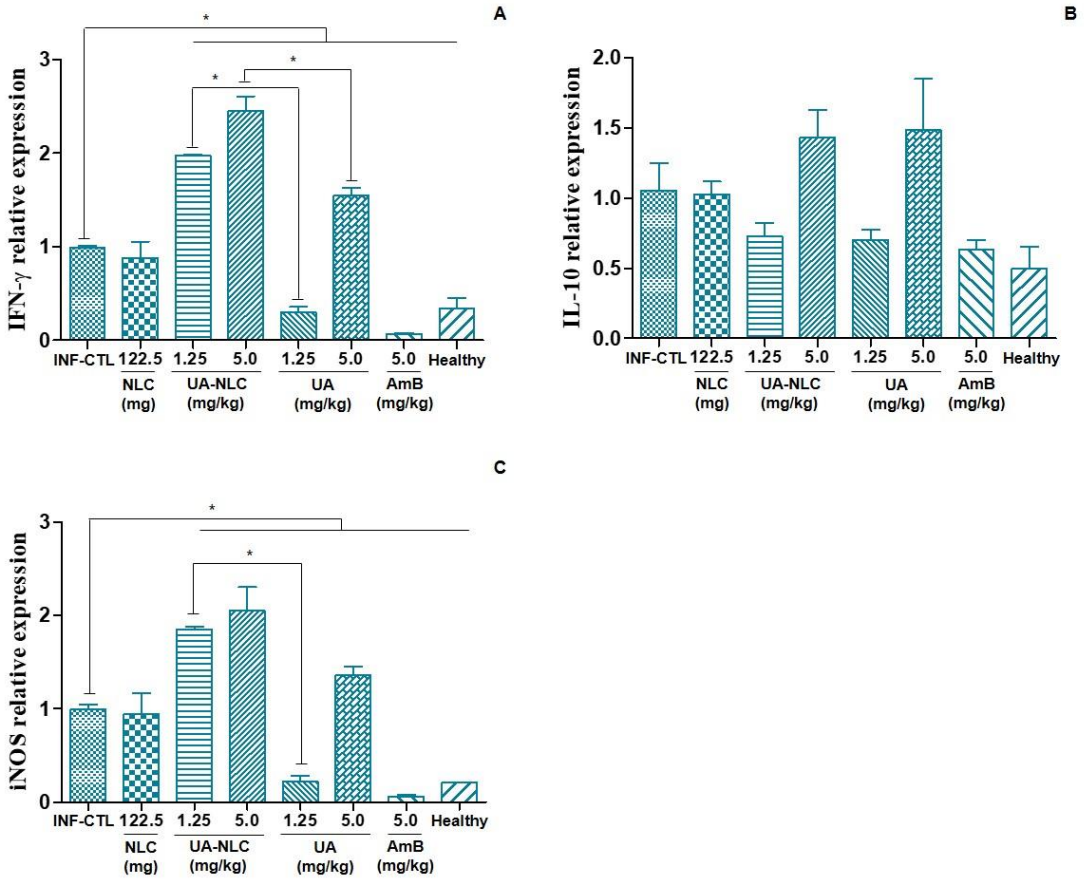

Figure 7 - Relative mRNA expression of IFN-γ (A), IL-10 (B) and iNOS (C) in the spleen of control and treated hamsters infected with *L. (L.) infantum*. * $p<0.05$ indicates statistical significance.

The analysis of the humoral immune response showed that only hamsters treated with 5.0 mg/kg UA-NLC produced a significant amount of antileishmanial IgG (Figure 8A) and IgG2 (Figure 8 B) in comparison to the infected control group ($p<0.05$).

