

UNIVERSIDADE FEDERAL DE MINAS GERAIS

Faculdade de Medicina

Programa de Pós-Graduação em Ciências da Saúde:

Infectologia e Medicina tropical

Isabela Amorim Gonçalves Pereira

**COMPOSTOS FENÓLICOS COMO ABORDAGEM TERAPÊUTICA PARA
LEISHMANIOSES TEGUMENTAR E VISCERAL**

Belo Horizonte - MG

2024

Isabela Amorim Gonçalves Pereira

**COMPOSTOS FENÓLICOS COMO ABORDAGEM TERAPÊUTICA PARA
LEISHMANIOSES TEGUMENTAR E VISCERAL**

Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical da Faculdade de Medicina da Universidade Federal de Minas Gerais como requisito parcial à obtenção do título de Doutora em Ciências da Saúde.

Linha de Pesquisa: Leishmanioses

Orientadora:

Dra. Denise Utsch Gonçalves

Coorientadores:

Dr. Eduardo Antonio Ferraz Coelho

Dra. Grasielle de Sousa Vieira Tavares

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ATA DE DEFESA DE TESE

Aos oito dezoito do mês de julho de dois mil e vinte e quatro (17/07/2024), às 13:00 (treze) horas, no Canal do CETES - Plataforma LIFESIZE, realizou-se a sessão pública para a 229ª defesa de tese de **ISABELA AMORIM GONÇALVES PEREIRA**, número de registro 2019658016, graduada no curso de BIOMEDICINA, como requisito parcial para a obtenção do grau de Doutor em CIÊNCIAS DA SAÚDE - INFECTOLOGIA E MEDICINA TROPICAL. A presidência da sessão coube a **PROFA. DENISE UTSCHE GONÇALVES**, orientadora principal. Inicialmente, a presidente fez a apresentação dos demais componentes da Comissão Examinadora, assim constituída: **PROFA. DENISE UTSCHE GONÇALVES - ORIENTADORA (UFMG)**, **DRA. GRASIELE DE SOUSA VIEIRA TAVARES – COORIENTADORA (UFMG)**, **PROF. EDUARDO ANTONIO FERRAZ COELHO - COORIENTADOR (UFMG)**, **PROFA. ELAINE SOARES COIMBRA (UFJF)**, **PROF. FERNANDO AÉCIO DE AMORIM CARVALHO (UFPI)**, **DR. JOÃO AUGUSTO OLIVEIRA DA SILVA (UFMG)**, **DRA. ANA THEREZA CHAVES LAGES (UFMG)**. Em seguida, a candidata fez a apresentação de sua Tese de Doutorado, intitulada: *"Compostos fenólicos como abordagem terapêutica para leishmanioses visceral e tegumentar"*. Seguiu-se a arguição pelos examinadores e, logo após, a Comissão reuniu-se, sem a presença da candidata e do público, e decidiu considerar a tese **APROVADA**. O resultado final foi comunicado publicamente à candidata e ao público, pelo presidente da Comissão. Conforme arts. 76 e 77 das Normas Gerais de Pós-Graduação da UFMG, as defesas de dissertação e tese são públicas. A aluna e os membros da banca estão cientes e autorizaram a gravação desta defesa, que ficará disponibilizada em acervo da Universidade. Nada mais havendo a tratar, a presidente encerrou a sessão, sendo lavrada a presente ata que, depois de lida e aprovada, foi assinada eletronicamente por todos os membros titulares da Comissão Examinadora presente através do SEI (Sistema Eletrônico de Informações) do Governo Federal.

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Esse trabalho foi desenvolvido no Laboratório de Biotecnologia Aplicada ao Estudo das Leishmanioses, Setor de Patologia Clínica do Colégio Técnico da Universidade Federal de Minas Gerais (COLTEC/UFMG), e no Laboratório de Pesquisa do Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical da Faculdade de Medicina da UFMG, sob orientação dos professores Dra. Denise Utsch Gonçalves, Dr. Eduardo Antonio Ferraz Coelho e Dra. Grasielle de Sousa Vieira Tavares. A coleta, extração e/ou síntese química dos compostos bioativos ocorreram nos Departamentos de Bioquímica e Biologia Molecular e Departamento de Química da Universidade Federal de Viçosa. As análises de mecanismo de ação dos compostos em *Leishmania* foram desenvolvidas no Departamento de Parasitologia, Microbiologia e Imunologia da Universidade Federal de Juiz de Fora. A leitura e a análise dos ensaios de citometria de fluxo foram realizadas no Laboratório Multiusuários de Citometria de Fluxo do Núcleo de Pesquisas em Ciências Biológicas da Universidade Federal de Ouro Preto.

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RESUMO

O tratamento das leishmanioses apresenta elevada toxicidade e alto custo, além da emergência de cepas resistentes dos parasitos e falha terapêutica. Assim, a identificação de novos compostos antileishmaniais se faz necessária, sendo os produtos naturais e seus derivados sintéticos uma alternativa atraente. No presente estudo, a atividade antileishmanial de três compostos fenólicos foi avaliada, de forma inédita. A primeira molécula, obtida a partir do extrato de *Maclura tinctoria*, denominada CMt (5, 7, 3', 4'-tetrahidroxi-6,8-diprenilisoflavona) foi analisada em ensaios *in vitro* contra formas promastigotas e amastigotas de *Leishmania infantum* e *L. amazonensis*. Resultados mostraram que esse composto foi mais ativo contra parasitos do que contra macrófagos murinos e hemácias humanas, com altos índices de seletividade mais expressivos que anfotericina B. Além disso, foi verificada a eficácia de CMt no tratamento de macrófagos infectados e na inibição da infecção utilizando parasitos previamente incubados com a molécula. O mecanismo de ação em *L. infantum* demonstrou a efetividade desse composto ao provocar distúrbios na mitocôndria do parasito. Outras duas moléculas do estudo, derivados sintéticos da vanilina, denominadas 3s [4-(2-hidroxi-3-(4-octil-1H-1,2,3-triazol-1-il)propoxi)-3-metoxibenzaldeído] e 3t [4-(3-(4-decil-1H-1,2,3-triazol-1-il)-2-hidroxipropoxi)-3-metoxibenzaldeído], bem como CMt foram avaliadas, em sua forma livre ou incorporadas em micelas poliméricas, em camundongos BALB/c infectados com *L. infantum* ou *L. amazonensis*. Os três compostos incorporados em micelas induziram melhor redução da carga parasitária nos órgãos avaliados (*L. infantum* e *L. amazonensis*) e tecido infectado (*L. amazonensis*) dos animais tratados, além do desenvolvimento de uma resposta imune celular e humoral do tipo Th1 com elevada produção de IFN- γ , IL-12, TNF- α , GM-CSF, nitrito e anticorpos IgG2a. Ademais, esses compostos fenólicos demonstraram baixa toxicidade renal e hepática, e na infecção por *L. amazonensis* reduziram o diâmetro médio das lesões dos animais tratados. As composições micelares apresentaram melhor resposta terapêutica em relação às moléculas livres e aos fármacos utilizados como controle, sendo miltefosina nos ensaios de CMt, e anfotericina B e AmBisome[®] para 3s e 3t. Avaliações parasitológicas e imunológicas foram realizadas um, 15 e/ou 30 dias pós-tratamento, e os resultados indicaram proteção contra a reinfecção. Desse modo, as moléculas CMt, 3s e 3t, principalmente incorporadas em micelas poliméricas, podem ser consideradas em estudos futuros para o tratamento das leishmanioses tegumentar e visceral.

Palavras-chave: tratamento; compostos fenólicos; moléculas sintéticas; leishmanioses; toxicidade; micelas poliméricas.

