UNIVERSIDADE FEDERAL DE UBERLÂNDIA
Instituto de Ciências Biomédicas
Programa de Pós-graduação em Imunologia e Parasitologia Aplicadas

NOVAS OPÇÕES TERAPÊUTICAS PARA O TRATAMENTO DAS LEISHMANIOSES

Iasmin Aparecida Cunha Araújo

Uberlândia
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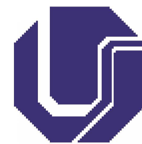

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DEDICATÓRIA

Dedico este trabalho ao meu grande mestre, orientador e amigo Sydnei Magno da Silva (*in memorian*). Nada disso seria possível sem que você me guiasse até aqui. Infelizmente a leitura desse texto não poderá ser feita por você, mas sei que esteve comigo.

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RESUMO

As leishmanioses são doenças negligenciadas causadas por protozoários do gênero *Leishmania*, que comprometem vísceras causando a leishmaniose visceral (LV), pele e mucosas causando a leishmaniose cutânea (LC). Na maioria dos casos os protocolos de tratamento atuais apresentam toxicidade e baixa efetividade. Além disso, esses parasitos estão se tornando mais resistentes aos métodos convencionais de tratamento. A presente tese é composta por três capítulos referentes ao estudo de novas opções terapêuticas no tratamento das leishmanioses. No primeiro capítulo avaliamos a eficácia do lapachol em modelos *in vitro* e *in vivo* de leishmaniose tegumentar e leishmaniose visceral. Nossos resultados mostraram que o lapachol possui uma baixa citotoxicidade em células HepG2, boa atividade anti-*Leishmania* e um favorável índice de seletividade (IS) contra promastigotas de *L. amazonensis* e *L. infantum*. A eficácia também foi avaliada em amastigotas intracelulares. Análises de citometria de fluxo mostraram que lapachol induz morte por apoptose em promastigotas de *Leishmania*. *In vivo*, a eficácia do lapachol foi confirmada em modelo murino de leishmaniose visceral e tegumentar, reduzindo a carga parasitária no fígado, baço e lesões de pele. No segundo capítulo avaliamos a eficácia in vitro de isoflavonoides (ácido xilópico, ácido caurenóico, (-)-α-bisabolol) e terpenos ((-)-duartina e (3R)-claussequinona). Dentre as substâncias testadas a claussequinona foi a mais efetiva em promastigotas de *L. infantum* e *L. amazonensis* e também mostrou boa atividade contra amastigotas intracelulares em de *L. infantum*. O terceiro capítulo mostra a atividade da Lectina do látex de *Synadenium carinatum* (ScLL) em modelos *in vitro* e *in vivo* de leishmaniose visceral. ScLL mostrou baixa citotoxicidade em fibroblastos NIH e mostrou atividade aglutinante em parasitos de *L. infantum*. Nos testes com amastigotas intracelulares, ScLL reduziu a porcentagem de infecção tanto nos testes que os parasitos foram tratados antes da infecção quantos nos testes que os macrófagos foram estimulados com lectina. *In vivo*, os animais que receberam parasitos tratados com ScLL por 1 hora mostraram uma diferença significativa na carga parasitária em relação ao grupo controle, tanto no baço quanto no fígado. Os animais que foram infectados com *L. infantum* e receberam tratamento 2 dias após a infecção (dpi) mostraram redução da carga parasitária no baço em relação ao grupo não tratado. Os animais que receberam tratamento 12 dpi mostraram redução no baço e no fígado. Nossos resultados mostram que lapachol, (3R)-claussequinona e ScLL podem ser considerados na busca de novas opções terapêuticas para o tratamento das leishmanioses.

Palavras-chave: Leishmaniose, Tratamento, Lapachol, (3R)-claussequinona, Lectina ScLL

ABSTRACT

Leishmaniasis are neglected diseases caused by protozoa of the genus *Leishmania*, which compromise viscera causing visceral leishmaniasis (VL), skin and mucous membranes causing cutaneous leishmaniasis (CL). In most cases, current treatment protocols present toxicity and low effectiveness. In addition, these parasites are becoming more resistant to conventional treatment methods. This thesis is composed of three chapters referring to the study of new therapeutic options in the treatment of leishmaniasis. In the first chapter we evaluated the efficacy of lapachol in in vitro and in vivo models of cutaneous leishmaniasis and visceral leishmaniasis. Our results showed that lapachol has a low cytotoxicity on HepG2 cells, good anti-*Leishmania* activity and a favorable selectivity index (SI) against *L. amazonensis* promastigotes and *L. infantum*. Efficacy was also evaluated in intracellular amastigotes. Flow cytometry analyzes showed that lapachol induces death by apoptosis in *Leishmania* promastigotes. In vivo, the efficacy of lapachol was confirmed in a murine model of visceral and tegumentary leishmaniasis, reducing the parasite load in the liver, spleen and skin lesions. In the second chapter we evaluated the in vitro efficacy of isoflavonoids (xylopic acid, kaurenoic acid, (-)-α-bisabolol) and terpenes ((-)-duartine and (3R)-claussequinone). Among the substances tested, claussequinone was the most effective in *L. infantum* and *L. amazonensis* promastigotes and also showed good activity against intracellular amastigotes in *L. infantum*. The third chapter shows the activity of *Synadenium carinatum* latex lectin (ScLL) in in vitro and in vivo models of visceral leishmaniasis. ScLL showed low cytotoxicity on NIH fibroblasts and showed agglutinating activity on *L. infantum* parasites. In tests with intracellular amastigotes, ScLL reduced the percentage of infection both in tests in which the parasites were treated before infection and in tests in which macrophages were stimulated with lectin. In vivo, animals that received parasites treated with ScLL for 1 hour showed a significant difference in parasite load compared to the control group, both in the spleen and liver. Animals that were infected with *L. infantum* and received treatment 2 days after infection (dpi) showed a reduction in spleen parasite load compared to the untreated group. Animals that received 12 dpi treatment showed a reduction in the spleen and in the liver. Our results show that lapachol, (3R)-claussequinone and ScLL can be considered in the search for new therapeutic options for the treatment of leishmaniasis.

Keywords: Leishmaniasis, Treatment, Lapachol, (3R)-claussequinone, Lectin ScLL

LISTA DE ABREVIATURAS

AIDS: Síndrome da Imunodeficiência Adquirida

AM: Antimoniato de meglumina

BOD: Demanda bioquímica de oxigênio

$CaCl_2$: Cloreto de cálcio

CC_{50}: Concentração citotóxica em 50%

ConA: Concavalina A

DMSO: Dimetilsulfóxido

DNA: Ácido desoxirribonucleico

DO: Densidade ótica

HIV: Vírus da imunodeficiência humana

HUFU: Herbário da Universidade Federal de Uberlândia

IC_{50}: Concentração inibitória em 50%

ICBIM: Instituto de Ciências Biomédicas

LC: Leishmaniose cutânea

LCD: Leishmaniose cutânea difusa

LCM: Leishmaniose cutâneomucosa

LT: Leishmaniose tegumentar

LTA: Leishmaniose tegumentar americana

LV: Leishmaniose visceral

LVC: Leishmaniose visceral canina

M: molar

mM: micro molar

MTT: Brometo de 3-(4,5-dimetiltiazol-2-il)- 2,5-difeniltetrazólio

NaCl: Cloreto de sódio

NaOH: Hidróxido de sódio

nM: nano molar

OMS: Organização Mundial da Saúde

PBS: Tampão fosfato salino

PBS: Tampão fosfato-salino

PCR: Reação em cadeia de polimerase

PHA: Lectina de *Phaseolus vulgaris*

PNPIC: Política Nacional de Práticas Integrativas e Complementares

PSG - *Promastigote Secretory Gel*

RPMI: *Roswell Park Memorial Institute medium*

ScLL: Lectina do látex de *Synadenium carinatum*

SFB: Soro fetal bovino

SI: Índice de seletividade

SINAN: Sistema de Informação de Agravos de Notificação

SMF: Sistema monocítoco fagocitário

SUS: Sistema Único de Saúde

V/V: volume/volume

WGA: Lectina de germe de trigo

WHO: *World Health Organization*

α-MEM: *Minimum Essential Medium Alpha modification*

µg: micrograma

µL: microlitro

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1. FUNDAMENTAÇÃO TEÓRICA

1.1. Leishmanioses

As leishmanioses compreendem um conjunto de doenças crônicas e infecciosas causadas por um protozoário intracelular obrigatório que pertence a classe Kinetoplastida, família Trypanosomatidae e gênero *Leishmania*, transmitida a humanos através da picada de fêmeas de flebotomíneos infectadas. Podem acometer pele, mucosa e vísceras, sendo o tipo de acometimento relacionado com a espécie do parasito responsável pela infecção e a resposta imune do hospedeiro infectado (LAINSON; SHAW, 2005; WHO, 2022). O termo leishmaniose está relacionado com a condição de adoecer por uma infecção causada por *Leishmania*, e não por o indivíduo estar infectado com o parasito, uma vez que a maioria das pessoas infectadas não desenvolvem nenhum sintoma durante a vida (WHO, 2022).

Os protozoários do gênero *Leishmania* são pleomórficos e apresentam em seu ciclo de vida as formas evolutivas promastigota e paramastigota, que se desenvolvem no trato intestinal de um inseto vetor, são móveis e com o flagelo aparente, e as formas amastigotas, que vivem e se multiplicam no interior de células do sistema mononuclear fagocitário (SMF) de um hospedeiro vertebrado, são imóveis e sem flagelo aparente (BATES, 2008). Da mesma forma que outros tripanossomatídeos, parasitos do gênero *Leishmania* possuem uma mitocôndria única e ramificada, que se estende por todo o corpo celular e tem um grande conteúdo de material genético localizado em uma região específica da organela, o cinetoplasto. Exibem um núcleo oval e cinetoplasto pequeno, em forma de bastão. A posição do cinetoplasto em relação ao núcleo varia, embora ele se localize preferencialmente, entre o núcleo e o maior volume de citoplasma (SIMPSON, 1986).

A transmissão do parasito para o ser humano pode ocorrer tanto da forma zoonótica, onde o parasito se encontra em um reservatório animal, passa pelo vetor e posteriormente para o humano quanto pela forma antroponótica, na qual a transmissão ocorre entre humanos, que atuam como hospedeiros e reservatórios pelo inseto vetor (DESJEUX, 2004; SECUNDINO et al., 2011; WHO, 2022). Uma grande variedade de mamíferos, silvestres e domésticos, atua como hospedeiros vertebrados de *Leishmania.* Os principais são animais pertencentes às ordens Edentata (tatus e preguiças), Carnívora (cães e gatos),

Rodentia (ratos), Primata (humanos e não humanos) e Marsupialia (gambás). Com algumas exceções, as leishmanioses são zoonóticas e a infecção em humanos é acidental (ASHFORD, 1996; SALIBA et al., 1999; GRAMICCIA et al., 2005; WHO, 2022).

As leishmanioses humanas podem apresentar distintas manifestações clínicas, sendo as duas formas básicas a leishmaniose visceral (LV) forma mais grave da doença e leishmaniose tegumentar (LT) (SILVEIRA et al., 2004; LAINSON & SHAW, 2005).

A LV, também conhecida como calazar, consiste em uma infecção causada por *Leishmania donovani* (Velho Mundo) e *Leishmania infantum* (Novo Mundo e Velho Mundo) (GRIENSVEN e DIRO, 2012). A LV é uma doença sistêmica grave que atinge as células do SMF de homens e animais, sendo o baço, fígado, linfonodos, medula óssea e pele os órgãos mais afetados. A maioria dos infectados é assintomático, porém, quando associada à má nutrição e/ou outras condições debilitantes, como co-infecções com o vírus da imunodeficiência humana (HIV), se manifesta na forma clínica clássica com febre, esplenomegalia, hepatomegalia, alterações do sistema hematopoiético como anemia, leucopenia e trombocitopenia, hipergamaglobulinemia. Sem o diagnóstico e o tratamento rápido e eficaz leva 90% dos pacientes a óbito (BADARÓ et al., 1996; CHAPPUIS et al., 2007). Sua grande importância na saúde pública se deve a expansão geográfica para áreas até então livres da doença e inclui, ainda, a urbanização, reemergência em focos endêmicos antigos e alta letalidade, principalmente em indivíduos não tratados e crianças desnutridas. É também considerada emergente em indivíduos com HIV, tornando-se uma das doenças mais importantes da atualidade (MINISTÉRIO DA SAÚDE, 2014).

A LT pode se manifestar como diversas síndromes clínicas no hospedeiro, dependendo da espécie de *Leishmania* que o acomete e da resposta imune do indivíduo, sendo classificada em: leishmaniose cutânea (LC), leishmaniose cutânea mucosa (LCM), leishmaniose cutânea difusa (LCD) ou anérgica (CHAPPUIS et al., 2007; RAMOS, et al., 2015). O indivíduo com LC apresenta uma ou várias úlceras na pele, sendo essa forma considerada mais frequente. Conhecida popularmente como espúndia, a LCM causa úlceras que são gradativamente destrutivas nas mucosas como boca, nariz, faringe e laringe. Essa manifestação clínica da doença é considerada grave e pode causar a mutilação da face e provocar sofrimento ao paciente. Feridas não ulceradas que apresentam uma grande quantidade de parasitos na pele do indivíduo e anergia ao teste

intradérmico de Montenegro, são manifestações clinicas de LCD (RIBAS-SILVA et al., 2013; MINISTÉRIO DA SAÚDE, 2017). A LT apresenta uma ampla distribuição mundial, estando presente em vários países. No continente americano, esta forma é denominada como leishmaniose tegumentar americana (LTA), sendo encontrados casos desde o sul dos Estados Unidos até o norte da Argentina (GONTIJO et al., 2003; MINISTÉRIO DA SAÚDE, 2017).

As leishmanioses ocorrem em 92 países distribuídos em quatro, dos seis continentes. De acordo com os dados da Organização Mundial de Saúde (OMS), cerca de 1 bilhão de pessoas vivem em áreas endêmicas para leishmaniose e estão expostas a contrair a doença, com incidência anual de aproximadamente 1 milhão de novos casos de LT e 90.000 de pessoas acometidas pela LV, das quais cerca de 20.000 a 30.000 dos indivíduos doentes evoluem para o óbito (ALVAR et al., 2012; CHAPPUIS et al., 2007; DESJEUX, 2004; WHO, 2022).

As leishmanioses constituem um grupo muito importante de doenças parasitárias. A LV é a segunda doença parasitária que mais mata no mundo, e a LT está entre as seis mais importantes doenças infecciosas combatidas pela OMS. Um dos pontos preocupantes é a baixa notificação da doença para o OMS, mesmo sendo compulsória em muitos países. Avanços relacionados a prevenção e diagnósticos das leishmanioses podem reduzir a taxa de casos graves e óbitos, porém a mortalidade e a morbidade ainda são muito preocupantes. Devido a isso, é necessário buscar ferramentas que melhorem o diagnóstico, controle e tratamento da doença, sendo na descoberta de novos produtos, produção de vacinas e melhoria nas técnicas de diagnóstico (WHO, 2022).

1.2. Transmissão e ciclo biológico

O ciclo de vida de parasitos do gênero *Leishmania* é heteroxênico, necessitando de dois hospedeiros, um invertebrado, representado por um flebotomíneo, e um mamífero pertencente as ordens Carnivora (cães e gatos), Xenarthra (tatus e preguiças), Marsupialia (gambás), Primata (humanos e não humanos), Rodentia (ratos) (LAINSON; SHAW 1987) (**Figura 1**). O gênero é dividido em dois subgêneros, de acordo com seu desenvolvimento no trato digestório do vetor, entre outros aspectos: *Leishmania* (Leishmania), com o desenvolvimento limitado ao intestino nas regiões média e anterior,

e *Leishmania* (Viannia), onde os parasitos se desenvolvem na região posterior e, migram paras as regiões média e anterior do intestino (LAINSON & SHAW, 1987).

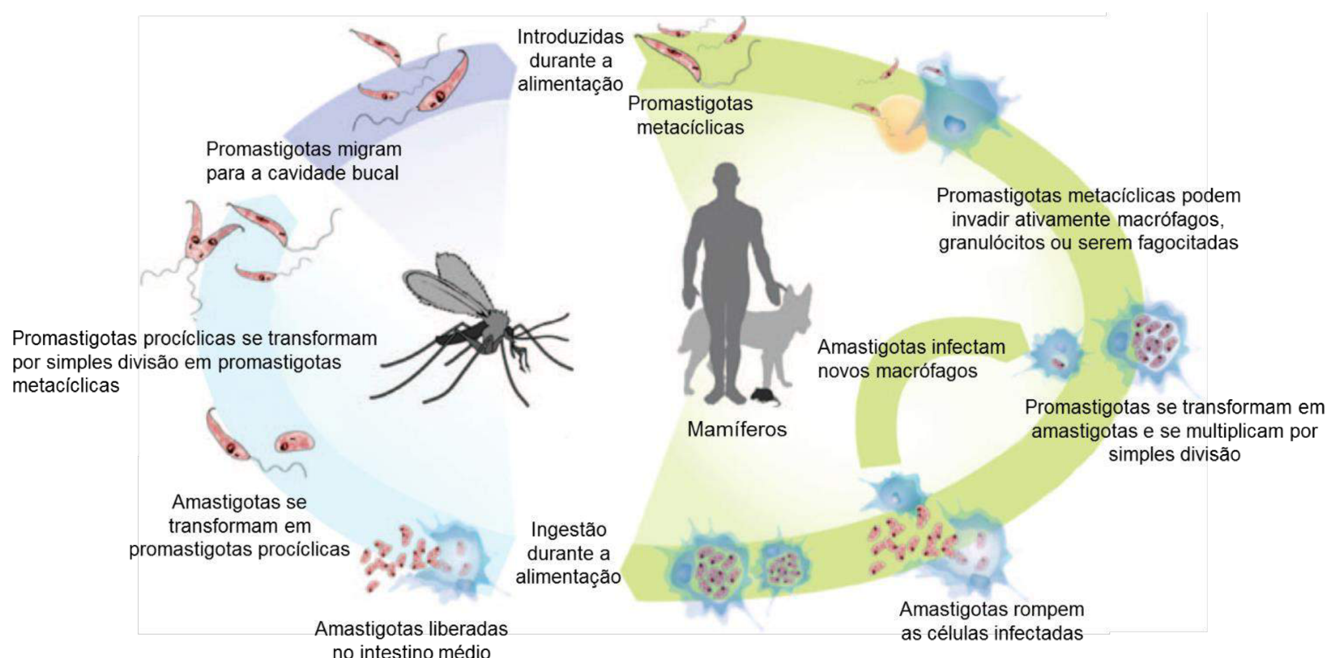

Figura 1 - Representação do ciclo de vida de *Leishmania* spp (Adaptado de Harhay et at., 2011). Os flebotomíneos ingerem células infectadas durante o repasto sanguíneo. No intestino médio do vetor, formas amastigotas se transformam em promastigotas, por simples divisão, se desenvolvem e migram para a probóscide. Durante o repasto sanguíneo as promastigotas são regurgitadas em hospedeiros humanos ou mamíferos vertebrados. As promastigotas são fagocitados por macrófagos e outros tipos de células fagocíticas e se transformam dentro dessas células em amastigotas, que se multiplicam por divisão simples e passam a infectar outras células fagocíticas mononucleares.

Algumas formas de transmissão das leishmanioses, como transfusão sanguínea, transplante de órgãos, acidentes de laboratório, compartilhamento de seringas e picada de carrapato ou pulga são descritas na literatura, porém a mais comum e de maior importância epidemiológica é a picada de fêmeas de flebotomíneos. Flebotmíneos são pequenos insetos pertencentes à ordem Diptera, família Psychodidae e subfamília Phlebotominae (BRUSCHI; GRADONI, 2018), possuem o hábito de vida crepuscular e pós-crepuscular, abrigando-se durante o dia em lugares protegidos e úmidos, sendo então encontrados em diferentes regiões geográficas com diferentes condições climáticas e ambientais. A maioria desses insetos habita o ambiente silvestre, porém pode se alimentar em homens e animais, cujas habitações estão próximas a regiões de mata. Algumas espécies apresentam acentuada antropofilia e domiciliação, o que as tornam

epidemiologicamente importantes na transmissão de doenças (LAINSON; SHAW, 1998; BRUSCHI; GRADONI, 2018). Mudanças na temperatura, precipitação e umidade podem ter fortes efeitos sobre vetores, alterando sua distribuição e influenciando sua sobrevivência e tamanho populacional (WHO, 2022).

São descritos em torno de 700 espécies de flebotomíneos, e cerca de 90 espécies são conhecidas por transmitir parasitos do gênero *Leishmania*, sendo que todas as espécies do parasito são transmitidas por flebotomíneos pertencentes aos gêneros *Lutzomyia*, encontrado no Novo Mundo, e *Phlebotomus*, no Velho Mundo (SCHLEIN, 1993; SACKS; KAMHAWI, 2001, BATES, 2008; MONGE-MAILLO; LÓPEZ-VÉLEZ, 2013).

A transmissão do parasito acontece durante a realização do repasto sanguíneo das fêmeas de flebotomíneos em um hospedeiro infectado, onde ocorre a ingestão de formas amastigotas, presentes no SMF da pele do mesmo. No intestino do vetor ocorre a transformação das formas amastigotas em formas paramastigotas, que se fixam, através do flagelo, à cutícula por meio de hemidesmossomos, e promastigotas que se replicam intensamente. Quando acontece o processo da metaciclogênese, as promastigotas migram para a parte anterior do tubo digestório do vetor e atingem o estágio infectivo, dando origem as formas metacíclicas. Essas formas infectantes se direcionam para o aparelho bucal do inseto e são introduzidas em um no hospedeiro durante o repasto sanguíneo (PIMENTA et al., 1992; SACKS; SHER, 2002, MICHALICK, et al., 2016).

As promastigotas metacíclicas, no tubo digestório do inseto vetor, produzem uma glicoproteína de consistência gelatinosa chamada PSG (*promastigote secretory gel*). Acredita-se que esse gel obstrua a passagem de alimento, quando há o repasto sanguíneo-tissular, o flebotomíneo é obrigado a regurgitar saliva juntamente com as formas promastigotas metacíclicas no local da picada (BATES, 2007).