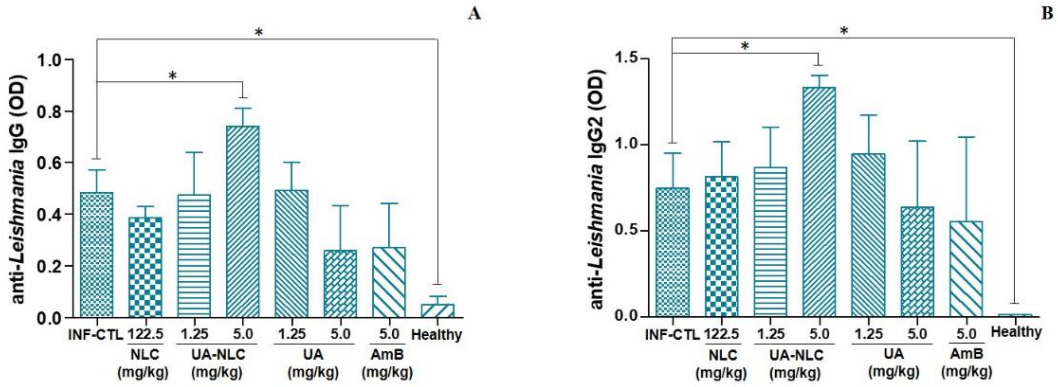

Figure 8 - Levels of antileishmanial IgG (A) and IgG2 (B) in the serum of hamsters infected with *L. (L.) infantum* and subjected to the treatment with 1.25 or 5.0 mg/kg UA loaded in NLC or UA; additionally, animals were treated with 5.0 mg/kg of AmB, as a standard treatment. * $p<0.05$ indicates statistical significance.

3.6 Biochemical analysis of hamsters infected and treated with UA-NLC

Animals treated with UA-NLC or UA did not change the levels of AST and ALT in comparison with the infected control (Figure 9 A and B); although animals treated with 5.0 mg/kg of UA-NLC displayed a significant reduction in the levels of AST in comparison with the infected control group ($p<0.05$), and AST level was similar with the healthy animals (Figure 9A). Animals treated with UA-NLC or UA exhibited a significant reduction in the levels of creatinine in comparison to the infected control ($p<0.05$) and these values were close to the normal levels, observed in the healthy group (Figure 9 C). Urea levels were similar in all treated animals (Figure 9 D), except in the healthy group that showed low levels of urea in comparison with the infected control ($p<0.05$).