ABSTRACT

Treatment of leishmaniasis presents high toxicity and cost, in addition to the emergence of parasite resistant strains and therapeutic failure. Therefore, the identification of new antileishmanial compounds is necessary, with natural products and their synthetic derivatives being a promising alternative. In the present study, antileishmanial activity of three phenolic compounds was evaluated for the first time. The initial molecule, CMt (5, 7, 3', 4'-tetrahydroxy-6,8-diprenylisoflavone), obtained from *Maclura tinctoria* extracts was evaluated in vitro against promastigote and amastigote stages of *Leishmania infantum* and *L. amazonensis*. Results showed that this compound was more effective against parasites than murine macrophages and red blood cells, with significantly higher selectivity index than amphotericin B. Moreover, CMt was effective in treating infected macrophages and inhibiting infection using parasites pre-incubated with the molecule. The mechanism of action in *L. infantum* revealed disturbances in the parasite's mitochondria. Two additional molecules, synthetic derivatives from vanillin: [4-(2-hydroxy-3-(4-octyl-1H-1,2,3-triazol-1-yl)propoxy)-3-methoxybenzaldehyde] or 3s and [4-(3-(4-decyl-1H-1,2,3-triazol-1-yl)-2-hydroxypropoxy)-3-methoxybenzaldehyde] or 3t, alongside CMt, were evaluated in BALB/c mice infected with *L. infantum* or *L. amazonensis*, either in pure form or incorporated into polymeric micelles. Micellar compositions induced a more significant reduction in parasite load in the distinct organs (*L. infantum* and *L. amazonensis*) and infected tissue (*L. amazonensis*) of treated animals. In addition, it triggered the development of a Th1-type cellular and humoral immune response, with higher levels of IFN- γ , IL-12, TNF- α , GM-CSF, nitrite, and IgG2a antibodies. These phenolic compounds showed low renal and hepatic toxicity, and in *L. amazonensis* infection, they also decreased the average diameter of lesions in treated animals. The micellar compositions exhibited a better therapeutic response compared to free molecules and the drugs used as controls, including miltefosine in CMt assays, and amphotericin B and AmBisome® for 3s and 3t. Parasitological and immunological evaluations were conducted at one, 15, and/or 30 days post-treatment, with results indicating protection against reinfection. Therefore, results suggest CMt, 3s and 3t, especially incorporated into polymeric micelles, could be considered for future studies as therapeutic candidates against cutaneous and visceral leishmaniasis.

Keywords: treatment; phenolic compounds; synthetic molecules; leishmaniasis; toxicity; polymeric micelles.

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LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

±	Mais ou menos
<	Menor que
%	Porcentagem
$\Delta\Psi_m$	Potencial de membrana mitocondrial
®/™	Marca registrada / <i>Trade Mark</i>
µg	Micrograma
µL	Microlitro
µm	Micrometro
µM/µmol	Micromolar
°C	Grau Celsius
3s	4-(2-hidroxi-3-(4-octil-1H-1,2,3-triazol-1-il)propoxi)-3-metoxibenzaldeído
3s/Mic	3s incorporada em micelas poliméricas
3t	4-(3-(4-decil-1H-1,2,3-triazol-1-il)-2-hidroxipropoxi)-3-metoxibenzaldeído
3t/Mic	3t incorporada em micelas poliméricas
ACTB	Beta-actina
ALT	Alanina aminotransferase
AmpB	Anfotericina B
ANOVA	Análise de variância
AST	Aspartato aminotransferase
ATP	Adenosina trifosfato
BIOPROS	Bioprospecção Molecular no Uso Sustentável da Biodiversidade
B/Mic	Micela vazia
BSA	Albumina sérica bovina
Ca ²⁺	Cálcio
CC ₅₀	Concentração citotóxica de 50% das células
CD4 ⁺	Marcador de superfície celular da subpopulação de linfócitos T auxiliares
CD8 ⁺	Marcador de superfície celular da subpopulação de linfócitos T citotóxicos
CDC	<i>Centers for Disease Control and Prevention</i>
cDNA	Ácido desoxirribonucleico complementar
CEUA	Comissão de Ética no Uso de Animais
CGen	Conselho de Gestão do Patrimônio Genético
CMC	Concentração micelar crítica
CMt	5,7,3',4'-tetrahidroxi-6,8-diprenilisoflavona
CMt/Mic	CMt incorporado em micelas poliméricas
¹³ C NMR	Ressonância magnética nuclear de carbono-13
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CO ₂	Dióxido de carbono
COBEA	Colégio Brasileiro de Experimentação Animal
COLTEC	Colégio Técnico da UFMG
CuSO ₄ .5H ₂ O	Sulfato de cobre penta
DLS	Espalhamento dinâmico de luz
DNA	Ácido desoxirribonucleico
EDTA	Ácido etilenodiamino tetra-acético
ELISA	Ensaio de imunoadsorção enzimática
ESI-MS	Espectrometria de massas com ionização por electrospray
FACS	<i>Fluorescence Assisted Cell Sorter</i>
FCCP	Carbonilcianeto 4-(trifluorometoxi)fenil-hidrazona
FSC	<i>Foward Scatter</i>

FVS450	<i>Fixable Viability Stain 450</i>
g	Grama
GAPDH	Gliceraldeído-3-fosfato desidrogenase
GM-CSF	Fator estimulador de colônia de granulócitos e monócitos
HCl	Ácido clorídrico
H ₂ DCFDA	Diacetato de 2',7'-diclorofluoresceína
HIV	Vírus da imunodeficiência humana
¹ H NMR	Ressonância magnética nuclear de hidrogênio-1
H ₂ O ₂	Peróxido de hidrogênio
H ₂ SO ₄	Ácido sulfúrico
IC ₅₀	Concentração inibitória de 50% de parasitos
ICB	Instituto de Ciências Biológicas
IFN-γ	Interferon-gama
IgG	Imunoglobulina G
IgG1	Imunoglobulina G subclasse 1
IgG2a	Imunoglobulina G subclasse 2a
IL-4	Interleucina 4
IL-5	Interleucina 5
IL-10	Interleucina 10
IL-12	Interleucina 12
IV	Infravermelho
kDNA	Ácido desoxirribonucleico do cinetoplasto
Kg	Quilograma
L	Litro
LC	Leishmaniose cutânea
LCD	Leishmaniose cutâneo-difusa
LM	Leishmaniose mucosa ou mucocutânea
LT	Leishmaniose tegumentar
LV	Leishmaniose visceral
mg	Miligrama
MHz	Megahertz
Mic	Micela vazia
mL	Mililitro
mm	Milimetro
m/m	Massa/massa
MMA	Ministério do Meio Ambiente
mmol	Milimol
MTT	Brometo de 3-[4,5-dimetil-tiazol-2-il]-2,5-difeniltetrazólio
m/v	Massa/volume
NH ₄ Cl	Cloreto de amônio
nm	Nanômetro
nmol	Nanomol
NO	Óxido nítrico
O ²⁻	Ânion superóxido
OE	Óxido de etileno
OH ⁻	Radical hidroxila
OMS	Organização Mundial de Saúde
OP	Óxido de propileno
OPAS	Organização Pan-Americana da Saúde
PA	Para análise

PBS	Solução salina tamponada com fosfato
PBS-T	Solução salina tamponada com fosfato com Tween®20
PEG	Polietilenoglicol
pg	Picograma
pH	Potencial hidrogeniônico
PI	Iodeto de propídeo
PMA	Forbol 12-miristato-13-acetato
PPG-IMT	Programa de Pós-Graduação em Infectologia e Medicina Tropical
qPCR	Reação em cadeia da polimerase quantitativa em tempo real
RBC ₅₀	Concentração inibitória de 50% de hemácias humanas
RNA	Ácido ribonucleico
rRNA	Ácido ribonucleico ribossômico
ROS	Espécies reativas de oxigênio
RPMI	<i>Roswell Park Memory Institute</i>
RT-qPCR	Reação em cadeia da polimerase com transcrição reversa quantitativa
SDS	Dodecil sulfato de sódio
SFB	Soro fetal bovino
SFM	Sistema fogocítico mononuclear
SI	Índice de seletividade
SLA	Extrato solúvel de <i>Leishmania</i>
Slc11a1	Gene carreadores de soluto da família 11 membro a1
SSC	<i>Side Scatter</i>
TGF-β	Fator de transformação de crescimento-beta
Th1	Linfócitos T <i>helper</i> do tipo 1
Th2	Linfócitos T <i>helper</i> do tipo 2
TNF-α	Fator de necrose tumoral-alfa
U	Unidade
UFMG	Universidade Federal de Minas Gerais
UFV	Universidade Federal de Viçosa
UV	Ultravioleta
v/v	Volume/volume
WHO	<i>World Health Organization</i>

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CONSIDERAÇÕES INICIAIS

Essa tese é requisito parcial para obtenção do grau de Doutor em Ciências da Saúde pelo Programa de Pós-Graduação em Infectologia e Medicina Tropical (PPG-IMT) da UFMG. O presente trabalho está inserido na linha de pesquisa “Leishmanioses”, a qual propõe investigar as características patogênicas, anátomo-patológicas, propedêuticas, terapêuticas e profiláticas das leishmanioses tegumentar e visceral através da avaliação, clínica e experimental, da eficácia e segurança da farmacoterapia da doença, aspectos imunológicos relacionados a nova forma de diagnóstico laboratorial, bem como na prevenção de novos fármacos ativos e menos tóxicos aos pacientes.