Após a infecção, os macrófagos atraídos pela reação inflamatória formada no local fagocitam os parasitos, que são internalizados no vacúolo parasitóforo, que se liga aos lisossomos, dando origem ao vacúolo fagolisossomal. No interior deste vacúolo, o parasito permanece em um ambiente hostil, contendo enzimas lisossomais e metabólitos reativos do oxigênio. Entre uma a quatro horas, as formas promastigotas se transformam em amastigotas, iniciando uma reprodução por divisão binária dentro do vacúolo

parasitóforo. Quando os macrófagos estão repletos com amastigotas, eles se rompem e liberam os parasitos que vão infectar outros macrófagos (PETERS et al., 2008; SACKS; SHER, 2002).

1.3. Leishmaniose tegumentar

A LT constitui um problema de saúde pública em 85 países, distribuídos em quatro continentes (Américas, Europa, África e Ásia), com registro anual de 0,7 a 1,3 milhão de casos novos (**Figura 2**) (WHO, 2022). É considerada pela OMS como uma das seis mais importantes doenças infecciosas, pela sua capacidade de produzir deformidades e também pelo envolvimento psicológico, com reflexos no campo social e econômico, uma vez que, na maioria dos casos, pode ser considerada uma doença ocupacional (DU et al., 2016; WHO, 2022).

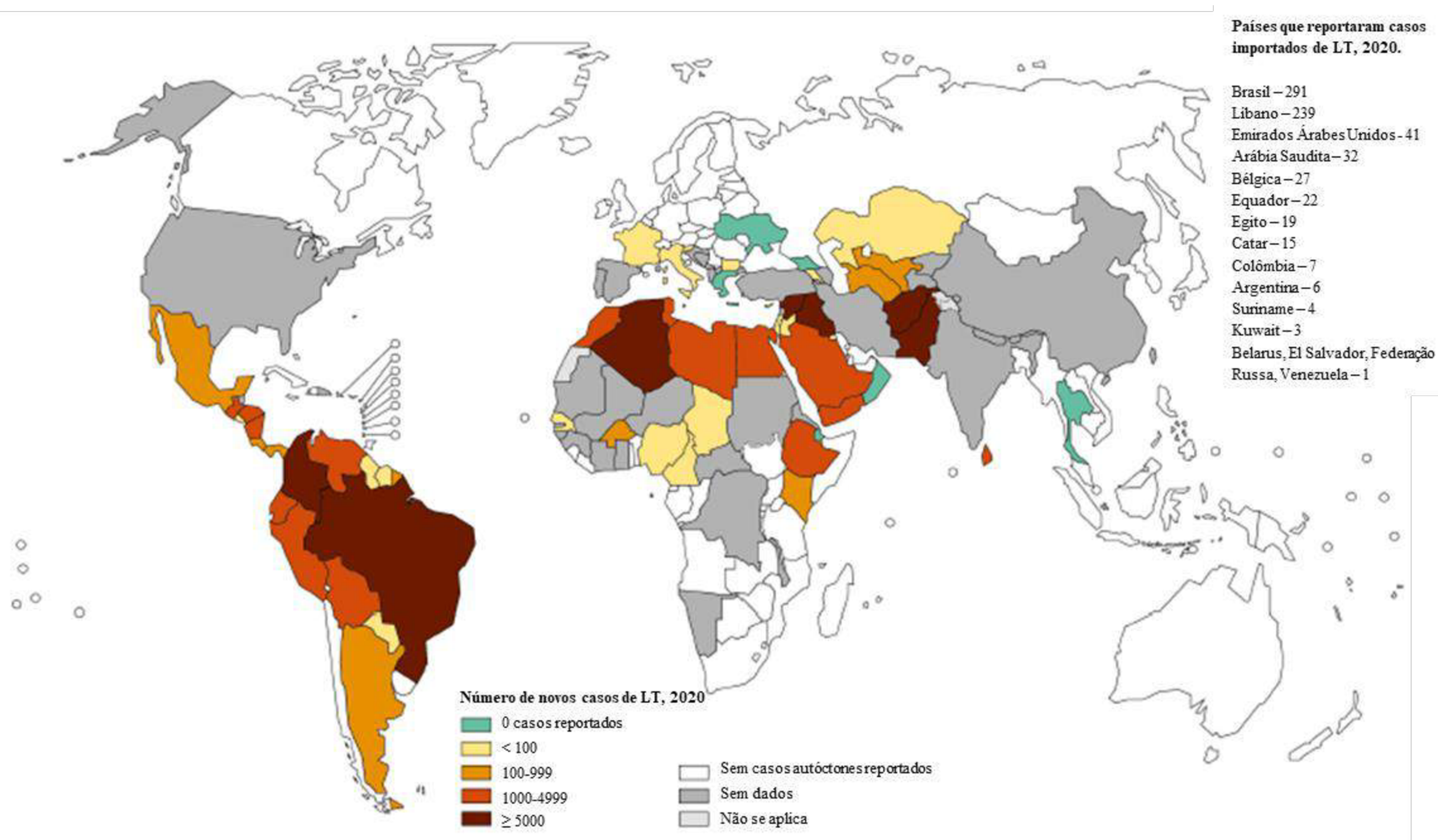

Figura 2 - Prevalência da leishmaniose tegumentar no mundo, 2020. Fonte: WHO (2022).

A LT pode se manifestar como diversas síndromes clínicas no hospedeiro, dependendo da espécie de *Leishmania* que o acomete e da resposta imune do indivíduo.

São descritas no continente americano 11 espécies dermotrópicas de *Leishmania* como agentes etiológicos da LTA que acometem o homem e animais, e oito que acometem apenas animais. No Brasil há sete espécies descritas que acometem o homem, seis do subgênero *Viannia: L. (V.) braziliensis, L. (V.) guyanensis, L. (V.) lainsoni, L. (V.) naiffi, L. (V.) lindenberg* e *L. (V.) shawi;* e uma do subgênero *Leishmania: L. (L.) amazonensis* (MINISTÉRIO DA SAÚDE, 2017). As de maior importância no país são: *L. (V.) braziliensis*, *L. (V.) guyanensis* e *L. (L.) amazonensis* (LAINSON e SHAW, 2005). Em relação às espécies de flebotomíneos, as mais importantes são: *Lutzomyia flaviscutellata*, *L. whitmani, L. umbratilis, L. intermedia, L. wellcomei* e *L. migonei*. *Lutzomyia neivai* e *L. fischeri* ainda não são vetores comprovados da LTA, porém estas espécies têm sido encontradas com frequência em ambientes domiciliares nas áreas de transmissão da doença (MINISTÉRIO DA SAÚDE, 2017; LAINSON e SHAW, 2005). Os reservatórios principais do parasito são os roedores, marsupiais, desdentados e canídeos (LAINSON e SHAW, 2005).

Casos autóctones de LTA são notificados em todas as regiões do Brasil desde 2003, sendo endêmica no Centro-Oeste, Sudeste, Norte e Nordeste. No ano de 2020, de acordo com o SINAN, o Brasil confirmou 16.813 casos, sendo a região Norte com o maior número de casos (8.060) e o Pará o estado com mais notificações com 2.981 casos (SINAN, 2022).

Como tem havido um aumento dos casos autóctones a cada ano, é notável as modificações no perfil de transmissão da doença. Primeiramente, a LTA era conhecida como zoonose apenas de animais silvestres e, portanto, transmitidas somente para indivíduos que tinham contato com matas e florestas. Em seguida, expandiu para as zonas rurais e periurbana. Nos dias atuais, são descritos três padrões de transmissão na epidemiologia da LTA: o silvestre, que ocorre em áreas de vegetação primária; o ocupacional ou de lazer, que é relacionada com a exploração desenfreada de matas e florestas para construção de estradas, atividades agropecuárias e de lazer; e o rural ou periurbano, onde há a adaptação do vetor ao peridomicílio (MINISTÉRIO DA SAÚDE, 2017).

O controle de LTA no Brasil é complexo e difícil de ser estabelecido, devido aos padrões epidemiológicos, a variedade de vetores, reservatórios, hospedeiros e agentes

etiológicos da doença, aliados ao insuficiente conhecimento sobre todos os aspectos da cadeia epidemiológica da doença (SILVEIRA et al., 2004).

1.4. Leishmaniose visceral

A LV é uma doença sistêmica grave que é fatal em 95% dos casos não tratados. A maioria dos casos ocorrem no Brasil, China, Etiópia, Eritreia, Índia, Quênia, Somália, Sudão do Sul, Sudão e Iêmen, mas é endêmica em 83 países (**Figura 3**). Devido ao potencial de surto e mortalidade é considerada pela OMS uma das principais doenças parasitárias, sendo relacionada a condições de pobreza (WHO, 2022). O número de casos pode ser subestimado, visto que somente 45% são relatados a OMS.

A maioria dos infectados podem ser assintomáticos, tendo uma variação no período de incubação de meses a anos, onde ocorre a disseminação do parasito pelo corpo (OLIVA et al., 2006). O surgimento da doença ocorre devido a um complexo de interações entre o parasito e a resposta imune do hospedeiro. Nos casos de LV, os parasitos possuem um tropismo tecidual e se estabelecem em órgãos viscerais como baço, fígado e medula óssea, a lesão inicial na pele é raramente observada e as infecções podem ser assintomáticas ou auto resolutivas (JAFFE; GREENBLATT, 1991; MANNA, 2006). Parasitos causadores de LV podem acometer indivíduos de qualquer faixa etária, porém a doença é mais frequente em crianças menores de 10 anos do sexo masculino (BRASIL, 2014).

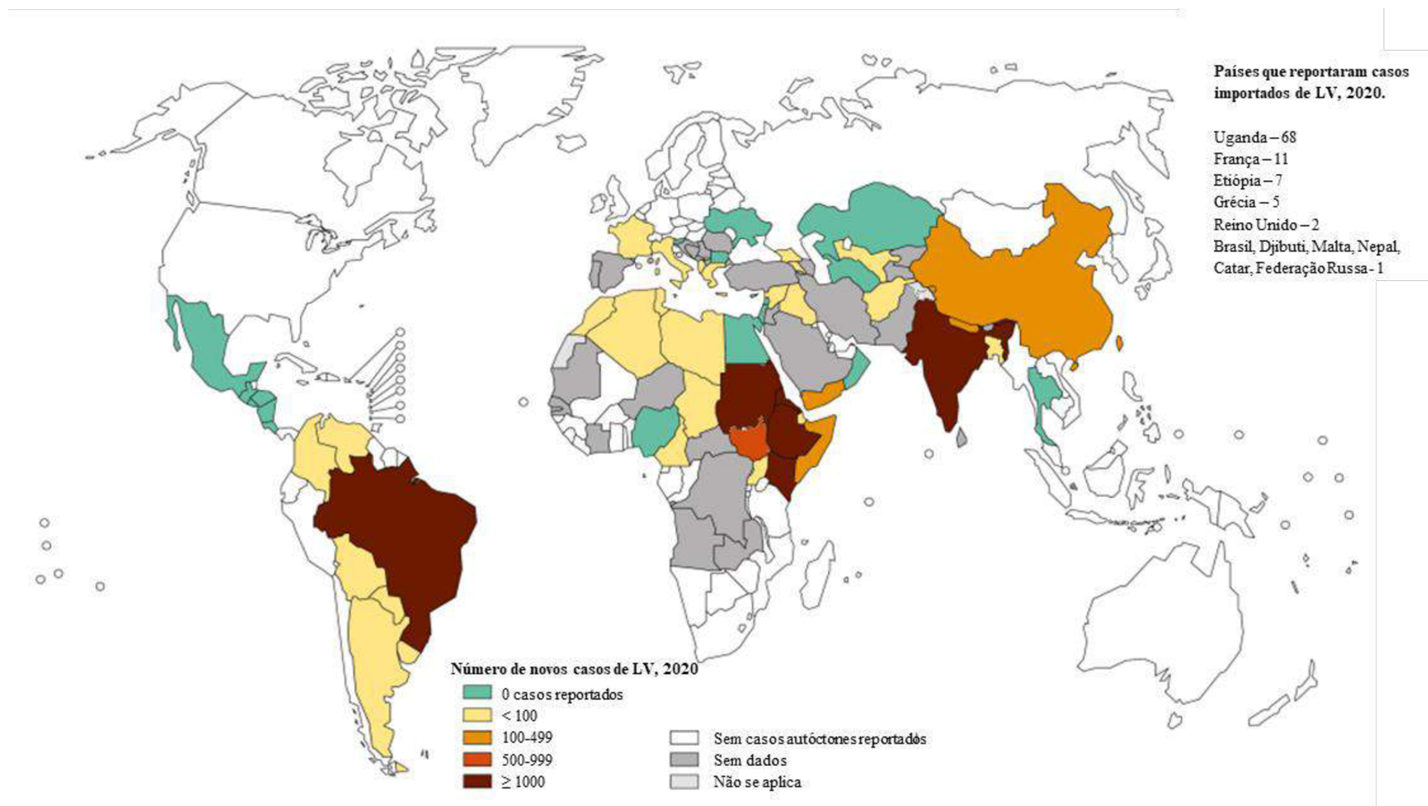

Figura 3 - Prevalência da leishmaniose visceral no mundo, 2020. Fonte: WHO (2022).

De acordo com a espécie de *Leishmania*, hospedeiros invertebrados e reservatórios envolvidos na transmissão da LV, em alguns países ou regiões, como na Índia, Nepal, Sudão, Quênia e nordeste da China a infecção pode apresentar um caráter antroponótico onde o homem é o reservatório do parasito, ou zoonótico, como em países europeus e nas Américas Central e do Sul. No Brasil, os principais reservatórios do parasito são membros da Família Canidae, tendo o cão doméstico (*Canis familiares*) como o mais importante devido ao desenvolvimento da doença no animal, conhecida como leishmaniose visceral canina (LVC) e o aumento do número de casos humanos, visto que a presença de casos caninos se correlaciona com aumento de casos humanos de LV (WHO, 2022). No ambiente silvestre os reservatórios mais comuns são as raposas e cachorros-do-mato (*Dusicyon vetulus* e *Cerdocyon thous*) e os marsupiais (*Didelphis albiventris*) (MINISTÉRIO DA SAÚDE, 2014).

Duas espécies de flebotomíneos, até o momento, estão relacionadas com a transmissão do parasito no Brasil, *Lutzomyia longipalpis*, que possui alta capacidade adaptativa e é o principal vetor no país, e *L. cruzi*, encontrado infectado por *L. infantum*

nos estados de Mato Grosso e Mato Grosso do Sul e que, até o momento, não tem importância epidemiológica confirmada. São conhecidos popularmente como mosquito palha, tatuquira, birigui, entre outros (SANTOS, et al., 1998; MISSAWA, et al., 2011).

Os principais fatores de risco estão relacionados a condições socioeconômicas, desnutrição, mobilidade da população, transformações ambientais e mudanças climáticas (WHO, 2022). A maioria da população acometida por LV são indivíduos que vivem em condições precárias de higiene e habitação. Locais com acúmulo de lixo e esgoto a céu aberto se tornam ambientes propícios para a reprodução de flebotomíneos. Casos de desnutrição, onde o paciente tem uma dieta pobre em proteínas, ferro e vitamina A estão mais propensos a ter uma evolução da doença (WHO, 2022).

A LV tem sido considerada emergente em pacientes coinfectados com HIV, pois esses pacientes tem uma maior chance de evoluir para uma forma grave da doença com taxas altas de mortalidade e recidiva. Em 2021, a coinfecção *Leishmania*-HIV foi relatada em 45 países, sendo Brasil, Etiópia e Bihar, na Índia, os que apresentam as maiores taxas. Desde 1985, há relatos da coinfecção ao longo da bacia do mediterrâneo, nesses casos as chances de o paciente adoecer por LV é 200 vezes maior e a replicação do HIV é favorecida, podendo desecandear/reagudizar a AIDS (Síndrome da Imunodeficiência Humana) (WHO, 2022; ZIJLSTRA, 2014).

O Brasil está entre os seis países que mais apresentam casos de LV no mundo e é o primeiro no América Latina, tendo uma representatividade de 90% dos casos no continente (WHO, 2022). No ano de 2020, 1.933 casos foram confirmados no Brasil, segundo o Sistema de Informação de Agravos de Notificações (SINAN), e o índice de letalidade gira em torno 9,5%, a maior nos últimos 20 anos. O estado do Maranhão, na região Nordeste, foi o que apresentou o maior número de casos da doença.

Antes da década de 1980 a LV era caracterizada como uma doença do meio rural, porém houve uma expansão para regiões periurbanas de vários municípios brasileiros (PASTORINO et al, 2002). A expansão atingiu todo o território brasileiro, principalmente em municípios com crescente urbanização e alto índice de pobreza (MARQUES et al., 2017). Como os fatores de risco para LV estão relacionados a condições ambientais, essa mudança de comportamento da doença pode estar relacionada ao desmatamento e a invasão humana em áreas florestais. Outro ponto muito importante gira em torno de

mudanças na temperatura, interferindo no ciclo de vida de vetores e reservatórios, permitindo que ocorra o desenvolvimento de promastigotas de *Leishmania* em flebotomíneos em áreas não endêmicas para LV. A seca e a fome podem fazer que ocorra migração de grandes populações para áreas onde a transmissão de *Leishmania* é bem estabelecida (WHO, 2022).

A cadeia de transmissão da LV no Brasil é complexa, em razão das características epidemiológicas e conhecimento insuficiente dos elementos que a compõe e as estratégias de controle desta doença têm se mostrado pouco efetivas. As principais formas de controle são o diagnóstico e tratamento precoce, redução de vetores e reservatórios domésticos e atividades de educação em saúde (BRASIL, 2014).

1.5. Tratamento convencional das leishmanioses e suas limitações

Uma das principais formas de controle das leishmanioses se concentra no tratamento dos casos humanos, e esse tratamento quando eficaz, pode reduzir a prevalência da doença. O tratamento deve ser realizado após o diagnóstico da doença.

Os principais medicamentos utilizados no Brasil e no mundo, são os antimoniais pentavalentes (Sb^{5+}), tendo duas versões disponíveis no mercado, o Glucantime® (antimoniato de N-metil d-glucamina) medicamento empregado no Brasil e no mundo há mais de 70 anos e o Pentostam® (estibogluconato de sódio) que não tem comercialização no Brasil. O mecanismo de ação dos antimoniais está relacionado à inibição de enzimas do parasito que são utilizadas na oxidação de ácidos graxos e glicólico, o que resulta na depleção dos níveis de ATP intracelular. A redução do antimônio pentavalente para trivalente (Sb^{5+} em Sb^{3+}), forma tóxica para os parasitos, capaz de interferir no metabolismo dos tióis (BALANA-FOUCE et al., 1998, ALVARENGA, et al. 2010; LIMA et al., 2015). Os antimoniais são aplicados por via endovenosa ou intramuscular com dose de 20mg de Sb5+ kg/dia, por no mínimo 20 e no máximo 40 dias, utilizando-se o limite máximo de 2 a 3 ampolas/dia do produto com bons índices de cura. Em casos que a doença está em níveis mais avançados não é possível observar uma melhora clínicas nos primeiros 20 dias, sendo necessário uma extensão no tempo de tratamento para que haja resultados satisfatórios (BRASIL, 2014).

Ainda que os antimoniais sejam o medicamento de primeira escolha no tratamento das leishmanioses, seu uso apresenta diversos efeitos colaterais, principalmente no final

do tratamento. O principal efeito colateral está ligado a problemas cardiovasculares, porém, incluem outros sintomas como náuseas, vômitos, diarreia, artralgias, mialgias, anorexia, elevação dos níveis de enzimas hepáticas, convulsões, pancreatite química e nefrotoxicidade (RIBEIRO et al., 2014).

Quando o paciente não apresenta melhora ao tratamento com o antimonial, ou é contraindicado em algumas situações (pacientes acima de 50 anos, portadores de cardiopatias, nefropatias, hepatopatias, doenças de Chagas e gestantes), a segunda opção mais utilizada no Brasil é a anfotericina b (ROCHA, et al., 2005; MINISTÉRIO DA SAÚDE, 2014, LIMA, et al., 2015).

A anfotericina B é a uma potente droga leishmanicida que atua nas formas promastigotas e amastigotas do parasito, tendo ótimos resultados tanto *in vitro* quanto *in vivo*. É um antibiótico da classe dos polienios, produzido a partir do actinomiceto *Streptomyces nodosus*. É primariamente uma droga fungicida, que possui atividade contra algumas espécies de protozoários, incluindo *Leishmania* spp., sendo o seu mecanismo de ação principal a ligação preferencial com ésteres (ergosterol ou episterol) que estão presentes na membrana plasmática da *Leishmania*, levando à formação de poros na membrana e consequente lise osmótica com morte do parasito (LEMKE et al., 2005; WHO, 2022). O Ministério da Saúde disponibiliza duas apresentações de anfotericina b: o desoxicolato de anfotericina b e a anfotericina b lipossomal (AmBisome®), sendo a eficácia dessas apresentações comparáveis, porém a anfotericina b lipossomal apresenta uma menor toxicidade (MEYERHOFF, 1998).

Mesmo que a anfotericina b seja uma droga eficaz, seu uso apresenta muitas limitações. Além do custo elevado da medicação, todos os pacientes em tratamento devem ser acompanhados por equipe médica para monitoramento dos efeitos colaterais, mesmo após o fim do tratamento. Os efeitos colaterais mais apresentados durante o uso da medicação são febre, cefaleia, náusea, vômitos, tremores, calafrios e dor lombar. Além desses efeitos colaterais citados, o desoxicolato de anfotericina b também pode causar anorexia, tremores, calafrios, flebite, cianose, hipotensão, hipopotassemia, hipomagnesemia, comprometimento da função renal, e distúrbios de comportamento (GONTIJO et al., 2003, BRASIL, 2014).

A miltefosina (hexadecilfosfocolina) é outro medicamento utilizado no tratamento das leishmanioses, sendo, inicialmente, este fármaco foi desenvolvido como droga antineoplásica e foi o primeiro medicamento a ser administrado por via oral no tratamento da LV. Este medicamento é usado em alguns países tanto para o tratamento da LV quanto para a LT (SINDERMANN et al., 2004; BHATTACHARYA et al., 2007). Apresenta efeitos colaterais importantes como nefrotoxicidade, teratogenicidade, e problemas gastrointestinais devido a sua administração por via oral. Também apresenta meia-vida prolongada, que acaba favorecendo a seleção de parasitos resistentes à droga (BHATTACHARYA et al., 2007).