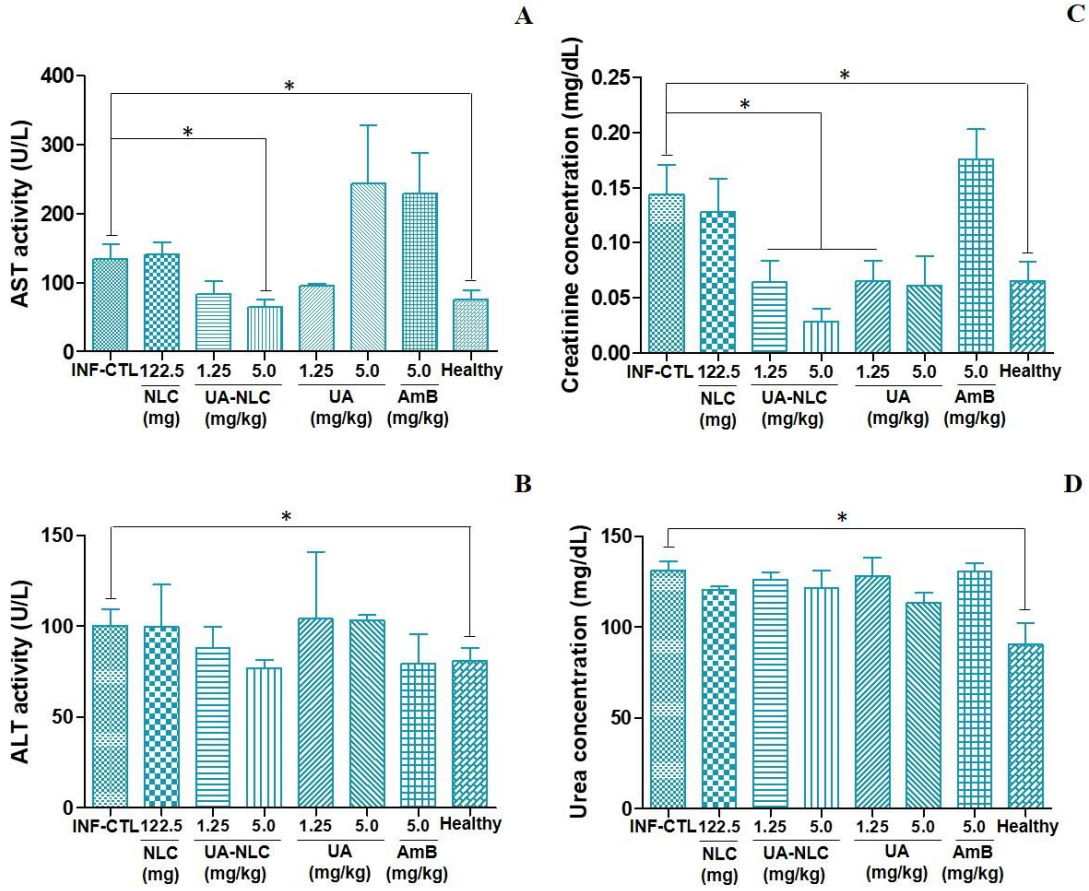

Figure 9 - Serum values of AST (A), ALT (B), creatinine (C) and urea (D) in infected controls, treated with NLC, UA, UA-NLC, or AmB for 10 consecutive days. * $p < 0.05$ indicates statistical significance.

4 Discussion

Nanotechnology has been considered an important tool to reduce side effects and increase the effectiveness and selectivity of drugs [26]. In this sense, NLCs have been elected as an interesting drug platform to treat different intracellular infections [27], because they can access the cytoplasm of cells and deliver the content directly into the target. This may account for the high efficacy of treatment performed with nanocarriers than drugs freely administered.

In the present study, high-pressure homogenization was used to formulate UA-NLC that exhibited a monodisperse distribution with suitable mean size (266 nm) and PDI of 0.18, which indicates a homogenous and narrow size distribution of the nanoformulations. Particles with a size between 100 and 500 nm may be used in human

and/or animal therapy, since they remain in circulation longer [28,29], enabling the distribution of nanoparticles through the tissues, including the liver and spleen, that are the main affected organs in visceral leishmaniasis. Furthermore, the steric stabilization on the surface of the nanoparticles led to negative zeta values, -26 and -29 mV for NLC and UA-NLC, respectively, suggesting that the samples remained dispersed and stable, with a reduced tendency to form aggregates due to the electrostatic repulsion [30]. These results are consistent with those obtained by other research groups, which produced NLCs with a similar range of negative zeta values [31,32]. Additionally, transmission electron microscopy showed that the incorporation of UA in NLCs preserved the size and morphology of the nanoparticles. Therefore, the physical properties obtained herein enabled the use of UA-NLC in *in vivo*.

The properties of UA-NLC on the visceral organs and on the metabolism of the liver and kidney of healthy golden hamsters were investigated. In the histopathological study, it was verified that the treatment of hamsters with UA-NLC did not change the morphology of the spleen, liver, kidney, lung and heart. Hepatic and renal functions of animals were also investigated, since these organs are responsible for the metabolization and excretion of drugs [33,34]. UA-NLC did not alter the levels of AST, ALT, urea or creatinine in golden hamsters, however, an increase in the levels of AST was observed in animals treated with 5.0 mg/kg of UA. AST is considered an important marker of structural damage of hepatocytes and is widely used in the assessment of liver damage caused by drugs or hepatotoxins [35]. This data suggests that UA given by the intraperitoneal route at 5.0 mg/kg damaged hepatocytes, however no structural changes were observed, pointing to an initial damage induced by UA. On the other hand, animals treated with NLC carrying 5.0 mg/kg of UA did not alter the levels of AST suggesting that UA was delivered to phagocytic cells, avoiding hepatocyte damage [33]. Previous studies showed that hamsters treated for sixteen days with 1.0 and 2.0 mg/kg of UA by intraperitoneal route did not present biochemical or histopathological changes in the liver; suggesting that UA is safe at low doses [10]. However, UA exhibited mild toxicity at higher doses, that may be abolished after encapsulation.

In experimental models of liver damage, it was observed that UA presented hepatoprotective potential when administered by the oral route [36–38]. In the present work, UA given by intraperitoneal route induced moderate liver toxicity, suggesting that the route of administration and dose may be associated with hepatotoxicity. In a clinical

trial, healthy volunteers and patients with solid tumors were treated with a single dose of UA entrapped in liposomes by intravenous route; although low and mild doses were safe to volunteers, high doses of the formulation induce several side effects, including the elevation of hepatic enzymes [39]. On the other hand, it has been demonstrated that UA has been safely used as a daily dietary supplement, without toxic events [40–42]. It is worth remembering that UA is naturally synthesized by different fruits and vegetables present in the human diet [43], therefore, UA toxicity may be related to the dosage, formulation, duration of treatment and route of administration.