Durante o doutoramento, foram desenvolvidos dois projetos distintos a partir de colaborações com os professores Dr. João Paulo Viana Leite, Departamento de Bioquímica e Biologia Molecular, e Dr. Róbson Ricardo Teixeira, Departamento de Química, da Universidade Federal de Viçosa (UFV). Inicialmente, foi proposto e realizado um estudo abordando um composto flavônico inédito extraído de planta típica de países tropicais como potencial estratégia terapêutica no tratamento da leishmaniose visceral. Diante os resultados positivos encontrados sobre a potencial atividade antileishmanial dessa molécula, o prosseguimento seria a avaliação também em modelo experimental da leishmaniose tegumentar.

Contudo, devido a contratempos internos do Laboratório de Biodiversidade coordenado pelo professor Dr. João Paulo, não foi possível realizar uma segunda extração do produto natural para proceder com a segunda etapa do projeto. Não obstante, esse primeiro projeto gerou, em 2020, a publicação intitulada “*Parasitological and immunological evaluation of a novel chemotherapeutic agent against visceral leishmaniasis*”, no periódico *Parasite Immunology*, além do depósito de patente de nº BR10201901835. Este projeto foi apresentado, ainda, em congressos, seminários e no cumprimento da qualificação do doutorado, dentro do prazo, em março de 2021.

Na ausência de terapias mais efetivas e menos tóxicas contra as leishmanioses, a identificação e a caracterização de novas drogas ou moléculas tornaram-se importantes alvos de estudo. Nesse contexto, tendo em vista a experiência em síntese orgânica do grupo de pesquisa coordenado pelo professor Dr. Róbson, bem como a lamentável descontinuação do primeiro projeto, foi proposto e elaborado um segundo estudo. O projeto foi desenvolvido a partir dos resultados preliminares, *in vitro*, que demonstraram a potencial atividade antileishmanial de duas moléculas sintéticas derivadas de vanilina contra diferentes espécies de

Leishmania spp. Assim sendo, esses derivados sintéticos foram avaliados, *in vivo*, em modelo murino da leishmaniose visceral (LV) e leishmaniose tegumentar (LT) experimental.

Perante o avanço do doutoramento, a realização do segundo projeto foi dividida entre os dois laboratórios de pesquisa coordenados pelo professor Dr. Eduardo Antonio Ferraz Coelho com participação ativa da autora da presente tese em ambos, porém com a concordância de que em um dos produtos científicos seria coautora. Desse modo, esse estudo gerou os artigos intitulados “*New synthetic molecules incorporated into polymeric micelles used for treatment against visceral leishmaniasis*”, publicado na revista *Cytokine* em fevereiro de 2024, e “*Treatment using vanillin-derived synthetic molecules incorporated into micelles is effective against infection caused by Leishmania amazonensis species*” no periódico *Experimental Parasitology* em março de 2024.

Diante desse cenário, optou-se por defender a tese apresentando os três trabalhos desenvolvidos. Apesar de serem moléculas distintas, um flavonoide extraído naturalmente e outros dois derivados produzidos quimicamente, são todos compostos fenólicos altamente bioativos com papel significativo no combate à infecção por *Leishmania*. É fundamental a divulgação de estudos que ressaltam a importância dos produtos naturais e seus derivados como alternativa a ser considerada na busca por um fármaco mais eficaz, seguro e economicamente viável para o tratamento das leishmanioses.

Ademais, durante o doutoramento, a aluna atuou como integrante em diferentes projetos de pesquisa coordenados pelo professor Dr. Eduardo, assim como em outras colaborações que visam o aprimoramento das condições de diagnóstico, prognóstico, tratamento e prevenção de importantes doenças tropicais negligenciadas utilizando ferramentas biotecnológicas como imunoproteômica, *phage display*, metabolômica, reposicionamento de fármacos e sistemas *drug delivery*. Ainda, atuou como monitora de disciplinas ofertadas pelo PPG-IMT e no COLTEC/UFMG e contribuiu com a orientação técnica de alunos de iniciação científica e mestrado, além da oportunidade de acompanhar pacientes com leishmaniose tegumentar ou enfermidades associadas à doença mucosa, sob a orientação da professora Dra. Denise Utsch Gonçalves, no Ambulatório São Geraldo do Hospital das Clínicas da UFMG.

A presente tese foi elaborada de acordo com as Diretrizes para Normalização de Trabalhos Acadêmicos (UFMG, 2023) e a Resolução nº 02/2013, de 18 de setembro de 2013, que regulamenta o formato dos trabalhos finais e de qualificação do PPG-IMT/UFMG.

1. INTRODUÇÃO

As leishmanioses são um complexo de doenças infecto-parasitárias causadas por protozoários do gênero *Leishmania* e transmitidas ao homem por vetores fêmeas flebotomíneos. Esse complexo de doenças é considerado um importante problema de saúde pública no mundo, sendo endêmico em 98 países (Burza *et al.*, 2018; PAHO/WHO, 2024). Conforme a espécie do parasito e a resposta imune do hospedeiro, a infecção pode ser assintomática ou desenvolver um amplo espectro de manifestações clínicas, tais como: leishmaniose tegumentar, que se apresenta sob as formas cutânea, menos severa com formação de úlceras autoresolutivas; cutâneo-difusa, com lesões nodulares disseminadas; e mucosa, caracterizada por lesões debilitantes, principalmente, na face, e leishmaniose visceral, forma mais grave da doença, que atinge órgãos como baço e fígado, resultando em morte se não for tratada (Alvar *et al.*, 2012; Uliana *et al.*, 2018; Kaye *et al.*, 2020).

O controle das leishmanioses requer a combinação de diagnóstico precoce e tratamento eficaz. O diagnóstico da doença baseia-se na avaliação clínica do paciente, nos dados epidemiológicos e na confirmação por exames laboratoriais parasitológicos, imunológicos e moleculares (Singh, 2006; Rodríguez-Cortés *et al.*, 2010). O tratamento atual inclui o uso de antimoniais pentavalentes, pentamidina, paramomicina, miltefosina, anfotericina B e sua formulação lipossomal. Tais fármacos, contudo, apresentam limitações como toxicidade, administração prolongada e efeitos adversos que provocam a não adesão dos pacientes à terapia, bem como o surgimento de cepas resistentes. Além disso, o custo elevado dificulta o tratamento (Freitas-Junior *et al.*, 2012; Sundar & Chakravarty, 2015; Ponte-Sucre *et al.*, 2017).

Na ausência de terapias mais efetivas, a identificação e a caracterização de novos compostos tornaram-se importantes alvos de estudo das leishmanioses. Há, entretanto, falta de interesse e de apoio financeiro das empresas farmacêuticas na pesquisa por novas estratégias terapêuticas, visto que se trata de uma doença tropical negligenciada, a qual atinge, principalmente, países subdesenvolvidos ou em desenvolvimento. Vale ressaltar que o processo de desenvolvimento de novos fármacos requer longo período de estudo e elevado investimento (Hughes *et al.*, 2011). Nesse contexto, os produtos naturais, especialmente metabólitos secundários extraídos de plantas e seus derivados sintéticos, são uma alternativa a ser considerada no tratamento das leishmanioses, dado o menor custo de produção, além das modificações químicas que permitem atingir máxima eficácia terapêutica e baixa toxicidade (Cortes *et al.*, 2020; Gervazoni *et al.*, 2020).

A diversidade do reino vegetal, essencialmente no Brasil, aliada à complexidade estrutural de cada espécie propiciam a descoberta de novas moléculas bioativas (Chaachouay & Zidane, 2024). Além disso, um composto natural pode servir como molde para a concepção de análogos sintéticos com modificações químicas estruturais, permitindo melhorar suas propriedades farmacológicas como biodisponibilidade e, conseqüentemente, aumentar a atividade biológica, bem como proporcionar alto rendimento de produção (Granchi, 2022).

Dentre os metabólitos secundários de plantas que apresentam atividade biológica, destacam-se os compostos fenólicos, em especial flavonoides e ácidos fenólicos como vanilina. Os flavonoides são abundantes em plantas da família Moraceae, particularmente na espécie *Maclura tinctoria*, típica de regiões tropicais e subtropicais. Com o mesmo perfil de distribuição geográfica, da orquídea *Vanilla planifolia* é obtido a vanilina, considerada principal componente da baunilha, mas que pode ser facilmente sintetizada (Genovese *et al.*, 2016; Zhang *et al.*, 2017). Esses polifenóis detêm alto valor farmacológico, principalmente, devido às propriedades antimicrobiana, antioxidante, anti-inflamatória e antialérgica (Boniface & Ferreira, 2019; Kumar & Goel, 2019; Santos *et al.*, 2019).