1.6. Novas opções terapêuticas para o tratamento das leishmanioses

Todas as terapias disponíveis atualmente para o tratamento das leishmanioses apresentam limitações importantes, quer seja pelo custo ou pela toxicidade (CORRAL et al., 2012; WHO, 2022). Devido às dificuldades do tratamento a OMS incentiva a busca de novas alternativas terapêuticas para o tratamento das leishmanioses, como: reposicionamento de fármacos, associação entre fármacos, rejuvenescimento farmacológico e o uso de produtos naturais (WHO, 2022).

1.6.1. Produtos Naturais

O interesse no uso de plantas medicinais e produtos naturais para a prevenção de doenças parasitárias vem crescendo nos últimos anos (BATISTA et al., 2009; LIMA et al, 2015). As plantas são fontes de descobertas de medicamentos, especialmente quando se trata de drogas antiparasitárias (ANTHONY et al., 2005). A eficácia e o mecanismo de ação dos medicamentos provenientes de vegetais vêm sendo estudados mais profundamente nos últimos 30 anos, e alguns autores pesquisam novas alternativas para o tratamento das leishmanioses, baseando-se na etnofarmacologia, onde utilizam do conhecimento tradicional em plantas medicinais para busca de novos medicamentos com menor toxicidade e menor custo (ANTHONY et al., 2005; VILA NOVA, 2012).

O Brasil possui uma grande diversidade genética vegetal, a maior do mundo, com mais de 55.000 espécies catalogadas (AZEVEDO; SILVA, 2006). Mesmo com tamanha diversidade, apenas 8% desse potencial biológico foi estudado para busca de compostos bioativos e 1.100 espécies vegetais foram avaliadas em suas propriedades medicinais

(SIMÕES et al., 2004). No Brasil foi criada a Política Nacional de Práticas Integrativas e Complementares (PNPIC) no Sistema Único de Saúde (SUS), que tem como objetivo ampliar as opções terapêuticas aos usuários do SUS, com garantia de acesso a plantas medicinais, a fitoterápicos e a serviços relacionados à fitoterapia, com segurança, eficácia e qualidade, na perspectiva da integralidade da atenção à saúde (BRASIL, 2006).

As plantas apresentam em sua composição metabólitos secundários das mais variadas classes: alcaloides, chalconas, flavonoides, lignanas, naftoquinonas e terpenos, diversos desses compostos descritos na literatura como efetivos na atividade leishmanicida (QUEIROZ, et al., 1996; ROCHA, et al., 2005).

1.6.2. Lectinas

Lectinas são glicoproteínas de base não-imune que fazem combinações de forma específica e reversível a carboidratos, resultando em aglutinação celular e precipitação de glicoconjugados. Não possuem atividade enzimática em seu ligante e são distintas de anticorpos e proteínas de transporte de oligossacarídeos livres. São amplamente distribuídas na natureza tendo estabilidade em plantas, fungos, vírus, bactérias, crustáceos, insetos e animais, porém as leguminosas são as principais fontes de lectina (PEUMANS; VAN DAMME, 1995; GABIUS et al., 2011; HIVRALE; INGALE, 2013).

As plantas constituem ricas fontes de lectina e podem ser encontradas nas raízes, folhas, flores, frutos, sementes, rizomas, tubérculos, bulbos, vagens, entrecascas, cerne, que servem como materiais de análise no isolamento dessas moléculas (RATANAPO et al., 2001; SÁ et al., 2008). As lectinas são purificas e isoladas principalmente de semente de leguminosas sendo que constituem aproximadamente 10% do total de proteínas solúveis em sementes, como únicas ou múltiplas formas moleculares (SHARON; LIS, 1990; PAIVA; COELHO, 1992; KONOZY et al., 2003).

Lectinas extraídas de plantas, principalmente da família das leguminosas, já são bastante estudadas em relação a suas propriedades biológicas (SHARON; LIS, 1990). Há mais de 130 anos existem estudos sobre essas lectinas, em 1888 Stillmark relatou uma proteína altamente tóxica encontrada em sementes de manona (*Ricinus communis* L.), que ficou conhecida como ricina e revelou ter atividade de hemaglutinação. Após essa descoberta, houve uma evolução sobre conhecimentos a cerca dessas proteínas,

mostrando que nem todas as proteínas aglutinantes extraídas de sementes eram tóxicas (TSANEVA; VAN DAMME, 2020; VAN DAMME, 2022).

Concavalina A (ConA), extraída de sementes de *Canavalia ensiformis*, foi a primeira lectina a ser purificada até a homogeneidade. Sumner (1919) descreveu que ConA era capaz de aglutinar células sanguíneas e que essa reação pode ser inibida com adição de sacarose. A partir desses dados, outros estudos foram realizados e marcaram a história das lectinas. A atividade hemaglutinante de lectinas vegetais pode ser diferente dependendo do tipo de células sanguíneas, e a adição de açúcares específicos para cada tipo de eritrócito pode bloquear a aglutinação causada pelas lectinas (RENKONEN, 1948; BOYD; REGUERA, 1949; WATKINS; MORGAN, 1952).

Com o passar dos anos, descobertas importantes foram realizadas com as lectinas. Nowell em 1960, descobriu a atividade mitogênica de uma lectina, isolada de *Phaseolus vulgaris*, denominada fitohemaglutinina (PHA). Aub et al., (1963; 1965) demonstraram que uma aglutinina de germe de trigo (WGA) reconhece e aglutina preferencialmente células malignas. Esses estudos mostraram que além da hemaglutinação existem outras atividades das lectinas.

Na literatura, trabalhos relatam que as lectinas vegetais têm um papel fundamental na imunologia e efeitos que podem ativar ou suprimir inflamação, inibir crescimento de tumores, facilitar a cicatrização de feridas e interferir na infecção de patógenos, vírus e parasitos (SHARON; LIZ, 2004; COELHO et al., 2017; TSANEVA; VAN DAMME, 2020).

Em leishmanioses, a lectina KM^+, extraída de sementes de jaca (*Artocarpus integrifolia*), quando injetada em camundongos BALB/c induz uma inversão no padrão de citocinas de Th2 para Th1, e esses camundongos se tornaram resistentes à infecção por *L. major* (PANUTO-CASTELO et al., 2001). Teixiera et al. (2006), avaliaram o uso de KM^+, ConBr (extraída de *Canavalia brasiliensis*) e PAA (extraída de *Pisum arvense*) como moléculas estimuladoras na vacinação contra *L. amazonensis*, os resultados mostraram que apesar de induzirem produção de IFN- γ, não houve redução da lesão, porém KM^+ em conjunto antígeno de *L. amazonensis* mostrou uma redução na carga parasitária.

Lectina do látex de *Synadenium carinatum* (ScLL) é uma lectina extraída de uma planta da família Euphorbiaceae conhecida popularmente como leiterinha. No Brasil é usada como planta ornamental e seu látex é empregado na medicina popular como tratamento para doenças infecciosas e câncer (AFONSO-CARDOSO et al., 2007). ScLL foi isolada e purificada por Souza et al., (2005) e mostrou que alta atividade hemaglutinante em diversos grupos sanguíneos humanos e animais. O uso de ScLL já foi testado em alguns modelos de doenças parasitárias.

Em toxoplasmose, camundongos infectados com *Toxoplasma gondii* e tratados com ScLL apresentaram uma redução na carga parasitária e maiores taxas de sobrevivência, resultados semelhantes com o tratamento com sulfadiazina (SOUZA et al., 2016). Cardoso et al., (2016) avaliaram o efeito adjuvante da ScLL na imunização de camundongos contra a neosporose, mostrando que a combinação de ScLL com antígeno de *Neospora caninum* foi capaz de alterar o perfil de citocinas para uma resporta Th1 resultando em alta proteção de camundongos desafiados com o parasito. Afonso-Cardoso et al., (2007; 2011) mostaram que ScLL tem efeito sobre a leishmaniose tegumentar, *in vitro* reduziu o crescimento de formas intracelulares de *L. amazonensis* não sendo citotóxica nas células de mamíferos e *in vivo* demonstrou um papel protetor em modelo murino de LT.

Como já mostrado, os tratamentos atuais para as leishmanioses possuem limitações importantes e são tóxicos aos pacientes, sendo assim, o desenvolvimento de novas terapias podem apresentar uma redução nos efeitos adversos e também diminuir os custos do tratamento.

2. OBJETIVOS

A presente tese foi dividida em manuscritos presentes nos capítulos I,II e III, tendo os objetivos de cada capítulo descritos abaixo.

CAPÍTULO I

1. Objetivo geral

Avaliar a atividade leishmanicida de lapachol por testes *in vitro* contra *Leishmania amazonensis* e *Leishmania infantum,* e *in vivo,* em modelo murino de leishmaniose tegumentar e leishmaniose visceral.

2. Objetivos específicos

- Avaliar a atividade leishmanicida do lapachol por testes *in vitro* em formas promastigotas de *L. amazonensis* e *L. infantum*;
- Avaliar a citotoxicidade do lapachol por testes *in vitro* em culturas de células derivadas de um hepatoblastoma primário humano (HepG2);
- Avaliar a atividade do lapachol em formas amastigotas intracelulares de *L. amazonensis* e *L. infantum*;
- Avaliar a externalização de Fosfatidilserina e alterações no ciclo celular em promastigotas de *L. amazonensis*;
- Avaliar a eficácia do lapachol em modelo murino de leishmaniose tegumentar e leishmaniose visceral.

CAPÍTULO II

1. Objetivo geral

Investigar os efeitos *in vitro* de terpenos e isoflavonóides isolados de plantas, a fim de avaliar sua atividade contra *L. infantum* e *L. amazonensis*.

2. Objetivos específicos

- Avaliar a atividade leishmanicida dos produtos naturais ácido kaurenôico, ácido xilópico, bisabolol, claussequinonona e duartina em promastigotas de *L. infantum* e *L. amazonensis*;

- Estabelecer a citotoxicidade e o índice terapêutico dos produtos naturais *in vitro*;

- Avaliar a eficácia do produto mais eficaz em amastigotas intracelulares de *L. infantum*;

- Avaliar a externalização de Fosfatidilserina em promastigotas de *L. infantum*.

CAPÍTULO III

1. Objetivo geral

Avaliar a eficácia de ScLL em modelos *in vitro* e *in vivo* de leishmaniose visceral.

2. Objetivos específicos

Extrair e purificar a a lectina extraída do látex de *S. carinatum* (ScLL);

- Avaliar a atividade aglutinante de ScLL em promastigotas de *L. infantum*;

- Avaliar a citotoxicidade de ScLL em células NIH;

- Avaliar a eficácia de ScLL em amastigotas intracelulares de *L. infantum*;

- Avaliar a eficácia de ScLL em modelos *in vivo* de leishmaniose visceral.

CAPÍTULO I - ARTIGO PUBLICADO

Efficacy of lapachol on treatment of cutaneous and visceral leishmaniasis

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2.1. Abstract

Leishmaniasis is one of the most important neglected diseases worldwide. It is a life-threatening disease and causes significant morbidity, long-term disability, and early death. Treatment involves disease control or use of intervention measures, although the currently used drugs require long-lasting therapy, and display toxicity and reduced efficacy. The use of natural products isolated from plants, such as lapachol, an abundant naphthoquinone naturally occurring in South American *Handroanthu*s species (*Tabebuia*, Bignoniaceae), is a promising option for the treatment of leishmaniasis. In this study, we investigated the leishmanicidal activity of lapachol in vitro and in vivo against *Leishmania infantum* and *L. amazonensis*, causative agents of visceral and cutaneous leishmaniasis, respectively. Low cytotoxicity in HepG2 cells (3405.8 ± 261.33 µM), good anti-*Leishmania* activity, and favorable selectivity indexes (SI) against promastigotes of both *L. amazonensis* (IC_{50} = 79.84 ± 9.10 µM, SI = 42.65) and *L. infantum* (IC_{50} = 135.79 ± 33.04 µM, SI = 25.08) were observed. Furthermore, anti-*Leishmania* activity assays performed on intracellular amastigotes showed good activity for lapachol (IC_{50} = 191.95 µM for *L. amazonensis* and 171.26 µM for *L. infantum*). Flow cytometric analysis demonstrated that the cytotoxic effect of lapachol in *Leishmania* promastigotes was caused by apoptosis-like death. Interestingly, the in vitro leishmanicidal effect of lapachol

was confirmed in vivo in murine models of visceral and cutaneous leishmaniasis, as lapachol (25 mg/kg oral route for 24 h over 10 days) was able to significantly reduce the parasitic load in skin lesions, liver, and spleen, similar to amphotericin B, the reference drug. These results reinforce the therapeutic potential of lapachol, which warrants further investigations as an anti-leishmaniasis therapeutic.

Keywords: *Leishmania infantum*; *L. amazonensis*; Leishmaniasis; Lapachol; Naphthoquinones; Natural products

2.2. Introduction

Leishmaniasis belongs to a group of complex diseases caused by more than 20 species of the genus *Leishmania*, an intracellular obligatory protozoa belonging to the class Kinetoplastida, family Trypanosomatidae (Lainson and Shaw, 2005; WHO, 2018). It is a neglected disease with a wide spectrum of clinical manifestations depending on the parasite "species", which are classified as cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL) (Chappuis et al., 2007; Desjeux, 2004; WHO, 2010). The transmission of the parasite to susceptible mammals occurs through the bite of infected female sandflies (Diptera: Psychodidade: Phlebotominae) of the genera *Lutzomyia* (New World) and *Phlebotomus* (Old World), which are ubiquitous in nature and are found in different ecological niches (Rangel and Lainson, 2003). It is estimated that ~ 1 million people are infected with the parasites causative of CL and MCL and 300,000 by parasites which cause VL, with 20,000–30,000 deaths per year.

Leishmaniasis are is placed mainly observed among the poverty neglected diseases the economically backward sections of societies worldwide, and it is estimated that about approximately 90% of the infected patients live on less than US $ 1 per day (Alvar et al., 2006). Over 90% of new CL and VL cases occur in Afghanistan, Algeria, Bangladesh, Bolivia, Brazil, Colombia, Ethiopia, India, Iran, Peru, South Sudan, Sudan, and Syria (WHO, 2018; Desjeux, 2004; WHO, 2018).

According to WHO, leishmaniasis must be prevented or controlled using intervention strategies, such as early diagnosis and effective management of human cases

to reduce the prevalence of the disease in endemic areas (WHO, 2018). Since the 1940s, all clinical forms of leishmaniasis are being treated using control measures, albeit several limitations. Pentavalent antimonials such as meglumine antimoniate (MA, Glucantime®) and sodium stibogluconate (SSG, Pentostam®) have been the first line of treatment in the last decades. However, the use of these drugs is limited because of serious side effects (Alvarenga et al., 2010; Balana-Fouce et al., 1998; Lima et al., 2015). The second-line drugs of choice are amphotericin B deoxycholate and its liposomal formulations, namely, miltefosine and pentamidine, which also present limited efficacy, toxicity, adverse side effects, drug-resistance, require long-term treatment, and/or are expensive (Ministry of Health Brazil, 2017; Rocha et al., 2005).

Owing to the limitations of the current treatment regimens of leishmaniasis, WHO encourages the search for new drugs and the use of natural products for treating this disease (WHO, 2018). This approach has been successfully utilized in the discovery and development of antimalarials (Oliveira et al. 2009). Furthermore, it is believed that the next generation of antiparasitic drugs or the scaffolds necessary for their synthesis may be obtained from plants used to treat tropical diseases (Newmann and Cragg, 2012). In this context, new therapeutic alternatives for leishmaniasis are currently being investigated. In particular, new drugs are being identified on the basis of ethnopharmacological concepts regarding the efficacy of medicinal plants and/or their active molecules or structurally similar analogues (Anthony et al., 2005; Davis et al., 2005; Mirsha et al., 2009; Rocha et al., 2005; Rohloff et al., 2013; Vila-Nova et al., 2011).

Plants of the Bignoniceae family are extensively studied owing to the presence of naphthoquinones. Naphthoquinones are antiprotozoal, the therapeutic applications of which are being evaluated. A study showed that structural differences in naphthoquinones may negatively interfere with leishmanicidal activity or benefit specific therapeutic applications (Ali, et al., 2011). Lapachol [2-hydroxy-3- (3-methyl-2-butenyl) -1,4-naphthoquinone], which occurs mainly in the genus *Handroanthus* (Cipriani et al., 2012; Lima et al., 2004) possesses wide spectrum of biological activities, such as anti-*Trypanosoma* (Salas et al., 2011), anti-*Leishmania* (Ali et al, 2011; Costa et al., 2017; Iwu et al., 1994; Lima et al., 2004; Odonne et al., 2017; Passero et al., 2014; Rocha et al., 2013; Teixeira et al., 2001), antimalarial (Hussain and Green, 2017), and antitumor and antitumor effects (Balassiano et al., 2005).

Owing to the absence of new effective drugs for treating leishmaniasis and considering the therapeutic potential of lapachol, a compound which is easily obtained from *Handroanthus* spp, the present study investigated the in vitro and in vivo leishmanicidal activity of lapachol against *Leishmania infantum* and *L. amazonensis*, the causative agents of VL and CL, respectively.

2.3. Material and methods

Chemicals

Amphotericin B deoxycholate (Amb), α-minimal essential medium (MEM), Roswell Park Memorial Institute (RPMI)-1640 medium, antibiotics penicillin and ampicillin, L-glutamine, HEPES, dimethyl sulfoxide (DMSO), resazurin sodium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), and lapachol [2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone] ($C_{15}H_{14}O_3$, CAS number 84-79-7) were purchased from Sigma-Aldrich Co. LLC. (USA). Fetal calf serum (FCS) was purchased from Thermo Fischer Scientific Inc. (USA). Stock solutions (50 mg/mL) of the substances were diluted in DMSO.

Parasites and cells

Reference strains of *L. infantum* (MCAN/BR/2002/BH401) and *L. amazonensis* (MHOM/BR/1989/Ba199) were maintained as promastigote forms in complete α-MEM supplemented with 10% (v/v) heat inactivated FBS, 100 µg/mL penicillin, 100 µg/mL ampicillin, 2 mM L-glutamine, and 20 mM HEPES, pH 7.2 at 24 ± 1°C in 25-cm^2 culture flasks on a B.O.D. incubator.

The HepG2 A16 cell line (ATCC, USA), derived from a human primary hepatoblastoma, and immortalized murine macrophages RAW 264.7 (ATCC, USA) were cultured in 25-cm^2 cell culture plastic bottles in complete RPMI-1640 medium (10% v/v FBS, 100 µg/mL penicillin, 100 µg/mL ampicillin, 2 mM L-glutamine, and 20 mM HEPES, pH 7.2). The cells were maintained at 37°C in an atmosphere of 5% CO_2 and 95% humidity.

Effect of lapachol on promastigote viability in vitro

The inhibitory concentrations (IC_{50}) of lapachol against promastigotes of *L. infantum* and *L. amazonensis* were determined using the resazurin-based colorimetric

assay (Corral et al., 2013). Log-phase promastigotes (2.5 × 10^5 parasites/well) were seeded in flat-bottom 96-well cell culture plates in complete α-MEM and incubated at 26°C. Lapachol was two-fold serially diluted over seven concentrations (from 412 μM to 6 μM) in complete α-MEM and each concentration was tested in triplicate. Amb was used as a positive control (0.54–0.008 μM). Non-treated parasites were used for comparing viability. The cells were incubated with the substances for 48 h, after which, resazurin solution (10% v/v) was added to the wells and the plates were incubated for 4 h. Fluorescence (Spectramax M2, Molecular Devices LLC, USA) was measured at 550 nm excitation and 590 nm emission wavelength. Fluorescence intensity was expressed as arbitrary units.

In vitro cytotoxicity of lapachol toward the HepG2 cell line

The cytotoxicity concentration (CC_{50}) of the substances against HepG2 cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). Cells were seeded in 96-well flat bottom plates at 5 × 10^4 cells/well in complete RPMI-1640 medium and maintained for 24 h at 37°C in a humidified 5% CO_2 atmosphere. Lapachol was serially diluted two-fold over seven concentrations (from 4.12 mM to 0.016 μM) and added to the plates, which were incubated for 48 h at 37°C in the presence of 5% CO_2. Subsequently, MTT solution (5 mg/mL, 50 μg/well) was added to the wells and the plates were incubated for additional 4 h. The supernatants were aspirated and the formazan crystals formed were dissolved in DMSO. Absorbance was determined in a spectrophotometer at 570 nm (Spectramax M2, Molecular Devices LLC, USA) (Dutta et al., 2005; Mosmann, 1983). Non-treated HepG2 cells and Amb (1.080 mM to 17 μM) were used as controls. Lapachol was tested both for leishmanicidal activity and cytotoxicity in technical triplicates on microplates and the results are representative of three independent experiments (biological triplicate).

Lapachol was tested both for leishmanicidal activity and cytotoxicity in technical triplicates on microplates and the results are representative of three independent experiments (biological triplicate).

Selectivity index

The selectivity index (SI) was determined from the CC_{50}/IC_{50} ratio. Samples with SI value > 20 were considered effective and selective for *L. amazonensis* and *L. infantum* (Nwaka and Hudson, 2006; Rocha et al., 2013; Velásquez et al., 2016).

Efficacy of lapachol against intracellular amastigotes

Immortalized murine macrophages, RAW 264.7, cultured in complete RPMI-1640 medium and maintained at 37°C in an atmosphere of 5% CO_2 and 95% humidity, were seeded (5 × 10^4/well) on a 24-well tissue culture plate containing circular coverslips in each well and incubated (37°C, 5% CO_2) for 4 h to allow cell adherence. Then, *Leishmania* promastigotes in late stationary growth phase were added to interact with the macrophages in the proportion of 10 promastigotes/macrophage/well for 24 h. Subsequently, two-fold serially diluted lapachol (ranging from 660 µM to 41 µM) and Amb (ranging from 0.54 to 0.03 µM) were added to the wells. After 48 h, the coverslips were removed and stained with rapid panoptic, mounted in glass slides using Canada balsam, and analyzed using light microscopy to determine the infection rate of macrophages. The values obtained for each concentration [(number of infected macrophages/300 counted macrophages) × 100] were used to obtain the amastigote intracellular IC_{50} value for lapachol. The IC_{50} for the effect of lapachol on the intracellular amastigotes was expressed as the concentration necessary to halve the number of the infected macrophages compared to non-treated control cells (Vermeersch et al., 2009).