In contrast to UA-NLC, it was observed that AmB caused morphological changes in the medullary area of the kidney along with an increase of creatinine levels in the blood, suggesting that the treated animals developed acute renal failure [44]. A common event in therapy with AmB is nephrotoxicity, that affects 49 to 65% of patients under treatment [45], that may show a reduced glomerular filtration rate, leading to renal dysfunction and failure [46,47]. Similarly, in the hamster model of visceral leishmaniasis, AmB also induced significant morphological and biochemical changes in the kidney [10], suggesting that in animal models, AmB also causes kidney failure. Taken together, these findings demonstrate that UA-NLC were not toxic for golden hamsters, while AmB, although recognized as an important leishmanicidal drug, induced severe side effects in golden hamsters.

In infected golden hamsters, it was verified that animals treated with UA-NLC or UA exhibited a significant reduction in splenic and hepatic parasitism, suggesting that both UA and UA-NLC presented high therapeutic potential. The number of parasites reduced as the UA dose increased, and this effect was maximized when UA was encapsulated. It was observed that treatment with UA at 1.25 mg/kg (cumulative total amount of 1.75mg) reduced parasitism by 74.20% in the spleen and 90.04% in the liver, while the same dose of UA loaded into NLC decreased splenic and hepatic parasitism by 98.63 and 99.78%, respectively. Animals treated with 5.0 mg/kg of UA (cumulative total amount of 7.0 mg) had a reduction in parasitism by 98.03% in the spleen and 99.79% in the liver; while a reduction of 99.92 and 99.98% in splenic and hepatic parasite loads, respectively, was observed in animals treated with 5.0 mg/kg of UA-NLC. These data suggest that NLC improved the efficacy of UA when compared to UA administered freely, reinforcing that nanoparticles can be good carriers of drugs, as it has been observed in different studies [48–51]. Das and collaborators [52] demonstrated that UA-loaded

NLC coated with chitosan oligosaccharides had high anti-leishmania activity *in vitro* and when administered orally to BALB/c infected with *L. (L.) donovani* this formulation was 94 times more active than miltefosine, 5 times more than sodium stibogluconate and 2 times more than paromomycin. According to a study conducted by Kar and collaborators [53], NLC potentiated the leishmanicidal effect of the sesquiterpene cedrol on amastigotes forms of *L. (L.) donovani* resistant or not to stibogluconate sodium and paromomycin. Additionally, it was observed that oral treatment with cedrol entrapped into NLC displayed higher efficacy in murine visceral leishmaniasis caused by wild or drug-resistant *L.* (*L.) donovani* than cedrol or miltefosine. Thus, the data shown herein indicate that NLC is an interesting platform to deliver drugs, and the formulation UA-NLC was more active than UA in visceral leishmaniasis.

In addition to the antileishmanial activity, UA-NLC also modulated the immune response of golden hamsters with visceral leishmaniasis, increasing the expression of IFN-γ transcripts more efficiently than the groups treated with UA. Effective immunity in leishmaniasis is mediated by IFN-γ, which activates macrophages to a leishmanicidal state [54]. If correctly activated by IFN-γ, macrophages will produce high amounts of the iNOS (inducible nitric oxide synthase) enzyme; that is able to convert nitric oxide (NO) from the precursor L-arginine [55], which has a potent microbicidal potential. In fact, in the present study, it was observed that the increase in IFN-γ mRNA transcript was directly associated with the increase in iNOS expression in animals treated with UA-NLC, suggesting that the expression of both genes along with the leishmanicidal activity of UA cooperated with the marked leishmanicidal activity of this molecule. Of note, in the present work the cytokine quantifications were performed with qPCR, that is an indirect estimate of the bioactive cytokine. Although the amount of RNA can have a direct association with the concentration of the protein, some post-transcriptional mechanisms can regulate the translation of the protein; thus, the real concentration of the bioactive cytokine may be lower than presented in Figure 7.