Os flavonoides, assim como os derivados da vanilina têm demonstrado ação contra vírus (Groweiss *et al.*, 2000; Rodrigues *et al.*, 2023), fungos (El-Sohly *et al.*, 2001; Gazolla *et al.*, 2023) e bactérias (Alvaro *et al.*, 2015; das Chagas Almeida *et al.*, 2019; Ellatar *et al.*, 2022). Embora não haja relatos na literatura sobre a atividade antiparasitária da *M. tinctoria*, Salustiano (2019) obteve em seu trabalho de bioprospecção de extratos dessa espécie vegetal diferentes metabólitos bioativos, destacando a molécula denominada CMt (5,7,3',4'-tetrahidroxi-6,8-diprenilisoflavona), a qual demonstrou em ensaios prévios potencial ação contra *Leishmania* spp. Em um estudo recente, dois derivados sintéticos da vanilina denominados 3s [4-(2-hidroxi-3-(4-octil-1H-1,2,3-triazol-1-il)propoxi)-3-metoxibenzaldeído] e 3t [4-(3-(4-decil-1H-1,2,3-triazol-1-il)-2-hidroxipropoxi)-3-metoxibenzaldeído] foram avaliados, preliminarmente, em experimentos *in vitro* contra *Leishmania* spp. Ambas moléculas foram efetivas contra as espécies de *Leishmania* e apresentaram mecanismo de ação similar, sugerindo morte do parasito por apoptose (Freitas *et al.*, 2023).

Portanto, tendo em vista a ausência de uma terapia mais eficaz, menos tóxica e de baixo custo, é necessário o desenvolvimento de novas estratégias de tratamento contra as leishmanioses. Assim sendo, o presente trabalho propôs avaliar a atividade biológica da molécula CMt em ensaios *in vitro*, e CMt, 3s e 3t na infecção experimental *in vivo* por *Leishmania infantum* e *L. amazonensis*, visando identificar potenciais candidatos a fármacos para as leishmanioses tegumentar e visceral.

2. REVISÃO DA LITERATURA

2.1. Aspectos biológicos da *Leishmania*

O gênero *Leishmania* compreende mais de 20 espécies de protozoários – Supergrupo Excavata, Grupo Euglenozoa e Subgrupo Kinetoplastea (classificação baseada na filogenia genética) (Adl *et al.*, 2005), capazes de causar um complexo de doenças denominado leishmanioses, que é transmitida ao hospedeiro vertebrado através da picada da fêmea de flebotomíneos, especialmente *Phlebotomus* e *Lutzomyia* (Teixeira *et al.*, 2013; PAHO/WHO, 2024). Além da transmissão vetorial, as leishmanioses também podem ser transmitidas por outras vias, mais raras, como compartilhamento de seringas, transfusão sanguínea, acidentes de laboratório e de forma congênita (Piscopo & Mallia Azzopardi, 2007; Alemayehu & Alemayehu, 2017). Diversas espécies de mamíferos selvagens, domésticos e sinantrópicos são considerados como hospedeiros e/ou reservatórios de *Leishmania* spp., sendo o cão um reservatório de grande importância veterinária (Ready, 2008; Steverding, 2017).

Esses tripanosomatídeos são parasitos intracelulares obrigatórios que exibem estágios distintos ao longo de sua vida que alternam entre hospedeiros vertebrados e invertebrados. No hospedeiro mamífero, o parasito apresenta-se no estágio amastigota, caracterizado por forma esférica ou oval, não móvel, localizada no interior de células do sistema fagocítico mononuclear (SFM), em especial monócitos e macrófagos. A fêmea do flebotomíneo, ao realizar o repasto sanguíneo no animal infectado, ingere sangue e/ou linfa intersticial contendo macrófagos parasitados por formas amastigotas de *Leishmania*. No intestino do inseto vetor, o parasito inicia um processo de diferenciação, transformando-se em promastigota procíclica até alcançar a forma infectante denominada promastigota metacíclica, morfologicamente alongada, flagelada e móvel (Bates & Rogers, 2004; Sunter *et al.*, 2017). Durante a hematofagia, o flebotomíneo inocula na derme de um hospedeiro saudável, juntamente com a saliva, as formas promastigotas metacíclicas, as quais são internalizadas no vacúolo parasitóforo de células do SFM onde se proliferam e se diferenciam em amastigotas. Logo, essas células se rompem e liberam as amastigotas que infectam novas células, disseminando a infecção (Bates, 2007; De Menezes *et al.*, 2016). Desse modo, os parasitos circulantes podem infectar um novo hospedeiro invertebrado durante o repasto sanguíneo, estabelecendo assim o ciclo biológico da *Leishmania* (Figura 1).

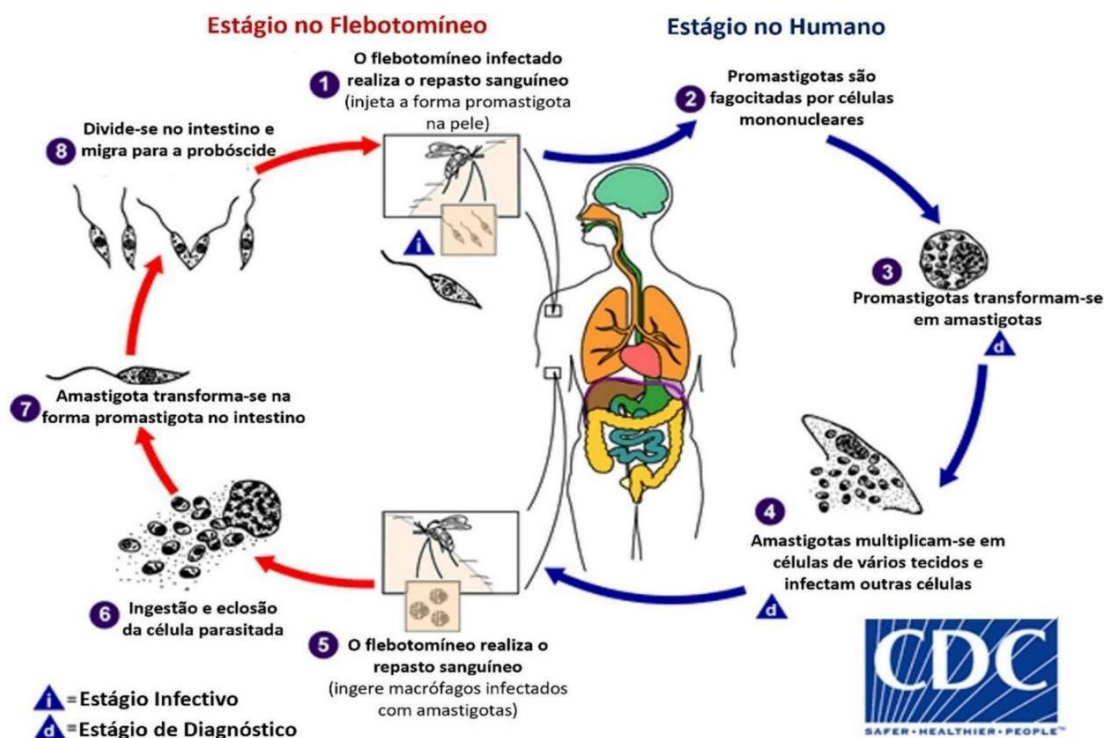


Figura 1. Ciclo biológico de *Leishmania* spp.

As leishmanioses são transmitidas pela picada de flebotomíneos fêmeas infectadas. Os flebotomíneos inoculam na derme do hospedeiro, pela probóscide, promastigotas metacíclicas durante o repasto sanguíneo (1). As formas promastigotas depositadas no local da picada são fagocitadas por macrófagos (2) e outras células do sistema fagocítico mononuclear (SFM). No interior do vacúolo parasitóforo dessas células, as promastigotas diferenciam-se em amastigotas (3) que se multiplicam por divisão simples. Após a lise dessas células, amastigotas são liberadas, infectando novas células do SFM (4). Os flebotomíneos são infectados ao ingerirem as células repletas de amastigotas durante o repasto sanguíneo (5). No intestino do inseto vetor, as amastigotas ingeridas são liberadas (6), diferenciam-se em promastigotas (7) e migram para a probóscide (8), completando assim o ciclo biológico da *Leishmania*.