Flow cytometry analysis

Externalized phosphatidylserine in *L. amazonensis* promastigotes

Exponential-phase *L. amazonensis* promastigotes (1 × 10^7cells/mL) were incubated with the test samples (at the pre-determined IC_{50} concentration) for 48 h at 24°C in a B.O.D. incubator. Cells were then centrifuged (900 × g for 10 min) at room temperature, washed in PBS, and resuspended in annexin V binding buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM $CaCl_2$]. Then, annexin V-FITC and propidium iodide (PI) (FITC Annexin V apoptosis detection kit, BD Biosciences, NJ, USA) were added according to the manufacturer's instructions. Data was acquired using a FACScan flow cytometer (BD Biosciences, NJ, USA) and analyzed using the FlowJo_V10™ software. Each experiment was performed at least three times in triplicate.

Cell cycle analysis

Promastigotes were treated with the pre-determined IC_{50} concentrations of the test substances and incubated for 48 h at 24 ± 1°C in 25 cm^2 culture flasks in a B.O.D. incubator. Then, 1 × 10^7/mL cells were washed once in PBS, fixed overnight in 70% (v/v)

ethanol, and stored at −20°C until analysis. Prior to analysis, promastigotes were washed in PBS, the resultant pellet resuspended in PBS containing 10 μg/mL of propidium iodide (PI) and RNase A, and incubated in the dark at 37°C for 30 min. The DNA content of PI-stained cells was analyzed in a FACScan flow cytometer (BD Biosciences) using the FlowJo_V10™ software. Each experiment was performed at least three times in triplicate.

In vivo efficacy of lapachol against leishmaniasis

The present study was approved by the Ethical Committee for Animal Experimentation of the Federal University of Uberlândia (protocol number 069/2013), and all procedures were performed according to the international guidelines (Principles of Laboratory Animal Care (1985)).

Cutaneous leishmaniasis model

Eighteen BALB/c mice (8-week-old females) were infected with 1×10^7 metacyclic promastigotes of *L. amazonensis* in the tail base via subcutaneous route. The mice were randomly divided into three groups 30 days after infection: (a) lapachol (n = 6; 25 mg/kg for 24 h oral route for 10 days), (b) Amb (n = 6; 5 mg/kg for 24 h; intraperitoneal route for 10 days), and (c) PBS (n = 6; oral route for 24 h over 10 days).

The animals were euthanized after the treatment and skin lesions were collected and used for determining parasitic load using quantitative polymerase chain reaction (qPCR).

Genomic DNA was extracted using a commercial kit (RealiapPrep ™ gDNA tissue miniprep system kit, Promega Co., USA) according to the manufacturer's instructions. DNA concentration and the 260 nm/280 nm ratio were determined using a spectrophotometer (NanoDrop™ Lite Spectrophotometer, Thermo Fisher Scientific, USA) and the samples were stored at −20ºC until further processing.

qPCR was performed in 96-well plates in triplicate and processed in a thermocycler (StepOne ™ Real-Time PCR System, Applied Biosystems, USA). To determine the parasite load, primers (L150 [5'-GGG (G/T)AG GGG CGT TCT(G/C)CG AA-3'] andL152 [5'-(G/C)(G/C)(G/C) (A/T)CT AT(A/T) TTA CAC CAACCC C-3']), which amplify a 120 bp fragment of the conserved region of *Leishmania* sp. kDNA minicircles, were used (Degrave et al., 1994). The reactions were performed in 25 μL final volume containing 100 nM of each primer, 1× SYBR Green PCR Master Mix (Applied Biosystems, USA), and 50 ng DNA template.

The number of *Leishmania* DNA copies in each sample was determined from linear regression after comparing to a standard curve generated with known amounts of total *Leishmania* DNA.

The gene encoding TNF-α is constitutively expressed in all murine cells and was hence used to verify DNA integrity of the samples using conventional PCR using primers (TNF-5241 5 'TCCCTCTCATCAGTTCTATGGCCCA 3' and TNF-5411 5 'CAGCA AGCATCTATGCACTTAGACCCC 3'), which amplify a 170 bp fragment (Cummings and Tarleton, 2003).

In all assays, the efficiency of the amplification was close to 100% and the standard curves presented correlation coefficients ranging from 0.97 to 0.99 (Bustin et al., 2009)

Visceral leishmaniasis model

Eighteen BALB/c mice (8-week-old females) were infected with 1×10^7 metacyclic promastigotes of *L. infantum* via the intraperitoneal route. Twenty days after infection, mice were randomly divided into three groups: (a) lapachol (n = 6; 25 mg/kg for 24 h oral route for 10 days), (b) amphotericin B (n = 6; 5 mg/kg for 24 h; intraperitoneal route for 10 days), and (c) PBS (n = 6; oral route for 24 h over 10 days).

The animals were euthanized after treatment, and liver and spleen were collected and used to determine the parasitic load using qPCR as described above.

Statistical analysis

The dose-response curves (IC_{50} and CC_{50}) were generated using the Origin 8.5 software (Originlab Co., USA). Flow cytometry and qPCR analyses were performed using the GraphPad Prism software version 6.0 (GraphPad Software, CA, USA). The samples distributions were verified using the Kolmogorov-Smirnov test, following the most appropriate test for each case. Differences between lapachol, Amb, and control group in the qPCR analysis and flow cytometry were evaluated using the one-way analysis of variance (ANOVA), followed by Tukey's post-hoc multiple comparison test. A significance level of 5% ($p < 0.05$) was observed in all tests.

2.4. Results and discussion

Lapachol presented high CC_{50} values in the in vitro assays using HepG2 cells, suggesting low cytotoxicity of this compound **(Table 1)**. The relatively low cytotoxicity

of lapachol toward HeG2 and other cell lines, such as murine macrophages and modified THP-1 cells has been reported previously (Costa et al., 2017; Rocha et al., 2013; Teixeira et al., 2001). The search for new leishmanicidal drugs with better efficacy, lower toxicity, and lesser adverse side effects than the current drugs is an important criterion to be considered (Oryan, 2015; WHO, 2010).

Table 1 - In vitro antileishmanial activity against *Leishmania infantum* and *Leishmania amazonensis* promastigotes, cytotoxicity against HepG2 cells and selectivity index of the lapachol

Substances	**CC_{50} µM (HepG2)**	**IC_{50} µM**		**SI**	
		L. infantum	*L. amazonensis*	*L. infantum*	*L. amazonensis*
Lapachol	3,405.8 ± 261.33*	135.79 ± 33.04	79.84 ± 9.10	25.08	42.65
Amphotericin b	36.75 ± 6.24	0.051 ± 0.02	0.059 ± 0.02	720.58	622.88

*Results are representative of three independent experiments preformed in triplicates

In addition to its low cytotoxicity, lapachol showed good leishmanicidal activity in vitro against the two *Leishmania* species tested. High CC_{50} and low IC_{50} against *L. amazonensis* and *L. infantum* resulted in SI values > 20 (42.65 and 25.08 for *L. amazonensis* and *L. infantum*, respectively), suggesting that lapachol was effective against and selective for the strains of *L. amazonensis* and *L. infantum* tested (**Table 1**). In contrast, the SI values for lapachol were lower than that of Amb as this naphthoquinone has higher IC_{50} against these two *Leishmania* species. Indeed, Amb is one of most effective leishmanicidal drug presently in clinical use; however, it is toxic (Amb desoxicholate) and expensive (liposomal Amb formulations) (Ministry of Health Brazil, 2011). Other studies have demonstrated that lapachol was effective against promastigote forms of *L. amazonensis* and *L. braziliensis* (Lima et al., 2004;Rocha et al., 2013; Costa et al., 2017),; however it is noteworthy that this is the first study showing the moderate in vitro efficacy of lapachol against *L. infantum*, a causative agent of VL (Costa et al., 2017; Lima et al., 2004; Rocha et al., 2013).

Natural products and plant extracts have been variably classified in literature based on their activities. For example, studies investigating the activity of lapachol against *Leishmania* show divergences. Costa et al. (2017) found lapachol to be active against *Leishmania* when the $IC_{50} \leq 100$ µg /mL (412 µM); however, Rocha et al. (2013) defined

lapachol as inactive because its IC_{50} was > 10 μg /mL and was toxic for the presented SI < 20. Lima et al. (2004) tested lapachol and its analogs against *L. amazonensis* and considered it to be an active product in the IC_{50} range of 1.6−7.8 μg/mL. Although naphthoquinones are considered cytotoxic and show relatively low SI, (Ali et al., 2011), lapachol showed SI > 20 for both *Leishmania* species and HepG2 cells in this study. Variation in the response of different *Leishmania* strains and cell lines toward different compounds (*i.e.* plant extracts or pure substances such as lapachol) should also be considered in this case.

After testing the cytotoxicity and leishmanicidal activity in promastigotes, the activity of lapachol was tested against intracellular amastigotes. Results suggest that lapachol was able to reduce the rate of infection of macrophages by both *L. amazonensis* and *L. infantum* intracellular amastigotes (IC_{50} = 191.95 μM and 171.26 μM, respectively) compared to the control. Previous studies have shown the efficacy of synthetic naphtoquinones against promastigotes and amastigotes of *L. infantum* (Pinto et al., 2014). However we demonstrated for the first time the moderate efficacy of lapachol against the amastigote forms of *L. infantum.*

The in vitro results obtained for *L. amazonensis* in this study differ from those reported previously irrespective of the parasite form evaluated. Rocha et al. (2013) obtained an IC_{50} of 15.48 μg/mL against intracellular amastigotes, whereas Lima et al. (2004) determined it to be 5.0 μg/mL, and Costa et al. (2017) showed that lapachol reduced macrophage infection, with the highest effect observed at 250 μg/mL (1.02 mM) in the 24 h period. These differences in IC_{50} values can be related to the different strains of *L. amazonensis* used in these studies and experimental conditions, as well as to differences in cell lines, parasite infection rate, and time of treatment.

We calculated the SI of lapachol from the results of the in vitro experiments. According to Velásquez et al. (2016), SI value close to 10 indicates that the compound should be evaluated for further studies. However, Rocha et al. (2013) demonstrated toxicity of lapachol when determining CC_{50} with peritoneal macrophages because it had SI < 20, which, according to Nwaka and Hudson (2005), indicates toxicity. According to Albernaz et al. (2010), high SI value suggests higher confidence regarding the compounds tested. Therefore, as SI was > 20 in our study, we consider lapachol to possess good leishmanicidal activity, and thus decided to proceed with the in vivo experiments.

Despite the anti-*Leishmania* activity of lapachol against promastigotes and amastigotes of *L. amazonensis* and *L. infantum*, this compound appears to be non-toxic to macrophages, as no morphological abnormalities were observed, even when treated with the highest concentration (4.12 mM) of lapachol. These results suggest that lapachol can be considered a promising candidate for therapeutic use as it appears to efficiently affect the intracellular parasites instead of the host cell (Oryan, 2015) (**Fig. 1**).

The currently used anti-leishmanial drugs induce parasite death by apoptosis (Moreira et al., 2011; Verma et al., 2007; Vincent et al., 2013). Apoptosis correlates with phosphatidylserine exposure and cell-cycle arrest at sub-G0/G1 phase. Hence, we determined whether lapachol induces apoptotic death of the parasite by assessing the expression of phosphatidylserine (a marker of apoptotic cell death).

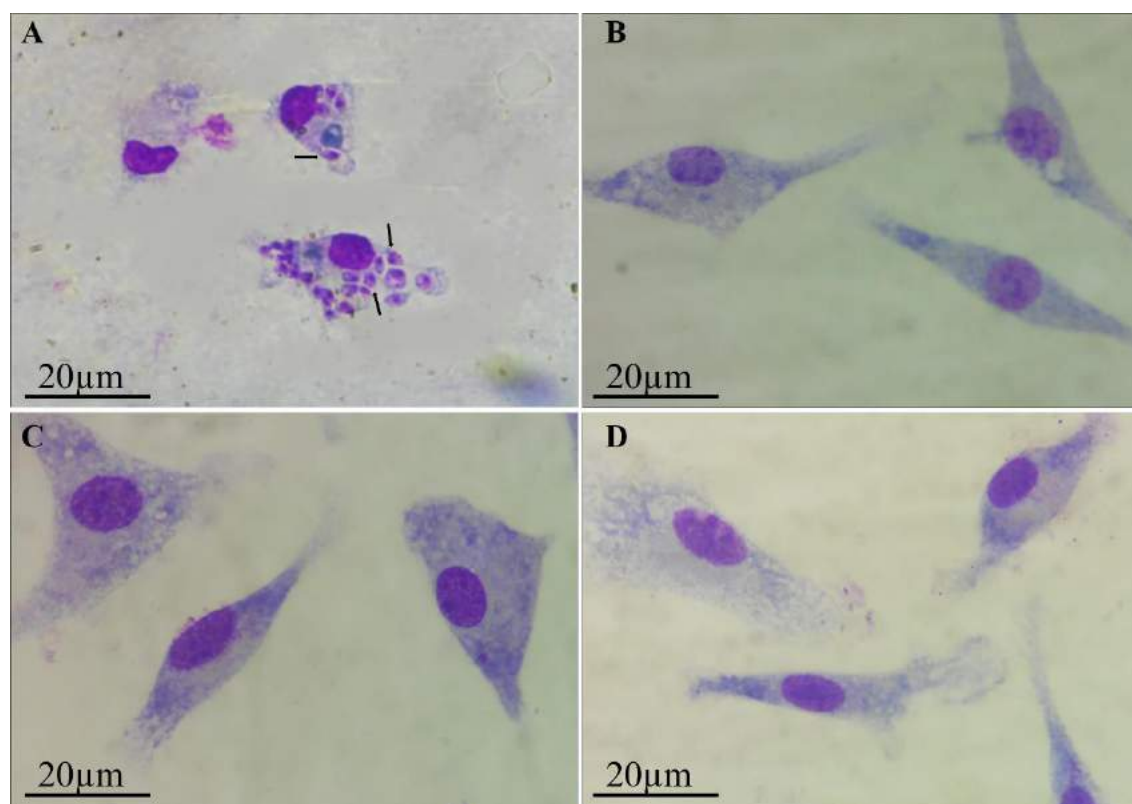

Fig 1. - Antiamastigote activity of lapachol. (A) Infected macrophage, (B) after treatment with amphotericin B (0.27 μM), (C) after treatment with lapachol (165 μM), and (D) uninfected macrophages treated with lapachol (660 μM). Magnification: 100x

Treated and untreated promastigotes were double-stained with FITC-annexin V and PI. Significant proportions (65.86%) of promastigotes treated with lapachol (38.23% in early apoptosis and 27.63% in late apoptosis) stained positively for annexin V, compared to 17.10% (9.15% in early apoptosis and 7.94% in late apoptosis) in untreated cells **(Fig.**

2A and B). The level of apoptosis after lapachol treatment was comparable to that triggered by the positive control Amb, which reached 79.10% (36.36% in early apoptosis and 42.75% in late apoptosis) **(Fig. 2C)**. These results suggest that lapachol exerts leishmanicidal activity probably via apoptosis

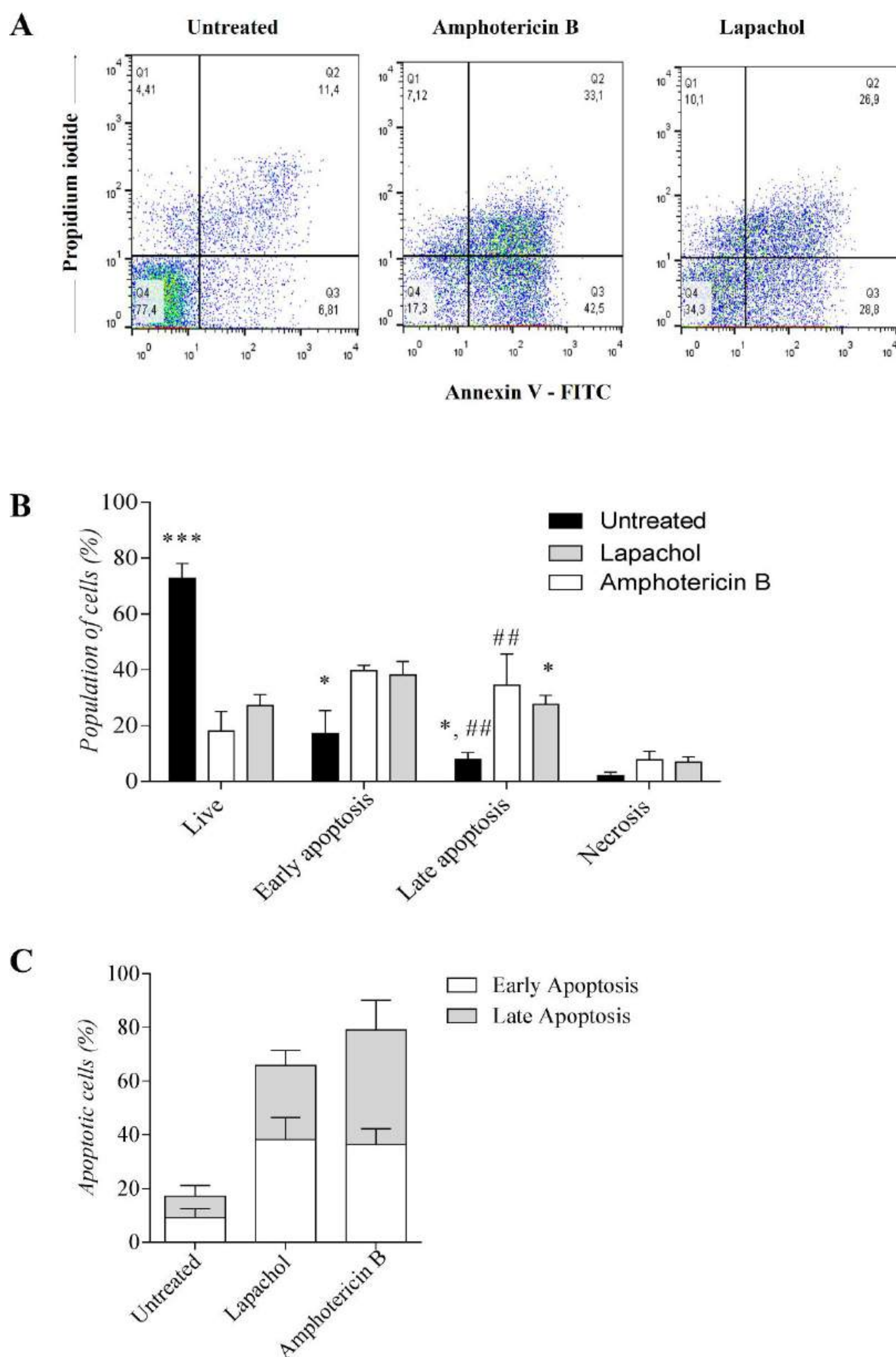

Fig. 2 – Externalization of phosphatidylserine in lapachol-treated *Leishmania amazonensis* promastigotes. Promastigotes (1×10^{7} cells/mL) were incubated with IC_{50} dose of lapachol (79.8 μM) for 48 h, co-stained with PI and annexin V–FITC, and analyzed using flow cytometry as described in Material and Methods. Amphotericin b

was used as the reference drug and parasites without treatment were used as control. (A) Dot plot; the lower-left quadrant (Q4) indicates the percentage of unstained cells (live), the upper-left (Q1) shows PI-positive cells (necrosis), the lower-right (Q3) shows annexin V-stained cells (apoptosis early), and the upper-right (Q2) shows PI- and annexin V-positive cells (apoptosis late). The figure shows a representative result of at least three experiments. (B) Percentage of live, apoptotic (early and late), and necrotic cells in three independent experiments. (C) Percentage of apoptotic cells (early and late) in three independent experiments. Data show means and SEM. * $p < 0.05$; ** $p < 0.01$; $^{\#\#}$ $p < 0.01$ (between untreated parasites and amphotericin B); *** $p < 0.01$, according to ANOVA, followed by Tukey´s *post-hoc* multiple comparison test.

In addition, we assessed the cell cycle of promastigotes treated with IC_{50} dose of the substances after 48 h. In promastigotes incubated with lapachol (79.8 μM for 48 h), the proportion of cells in the sub-G0/G1 phase increased to 41.10% compared to 12.4% for control cells and 85.56% for Amb-treated cells (**Fig. 3**). This lapachol-induced increase in the sub-G0/G1 phase was accompanied by a decrease in the number of cells in the G0/G1 (37.10 %) phase compared to untreated cells (62.53 %) at 48 h. Amb-treated cells showed reduction in cell number in all other phases **(Fig. 3)**. Thus, these results indicated that lapachol arrests *Leishmania* promastigote proliferation, with an increase in sub-G0/G1 phase population.

Taken together, the induction of phosphatidylserine exposure and cell-cycle arrest at sub-G0/G1 phase, both hallmarks of classic apoptosis, confirm the apoptotic-like cell death in *L. amazonensis* promastigotes upon lapachol treatment. Different substances are able to cause apoptosis in *Leishmania* parasites, including Amb (Moreira et al., 2011), miltefosine (Verma et al., 2007), and staurosporine (Arnoult et al., 2002). In addition, other natural products such as *Aloe vera* leaf exudate (Dutta et al., 2007) and artemisinin (Sen et al., 2007) also lead to apoptosis in *Leishmania* sp..