In addition to the elevation of the cell immune response, it was observed that infected animals treated with 5.0 mg/kg of UA-NLC increased the levels of anti-*Leishmania* IgG, that subsequently were classified as IgG2 isotype. In experimental murine leishmaniasis, antibodies can be used as markers of resistance, as is the case of IgG2 isotype, that is produced upon the increase of IFN-γ, therefore it has been associated with resistance and Th1 immune response development [56,57]. In the hamster model,

few studies performed this association; however, works of vaccination showed that immunized hamsters develop a Th1 immune response and increase the levels of IgG2 upon challenge with henipavirus [58] or *L. (L.) donovani* [59], suggesting that IgG2 antibody can be a good marker of Th1 immune response as well as resistance in hamsters infected with *L. (L.) infantum*.. In contrast, it was observed that animals treated with AmB displayed a significant decrease of IFN-γ and iNOS gene expression, which may be associated with an inhibition of the inflammatory response, caused by the low number of parasites [60]. The treatment with UA-NLC also decreased the number of tissue parasites in the spleen and liver of animals, and contrary, it stimulated the immune response. Although some works showed that an active infection would maintain an active immune response [60,61], it is still important to observe that UA as well as related pentacyclic triterpene are able to modulate innate and acquired immune responses [62], as observed in treated animals.

In the histopathological study it was observed that the spleen of the infected control showed expansion of the red pulp, associated with the presence of an elevated number of polymorphonuclear cells; additionally a higher frequency of macrophage nodules containing intracellular parasites was observed, indicating high disease severity [10]. In contrast, animals treated with UA-NLC showed preservation of white and red pulp, with a low frequency of macrophage nodules, suggesting that this treatment was efficient at decreasing parasitism and controlling the inflammatory response in comparison to the histological sections of animals treated with UA. In the liver of the infected control group, an inflammatory infiltrate was observed in the portal space, however, the intensity of the decreased according to the type of treatment employed, being less intense in the animals treated with UA-NLC when compared with UA. In animals treated with UA-NLC or AmB, a focal inflammatory process was observed, and it was characterized by a discrete infiltration of mononuclear cells in the portal space with low parasitism. The increased leishmanicidal activity and reduced inflammatory process observed in the spleen and liver of animals treated with UA-NLCs in comparison with animals treated with UA can be associated with the uptake of nanoparticles by macrophages, destruction of amastigote forms and inhibition of inflammation as the number of parasites drastically decreased in the spleen and liver [63]. These data suggest that treatment performed with UA-NLCs preserved the histology of the liver and spleen, since the animals displayed small areas of inflammation in these organs and developed a

potent immune response compared to animals treated with UA and nontreated infected groups.

Furthermore, the biochemical parameters related to the assessment of liver and kidney functions reinforce that UA-NLC is safe and can be used in the treatment of visceral leishmaniasis. In fact, it was observed that UA-NLC accentuated the reduction of AST and creatinine in animals with visceral leishmaniasis at levels close to normality. On the other hand, the treatment of infected hamsters with AmB did not normalize the levels of hepatic and renal enzymes, suggesting that treatment, parasite persistence and inflammation are factors associated with liver and kidney damage [64,65]. Although AmB was active in experimental visceral leishmaniasis, indeed the toxicity limits its use. As an alternative, the liposomal version of AmB (AmBisome) should be employed in the present study, allowing for a more robust comparison between the efficacy of UA-NLC and the highly active and safer version of AmB [66,67]. Although, it is still important to note that the costs related to the acquisition of AmBisome are too high, even in the context of preclinical studies. Furthermore, in low-income countries, such as Brazil [68], the treatment of visceral leishmaniasis is performed with conventional AmB, thus the liposomal version is extremely expensive to be acquired and offered in public medical services.

Taken together, the results obtained herein demonstrate that UA-NLC was stable and showed a homogeneous morphology and size. Additionally, UA-NLC proved to be safe for use in golden hamsters and it showed therapeutic activity in experimental animals with visceral leishmaniasis caused by *L. (L.) infantum*. Importantly, the nanoformulation exhibited superior therapeutic activity than UA given in free form and AmB, which, although it has a significant therapeutic activity, is nephrotoxic, limiting its use in infected animals as well as humans. Thus, the UA loaded into NLCs can be considered an important and promising approach in the treatment of leishmaniasis.

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and L.F.D.P.; visualization, J.A.J.; M.D.L.; L.A.; P.C.C.; and L.F.D.P.; supervision, L.F.D.P; P.C.C. and D.C.F.; project administration, L.F.DP.; funding acquisition, L.F.D.P; D.C.F.; M.D.L.; L.A. All authors have read and agreed to the published version of the manuscript.