Fonte: Adaptado de Centers for Disease Control and Prevention (CDC), 2017.

As distintas espécies de *Leishmania*, assim como outros tripanossomatídeos exibem organelas eucarióticas típicas como membrana plasmática, retículo endoplasmático e complexo de Golgi, mas também apresentam estruturas peculiares (Teixeira *et al.*, 2013) como os glicossomos, sendo responsáveis por apresentar um grande número de enzimas importantes da via glicolítica (Banerjee *et al.*, 2019); megassomos, com hidrolases lisossomais, e acidocalcissomos que funcionam para armazenamento de íons com papel na resposta ao estresse osmótico e na manutenção do pH intracelular (Do Campo *et al.*, 2010) (Figura 2).

Fonte: Adaptado de Teixeira *et al.*, 2013.

A respiração mitocondrial ocorre via cadeia transportadora de elétrons, a qual é composta por complexos enzimáticos que funcionam como bombas de prótons estabelecendo um gradiente/potencial através da membrana ($\Delta\Psi_m$) mitocondrial interna para a síntese de trifosfato de adenosina (ATP) por meio da ATP sintase em um processo acoplado à fosforilação oxidativa (Mather *et al.*, 2007; Dey *et al.*, 2010). Durante esse processo, ocorre a formação de espécies reativas de oxigênio (ROS), no qual parte do oxigênio é reduzido a ânions superóxido

(O_2^-) e, conseqüentemente, peróxido de hidrogênio (H_2O_2) e radicais hidroxila ($OH^\cdot$) (Turrens, 2004). No entanto, distúrbios no $\Delta\Psi_m$ estão associados a uma produção excessiva de ROS, provocando estresse oxidativo na célula. Essas espécies reativas atravessam a membrana mitocondrial e se espalham pelo citosol e para outras organelas, com efeitos deletérios para macromoléculas como proteínas, lipídeos e DNA, e culminam na morte celular (Jin *et al.*, 2018; Reverte *et al.*, 2022).

O aumento da produção de ROS nos tripanossomatídeos é acompanhado por perda do $\Delta\Psi_m$ e aumento de cálcio citosólico (Ca^{2+}), interferindo no processo metabólico e na permeabilidade não-seletiva da membrana mitocondrial interna, respectivamente, (Kowaltowski *et al.*, 2001) e acompanhado, ainda, da exposição de fosfatidilserina na membrana plasmática externa. Além disso, essas alterações potencializam a liberação de citocromo C (componente importante da cadeia transportadora de elétrons) e a ativação de proteases e nucleases, ocasionando a fragmentação do DNA e o desmantelamento celular. Todo esse processo representa uma das principais vias de morte celular em tripanossomatídeos – apoptose, induzida por choque térmico, carência de nutrientes, desregulação do ciclo celular e fármacos antiparasitários (Smirlis *et al.*, 2010; Dos Anjos *et al.*, 2016).

A apoptose é um processo fisiológico essencial responsável pela homeostase dos tecidos através da remoção de células danificadas ou não funcionais, renovação celular e tecidual, e prevenção de uma resposta inflamatória a partir do desenvolvimento e funcionamento adequado do sistema imune (Voll *et al.*, 1997; Danial & Korsmeyer, 2004; Martinez *et al.*, 2010). Esse processo de morte determina a sobrevivência e a manutenção do ciclo de vida de protozoários com o desenvolvimento do parasito no hospedeiro mamífero e no inseto vetor, diferenciação celular e modulação da resposta imune do hospedeiro (Lüder *et al.*, 2010; Gannavaram & Debrabant, 2012). No entanto, em um cenário de infecção por *Leishmania*, a apoptose desempenha papel fundamental na defesa celular, compondo um mecanismo de controle da disseminação do parasito e, por conseguinte, desenvolvimento da doença (Basmaciyan & Casanova, 2019).

Assim sendo, a mitocôndria por ser única e crucial para a sobrevivência de *Leishmania*, a torna alvo de ação de fármacos. O entendimento da biologia celular desse protozoário, do mesmo modo que dos fatores que desencadeiam a sua morte, concede informações primordiais para o desenvolvimento de estudos sobre as leishmanioses no âmbito de suas manifestações clínicas, resposta imune do hospedeiro e ação de fármacos utilizados no tratamento da doença, além de propiciar a busca por novas estratégias terapêuticas.

2.2. Aspectos gerais das leishmanioses

As leishmanioses apresentam um amplo espectro de manifestações clínicas que variam conforme a espécie infectante, o estado imunológico do hospedeiro e outros fatores relacionados ao vetor e ao ambiente (Vargas-Martinez *et al.*, 2013; Natarajan *et al.*, 2014). Essas manifestações podem ser assintomáticas ou clinicamente evidentes, classificadas em duas principais categorias: leishmaniose tegumentar (LT) e leishmaniose visceral (LV). A LT compreende, ainda, as formas clínicas: (i) leishmaniose cutânea (LC), que são observadas lesões na pele, principalmente, úlceras cutâneas; (ii) leishmaniose mucosa ou mucocutânea (LM), que leva à destruição total ou parcial de mucosas do nariz, boca e garganta e; (iii) leishmaniose cutâneo-difusa (LCD), com lesões nodulares não ulcerativas disseminadas em todo o corpo. Essas distintas manifestações da doença são causadas por *L. major*, *L. tropica*, *L. aethiopica*, *L. mexicana*, *L. braziliensis*, *L. amazonensis* e *L. guyanensis* (Burza *et al.*, 2018; WHO, 2023a).

A LC é a manifestação mais frequente da doença, responsável por mais de 95% dos casos de LT, que atinge as áreas mais expostas do hospedeiro e de fácil acesso ao inseto vetor tais como face, pescoço e membros. Esta forma clínica é caracterizada por uma pápula inicial que evolui, após semanas ou meses, para nódulos com bordas elevadas e endurecidas formando úlceras granulomatosas (Lainson & Shaw, 2010; Aara *et al.*, 2013). Normalmente, na ausência de infecção secundária, a lesão é indolor (Handler *et al.*, 2015). Contudo, verifica-se cura espontânea na maioria dos casos, resultando na formação de cicatrizes permanentes que levam à estigmatização social (Pearson & Sousa, 1996; Yanik *et al.*, 2004; Bennis *et al.*, 2017).

A leishmaniose disseminada é uma forma relativamente rara que ocorre devido à disseminação hematogênica e/ou linfática do parasito, caracterizada por numerosas e pequenas lesões ulceradas na pele que acometem diversas partes do corpo (Silveira *et al.*, 2009). Ainda, a LCD é uma forma atípica e severa de LT, que atinge indivíduos com deficiência na resposta imune celular aos antígenos de *Leishmania*, e se inicia com lesão única que progride, cronicamente, para a formação de múltiplas nodulações não ulceradas por todo o corpo (Scott & Novais, 2016). Frequentemente, essas lesões nodulares não apresentam cicatrização espontânea e, ainda, são resistentes ao tratamento (Burza *et al.*, 2018).

Em áreas endêmicas, 1-10% dos casos de LC resulta em LM, após resolução das lesões primárias (Torres-Guerrero *et al.*, 2017). Essa forma clínica é determinada pela habilidade do parasito de atingir mucosas, especialmente trato respiratório superior, via hematogênica ou linfática (Brushi & Gradoni, 2018). Primeiramente, observa-se inflamação e

entupimento nasal, seguida por ulceração da mucosa e perfuração do septo. Além disso, em alguns casos, pode envolver lábios, bochechas, palato mole, faringe ou laringe. A LM não apresenta cura espontânea, sendo difícil tratar e, conseqüentemente, ocasionando grave incapacidade (Amato *et al.*, 2009; Guerra *et al.*, 2011). Além disso, há a leishmaniose recidiva, forma crônica rara e recorrente de LT, que ocorre após infecção primária por *L. tropica*, *L. major* ou *L. braziliensis* (Pearson & Sousa, 1996; Marovich *et al.*, 2001; Sharifi *et al.*, 2010), apresentando novas lesões ao redor da antiga cicatriz que, possivelmente, se expandem (van Griensven *et al.*, 2014).