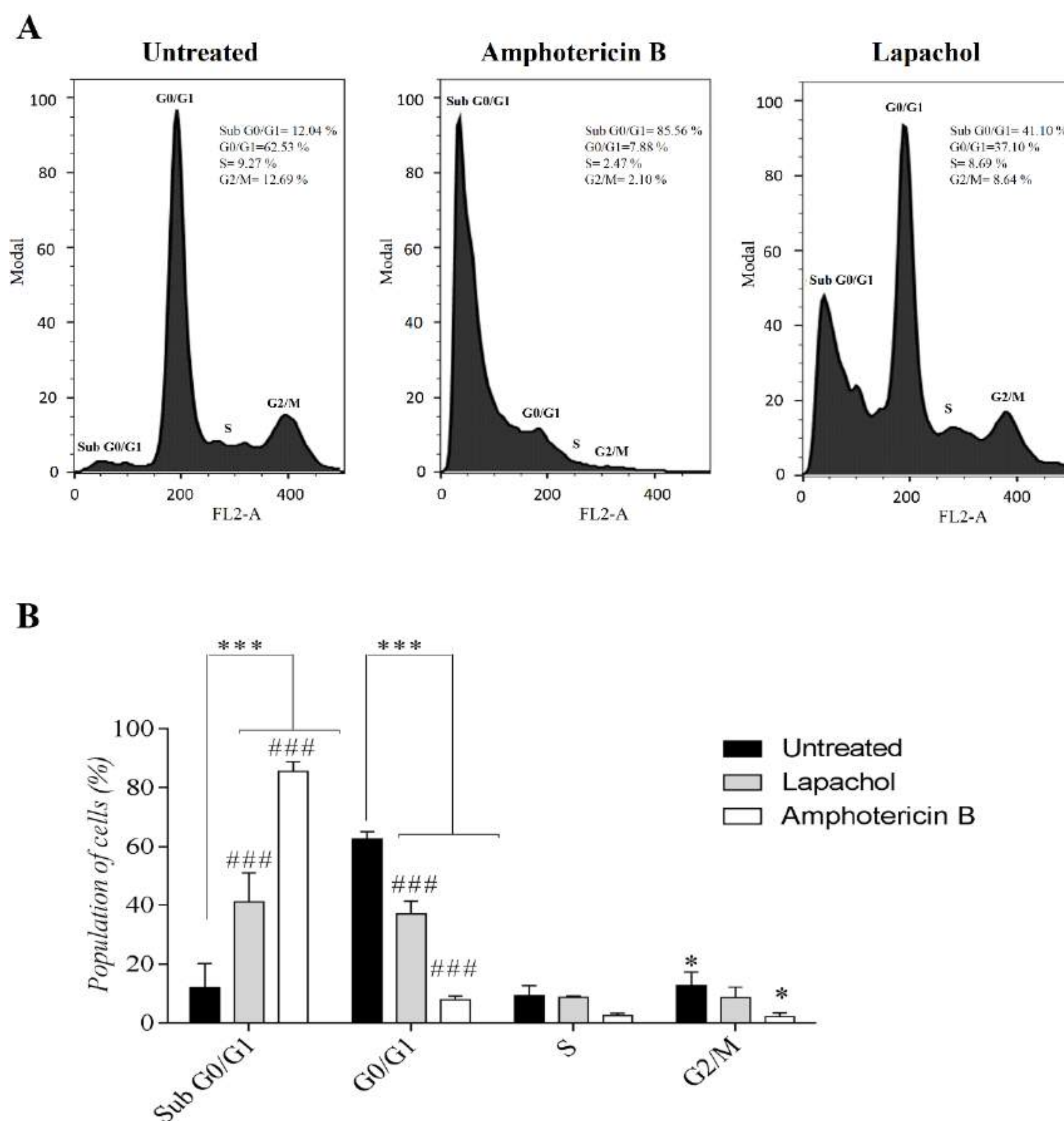

Fig. 3 – Effect of lapachol on the cell cycle of *Leishmania amazonensis* promastigotes. *L. amazonensis* promastigotes (1×10^7cells/mL) were treated with IC_{50} dose of lapachol (79.8 µM) for 48 h. Amphotericin b was used as the reference drug and parasites without treatment were used as control. Cells were processed for cell-cycle analysis as described in Material and Methods. (A) Typical cell cycle profile showing each phase. (B) Percentage of cells in the sub G0/G1, G0/G1, S, and G2/M phases, obtained in three experiments, are indicated in the histogram. Data represent mean ± SEM. * $p < 0.05$; ***$p < 0.01$; $^{###}p < 0.001$, according to ANOVA, followed by Tukey´s *post-hoc* multiple comparison test.

Although lapachol exhibits a wide spectrum of biological activities, such as analgesic (Grazziotin et al., 1992), anti-inflammatory (Almeida et al., 1990), antitumoral

(Eyong et al., 2008; Maeda et al., 2008), molluscidal (Dos Santos et al., 2000), antimicrobial, and antiprotozoal activities (Carvalho et al., 1988; Salas et al., 2008), reports on the cytotoxic effects of lapachol in *Leishmania* leading to apoptosis-like death are lacking. Here we demonstrated for the first time that lapachol causes apoptosis-like death of *L. amazonensis*. This is important, as knowledge regarding the mechanism of action of candidates used for the treatment of any infectious disease is critical for the development of new drugs.

To evaluate the effect of *Leishmania* on host tissues, murine models of CL and VL were submitted to a therapeutic protocol using orally administered lapachol. The animals were euthanized after the treatment. No significant differences in lesion size and macroscopic changes in liver or spleen were observed during sample collection (data not shown). The treatment of CL with lapachol or Amb significantly reduced parasite load in mice skin compared to the control group (negative treatment group) ($p < 0.05$).The mean number of parasites in the skin lesion (5.4×10^8 parasites/mg) determined for the lapachol group was approximately 24.5 times lower than that in the non-treated group (1.324×10^{10} parasites/mg). Similarly, animals treated with Amb (positive treatment group) showed 33.5 times fewer parasites (3.95×10^8) per milligram of skin compared to the non-treated control group **(Fig. 4B).**

Another study demonstrated that oral application of lapachol in hamsters infected with *L. braziliensis* did not significantly reduce the parasite load in the lesion (Teixeira et al., 2001). Although lapachol was administered orally in this study, the model of CL (BALB/c mice instead hamsters), *Leishmania* species, therapeutic protocol, and methodology for evaluating parasitic load were different from that of the previous study, which might explain the differences in the results.

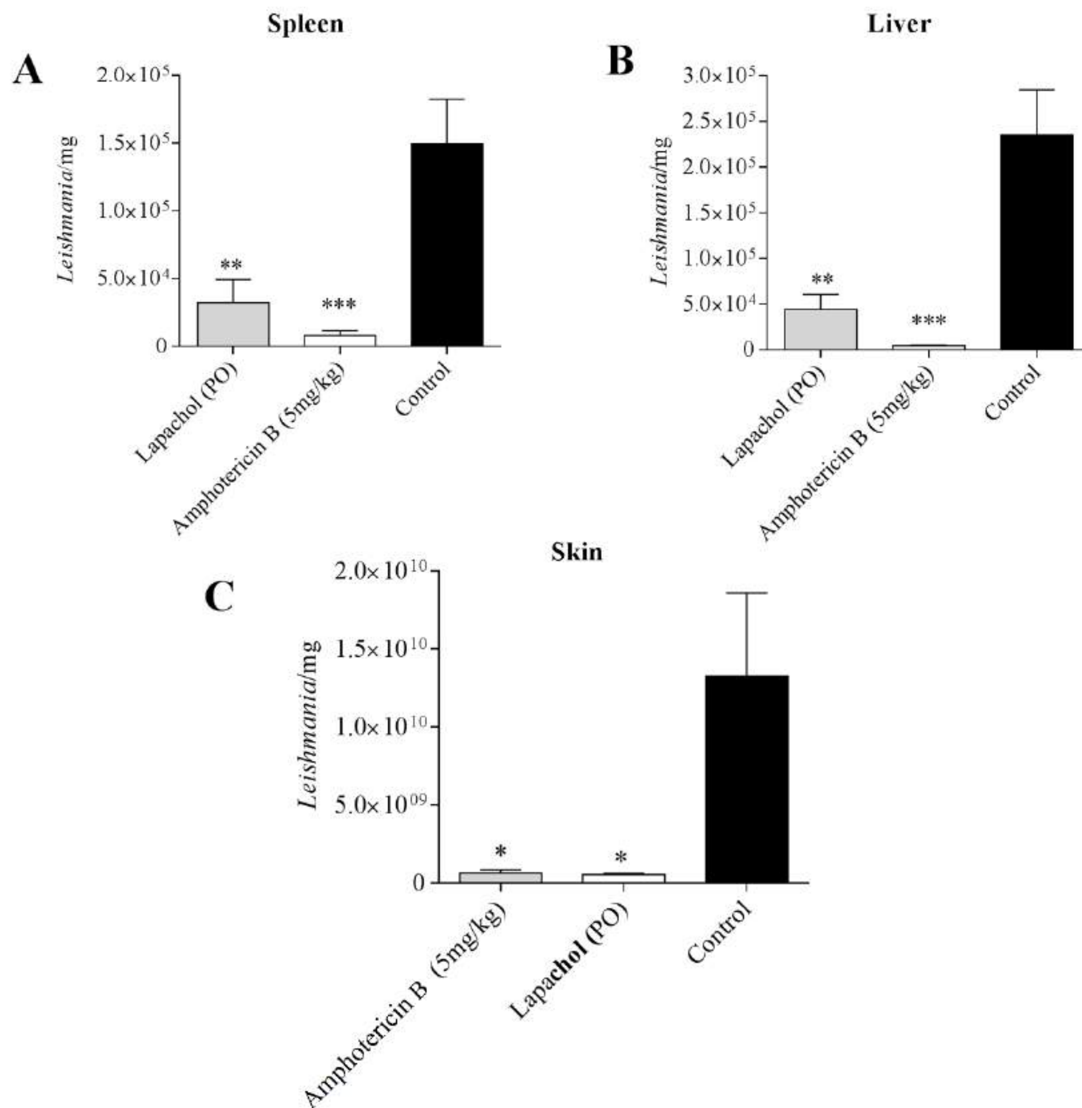

Fig. 4 - Parasite burden in the spleen, liver, and skin of BALB/c mice infected with *Leishmania infantum* (A and B) and *Leishmania amazonensis* (C) after treatment with lapachol (25 mg/kg orally for 24 h over 10 days). Amphotericin b deoxicholate (5 mg/kg via intraperitoneal route for 24 h over 10 days) was used as reference drug treatment and non-treated animals were used as controls (PBS administered orally for 24 h over 10 days). Parasite burden was determined using qPCR as described in Material and Methods. Data represent means ± SEM of each group (n = 6). * $p < 0.05$; **$p < 0.01$; ***$p < 0.01$, according to ANOVA, followed by Tukey´s *post-hoc* multiple comparison test.

Strikingly, lapachol treatment of mice with VL significantly reduced the parasitic load in the spleen and liver of the animals ($p < 0.05$). Mice treated with lapachol presented approximately 4.6 and 5.3 fewer parasites in the spleen and liver, respectively, compared to non-treated animals. When treated with Amb, mice presented approximately 18.6 and

54.4 lesser parasites in the spleen and liver respectively, compared to non-treated animals (**Fig. 4A**).

The search for an oral treatment for leishmaniasis is important since this type of protocol avoids parenteral injections and its complications, reducing expenses associated with ambulatory procedures and hospital care. The development of an effective and inexpensive oral treatment for leishmaniasis should be prioritized when control strategies and guidelines are discussed, as leishmaniasis, a neglected disease, is one of the "diseases of the poorest of the poor" (Alvar et al., 2006).

2.5. Conclusion

We demonstrated for the first time the moderate in vitro efficacy of lapachol against *L. infantum*, and confirmed its moderate in vitro efficacy against *L. amazonensis*, the causative agents of VL and CL, respectively. The leishmanicidal effect of lapachol were confirmed in vivo using murine models of VL and CL where lapachol was able to significantly reduce the parasitic load in the skin lesions, liver, and spleen of treated mice, similar to the reference drug amphotericin B. Furthermore, flow cytometry demonstrated that the cytotoxic effect of lapachol in *Leishmania* promastigotes was due to apoptosis-like cell death. The results reported here represent an advancement in studies on lapachol as a leishmanicidal drug candidate and might promote pre-clinical investigations on toxicity, especially renal and hepatic effects, mechanism of action, and the immune response of treated mice to determine an adequate therapeutic protocol for curing infected mice, ultimately aiming toward the development of new low-cost medicines for the treatment of leishmaniasis. The low cytotoxicity of lapachol, at least against HepG2 cells, will promote new experiments with higher doses of this naphthoquinone, which can be obtained from the lumber industry waste, as Brazil is an exporter of *Tabebuia* sp. wood, a botanical species with high contents of lapachol.

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CAPÍTULO II – ARTIGO PUBLICADO

In vitro efficacy of isoflavonoids and terpenes against *Leishmania (Leishmania) infantum* **and** *L. amazonensis*

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3.1. Abstract

The main form of control of leishmaniasis is the treatment, however various side effects and poor efficacy are associated with presently available drugs. The investigation of bioactive natural products for new antileishmanial drugs is a valid approach. The present study reports the *in vitro* efficacy of natural isoflavonoids and terpenes against *Leishmania infantum* and *L. amazonensis* and their cytotoxicity against HepG2 cells. *L. infantum* and *L. amazonensis* promastigotes were exposed to the terpenes kaurenoic acid, xylopic acid, and (-)-α-bisabolol and to the isoflavonoids (-)-duartin and (3R)-claussequinone for antileishmanial activity and to cytotoxicity to HepG2 cells. The most effective substance against both *L. infantum* and *L. amazonensis* species was (3R)-claussequinone (IC_{50} = 3.21μg/mL and 2.47μg/mL, respectively) that disclosed low cytotoxicity against HepG2 cells (CC_{50} = 387.79μg/mL). The efficacy of (3R)-claussequinone against intracellular amastigotes of *L. infantum* and the externalization of phosphatidylserine in promastigotes of this isoflavanoid were investigated by infection of Raw 264.7 macrophages and marking with Annexin V-FITC and propidium Iodide for flow cytometry analysis. The results for amastigotes showed that (3R)-claussequinone was able to reduce the rate of infection with IC_{50} = 4.61μg/mL and did not alter the externalization of phosphatidylserine. In conclusion it is presently reported, for the first time, the striking antileishmanial activity of (3R)-claussequinone against *L. infantum* and

L. amazonensis associated to low cytotoxicity. Furthermore, these results suggest that (3R)-claussequinone is a new hit aiming to develop new therapeutic alternatives.

Keywords: Isoflavonoids, Terpenes, Claussequinone, Bisabolol, *Leishmania*

3.2. Introduction

Leishmaniasis is a complex of diseases caused by the invasion of protozoan parasites of the genus *Leishmania* into the mononuclear phagocytic system of mammalian hosts that can compromise viscera, skin and mucosa. This vector-borne disease threatens ~350 million people worldwide mainly in the tropical and subtropical areas. It is estimated that ~0.9 and 1.7 million people are infected with 20,000–30,000 deaths per year. Over 90% of new leishmaniasis cases occur in Afghanistan, Algeria, Bangladesh, Bolivia, Brazil, Colombia, Ethiopia, India, Iran, Peru, South Sudan, Sudan and Syria (Desjeux, 2004; WHO, 2021). Leishmaniasis are one of the neglected poverty related diseases and it is estimated that the majority of infected patients live with less than US$ 1 per day (Alvar et al., 2006).

The diagnosis and effective treatment of patients can reduce the prevalence of the disease (WHO, 2021). Despite of its limitations, chemotherapeutical treatment still remains as the main control measure for all clinical forms of leishmaniasis (WHO, 2021). Even though the use of pentavalent antimonials, such as meglumine antimoniate (MA, Glucantime®) and sodium stibogluconate (SSG, Pentostam®), present several limitations, these drugs have been used for more than half a century in the therapy of leishmaniasis as first-line drugs (González et al., 2009; Name et al., 2005).

The large-scale use of antimonials has led to the selection of parasites that have mechanisms of resistance to these drugs. Therefore, other drugs are used for the treatment of leishmaniasis. Main drugs used for the treatment of leishmaniasis are amphotericin b deoxycholate, liposomal amphotericin B, miltefosine and pentamidine (Chappuis et al., 2007; Croft and Coombs, 2006). However, these current antileishmanial agents present several limitations including low efficacy, toxicity, adverse side effects, drug-resistance, long-term treatment and high cost (Kedzierski et al., 2009; Sundar et al., 2012).

In the light of the limitations of the current therapeutic arsenal and strategies, the WHO strongly recommends and supports research into new drugs against leishmaniasis

(Ridley, 2003). However, a lack of commercial return from drug development and of political support in the case of neglected diseases, such as leishmaniasis, has resulted in insufficient funding and commitment from both public sector agencies and the pharmaceutical industry (Ridley, 2003). Another factor is that most of the medications used in the treatment of leishmaniasis are not new chemical entities, but reused treatments (Olías-Molero et al., 2021).

In this context, new therapeutic options are being researched for the treatment of leishmaniasis, seeking a more effective and less toxic surgery. Research in the field of vaccine, pharmacological repositioning and rejuvenation, drug association, search for new synthetic and/or natural products derived from flora represents a valid technique in the search for new antileishmanials (Santiago et al., 2021; WHO, 2021). Indeed, the interest in the investigation of medicinal plants and natural products for the treatment of leishmaniasis and others parasitic diseases has been grown in recent years (Batista et al., 2009; Lima et al., 2015).

Brazil has a great diversity of flora throughout its territory, estimated at around 20% of the total number of species on the planet (Ministry of the Environment Brazil, 2021). The *National Policy on Integrative and Complementary Practices* (NPICP) was created by the public health system aiming to expand the therapeutic options, with a guarantee of access to medicinal plants, herbal medicines and services related to phytotherapy, with safety, efficacy and quality, from the perspective of integral health care (Ministry of Health Brazil, 2006). Several plants are used by native populations, in different regions of the country, to treat various infectious diseases, such as malaria and leishmaniasis (Brito and Brito, 1993; Alves et al., 2000). This popular\traditional knowledge, or ethnopharmacology, may be useful in the search for new active compounds and development of phytomedicines against these diseases, with reduced side effects and low cost.

The present study investigated the *in vitro* effects of five different natural products isolated from plants, in order to evaluate their activity against *L. infantum* and *L. amazonensis*. Their inhibitory concentrations (IC_{50}) against promastigote forms, as well as their cytotoxic effects (CC_{50}) on cells derived from a human primary hepatoblastoma (HepG2), were determined and the selectivity index of each substance was calculated (SI = CC_{50} / IC_{50}).

3.3. Material and methods

Chemicals

Amphotericin B deoxycholate, culture medium α-MEM (Minimum Essential Medium Eagle) and RPMI 1640 (Roswell Park Memorial Institute), antibiotics penicillin and ampicillin, L-glutamine, hydroxyethyl piperazine ethanesulfonic (HEPES), dimethyl sulfoxide (DMSO), resazurin sodium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchase from Sigma-Aldrich Co. LLC. (USA). Gibco® fetal calf serum (FCS) was purchased from Thermo Fischer Scientific Inc. (USA).

Assayed natural products

The diterpenes kaurenoic acid and xylopic acid were isolated from *Xylopia frutescens* (Melo et al., 2001; Takahashi et al., 1995), (-)-α-bisabolol was isolated from *Eremanthus erythropappus* (Atina, 2021). The isoflavonoids (-)-duartin and (3R)-claussequinone were isolated from *Machaerium villosum* (Kurosawa et al., 1968; Oliveira et al., 1968) and *Cyclolobium clausseni* (Oliveira et al., 1971), respectively. The chemical structures of these compounds are represented in Fig 1. DMSO was the solvent for stock solutions (50mg/mL) of the substances.

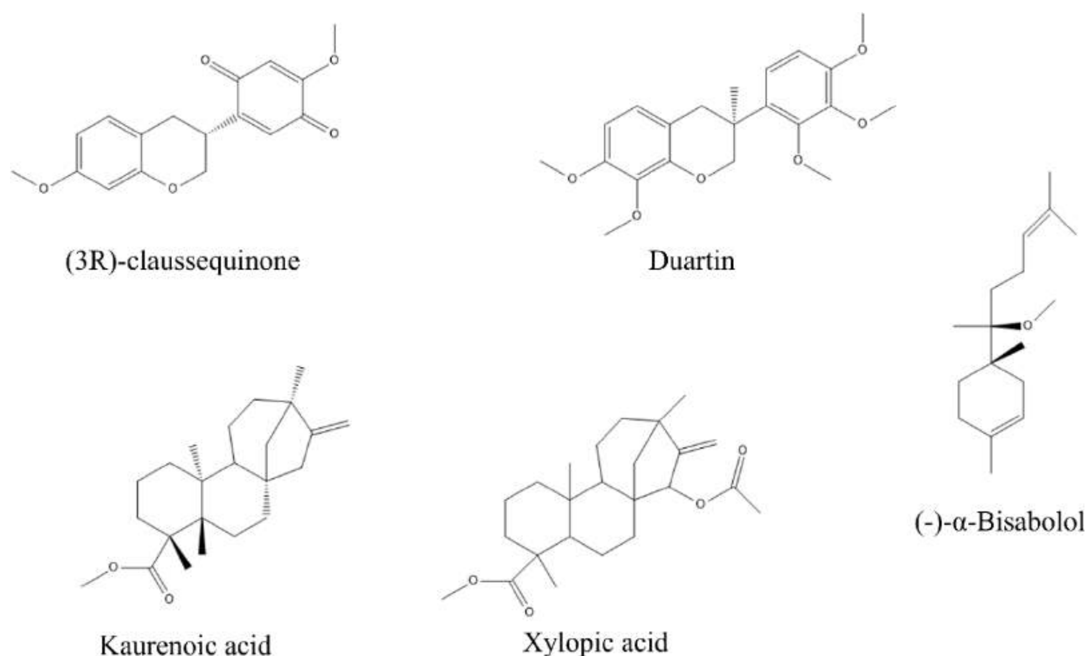

Figure 1 - Chemicals structures of substances (3R)-claussequinone, Duartin, Kaurenoic acid, Xylopic acid and (-)-α-Bisabolol.

Parasites and cells

WHO reference strains of *Leishmania (Leishmania) infantum* (MCAN/BR/2002/BH401) and *L. amazonensis* (MHOM/BR/1989/Ba199), were maintained as promastigotes forms in complete α-MEM supplemented with 10% (v/v) heat inactivated FBS, 100µg/mL penicillin and 100µg/mL ampicillin, 2mM L-glutamine, 20mM HEPES, pH 7.2, at 24±1°C in 25cm^2 culture flasks on BOD incubator.