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DISCUSSÃO GERAL

8 DISCUSSÃO GERAL

O primeiro artigo publicado demonstra a eficácia terapêutica do AU combinado com os fármacos convencionais anfotericina B ou glucantime na leishmaniose visceral e cutânea experimental. Na leishmaniose visceral, os animais tratados com a combinação terapêutica do AU com AmB tiveram redução da carga parasitária esplênica e hepática enquanto na leishmaniose cutânea, foi observado uma redução no tamanho lesão no tratamento AU associado ao glucantime superior a monoterapia. Vale a pena ressaltar que inicialmente tivemos um atraso com a importação dos triterpenos que fariam parte do estudo, então, durante o período que estivemos à espera das demais moléculas, decidimos trabalhar com os reagentes disponíveis naquele momento, e felizmente obtivemos dados importantes que sugerem que a associação de fármacos para o tratamento da leishmaniose pode aumentar a eficácia do tratamento e reduzir a toxicidade associada aos medicamentos convencionais.

O segundo artigo publicado traz importantes resultados sobre o potencial leishmanicida do AU, Be e Lu, assim como os alvos celulares destas moléculas em *L. (L.) infantum*. Além disso, o potencial terapêutico do AU e Lu foi avaliado na leishmaniose visceral experimental. O estudo demonstra que os esses triterpenóides eliminaram formas amastigotas de *L. (L.) infantum* com alta seletividade, sendo superior àquela da miltefosina. Por outro lado, o triterpeno Be não foi ativo. Além disso, foi possível verificar o provável mecanismo de ação do AU e Lu sobre formas promastigotas de *L. (L.) infantum.* A principal organela afetada foi a mitocôndria, que se mostrou fragmentada e seu potencial de membrana mostrou-se alterado após 30 minutos de incubação com os triterpenos, sugerindo que estes compostos induzem morte celular programada.

Os resultados mostrados no artigo II também revelaram dados importantes quanto a toxicidade do AU e Lu em hamsters saudáveis e o potencial *in vivo* desses triterpenos em hamsters infectados. Os triterpenos AU e Lu apresentaram redução expressiva da carga esplênica e hepática, a qual foi correlacionada com o aumento da resposta imune celular Th1, conferida pelo aumento dos níveis de IFN-γ. Os resultados indicaram que esses triterpenos são interessantes alvos para o desenvolvimento de novas classes de fármacos contra a leishmaniose visceral.

O terceiro artigo publicado demonstra o potencial do triterpeno AM isolado das folhas de *Hyptidendron canum* (Lamiaceae) e seu mecanismo de ação *in vitro*. Neste caso,

observou-se que o AM eliminou de forma seletiva formas promastigotas e amastigotas de *L. (L.) infantum* sem apresentar qualquer toxicidade a macrófagos peritoneais. Contrariamente, os fármacos utilizados na terapia da leishmaniose, anfotericina B e miltefosina, foram citotóxicos. Adicionalmente, os resultados obtidos indicam que esse triterpenóide também elimina os parasitos através de morte celular programada.

Do ponto de vista crítico, o ideal seria publicar os dados do AM no artigo II, pois são dados complementares e obviamente, enriqueceriam mais ainda o artigo II. Entretanto, todos os triterpenos foram adquiridos comercialmente, e no momento da aquisição o valor do AM aumentou muito. Atualmente, na empresa Sigma-Aldrich, um frasco contendo 5 mg de AM custa R$ 1584,00. Para a realização de estudos *in vivo* em hamster, seria necessária uma massa de 200 mg para o tratamento contínuo por 10 dias, utilizando uma dose diária por animal na concentração de 2,5 mg/kg. Ou seja, precisaríamos utilizar a verba de R$ 63.360,00 para produzir apenas um experimento. Devido às condições econômicas não foi possível adquirir este triterpeno para realização de experimentos envolvendo tratamento experimental. Em colaboração com o Prof. João Lago, foi possível obter o AM a partir de uma planta do bioma Cerrado, entretanto, a concentração ainda foi baixa, o que nos limitou aos estudos *in vitro*. De toda forma, o conhecimento gerado foi publicado, e se tornou mais um trabalho reforçando o potencial de plantas medicinais assim como deste triterpeno na terapia das doenças negligenciadas, sobretudo no tratamento da leishmaniose.