A LV, também conhecida como calazar, é uma doença crônica e sistêmica causada por *L. donovani* e *L. infantum* (Singh, 2006; Kevric & Cappel, 2015). Essa doença constitui a manifestação clínica mais grave que afeta, principalmente, linfonodos, baço, fígado e medula óssea comprometendo, após disseminação, o sistema retículoendotelial (Wilson *et al.*, 2005). Inicialmente, os pacientes com LV são assintomáticos, mas após o período de 10 dias a 24 meses podem desenvolver uma condição sistêmica com esplenomegalia, hepatomegalia, anemia, febre persistente e perda de peso. Além disso, os doentes apresentam altos níveis de anticorpos e intenso parasitismo em órgãos-alvo (Kaye, 2002; MS, 2014; Barani *et al.*, 2020), mas se não tratados podem evoluir para infecções secundárias e morte (Bern *et al.*, 2008). Não obstante, indivíduos com LV tratados (aparente cura clínica) podem, ainda, desenvolver uma forma crônica de LC denominada leishmaniose dérmica pós-calazar, na qual apresentam máculas hipopigmentadas ou exantema maculopapular eritematoso ao redor da boca e do tronco, e que se estendem por todo o corpo (Zijlstra *et al.*, 2003; Zijlstra, 2014).

As manifestações clínicas, a manutenção da carga parasitária e o processo patológico da doença estão estreitamente relacionados à resposta imune do hospedeiro frente à infecção por *Leishmania*. Nesse sentido, um perfil de resistência à infecção e, conseqüentemente, ao desenvolvimento da doença está associado ao estabelecimento da imunidade celular, especificamente pela ativação e diferenciação de linfócitos T que estimulam a produção de citocinas pró-inflamatórias como interferon-gama (IFN- γ), fator de necrose tumoral-alfa (TNF- α), interleucina-12 (IL-12) e fator estimulador de colônia de granulócitos e macrófagos (GM-CSF), as quais induzem a ativação de fagócitos mononucleares infectados como os macrófagos (Atri *et al.*, 2018). Logo, os macrófagos ativados são capazes de controlar a infecção através da produção de óxido nítrico (NO) e ROS, culminando na eliminação do parasito (Novais & Scott, 2016). Esse perfil de resposta imune do tipo T *helper* 1 (Th1) também está associado aos baixos títulos de imunoglobulinas G (IgG) circulantes. No entanto, uma resposta celular suprimida do tipo Th2, caracterizada por aumento dos níveis de IL-10, IL-5, IL-4 e fator de crescimento

transformador-beta (TGF- β) que regulam negativamente os níveis de TNF- α e de ROS, além de alta concentração de anticorpos IgG1, favorecem a proliferação intracelular do parasito e a progressão da doença (Li *et al.*, 2013; Muxel *et al.*, 2018; Giunchetti *et al.*, 2019).

Os processos imunológicos de resistência/susceptibilidade à infecção, bem como o amplo espectro de manifestações clínicas com potencial de morte que atinge, principalmente, comunidades marginalizadas, caracterizam as leishmanioses como um grave problema de saúde pública no mundo com mais de 12 milhões de infectados. As leishmanioses estão distribuídas em regiões tropicais e subtropicais, atingindo 98 países na América, África, Ásia e Europa, com incidência anual de 0,7 a um milhão de novos casos (WHO, 2023a).

De 2017 a 2022, foram notificados mais de 250 mil casos de LT, com média anual de 42.166 casos. Destes, aproximadamente 97% correspondem à região Andina (41%), Brasil (37%) e América Central (19%). Somente no ano de 2022, o Brasil registrou 12.878 casos de LT. No continente americano, a LV é descrita em 13 países, sendo o Brasil representante de 92% dos casos (OPAS, 2023). No período de 2001 a 2021, foram registrados nas Américas quase 70 mil novos casos de LV, com média anual de 2.488 casos e taxa de letalidade de 8% (PAHO/WHO, 2024). Esses dados são, portanto, preocupantes e ressaltam a necessidade de medidas de prevenção e controle efetivas objetivando reduzir a ocorrência de casos, bem como evitar mortes.

2.3. Tratamento das leishmanioses

As principais medidas de controle das leishmanioses recomendadas visam a redução da população de flebotomíneos no peri e intradomicílio, eliminação dos reservatórios (eutanásia de cães infectados), atividades de educação em saúde, assim como diagnóstico precoce e tratamento eficaz (WHO, 2023a). Todavia, essas medidas apresentam resultados controversos, sendo que seus efeitos são somente observados a longo prazo ou apresentam falhas devido aos distintos perfis epidemiológicos, às características da doença (manifestações clínicas, variedade de espécies, vetores e reservatórios) e às dificuldades e/ou limitações de diagnóstico e tratamento atuais (Barreto *et al.*, 2011). Embora existam diferentes fármacos utilizados no tratamento contra as leishmanioses, a maioria não são adequadas e/ou suficientes para erradicar a infecção e/ou reverter a doença.

Nos últimos 70 anos, a primeira escolha de tratamento das leishmanioses, especialmente LV, LC e LM, eram os antimoniais pentavalentes, disponíveis em duas formulações: antimoniato de metilglucamina (Glucantime[®]) e estibogluconato de sódio (Pentostam[®])

(Marsden & Jones, 1985). Esses compostos são administrados via parenteral na dose de 20mg/Kg por um mês, podendo ser utilizado em associação com outros fármacos (Akbari *et al.*, 2017). Acredita-se que os antimoniais atuem interferindo na bioenergética das formas amastigotas do parasito, inibindo a atividade glicolítica e a via oxidativa dos ácidos graxos e, consequentemente, reduzindo a produção de ATP e biossíntese molecular (Wyllie *et al.*, 2004; Croft *et al.*, 2006). No entanto, após décadas de uso, os parasitos desenvolveram mecanismos de resistência tornando-se um desafio no tratamento das leishmanioses (Mohapatra, 2014) somado, ainda, aos efeitos adversos como arritmia cardíaca, pancreatite, toxicidade renal e hepática. Com isso, esses compostos não são indicados para gestantes, idosos e indivíduos com doença cardíaca, renal ou hepática (Oliveira *et al.*, 2011; Marques *et al.*, 2019).

Contudo, na ausência de uma resposta terapêutica satisfatória ou uso não recomendado dos antimoniais, a terapia de segunda escolha consiste em anfotericina B, pentamidina, paramomicina e miltefosina. A anfotericina B é um antibiótico poliênico, normalmente, utilizado no tratamento de infecções fúngicas sistêmicas, mas que apresenta atividade antileishmanial com taxa de cura de 97% para LV e LT considerando as espécies de *Leishmania* envolvidas (Croft & Yardley, 2002). Esse fármaco é capaz de controlar a infecção através de dois mecanismos: auto-oxidação com produção de radicais livres e ligação aos esteróis da membrana do parasito, formando poros que aumentam a permeabilidade e o influxo de íons.

Ademais, verifica-se a interação seletiva da anfotericina B com o colesterol da membrana de macrófagos, bloqueando a entrada dos parasitos nas células e impedindo a disseminação da infecção (Paila *et al.*, 2010; Purkait *et al.*, 2012; Marek & Timmons, 2019). Esse fármaco apresenta baixa biodisponibilidade oral, sendo assim administrado via intravenosa em ambiente hospitalar. Além dessa limitação, o tratamento com anfotericina B abrange efeitos adversos como febre, dor de cabeça, náusea, anemia, leucopenia, hipotensão, hipocalcemia, hipomagnesemia, nefrotoxicidade e miocardite (Laniado-Laborín & Cabrales-Vargas, 2009; Anjum *et al.*, 2023).

Com o intuito de reduzir a toxicidade e os efeitos colaterais, foi desenvolvida uma formulação lipossomal de anfotericina B (AmBisome®). Os lipossomas consistem em vesículas esféricas com núcleo aquático cercado por uma bicamada lipídica, no qual o componente lipídico conduz o fármaco para o meio intracelular favorecendo a interação com o ergosterol do parasito sobre o colesterol do hospedeiro. Embora o AmBisome® tenha alto custo, exibe altos níveis plasmáticos e tempo de meia-vida mais curto, atingindo altas concentrações no fígado e no baço (Berman, 1997; Balasegaram *et al.*, 2012). A anfotericina B lipossomal é recomendada para pacientes com LV e LT, gestantes e indivíduos com complicações cardíacas,

renais ou com co-infecção pelo HIV não responsivos ao tratamento de primeira escolha (OPAS, 2022). Ademais, existem outras formulações lipídicas da anfotericina B como complexo lipídico (Abelcet®) e dispersão coloidal (Amphocil®), que apresentam eficácia semelhante, mas diferem em sua estrutura, tamanho, forma, composição e conteúdo (Croft *et al.*, 2006; Goto & Lindoso, 2010).