The HepG2 A16 cell line (ATCC, USA), derived from a human primary hepatoblastoma, was cultured in 25cm^2 cell culture plastic bottles in complete RPMI 1640 medium (10% v/v FBS, 100µg/mL penicillin and 100µg/mL ampicillin, 2mM L-glutamine, 20mM HEPES, pH 7.2). The cells were maintained at 37°C, 5% CO_2 and 95% humidity.

In vitro efficacy of substances on the viability of *L. infantum* and *L. amazonensis* promastigotes

The inhibitory concentration (IC_{50}) of the substances kaurenoic acid, xylopic acid, (-)-α-bisabolol, (-)-duartin and (3R)-claussequinone against promastigotes of *L. infantum* and *L. amazonensis* were evaluated by the resazurin-based colorimetric assay (Corral et al., 2013). Log-phase promastigotes (2.5x10^5 parasites/well) were seeded in flat-bottom 96-well cell culture plates in complete α-MEM medium and incubated at 26°C. The substances were 2-fold serially diluted over eight concentrations (200 to 1.56µg/mL) in complete α-MEM medium and tested in triplicate at each concentration. Amphotericin B deoxycholate was used as a positive control (1000-15.6ng/mL). Non-treated parasites were used for viability comparison. Then the substances were incubated for 48h. After these incubation period 10% v/v of a resazurin solution (0.15mg/mL) was added on the wells and plates were incubated for 4h. Then fluorescence was measured at 550nm excitation and 590nm emission wavelength (Spectramax M2, Molecular Devices LLC, USA). Fluorescence intensity was expressed as arbitrary units.

In vitro cytotoxicity of the substances on the HepG2 cell line

The cytotoxicity concentration (CC_{50}) of the substances kaurenoic acid, xylopic acid, (-)-α-bisabolol, (-)-duartin and (3R)-claussequinone against HepG2 was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). Cells were seeded in 96-well flat bottom plates at 5x10^4 cells/well in

complete RPMI 1640 medium and maintained for 24h at 37°C in a humidified 5% CO_2 atmosphere. The substances were 10-fold serially diluted over four concentrations (1000 to 1µg/mL), added to the plates which were incubated for 48 hours (37°C, 5% CO_2). After the incubation period MTT solution (5mg/mL, 50µg/well) was added to the wells and the plates were incubated for additional 4h. Supernatants were aspirated and formazan crystals formed were dissolved in DMSO. Absorbance was determined in spectrophotometer at 570nm (Dutta et al., 2005; Mosmann, 1983).

All substances were tested for antileishmanial activity and cytotoxicity in technical triplicates on microplates and the results are representative of three independent experiments (biological triplicate).

Selectivity index

The selectivity index (SI) was determined by the ratio CC_{50}/IC_{50}. Samples with SI values greater than 10 were considered effective and selective for *L. amazonensis* and *L. infantum* (Lenta et al., 2007; Ribeiro et al., 2014).

By account to its greater clinical-epidemiological importance, *L. infantum* was chosen for the following experiments.

Efficacy of (3R)-claussequinone against intracellular *L. infantum* amastigotes

Immortalized murine macrophages RAW 264.7 was cultured in complete RPMI 1640 medium and maintained at 37°C, 5% CO_2 and 95% humidity. The macrophages were seeded (5×10^4/well) on a 24-well tissue culture plate contained a circular coverslip per well and incubated (37°C, 5% CO_2) for cell adherence (4h). Then *Leishmania* promastigotes in late stationary growth phase were added to interact with the macrophages in the proportion of 10 promastigotes/macrophage/well for 24 hours. After this period, (3R)-claussequinone were added to the wells in two-fold diluted concentrations ranging from 20 to 1.25µg/mL. Amphotericin B deoxycholate was used as a positive control (500-31.25 ng/mL). The other controls of the experiment were: infected macrophages incubated only with complete medium, and macrophages not infected with (3R)-claussequinone and amphotericin B. After 48h the coverslips were removed and stained with rapid panoptic, mounted in glass slides using Canada balsam and analyzed on light microscope in order to determine the infection rate of macrophages.

The values obtained for each concentration were used to obtain the amastigote intracellular IC_{50} value for (3R)-claussequinone.

The percentage of infected macrophages (number of infected macrophages x 100 / number of macrophages counted) and the mean number of intracellular amastigotes (number of amastigotes per cell/number of infected macrophages) were determined after counting 300 macrophages per coverslip, in triplicate. (3R)-claussequinone and amphotericin B were compared to the infection rate of the control without the drugs (100 % infection). The values obtained for each concentration were used to obtain the IC_{50}.

Flow cytometry analysis

Flow cytometry experiments were performed in the Laboratory of Flow Cytometry at the Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil (http://labs.icb.ufmg.br/citometria/).

Externalized phosphatidylserine in *L. infantum* promastigotes

Exponential-phase *L. infantum* promastigotes (1×10^7/mL) were incubated with an IC_{50} concentration for 48h, of each substance. Cells were centrifuged (900g for 10min), washed in PBS and resuspended in annexin V binding buffer [10mM Hepes/NaOH (pH 7.4), 140mM NaCl, 2.5mM $CaCl_2$]. Annexin V-FITC and propidium Iodide were then added according to the manufacturer's instructions. Data acquisition was carried out on a FACScan flow cytometer (BD Biosystems) using FlowJo_V10™ software. Each experiment was performed at least three times in triplicate.

Statistical Analysis

The results were evaluated in the Origin 8.5 program with determination of the dose-response curves. The growth inhibitory concentrations of 50% of the parasites (IC_{50}) and cells (CC_{50}) were compared to drug-free controls.

3.4. Results and discussion

Evaluation of natural products extracted of plants from against *Leishmania* sp. and other parasites has disclosed potent antiparasitic effects providing promising leads for the development of new drugs and supporting the use of herbal medicines in the treatment of some parasitic diseases (Andrews et al., 2014; Ndjonka et al., 2013; Singh et al., 2014; WHO, 2021).

The results obtained in this study demonstrated that, excepting the diterpenes kaurenoic acid and xylopic acid, all natural products presently assayed showed activity against promastigote forms with $SI \geq 10$. Due to the low activity of kaurenoic acid and xylopic acid the next experiments were not performed with these substances. Thus (-)-α-bisabolol, (-)-duartin and (3R)-claussequinone were considered effective against *L. infantum* and *L. amazonensis*, in the *in vitro.* Since the results of $SI > 10$ suggest that the substances are promising against *Leishmania* species. The IC_{50}, CC_{50} and SI values of the substances are shown in Table 1. Among all the substances tested, (3R)-claussequinone presented a higher SI for this reason it was tested in amastigote forms of *L. infantum* were tested. The highest concentrations of (3R)-claussequinone showed a significant difference in macrophages infected and in the reduction of amastigotes when compared to the control group. All concentrations reduced amastigotes (IC_{50}: 4.16 µg/mL) without altering macrophage morphology. Likewise, the positive control amphotericin B showed a reduction in the amastigote forms at all concentrations tested when compared to the control group (IC_{50}< 31.25 ng/mL) (**Fig 2** and **Table 2**).

(3R)-Claussequinone is an isoflavonoid isolated from *Cyclolobium clausseni* (Oliveira et al., 1971). Chiari et al. (1991) and Sepúlveda-Boza & Cassels (1996) firstly demonstrated the antiparasitic activity of (3R)-claussequinone against *Trypanosoma cruzi*. Takahashi et al. (2006) tested (3R)-claussequinone against *L. major* promastigotes and found moderate activity.

For promastigotes, (3R)-claussequinone presented the lowest IC_{50} for both *Leishmania* species and higher CC_{50} resulting into a remarkable antileishmanial activity against *L. infantum* ($SI > 120$) and *L. amazonensis* ($SI > 150$). For intracellular amastigotes of *L. infantum*, the results suggest that (3R)-Claussequinone was able to reduce the rate of infection of macrophages in relation to the control non-treated (IC_{50}: 4.61µg/mL). These striking results suggest an urgent need of testing (3R)-claussequinone against models of visceral and cutaneous leishmaniasis, in vivo, as well studies to elucidate its mechanism of action and potential synergistic/additive effects when associated with antileishmanial drugs, such as pentavalent antimonials, miltefosine and amphotericin b. To the best of our knowledge, this is the first experimental demonstration of the *in vitro* efficacy of (3R)-claussequinone against *L. amazonensis* and *L. infantum*.

Table 1 - In vitro antileishmanial activity against *L. infantum* and *L. amazonensis* promastigotes, cytotoxicity against HepG2 cells and selectivity index of the terpenes and isoflavanoids isolated from plants*

Substances	**Class**	**Plants**	**IC_{50} µg/mL**		**CC_{50} µg/mL**	**SI**	
			L. infantum	***L. amazonensis***	**HepG2**	***L. infantum***	***L. amazonensis***
Kaurenoic acid	Terpene	*Xylopia frutescens*	50.38 ± 2.89	51.75 ± 4.28	268.7 ± 30.49	5.33	5.19
Xylopic acid	Terpene	*Xylopia frutescens*	52.51 ± 7.46	>100	294.87± 56.66	5.61	>2.94
Bisabolol	Terpene	*Matricaria chamomilla*	4.45 ± 2.84	4.77 ± 1.98	300.55 ± 123.28	67.53	63
Claussequinone	Isoflavonoid	*Cyclolobium claussenii*	3.21 ± 0.49	2.47 ± 0.92	387.79 ± 25.93	120.8	157
Duartin	Isoflavonoid	*Machaerium villosum*	34.14 ± 10.97	37.15 ± 2.43	346.41 ± 40.99	10.14	9.32
Amphotericin b	-	-	0.048 ± 0.02	0.055 ± 0.02	35,24 ± 2.78	734.16	640.72

*Results are expressed as mean ± standard deviation (SD) of three independent experiments, performed in triplicates.

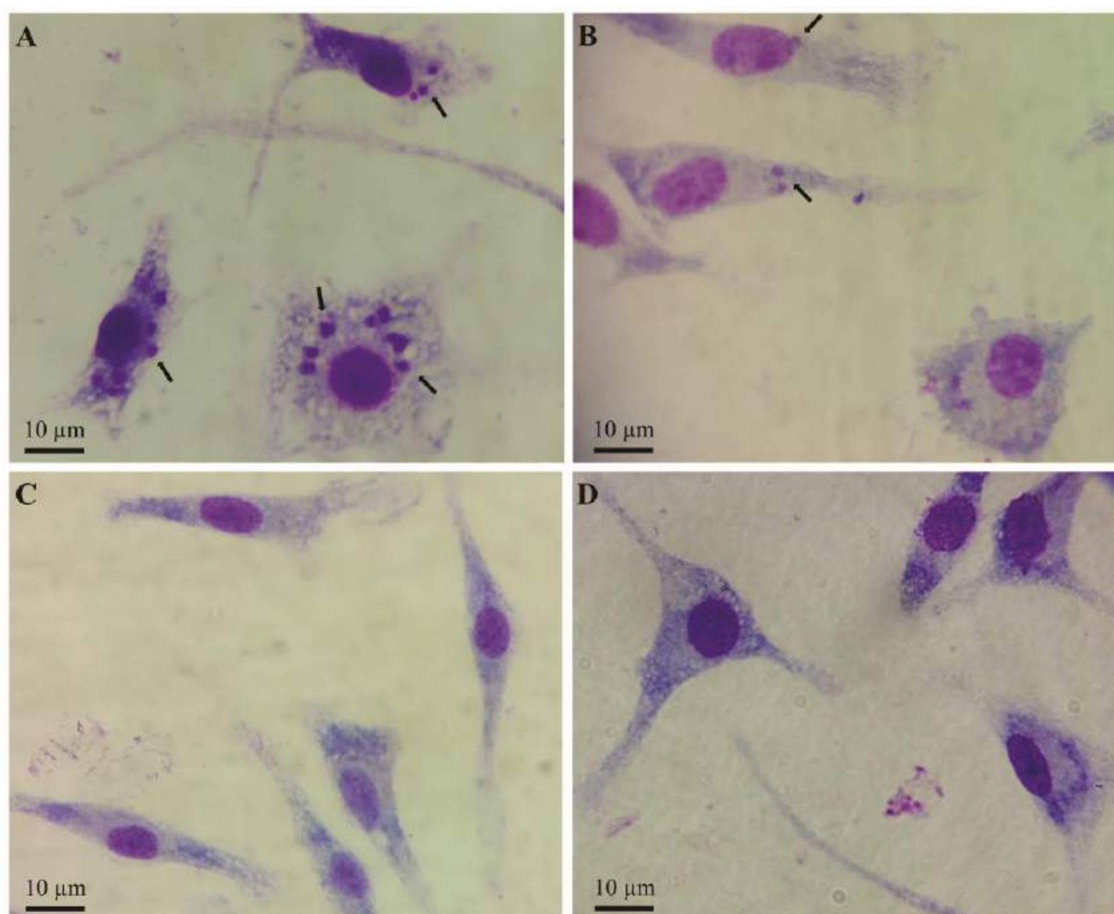

Figure 2 - Antiamastigote activity of (3R)-claussequinone. (A) Untreated infected macrophage; (B) treatment (3R)-claussequinone 1,25µg/mL; (C) treatment (3R)-claussequinone 20µg/mL; (D) macrophages non-infected treated with (3R)-claussequinone (20µg/mL). Magnification: 100x.

Table 2 - Parameters of macrophage infection with L. infatum after treatement with (3R)-claussequinone and amphotericin b. Results are expressed as mean ± standard deviation (SD)

Substances	Concentration (µg/mL)	% Infection	Amastigotes/cell (average)	IC_{50} (µg/mL)
Medium		100.0		
	20	27.94 ± 0.80	2,30 ± 0.10	
	10	30.64 ± 0.49	2,46 ± 0.08	
Claussequinone	5	33.09 ± 0.20	2,73 ± 0.45	4.16 ± 0.67
	2.5	41.1 ± 0.17	3,30 ± 0.20	
	1.25	50.54 ± 0.05	4,17 ± 0.50	

	0.5	27.57 ± 0.15	2,28 ± 0.18	
	0.25	41.67 ± 0.35	2,30 ± 0.28	
Amphotericim b	0.12	44.19 ± 0.26	2,83 ± 0.15	0.031 ± 0.04
	0.062	46.54 ± 0.36	3,80 ± 0.60	
	0.031	49.80 ± 0.21	4,07 ± 0.50	

The terpenes present a wide spectrum of biological activities such as antifungal, antibacterial, antitumor, antiviral, antiparasitic, analgesic, anti-inflammatory and antioxidant (Coloma et al., 2011; Lima et al., 2015). The antileishmanial activity of terpenes was demonstrated by Lima et al. (2015), when *Croton cajucara* terpenes were assayed. However, our results showed that the diterpenes kaurenoic acid and xylopic acid, previously isolated from *Xylopia frutescens* Aubl. (Annonaceae) (Saúde-Guimarães and Faria, 2007; Takahashi et al., 2014) were not active against promastigote forms of *L. infantum* and *L. amazonensis*. (-)-α-Bisabolol, that is produced in Brazil from *Eremanthus erythropappus* (Asteraceae) (Atina, 2021) essential oil, was shown to be active against the promastigote forms of *L. infantum* e *L. amazonensis*, presenting high SI in both cases (67.59 and 63, respectively), corroborating the results of Rottini et al. (2015). Corpas-López et al. (2015) showed that (-)-α-Bisabolol was effective against *L. infantum* and *L. donovani* in vitro and was more effective than meglumine antimoniate in a murine model of visceral leishmaniasis in the oral treatment. Further studies should be conducted in order to evaluate its efficacy against in vivo models of leishmaniasis.

In an attempt to understand how the substances caused the death of the parasite, it was evaluated whether they induce the apoptotic death of the parasite. Thus we evaluated whether our compounds induce the parasite apoptotic death. Then, we evaluated the expression of phosphatidylserine, which is a marker of cell death by apoptosis. Our results demonstrate that bisabolol and duartin induced the externalization of phosphatidylserine in *L. infantum* promastigotes after 48h of exposure. Furthermore, bisabolol showed a percentage of labeled cells only with annexin V and similar results were observed for annexin V and propidium iodide in relation to the percentage for amphotericin B treatment. Although claussequinone did not alter the induction of phosphatidylserine externalization in the parasite under these experimental conditions, we cannot rule out the induction of death by apoptosis, since we used only annexin V and propidium iodide to assess cell death (**Fig 3**).

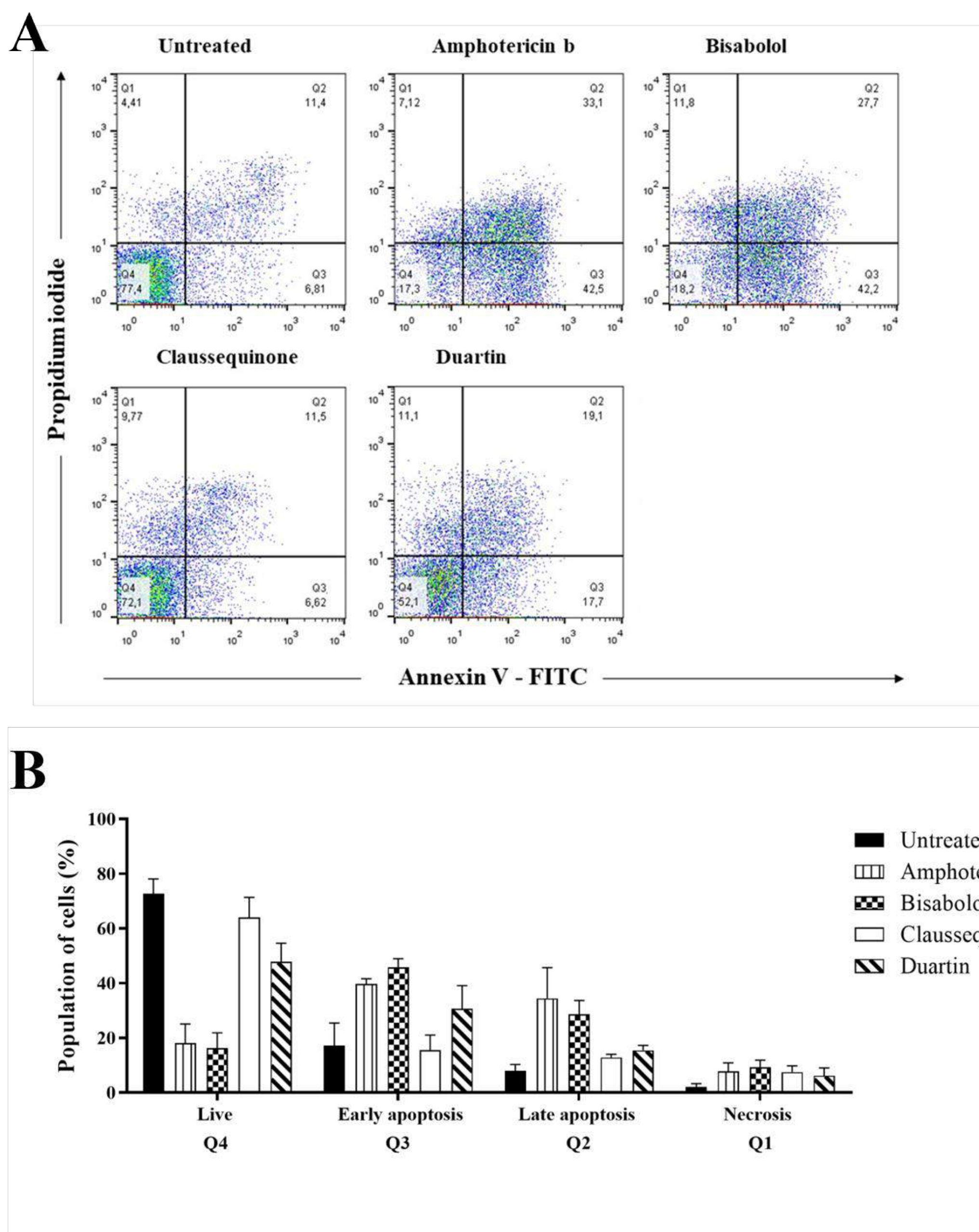

Figure 3 - Induction of apoptosis in promastigote treated. (A) Externalization of phosphatidylserine detected in *L. infantum* promastigotes. Dot plots represents of one of three similar results. Q1 quadrant (population in the top left) represent necrotic cells, Q2 quadrant (population in the top right) indicate late apoptosis cells, Q3 quadrant (population in the bottom right) exhibit early apoptosis cells, whereas Q4 quadrant (population in the bottom left) showed live cells. (B) Bar graph showing the percentage live, apoptotic (early and late) and necrotic cells. The results represent the mean ± standard deviation of three independent experiment performed in triplicate.

The present investigation highlights the antileishmanial activity of some naturally occurring isoflavonoids and terpenes. (-)-α-Bisabolol, duartin and (3R)-claussequinone showed high activity against *L. infantum* and *L. amazonensis*. (3R)-claussequinone

represents a new hit for the research aiming the development of new medicines for the treatment of leishmaniasis and several approaches are envisaged: (i) use of standardized *C. clausseni* extracts as phytomedicines, which would represent reduced cost-lines for large scale production; (ii) use of the pure substance, which is the major constituent of *C. clausseni* ethanol extract and is easily isolated, (iii) use of the naturally occurring compound as scaffold for structural modifications in order to produce (3R)-claussequinone derivatives aiming to discover compounds with better pharmacological profile, (iv) use of synthetic racemic claussequinone for which a viable route was reported (Oliveira et al., 1975) and that could be adapted for the synthesis of analogues

Furthermore, this study reports for the first time the striking antileishmanial activity of the isoflavonoid (3R)-claussequinone against both *Leishmania* species associated to lower cytotoxicity. In vivo efficacy and toxicity studies using visceral and cutaneous leishmaniasis models should be conducted to confirm these promising results which could stimulate the development of new therapeutic alternatives (different routes of administration and association with other drugs) derived from natural products to treat leishmaniasis with high efficacy and reduced side effects.