O artigo IV foi publicado após a caracterização da ação *in vitro* e *in vivo* dos triterpenos em *L. (L.) infantum*. Este estudo foi publicado em colaboração com pesquisadores da Universidade do Porto, Portugal. Para a realização desta etapa, fomos contemplados com a bolsa BEPE (2018/04080-2) da FAPESP, para realização do estágio de pesquisa com o projeto intitulado "Preparação e caracterização físico química de Nanopartículas Lipídicas como carreadores de triterpenos para o tratamento da Leishmaniose Visceral", junto ao Laboratório de Tecnologia Farmacêutica da Faculdade de Farmácia da Universidade de Porto (FFUP) em Portugal, sob a supervisão do Prof. Dr. Domingos de Carvalho Ferreira e Prof. Dr. Paulo Cardoso da Costa. A colaboração com o grupo do Prof. Dr. Domingos proporcionou a oportunidade de estabelecer uma parte importante deste projeto de doutorado, envolvendo o desenvolvimento de nanosistemas de biodistribuição (SLN e NLC) para o tratamento experimental da leishmaniose visceral.

Esta pesquisa colaborativa foi fundamental para melhorar a eficiência terapêutica dos agentes leishmanicidas estudados no artigo II e III.

Nesse período na Universidade do Porto, os dois tipos de nanosistemas foram desenvolvidos e caracterizados (SLN e NLC). Todos se mostraram homogêneos e estáveis. Embora não tenha sido mostrado nos artigos, verificamos que as SLN e NLC carreando os triterpenos foram estáveis durante 180 dias, a 4°C, e durante este período não foram detectadas variações físicas e químicas significantes, sugerindo que a formulação pode ser armazenada por um longo período em temperatura de geladeira, o que facilitaria a distribuição e armazenamento em países tropicais. Embora dois tipos de nanopartículas tenham sido produzidas, os estudos *in vivo* foram efetuados somente com NLC, pois os estudos *in vitro*, mostraram a alta susceptibilidade de formas amastigotas às NLC carregadas.

Na leishmaniose visceral experimental foi observado que NLC-AU foi altamente ativo na eliminação de amastigotas em baço e fígado de hamsters dourados infectados com *L. (L.) infantum* e estes resultados estiveram associados ao aumento da expressão de IFN-γ, uma importante citocina que ativa macrófagos. Portanto, os dados obtidos com NLC-AU mostram que há uma ação direta da molécula ativa nos parasitos, e também ocorre ativação da resposta imune, o que de fato, induz uma forte resposta terapêutica, que é superior àquela da anfotericina B. Outro importante achado está ligado a ausência de efeitos tóxicos da nanoformulação em hamsters, e reforça a hipótese de que a formulação contendo ácido ursólico pode ter uma importante aplicação clínica nas leishmanioses.

Embora não tenha sido publicado artigo científico, os dados referentes à nanopartícula NLC contendo Lu já foram compilados e analisados. O manuscrito se encontra em fase final de elaboração. De maneira similar ao AU, verificou-se que animais infectados com *L. (L.) infantum* e tratados com o Lu veiculado em NLC tiveram expressiva redução do parasitismo tecidual, e não apresentaram efeitos colaterais ao tratamento.

A obtenção de todos estes dados, estejam publicados ou não, e que compõem esta tese, contribui sem dúvida alguma para a expansão dos meus conhecimentos acerca da terapia das doenças infecciosas e fortalece a continuidade do meu trabalho na busca árdua de novas opções terapêuticas, que possam ser mais efetivas, menos tóxicas e mais acessíveis ao governo e população afetada pela doença. Além disso, os resultados indicam

que os estudos podem avançar para a próxima etapa, e ser avaliados do ponto de vista clínico para o tratamento da leishmaniose visceral.

CONCLUSÃO

9 CONCLUSÃO

1) Os triterpenos AM, AU e Lu foram ativos contra formas promastigotas e amastigotas de *L. (L.) infantum*, apresentando citotoxicicidade baixa a moderada, e alta seletividade em relação aos parasitos;

2) Formas promastigotas de *L. (L.) infantum* incubadas com AM, AU e Lu apresentaram alterações morfológicas e fisiológicas na mitocôndria, sugerindo que estes compostos induzem morte celular programada;

3) Os nanosistemas do tipo NLC produzidos por HP quente foram os mais promissores para a terapia *in vivo*, pois apresentaram alta eficiência de encapsulação de triterpenos, tiveram uma distribuição de tamanho uniforme, são sistemas estáveis, com reduzido índice de polidispersão (IP<0,25);