Outro tratamento das leishmanioses de segunda escolha é o uso da pentamidina, indicada aos pacientes que não respondem aos antimoniais pentavalentes e em casos de LC e LCD (Andersen *et al.*, 2005; OPAS 2022). Esse fármaco atua no cinetoplasto e na membrana mitocondrial, interferindo na síntese de kDNA e assim, culminando na morte do parasito. Porém, as limitações do uso da pentamidina incluem efeitos adversos como hipotensão, hipoglicemia, mialgia e diabetes mellitus (Brindha *et al.*, 2021). Em adição aos efeitos adversos, destaca-se o surgimento da resistência parasitária que diminuiu a eficácia desse tratamento ao longo do tempo (Croft & Sundar, 2006).

A paramomicina é um antibiótico aminoglicosídeo de amplo espectro utilizado no tratamento de infecções bacterianas e parasitárias (amebíase e giardíase), mas que também demonstra atividade antileishmanial ao afetar a atividade ribossomal do parasito, inibindo a síntese proteica e o $\Delta\Psi_m$ e, logo, a respiração (Chawla *et al.*, 2011). Esse fármaco apresenta baixo custo e efeitos colaterais como nefro- e oto-toxicidade, considerados raros. Dessa forma, a paramomicina torna-se uma alternativa para os casos de resistência aos outros tratamentos (Croft & Yardley, 2002). Contudo, observa-se variações na sua eficácia em casos de LV (Hailu *et al.*, 2010), mas é recomendado o uso em pacientes pediátricos com LC (OPAS, 2022).

Ademais, tem-se a miltefosina também utilizada na prática clínica para o tratamento das leishmanioses. Inicialmente desenvolvida como terapia para o câncer, a miltefosina é um medicamento, via oral, utilizado contra LV na Índia, desde 2002, que apesar de relativa eficácia, registra recidiva em quase 20% dos pacientes (Sundar *et al.*, 2002; Rijal *et al.*, 2013), sendo também utilizada contra LC e LM na América do Sul com diferenças consideráveis na porcentagem de sucesso da terapia (Soto *et al.*, 2008; Pinart *et al.*, 2020). A miltefosina atua principalmente inibindo a biossíntese de fosfolipídios e esteróis em *Leishmania* (Goto & Lindoso, 2010; Seifert, 2011). No entanto, apresenta toxicidade para o sistema gastrointestinal, hepático e renal, teratogenicidade e custo relativamente alto, além do desenvolvimento de resistência por algumas cepas de *Leishmania* devido ao seu longo tempo de meia-vida (Deep *et al.*, 2017).

Assim sendo, como as terapias farmacológicas contra as leishmanioses apresentam limitações como alto custo, toxicidade e resistência parasitária, faz-se urgente o desenvolvimento de novos fármacos que possam substituir ou complementar as alternativas disponíveis. As investigações de novos produtos, formulações e/ou esquemas terapêuticos se baseiam, principalmente, em estudos envolvendo reposicionamento de fármacos, substâncias comumente utilizadas pela medicina popular e compostos isolados de microrganismos e plantas (Santos *et al.*, 2008). Além disso, a nanotecnologia, aliada à medicina, têm permitido avanços significativos no tratamento de doenças através dos sistemas de liberação controlada de fármacos.

2.4. Produtos naturais obtidos de plantas como abordagem terapêutica

Desde a antiguidade, produtos naturais obtidos de plantas têm sido amplamente empregados no tratamento e na prevenção de diversas doenças considerando os achados arqueológicos da era Paleolítica, cerca de 60.000 anos atrás (Aboelsoud, 2010; Sam, 2019), registros escritos sumérios de plantas de uso medicinal há mais 5.000 anos (Yaniv, 2014), tratado médico egípcio Papiro Ebers, datado de aproximadamente 1.550 a.C. (David & Forshaw, 2023), bem como a tradicional medicina chinesa, que perdura até os dias atuais (Fan *et al.*, 2012; He, 2013). Portanto, o significado histórico do uso de plantas na medicina determina a longa e forte relação entre o mundo natural e o homem na busca pela saúde e bem-estar (Reis, 2001). Essas tradições moldaram, significativamente, o avanço da farmacologia e das práticas de saúde.

Com isso, nos últimos anos, produtos naturais se tornaram populares em muitos países ocidentais devido aos seus efeitos terapêuticos, segurança e acessibilidade, sendo o ponto de partida na identificação e na descoberta de novas drogas (Hu *et al.*, 2012). De acordo com a Organização Mundial de Saúde (OMS), cerca de 65% a 80% da população dos países em desenvolvimento utiliza plantas e/ou seus derivados como conduta primária no tratamento de determinadas doenças. Além disso, estima-se que cerca de 40% dos produtos farmacêuticos produzidos originam de plantas (WHO, 2011; 2023b).

O desenvolvimento de novos produtos a partir de fontes naturais é incentivado, principalmente, devido a estimativa de 300.000 espécies de plantas existentes no mundo (De Luca *et al.*, 2012). Dessa abundante variedade, o Brasil se destaca como um dos países com maior biodiversidade, abrigando mais de 20% do total de espécies do planeta. Suas diferentes zonas climáticas somado à sua extensão territorial, quase metade de todo o continente sul-

americano, propiciam a formação de zonas biogeográficas (biomas) que compreendem muitas espécies vegetais endêmicas e de importância econômica mundial (MMA, 2024).

Assim sendo, a pluralidade do reino vegetal aliada à química diversa da estrutura de cada espécie, em sua maioria ainda não explorada, sumariza a importância dos estudos sobre produtos naturais como potenciais candidatos no tratamento de doenças (Chaachouaey & Zidane, 2024). As plantas são fonte de moléculas que exibem atividade biológica devido aos seus constituintes químicos sintetizados através de precursores inorgânicos. De acordo com as funções atribuídas a esses compostos químicos são classificados em metabólitos primários e secundários, e hormônios. Os metabólitos primários incluem carboidratos, proteínas, lipídeos e ácidos nucleicos necessários para o crescimento das plantas. Os metabólitos secundários protegem as plantas contra estresses bióticos (microrganismos, insetos e animais) e abióticos (temperatura, umidade e luminosidade). Os hormônios regulam os processos biológicos do organismo como crescimento e desenvolvimento das plantas, além de auxiliar a adaptação em condições de estresse ambiental (Atanasov *et al.*, 2015; Varsha *et al.*, 2017).

Os metabólitos secundários compõem o maior grupo de compostos bioativos, estruturalmente diverso, que são formados durante a biossíntese de metabólitos primários e intermediários. Assim, baseado em sua via biossintética, os compostos secundários são classificados em terpenos, alcaloides, esteróis, fenólicos e flavonoides (Verma *et al.*, 2015). Além disso, essa diversidade estrutural química desses compostos confere a esse grupo inúmeras atividades biológicas como antimicrobiana (Bahman *et al.*, 2019), antifúngica (Samadi *et al.*, 2019), antioxidante (Anwar *et al.*, 2018), antitumoral (Davatgaran-Taghipour *et al.*, 2017; Salehi *et al.*, 2018) e anti-inflamatória (Alvarez *et al.*, 2012).

Ademais, os metabólitos secundários também têm demonstrado potencial efeito terapêutico frente a doenças tropicais (Ndjonka *et al.*, 2013; Katsuno *et al.*, 2015; Sundar & Chakravarty, 2015; Zulfiqar *et al.*, 2017), especialmente ação anti-*Leishmania* (Lage *et al.*, 2016a; Sousa *et al.*, 2019; Tavares *et al.*, 2019, 2020; Freitas *et al.*, 2021; Mendonça *et al.*, 2018, 2019, 2022).

Os compostos fenólicos, também denominados polifenóis, constituem um amplo e importante grupo de metabólitos secundários de ocorrência natural em raízes, folhas, polpa de frutas e cascas de todo o reino vegetal. Até o momento, mais de 8.000 polifenóis foram identificados e isolados (Tian *et al.*, 2021). Esses compostos apresentam uma estrutura química comum que compreende um anel aromático com um ou mais substituintes hidroxila (OH), que são divididos, de acordo com os diferentes grupamentos conjugados a essa estrutura principal,

em flavonoides, ácidos fenólicos, taninos, estilbenos e lignanas (Alu'datt *et al.*, 2017). Dentre esses, as maiores classes de compostos fenólicos são flavonoides e ácidos fenólicos.