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CAPÍTULO III – ARTIGO A SER SUBMETIDO

Effect of the *Synadenium carinatum* latex lectin (ScLL) against *Leishmania infantum* in vitro and in vivo

4.1. Abstract

Leishmaniasis are neglected diseases caused by protozoa of the genus *Leishmania,* which compromise viscera causing visceral leishmaniasis (VL), skin and mucous causing cutaneous leishmaniasis (CL). In most cases the current treatment protocols present toxicity and poor effectiveness. Furthermore, these parasites are becoming more resistant against those conventional treatment methods. Therefore, the main objective of this work is to evaluate the therapeutic potential of ScLL (*Synadenium carinatum* latex lectin) in a visceral leishmaniasis model, both in vitro and in vivo. Our results showed that ScLL was extracted from *S. carinatum* and showed hemagglutinating activity at concentrations of 30 - 0.45 µg. Showed a low toxicity was not toxic in NIH cells (CC_{50}: 566.35 µg/mL) and bound to the *Leishmania* membrane as observed in the agglutination and immunofluorescence assay. In the tests of interaction of promastigotes with macrophages, both in the assay in which the parasites were treated before interacting with ScLL and in the assays in which macrophages were stimulated, we observed a reduction in the percentage of infection when compared to the control group. In vivo we observe this same pattern. It was observed that there was a significant reduction ($p < 0.05$) of the parasite load in the animals that received parasites treated with ScLL, both in the spleen and in the liver (3.56 and 3.25-fold, respectively). In animals treated with ScLL 2 days after infection (dpi) a 3.25-fold reduction was observed in the spleen and animals that received ScLL treatment 12 dpi showed a 3.21-fold reduction in the spleen and 3.42-fold in the liver. These results show that ScLL is a possibility for the search for alternative ways in the treatment of visceral leishmaniasis.

Keywords: *Synadenium carinatum*, Lectin, Leishmanicidal activity, *Leishmania*, Visceral leishmaniais.

4.2. Introduction

Leishmaniasis comprises a complex of chronic and infectious diseases with great worldwide prevalence, caused by parasites of the genus *Leishmania* (Ross, 1903; Charlton et al., 2018). They constitute a complex of diseases that affect man, wild and domestic animals. Human leishmaniasis can present different clinical manifestations in the vertebrate host, the basic clinical forms being visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL) (Braga, 2019). VL is a chronic disease and in most people, it is asymptomatic. It is an infection caused by *Leishmania donovani* (Old World) and *Leishmania infantum* (New World) (Griensven; Diro, 2012; Bush et al., 2017).

VL is a serious public health problem that affects the most economically vulnerable population (Alvar et al., 2006). It has a worldwide distribution and approximately 310 million people are exposed to contracting the disease. Approximately 70,000 cases are recorded every year with Brazil, East Africa and in India, being the places with the highest number of cases. In 2020, 95% of cases in America were in Brazil (Desjeux, 2004; WHO 2022).

Early diagnosis and effective treatment are among the main ways to control the disease (WHO, 2022). Treatment is carried out according to the patient's condition and the species causing the disease. Pentavalent antimonials (sodium stibogluconate and N-methyl-d-glucamine antimoniate) are the first-choice drugs, however, due to adverse reactions caused by these drugs or the ineffectiveness of the treatment, other drugs are used (Alvarenga et al., 2010; Balana-Fouc et al., 1998; Lima et al., 2015). Amphotericin B deoxycholate, liposomal amphotericin B and miltefosine are drugs used in the treatment of patients, but they all have serious adverse reactions, in addition to their high cost.

The complex treatment leads to the search for new therapeutic alternatives for the control of VL. The use of plants in the treatment of diseases is historical and, as Brazil is the country with the greatest plant genetic diversity in the world, it is interesting to carry out studies that assess the therapeutic potential of plants against diseases (Sales et al., 2015). In recent years, has been a breakthrough in the investigation of properties of plant

species and many compounds have been isolated from native Brazilian species, with lectins being a group studied for their properties (Afonso-Cardoso et al., 2011).

Lectins are widely found in several species of living organisms being a group of proteins that recognize and bind to carbohydrates (Katoch & Tripathi, 2017). It has been widely studied due to its biochemical and physicochemical properties, evolutionary relationships, molecular structure and carbohydrate specificity and play important cellular and biological functions (Van Damme 2014; Cavada et al., 2019; Katoch & Tripathi, 2021).

Synadenium carinatum latex lectin (ScLL) is a lectin extracted from the latex of *Synadenium carinatum* popularly known as “leiteirinha”, used in Brazil as an ornamental plant. Isolated and purified by Souza et al. (2005), there are studies showing the effect of lectin in models of cutaneous leishmaniasis (Afonso-Cardoso et al., 2007; 2011), toxoplasmosis (Souza et al., 2016), neosporosis (Cardoso, et al., 2012), asthma (Rogerio et al., 2007) and wound healing. Its role has already been described in a cutaneous leishmaniasis model as a vaccine adjuvant. Differently from that, the main objective of this work is to evaluate the therapeutic potential of ScLL in a visceral leishmaniasis model, both in vitro and in vivo.

4.3. Material and methods

Purification of the lectin and determination of the hemagglutinating activity of ScLL

Synadenium carinatum latex was collected from the stems of plants, which were grown under natural conditions in the University Campus localized in Uberlândia, Minas Gerais, Brazil (registered in the Herbarium of the Federal University of Uberlandia HUFU 38354). The latex was diluted in phosphate buffered saline (PBS) in a 1:5 ratio and kept under agitation for 24 hours at 4°C. After complete homogenization, it was kept in a -80° C freezer. The mixture was centrifuged at 10,000g for 20 minutes at 4°C and filtered through nitrocellulose membranes (0.22 mm pore size), giving rise to the crude extract (Souza et al., 2005).

ScLL was purified on immobilized D-galactose agarose column (Pierce, Rockford, Illinois, USA), balanced with PBS, pH 7.2. The ScLL was eluted with 0.4 M D-galactose in PBS and dialyzed against Tris buffer (pH 7.2). The electrophoretic profile of this lectin was visualized by SDS-PAGE (12%). The protein concentration was determined using a

spectrophotometer (NanoDrop™ Lite Spectrophotometer, Thermo Fisher Scientific, USA). Hemagglutination assays using human type A+ erythrocytes, were performed to confirm ScLL lectin activity, as previously described (Souza et al., 2005). ScLL aliquots were stored at -80°C until using in vitro and in vivo assays.

Gel electrophoresis

The samples were electrophoresed on 1-mm thick slab gels with a 12% polyacrylamide in the running gel, and a stacking gel of 5% acrylamide, using a discontinuous SDS buffer system. Crude extract of *S. carinatum* and ScLL were diluted in sample buffer (125 mM Tris-HCl [pH 6.8], 4% sodium dodecyl sulphate [SDS], 25% glycerol, 5% (vol/vol) 2-mercaptoethanol, 0.02% bromophenol blue). The molecular weight markers (BenMark™ Protein Ladder) and samples were heated for 5 min and applied to the gel. The proteins were visualized using the Coomassie brilliant blue (R 250).

Citotoxicity assay

The cytotoxicity concentration (CC_{50}) of the ScLL against NIH/3T3 fibroblast cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). NIH/3T3 cells were seeded in 96-well flat bottom plates ($2x10^4$ cells/well), being incubated (37ºC, 5% CO_2 for 24 hours) until their complete adhesion. After the incubation period, the wells were depleted and compounds diluted in complete RPMI were added at concentrations of 600 a 30 µg/mL of ScLL. Cell cultures remained exposed to compounds for 24 hours (37ºC, 5% CO_2). Subsequently, the wells were depleted and an MTT solution (50 µg/well) was added. The plates were incubated for an additional 1 hour and 30 minutes (37ºC, 5% CO_2). DMSO was added to solubilize the generated formazan crystals and the plates were subjected to reading on a spectrophotometer (570nm emission, on Spectramax M2). Each experiment was repeated three times, and the optical density (OD) was obtained by the value of the mean ± standard deviation of the sample readings, performed in triplicate, subtracted from the average OD value of the wells containing only cells incubated with complete medium (considered 100% viability).

Parasites and animals

Reference strain of *L. infantum* (MCAN/BR/2002/BH401) were maintained as promastigote forms in complete α-MEM supplemented with 10% (v/v) heat inactivated FBS, 100 µg/mL penicillin, 100 µg/mL ampicillin, 2 mM L-glutamine, and 20 mM HEPES, pH 7.2 at 24 ± 1°C in 25-cm^2 culture flasks on a B.O.D. incubator.

In this study, female BALB/c mice with 6 weeks of age and weight varying between 20-25g were used. The present study was approved by the Ethical Committee for Animal Experimentation of the Federal University of Uberlândia (protocol number 077/2019), and all procedures were performed according to the international guidelines (Principles of Laboratory Animal Care, 1985).

ScLL-mediated agglutination of *L. infantum*

L. infantum promastigotes ($5x10^7$ promastigotes/mL) were incubated in 48-well plates, in equal volumes, with ScLL at different concentrations. The suspensions were incubated for 1 hour at room temperature at the following concentrations: 300; 150; 75; 37.5; 18.75; 9.37; 4.68 and 2.34 µg/mL. Then, the reading was performed under a microscope. Promastigotes incubated in the absence of ScLL were used as a negative reaction control.

Efficacy of ScLL against intracellular amastigotes

Peritoneal macrophages were harvested from BALB/c mice previously inoculated with starch 2% (Sigma Aldrich) (24h before collection). Cells were seeded ($5x10^4$/well) on a 24-well tissue culture plate containing circular coverslips in each well and incubated cultured in complete RPMI medium (37°C, 5% CO_2) for 4 hours to allow cell adherence.

The infection assay was performed with two variables: (i) *L. infantum* promastigotes in late stationary growth phase treated with ScLL (300, 100 and 30 µg/mL) for 1 hour at room temperature, after which they were washed with PBS and were added to interact with the macrophages in the proportion of 10 promastigotes/macrophage/well for 24, 48 and 72 hours. (ii) *L. infantum* promastigotes in late stationary growth phase were added to interact with the macrophages in the proportion of 10 promastigotes/macrophage/well for 24 hours. Subsequently, three-fold serially diluted ScLL (ranging from 300 µg/mL to 30 µg/mL) was added to the wells. After 24, 48 and 72 hours, the coverslips were removed and stained with rapid panoptic, mounted in glass slides using Canada balsam, and analyzed using light microscopy to determine the

infection rate of macrophages. The values obtained for each concentration [(number of infected macrophages/100 counted macrophages) × 100] were used to obtain the amastigote intracellular IC_{50} value for ScLL. The IC_{50} for the effect of ScLL on the intracellular amastigotes was expressed as the concentration necessary to halve the number of the infected macrophages compared to non-treated control cells (Vermeersch et al., 2009).

Infection and treatment of mice

Five BALB/c mice (6-week-old females) were infected with $1x10^7$ metacyclic promastigotes of *L. infantum* previously treated with ScLL (100 µg/mL) for 1h in room temperature, via the intraperitoneal route.

Five BALB/c mice (6-week-old females) were infected with $1x10^7$ metacyclic promastigotes of *L. infantum* previously treated with PBS for 1 hour in room temperature, via the intraperitoneal route.

Ten BALB/c mice (6-week-old females) were infected with $1x10^7$ metacyclic promastigotes of *L. infantum* via the intraperitoneal route. Two days after infection, mice were randomly divided into two groups: (a) ScLL (n = 5; 100 µg/mL/every 24 h intraperitoneal route for 10 days) and (b) PBS (n = 5; intraperitoneal route every 24 h over 10 days).

Ten BALB/c mice (6-week-old females) were infected with $1x10^7$ metacyclic promastigotes of *L. infantum* via the intraperitoneal route. Twelve days after infection, mice were randomly divided into two groups: (a) ScLL (n = 5; 100 µg/mL every 24 h intraperitoneal route for 10 days) and (b) PBS (n = 5; intraperitoneal route every 24 h over 10 days).

The animals were euthanized after treatment, and liver and spleen were collected and used to determine the parasitic load using qPCR.

Parasite burden

Genomic DNA was extracted using a commercial kit (RealiapPrep ™ gDNA tissue miniprep system kit, Promega Co., USA) according to the manufacturer's instructions. DNA concentration and the 260 nm/280 nm ratio were determined using a spectrophotometer (NanoDrop™ Lite Spectrophotometer, Thermo Fisher Scientific, USA) and the samples were stored at −20ºC until use.

qPCR was performed in 96-well plates in triplicate and processed in a thermocycler (StepOne ™ Real-Time PCR System, Applied Biosystems, USA). To determine the parasite load, primers (L150 [5'-GGG (G/T)AG GGG CGT TCT(G/C)CG AA-3'] andL152 [5'-(G/C)(G/C)(G/C) (A/T)CT AT(A/T) TTA CAC CAACCC C-3']), which amplify a 120 bp fragment of the conserved region of *Leishmania* sp. kDNA minicircles, were used (Degrave et al., 1994). The reactions were performed in 25 μL final volume containing 100 nM of each primer, 1× SYBR Green PCR Master Mix (Applied Biosystems, USA), and 50 ng DNA template.

The number of *Leishmania* DNA copies in each sample was determined from linear regression after comparing to a standard curve generated with known amounts of total *Leishmania* DNA.

The gene encoding TNF-α is constitutively expressed in all murine cells and was hence used to verify DNA integrity of the samples using conventional PCR using primers (TNF-5241 5 'TCCCTCTCATCAGTTCTATGGCCCA 3' and TNF-5411 5 'CAGCA AGCATCTATGCACTTAGACCCC 3'), which amplify a 170 bp fragment (Cummings and Tarleton, 2003).

In all assays, the efficiency of the amplification was close to 100% and the standard curves presented correlation coefficients ranging from 0.97 to 0.99 (Bustin et al., 2009).

Statistical analysis

The dose-response curve (CC_{50}) was generated using the Origin 8.5 software (Originlab Co., USA). qPCR analyses were performed using the GraphPad Prism software version 8.0 (GraphPad Software, CA, USA). The samples distributions were verified using the Kolmogorov-Smirnov test, following the most appropriate test for each case. Differences between ScLL and control group in the qPCR analysis were evaluated using Mann-Whitney test. A significance level of 5% ($p < 0.05$) was observed in all tests.

4.4. Results and discussion

This study aimed to evaluate the effect of ScLL against *L. infantum*, in order to reduce side effects and cost related to current treatment. Leishmaniasis is a neglected disease closely related to poverty. The majority of the population that is sick or at risk of contracting the disease live on less than US$1 per day (Alvar, 2006). Treatment of leishmaniasis still remains mainly as control measures for all the clinical forms. Even

though, the drugs to treat leishmaniasis present several limitations and these compounds are the only therapeutic options currently available (Andrade et al. 2016). Therefore, seeking therapeutic alternatives that help reduce treatment costs is essential (WHO, 2022). ScLL is a single extraction lectin and the interaction of these proteins specifically with carbohydrates allows them to be used in various scientific researches (Afonso-Cardoso et al., 2007).

ScLL was extracted of *S. carinatum*. After affinity column chromatography on D-galactose, the aqueous extract of *S. carinatum* latex was eluted in D-galactose buffer, concentrated and dialyzed in PBS. The ScLL concentration was 0.6 mg/mL. Electrophoresis gel (**Fig 1 A**) analysis showed that the crude extract contained (lane 2) a diversity of proteins with different molecular weights, ranging from 10 to 40 kDa. When this material was eluted in D-galactose, the ScLL (lane 3) profile was observed as previously described by Souza et al. (2005).

Another method used to identify this lectin was incubation with human type A+ erythrocytes to measure its hemagglutinating activity. Lectins bind to a specific carbohydrate or carbohydrate group on oligosaccharides or glycoproteins. Its specificity is defined as a function of the monosaccharide that, in lower concentration, has the greatest ability to inhibit its hemagglutinating activity. The hemagglutination assay is the most used to visualize the property of lectin to agglutinate cells (Hivrale and Ingale, 2013; Sharon and Lis, 2002). In **Fig 1 B** we can see that ScLL showed hemagglutinating activity at concentrations of 30 - 0.45 μg.

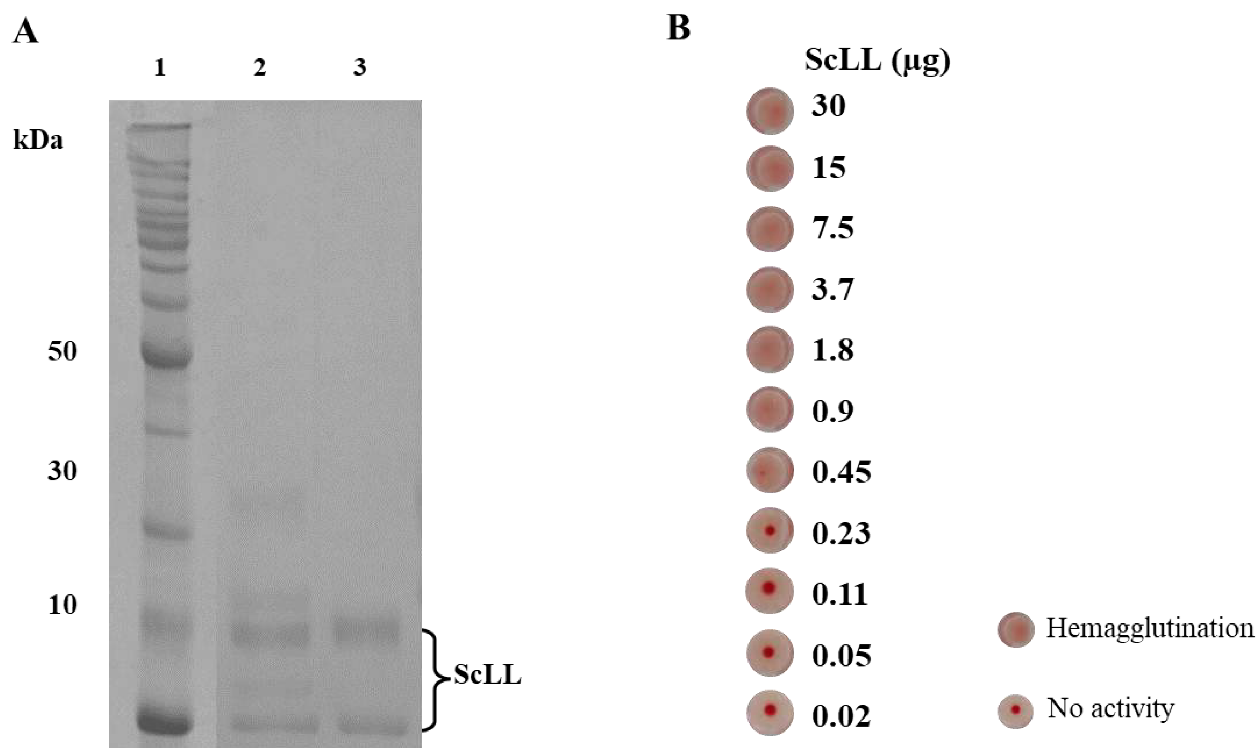

Figure 1 – Purification of ScLL lectin from *S. carinatum* and its hemagglutinating. (A) SDS-PAGE from *S. carinatum*: lane 1: molecular weight markers; lane 2: crude extract; lane 3: aqueous extract of *S. carinatum* latex was eluted in D-galactose. (B) ScLL lectin presented hemagglutinating activity (30-0.45 µg).

An important criterion in the development of new therapeutic options for VL is the search for substances with less toxicity than the drugs used today. NIH cells treated with ScLL showed a low cytotoxicity (CC_{50}: 566.35 µg/mL) a very important point to be analyzed is cytotoxicity, since the conventional treatment is very toxic to the patient. Previously, it was evaluated the effect of ScLL on viability, proliferation and release of IL-10 in human gingival fibroblasts stimulated with lipopolysaccharide. The lectin did not demonstrate cytotoxicity, which corroborates with our results (Reis et al., 2016). Some lectins can be toxic to mammals when they are in high concentrations in certain foods (Hivrale and Ingale, 2013). On the other hand, this toxicity is being studied as an alternative way to treat cancer. Studies have already shown that *Musa basjoo* lectin stimulates macrophages and inhibits the proliferation of leukemic cells, also having an effect on prostate cancer (Wong and Ng, 2006; Gabor et al., 2001).

To understand whether ScLL has an effect on *Leishmania* promastigotes preventing cell invasion, we performed the assay ScLL-mediated parasite agglutination. The

agglutination reaction (**Fig 2**) shows that ScLL is able to agglutinate *L. infantum* promastigotes at the highest concentrations tested (300 - 37.5 μg/mL). Therefore, we observed that it is possible that ScLL binds to the membrane of the parasite due to the specificity of binding to the sugar on the surface of *L. infantum*. Members of the Trypanosomatidae family are organisms rich in glycoproteins, which correspond to approximately 10% of the total proteins. The oligosaccharide structures of these glycoproteins, in some cases, may even include complex oligosaccharides, such as fucose, sialic acid residues and α-galactose (Parodi, 1993; Clayton, 1999). Due to the fact that lectins bind to carbohydrates, ScLL can bind on the parasite's membrane allowing the interaction of ScLL with *L. infantum* promastigotes.

The effect of ScLL on *L. infantum* amastigotes was performed in two ways, both in the treatment of promastigotes and with stimulation of peritoneal macrophages. The percentages of infection are shown in **Table 1**. Based on CC_{50} and on published work using ScLL and *Leishmania* (Afonso-Cardoso et al., 2007; 2011) we used an initial concentration 300 μg/mL, being effective both in the treatment of parasites and in the stimulation of macrophages. In the three time periods it was possible to observe a rate of 0.0% when macrophages received treatment with ScLL at the highest concentration, which differs from macrophages that received treated parasites. Macrophages that received treated parasites showed an infection profile that increased over time, but macrophages that were treated with ScLL had a decrease in the percentage of infection over the course of the experiment. These results corroborate those of Afonso-Cardoso et al. (2011), in the analysis of infection with *L. amazonensis*, and show that treatment with ScLL can interfere with the phagocytosis process, preventing a successful infection of the parasite. The longer the time of interaction of the parasites with the macrophages, the rate of infection decreases. As described by Afonso-Cardoso et al. (2011) these data may suggest that macrophages that received treatment with ScLL have a modification in the phagocytosis process and thus the infection by parasites is not successful. Despite the anti-*Leishmania* activity of ScLL against amastigotes of *L. infantum*, this compound appears to be non-toxic to macrophages, as no morphological abnormalities were observed (**Fig 4**). These results suggest that ScLL can be considered a promising candidate for therapeutic use as it appears to efficiently affect the intracellular parasites instead of the host cell.