4) A análise por FTIR mostrou que não ocorreu deslocamento das bandas de absorção dos terpenos, corroborando com as análises de DSC, revelando que os triterpenos se encontram dissolvidos (molecularmente dispersos) na matriz lipídica das nanopartículas. Além disso, análises de DSC forneceram evidências da redução da cristalinidade nos NLC a partir de das matrizes lipídicas sólidas (CP) em comparação com SLN;

5) Hamsters saudáveis tratados com AU e Lu veiculados em NLC não apresentaram alterações morfológicas significativas em baço, fígado, rim, pulmão e coração;

6) Os potenciais terapêuticos do AU e Lu na leishmaniose visceral experimental foram maximizados após encapsulação em NLC; além disso, as nanoformulações apresentaram eficácia superior a AmB;

7) O tratamento com os triterpenos veiculados em NLC desencadeou respostas imune do tipo Th1, a qual se associou a uma redução significativa da carga parasitária esplênica e hepática.

8) Tomados em conjunto, esses dados demonstram que os triterpenos AU e Lu veiculados em NLC podem ser considerados uma abordagem importante e promissora no tratamento da leishmaniose.

ANEXOS

10 ANEXOS

ANEXO A – Aprovação da Comite de Ética no Uso de Animais (CEUA/FMUSP)

Faculdade de Medicina da Universidade de São Paulo
Avenida Dr. Arnaldo, 455
Pacaembu – São Paulo – SP

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Certificamos que o projeto intitulado **"Nanopartículas Lípidicas como carreadores de triterpenos para o tratamento da Leishmaniose Visceral Experimental",** protocolo nº **056/16** sob a responsabilidade de **Luiz Felipe Domingues Passero** e **Jéssica Adriana de Jesus**, apresentado pelo Departamento de Patologia - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSAO DE ÉTICA NO USO DE ANIMAIS (CEUA) da Faculdade de Medicina da USP em reunião de 22.06.16.

Finalidade	( ) Ensino (x) Pesquisa Científica
Vigência da autorização	Junho/2019
Espécie/linhagem/raça	Hamster
Nº de animais	**156**
Peso/Idade	8 semanas
Sexo	Macho
Origem	Anilab

CEUA-FMUSP, 22 de Junho de 2016

Dr. Eduardo Pompeu
Coordenador
Comissão de Ética no Uso de Animais

ANEXO B – Aprovação da Comite de Ética no Uso de Animais (CEUA/IMT)

UNIVERSIDADE DE SÃO PAULO
INSTITUTO DE MEDICINA TROPICAL DE SÃO PAULO
Av. Dr. Enéas de Carvalho Aguiar, 470
CEP 05403-000 - São Paulo - Brasil - e-mail: cpq-imt@usp.br
Telefones: (55) 11-3061-8650, FAX (55) 11-3064-5132

São Paulo, 02 de Março de 2018

Ilmo(a)
Dr(a). Luiz Felipe Domingues Passero
(aos cuidados de Jéssica Adriana de Jesus)

Certificamos que a proposta intitulada **" Nanopartículas Lipídicas como carreadores de triterpenos para o tratamento da Leishmaniose Visceral Experimental."**, registrada com o nº **000344A**, sob a responsabilidade de **Luiz Felipe Domingues Passero** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **APROVADA** pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA-IMT) do Instituto de Medicina Tropical de São Paulo, em reunião na presente data.

Cabe ao pesquisador elaborar e apresentar à CEUA-IMT, o relatório final sobre a pesquisa.

Vigência da Autorização: 01/07/2016 a 01/07/2019							
Finalidade	Pesquisa						
Espécie	**Linhagem**	**Idade**	**Peso**	**Material**	**Quantidade**		
					M	**F**	**M+F**
Hamster	ROE-golden	8 semanas	90-120g	*a ser Coletado*	156	0	156
Origem: Biotério Externo - Empresa comercial ANILAB					**TOTAL**		156

Atenciosamente,

p/ Thilma Aug Oka

Prof. Dr. Jorge Simão do Rosário Casseb
Presidente da Comissão de Pesquisa e Ética do IMT-USP

Dra. Luciana Regina Meireles Jaguaribe Ekman
Coordenadora da Comissão de Ética no Uso de Animais em Pesquisa do IMT-USP

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11 REFERÊNCIAS

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