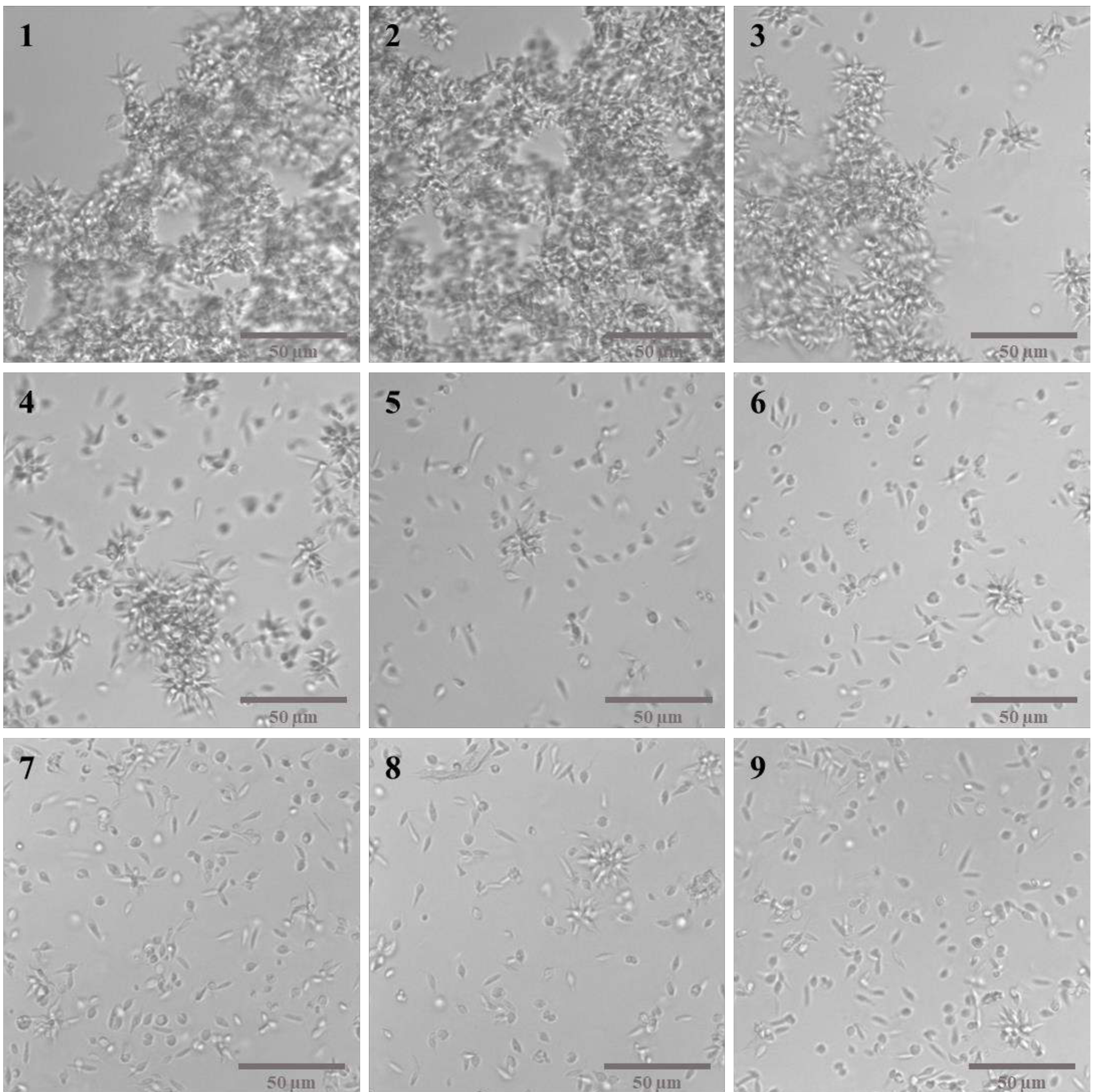

Figure 2 - ScLL-mediated agglutination of *L. infantum*. Agglutination was observed at the highest concentrations (1: 300 µg/mL; 2: 150 µg/mL; 3: 75 µg/mL; 4: 37.5 µg/mL; 5: 18.75 µg/mL; 6: 9.37 µg/mL; 7: 4.68 µg /mL; 8: 2.34 µg/mL; 9: negative control).

Table 1 - Percentage of infection of murine macrophages infected with *L. infantum* after 24, 48 and 72h of infection. Results are expressed as mean ± standard deviation (SD).

		Infection %					
		Macrophages treated			Parasite treated		
		24 h	48 h	72 h	24 h	48 h	72 h
ScLL (µg/mL)	300	0.0	0.0	0.0	0.0	34.21 ± 8.41	40.40 ± 5.65
	100	42.05 ± 2.12	50.0 ± 1.41	30.34 ± 2.12	36.59 ± 1.41	39.74 ± 0.70	58.59 ± 8.48
	30	48.86 ± 0.71	58.33 ± 2.82	43.82 ± 3.53	30.49 ± 0.71	72.37 ± 0.71	75.76 ± 2.12
Medium			100.0			100.0	

After the results of the in vitro assays demonstrate that ScLL interferes in the dynamics of macrophage infection and binds to the parasite membrane, we sought to evaluate the effect of the lectin in vivo. As a primary trial, we treated *L. infantum* metacyclic promastigotes with ScLL and PBS before infection in mice. During the 12 days of infection, no changes were observed in behavior parameters, water and food intake. As can be seen in **Fig 4A** the group that received the parasites treated with ScLL showed a significant decrease ($p < 0.05$), both in the spleen and in the liver, in relation to the group that received parasites treated with PBS group. The group treated with ScLL showed an approximately 3.5-fold decrease in parasite burden in the spleen and liver. The effect of ScLL had not been tested against promastigotes of *L. infatum*, the species responsible for causing VL, and against other species of *Leishmania*, this aspect had not been studied. These data are very interesting, because it is possible that ScLL has the ability to bind to the parasite and thus prevent a successful infection in the host, however, experiments that directly show this effect are necessary.

The therapeutic potential of ScLL was also tested. Animals that were infected with *L. infatum* and received treatment two days after infection (**Fig 4B**) also had a significant reduction in spleen and liver parasite burden ($p < 0.05$) compared to the untreated group, when we analyzed the parasite load in the liver of the animals, it was possible to observe that there was a reduction (1.43-fold) but these results were not significant. We also evaluated the therapeutic efficacy of ScLL after well-established infection in animals starting daily treatment 12 days after infection (**Fig 4C**). This is a very interesting point

to be evaluated. In the qPCR analysis, a significant reduction ($p < 0.05$) in the parasite load was observed in the groups that received the treatment with ScLL, showing a 3.2-fold reduction in the spleen and 3.4 in the liver. These results show that ScLL is effective in the treatment of VL. Souza et al. (2016) performed a treatment with ScLL in mice infected with *Toxoplama gondii* and observed a significant difference in brain parasite burden with comparable efficacy to conventional treatment performed with sulfadiazine. As pharmacological therapy is the main form of VL control, more effective therapeutic regimens are needed (Brasil, 2014; Chouhan et al., 2014; Hamill, 2013; Van Griensven et al., 2010).

The therapeutic potential of ScLL was also tested. Animals that were infected with *L. infatum* and received treatment two days after infection (**Fig 4B**) also had a significant reduction in spleen and liver parasite burden ($p < 0.05$) compared to the untreated group, when we analyzed the parasite load in the liver of the animals, it was possible to observe that there was a reduction (1.43-fold) but these results were not significant. We also evaluated the therapeutic efficacy of ScLL after well-established infection in animals starting daily treatment 12 days after infection (**Fig 4C**). This is a very interesting point to be evaluated. In the qPCR analysis, a significant reduction ($p < 0.05$) in the parasite load was observed in the groups that received the treatment with ScLL, showing a 3.2-fold reduction in the spleen and 3.4 in the liver. These results show that ScLL is effective in the treatment of VL. Souza et al. (2016) performed a treatment with ScLL in mice infected with *Toxoplama gondii* and observed a significant difference in brain parasite burden with comparable efficacy to conventional treatment performed with sulfadiazine. As pharmacological therapy is the main form of VL control, more effective therapeutic regimens are needed (Brasil, 2014; Chouhan et al., 2014; Hamill, 2013; Van Griensven et al., 2010).

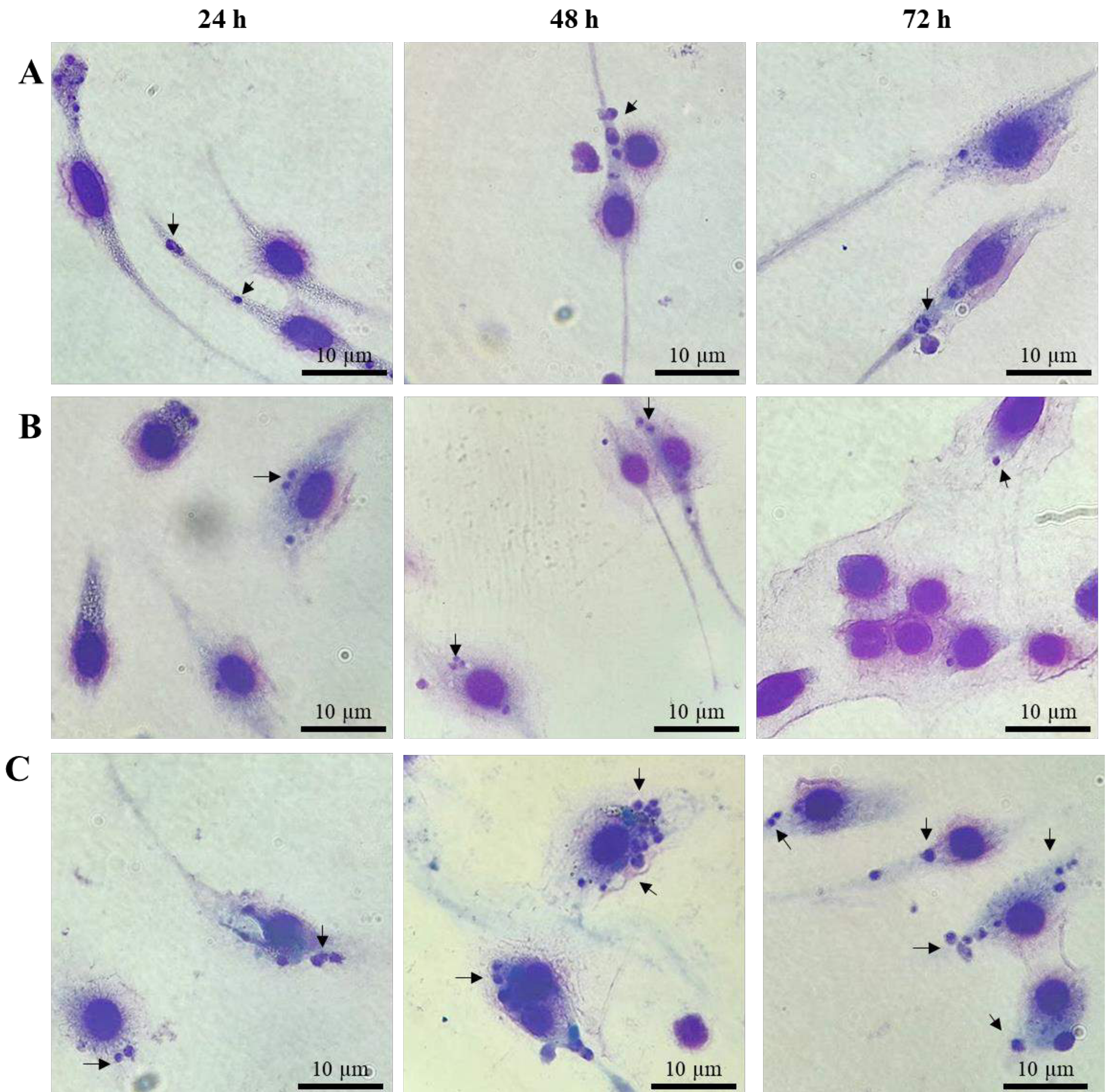

Figure 3 - Antiamastigote activity of ScLL (100 ug/ml). (A) Macrophages infected with parasites of *L. infantum* treated with ScLL. (B) Macrophages treated with ScLL for 24h e infected with *L. infantum*. (C) Macrophages infected no treatment.

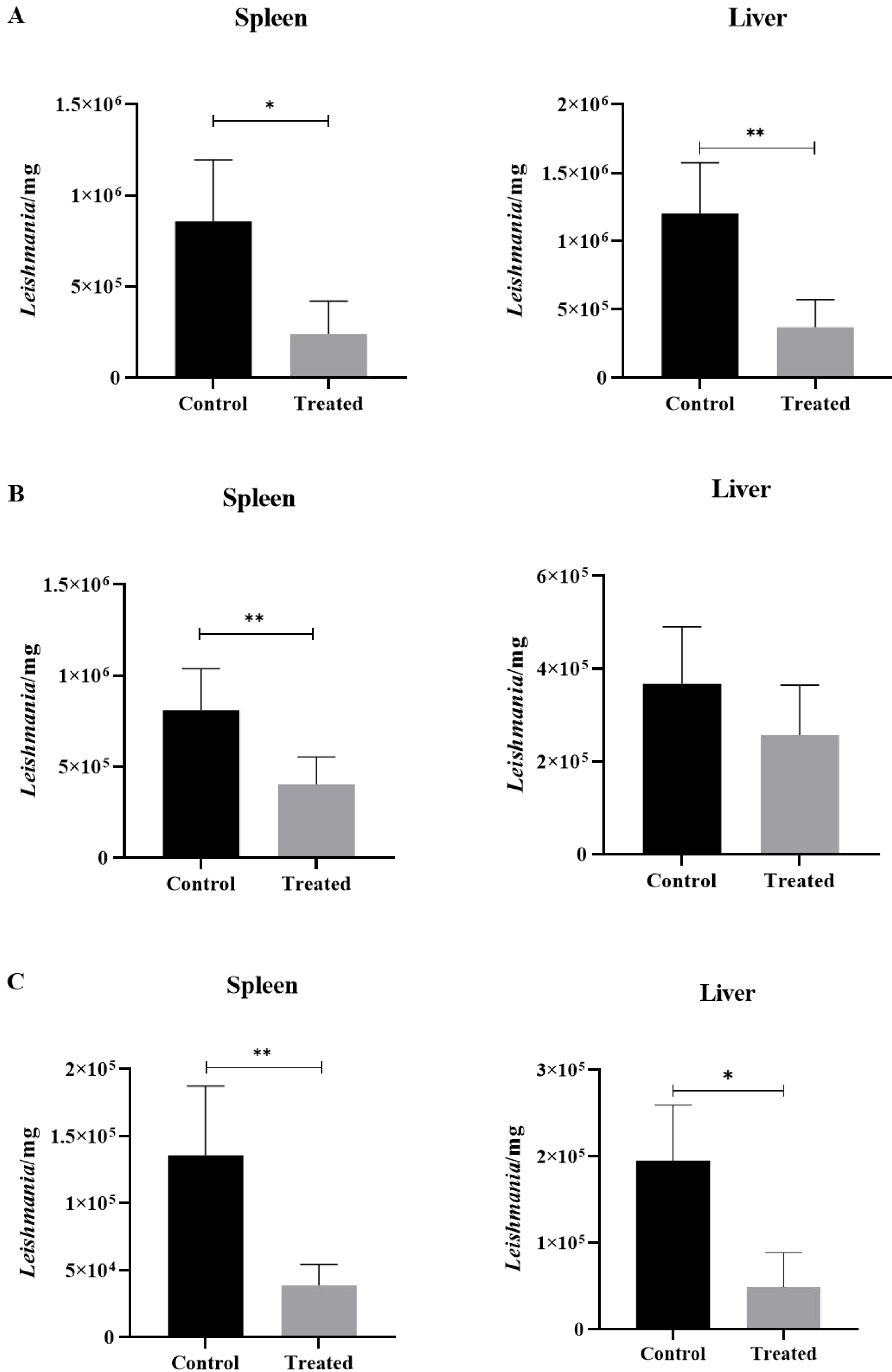
A
Spleen
Liver
1.5×10⁶
1×10⁶
5×10⁵
0
*
Leishmania/mg
Control
Treated
2×10⁶
1.5×10⁶
1×10⁶
5×10⁵
0
**
B
Spleen
Liver
1.5×10⁶
1×10⁶
5×10⁵
0
**
6×10⁵
4×10⁵
2×10⁵
0
C
Spleen
Liver
2×10⁵
1.5×10⁵
1×10⁵
5×10⁴
0
**
3×10⁵
2×10⁵
1×10⁵
0
*

Figure 4 - Parasite burden in the spleen and liver of BALB/c mice infected with *Leishmania infantum*. (A) Animals that received parasites treated with ScLL for 1h (B) Animals treated with ScLL 2 days post infection during 10 days (C) Animals treated with ScLL 12 days post infection during 10 days. Parasite burden was determined using qPCR as described in Material and Methods. Data represent means ± SEM of each group (n = 5). * $p < 0.05$; ** $p < 0.008$, according Mann-Whitney test.

ScLL and other lectins have already been studied as adjuvants or immunostimulators in different infections (Teixeira et al., 2006; Cardoso et al., 2011; Afonso-Cardoso et al., 2007; Panunto-Castelo et al., 2011). As they are carbohydrate-binding proteins, lectins can function as pattern recognition receptors (PRRs) on host cells and as a consequence activate these cells. As demonstrated by Afonso-Cardoso et al. (2007) ScLL demonstrated a protective effect on *L. amazonensis* infection, decreasing the lesion in infected animals and significantly reducing the parasite load. In the investigation of ScLL as an adjuvant in the immunization of mice against neosporosis, positive results were also observed when ScLL was associated with *Neospora caninum* antigen, resulting in the protection of mice that were immunized and challenged with the parasite (Cardoso et al., 2012).

We demonstrated for the first time the therapeutic potential of ScLL against *L. infantum*. Being a parasitic disease of great importance, the search for new tools to control the disease is essential. In this work, we saw that ScLL did not demonstrate cytotoxicity in NIH cells and was effective in assays with intracellular amastigotes of *L. infantum*. In the in vivo assays, there was a reduction in the parasite load, both in the spleen and in the liver, in mice that received daily treatment and in mice that received parasites treated with ScLL. Future studies related to the use of ScLL as a vaccine adjuvant in an experimental model of VL will be carried out, as it was effective in models of cutaneous leishmaniasis.

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4. CONCLUSÕES

Capítulo II

- Lapachol demonstrou atividade leishmanicida *in vitro*;
- Em promastigotas de *Leishmania*, o lapachol induziu a morte semelhante a apoptose;
- O lapachol *in vivo* reduziu a carga parasitária nas lesões cutâneas, no fígado e no baço.

Capítulo III

- Bisabolol, duartina e (3R)-claussequinona mostraram alta atividade contra promastigotas de *Leishmania*;
- (3R)-claussequinona apresentou alta atividade contra amastigotas de *Leishmania infantum*;
- Em promastigotas de *Leishmania*, bisabolol e duartina induziram a morte semelhante à apoptose.

Capítulo IV

- . ScLL mostrou baixa citotoxicidade em fibroblastos;
- ScLL é capaz de aglutinar promastigotas de *L. infantum*;
- ScLL reduziu a infecção em macrófagos estimulados e infectados com *L. infantum*;
- ScLL reduziu a carga parasitária em modelos murinos de leishmaniose visceral.

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ANEXO

SERVIÇO PÚBLICO FEDERAL
UNIVERSIDADE FEDERAL DE UBERLÂNDIA
Pró-Reitoria de Pesquisa e Pós-Graduação
Comissão de Ética na Utilização de Animais

CERTIFICADO

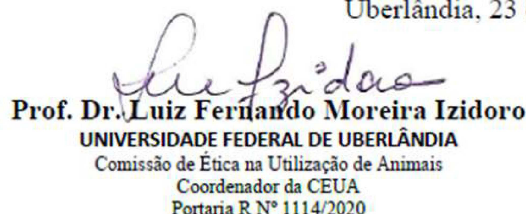
Certificamos que o projeto intitulado "Associação de produtos naturais e fármacos para o tratamento das leishmanioses'', protocolo nº **077/19**, sob a responsabilidade de **Iasmin Aparecida Cunha Araújo** – que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata, para fins de pesquisa científica – 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 NA UTILIZAÇÃO DE ANIMAIS (CEUA) da UNIVERSIDADE FEDERAL DE UBERLÂNDIA, em reunião **23 de novembro de 2021.**

(We certify that the project entitled "Associação de produtos naturais e fármacos para o tratamento das leishmanioses.",protocol **077/19**, under the responsibility of **Iasmin Aparecida Cunha Araújo** - involving the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata, for purposes of scientific research - is in accordance with the provisions of Law nº 11.794, of October 8th, 2008, of Decree nº 6.899 of July 15th, 2009, and the rules issued by the National Council for Control of Animal Experimentation (CONCEA) and it was approved for ETHICS COMMISSION ON ANIMAL USE (CEUA) from FEDERAL UNIVERSITY OF UBERLÂNDIA, in meeting of **November 23th, 2021**).

Finalidade	( ) Ensino (x) Pesquisa Científica
Vigência do Projeto	Início: 20/09/2020_Término: 20/08/2022
Espécie / Linhagem / Grupos Taxonômicos	Camundongo isogênico BALB/c
Número de animais	152
Peso / Idade	25g / 4-8 semanas
Sexo	Fêmea
Origem / Local	REBIR
Local onde serão mantidos os animais:	REBIR

Alteração Realizada: Alteração na data término de 20/09/2021 para 20/08/2022.

Uberlândia, 23 de novembro de 2021.



Prof. Dr. Luiz Fernando Moreira Izidoro
UNIVERSIDADE FEDERAL DE UBERLÂNDIA
Comissão de Ética na Utilização de Animais
Coordenador da CEUA
Portaria R Nº 1114/2020