2.4.1. Flavonoides

Os flavonoides estão presentes, principalmente, nas plantas superiores em suas folhas, flores, caules e sementes, nos quais estão envolvidos em processos de fixação de nitrogênio e pigmentação (Samanta *et al.*, 2011; Shen *et al.*, 2022). Atualmente, mais de 6.000 moléculas de flavonoides já foram descritas. Esses compostos fenólicos apresentam estrutura básica central do tipo C6-C3-C6, ou seja, dois anéis de benzeno conectados entre si por um anel pirano heterocíclico (Figura 3).

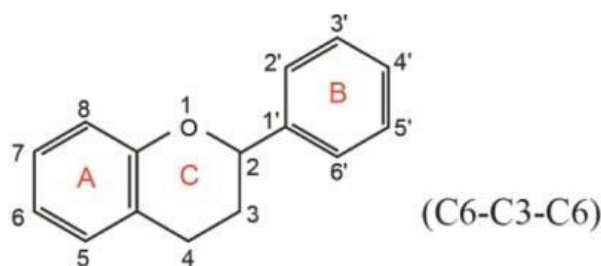


Figura 3. Estrutura química geral de flavonoides.

Estrutura básica dos flavonoides consiste em 15 átomos de carbono (C15) distribuídos em dois anéis aromáticos (A e B), os benzenos, interligados a um anel heterocíclico central, o pirano (C), no qual o primeiro benzeno é condensado com o sexto carbono do pirano que na posição 2 carrega um grupo fenila.

Fonte: Adaptado de Shen *et al.*, 2022.

O grau de insaturação e oxidação do heterociclo central determina as diferentes subclasses de flavonoides: flavonóis, flavanóis, flavonas, isoflavonas, antocianidinas, flavanonas e chalconas (Shen *et al.*, 2022). Esses compostos detêm alto valor farmacológico devido às diversas propriedades bioativas associadas como antimicrobiana, larvicida, antialérgica e anti-inflamatória. Ademais, os flavonoides têm demonstrado ação anti-leishmaniana (Boniface & Ferreira, 2019; Cataneo *et al.*, 2019; Santos *et al.*, 2019).

Entretanto, tem se destacado um grupo único de flavonoides denominado flavonoides prenilados. Esses derivados flavonoides são caracterizados pela presença de uma cadeia lateral prenilada (prenil, geranil e lavandulil) ligada ao esqueleto da estrutura química por prenilação. Essas cadeias laterais apresentam potencial para modificações adicionais como oxidação,

ciclização e hidroxilação, que enriquecem a diversidade estrutural e biológica dos flavonoides prenilados (Shi *et al.*, 2021). Desse modo, esse grupo específico de flavonoides são, geralmente, mais bioativos em comparação aos seus precursores não prenilados, apresentando maior solubilidade lipídica, afinidade pela membrana celular e capacidade de absorção gastrointestinal (Hošek *et al.*, 2011; Jakimiuk *et al.*, 2021). Portanto, os flavonoides prenilados exibem maior potencial de interação com diversos alvos celulares, atraindo, assim, a atenção de pesquisas farmacológicas (Lv *et al.*, 2023).

Os flavonoides prenilados são encontrados em abundância em plantas da família Moraceae, especialmente no gênero *Maclura* (Graweiss *et al.*, 2000; El-Sohly *et al.*, 2001; Genovese *et al.*, 2016). Essa família compreende cerca de 1.500 espécies e 63 gêneros, amplamente distribuídos em regiões tropicais e subtropicais, que inclui árvores, arbustos e cipós. O gênero *Maclura* é composto por 40 espécies, desde a América do Norte à América do Sul (Battilani *et al.*, 2006; Lamounier *et al.*, 2012). Porém, no Brasil foram identificadas apenas duas espécies: *M. brasiliensis* e *M. tinctoria* (Pederneiras, 2020).

M. tinctoria (L.) D. Don ex Steud (Fortunato, 1986), popularmente conhecida como amoreira, taiúva ou amarelinho, desenvolve-se em solos úmidos de diversos domínios fitogeográficos. É uma espécie arbórea frondosa que produz um fruto comestível polposo, adocicado e rico em sementes (Carvalho, 2003; Barroso *et al.*, 2004; Lorenzi, 2020) (Figura 4).



Figura 4. *Maclura tinctoria* (L.) D. Don ex Steud.

Parte aérea com frutos de *M. tinctoria* (A); indivíduo de *M. tinctoria* *in situ* plaqueado da coleção do Programa de Bioprospecção Molecular no Uso Sustentável da Biodiversidade (BIOPROS) (B).

Fonte: Adaptado de Salustiano, 2019.

Os primeiros relatos sobre o uso medicinal da *M. tinctoria* são de comunidades indígenas, especialmente, do Brasil e da Bolívia, as quais preparavam infusões a partir da casca ou das folhas para alívio de dor e inchaço após extrações dentárias (Bueno *et al.*, 2005; Hadju & Hohmann, 2012).

M. tinctoria apresenta uma quantidade substancial de compostos fenólicos como flavonas, flavonóis e isoflavonas na casca e nas folhas (Pires *et al.*, 2021). A literatura científica sobre a atividade biológica dos compostos dessa espécie ainda é limitada. No entanto, os flavonoides extraídos dessa planta exibiram ação contra vírus da imunodeficiência humana (HIV) (Groveiss *et al.*, 2000), fungos oportunistas e patogênicos como *Candida albicans* e *Cryptococcus neoformans* (El-Sohly *et al.*, 2001) e, ainda, bactérias como *Staphylococcus aureus* (das Chagas Almeida *et al.*, 2019), *Streptococcus mutans*, *Porphyromonas gingivalis* e outras (Lamounier *et al.*, 2012; Alvaro *et al.*, 2015). Além disso, estudos apontam também atividade anticancerígena (Chen & Zhang, 2019; Gor *et al.*, 2022), antidepressiva e neuroprotetora (Zhang *et al.*, 2010; Lee *et al.*, 2016; Camargo *et al.*, 2022). Não há estudos sobre a potencial atividade antiparasitária da *M. tinctoria*. Contudo, espécies pertencentes à mesma família Moraceae, especificamente do gênero *Ficus*, demonstraram ação contra *Leishmania major*, em ensaios *in vitro* e *in vivo*, com redução do tamanho das lesões cutâneas e da carga parasitária (Siyadatpanah *et al.*, 2022).

As plantas utilizam flavonoides prenilados como mecanismo de defesa contra microrganismos patogênicos e outras pragas, além de neutralizar o estresse abiótico provocado pelo ambiente como estresse oxidativo ou dessecação. Assim sendo, esses compostos são constitutivos das plantas e, frequentemente, isoflavonoides prenilados são formados em resposta ao dano ou ataque patogênico (Yazaki *et al.*, 2009).

Apesar do potencial farmacológico dos flavonoides prenilados, ainda não são bem explorados. *Cudrania tricuspidata*, família Moraceae, apresenta isoflavonas preniladas que apontaram atividade anti-*Trypanosoma brucei* (Sun *et al.*, 2016) e ação neuroprotetora (Kim *et al.*, 2017). Os dados na literatura científica sobre os aspectos toxicológicos dos flavonoides são limitados (Boniface & Ferreira, 2019). No entanto, alguns estudos apontaram a baixa toxicidade de extratos flavônicos, obtidos de diferentes espécies vegetais, em células murinas e humanas (Sun *et al.*, 2016; Cataneo *et al.*, 2019; Santos *et al.*, 2019).

Em geral, as limitações do uso dos flavonoides nos campos médico/farmacêutico residem em sua baixa solubilidade e biodisponibilidade, além do baixo rendimento de produção vegetal (Sajid *et al.*, 2021). No entanto, uma solução para essas desvantagens é a nanotecnologia, aumentando a estabilidade e o controle desses compostos secundários

encapsulados (Liga *et al.*, 2023). Além disso, os flavonoides prenilados se mostram vantajosos no desenvolvimento de novos fármacos pela sua melhor biodisponibilidade em relação aos não prenilados.

2.4.2. Ácidos fenólicos

Outra importante classe de polifenóis encontrada em plantas são os ácidos fenólicos, caracterizados por constituir vários grupos OH em anéis aromáticos. Esses compostos podem ser divididos em dois subgrupos: ácido benzoico, contendo sete átomos de carbono (C₆-C₁) e ácido cinâmico, com nove átomos de carbono (C₃-C₆). Os derivados do ácido cinâmico são ácidos cumárico, caféico, ferúlico e sinápico. No entanto, os derivados do ácido benzoico incluem ácidos p-hidroxibenzoico, protocatequico, vanílico, gálico e sirínico (Zhang *et al.*, 2016).

A vanilina (4-hidroxi-3-metoxibenzaldeído) (Figura 5A) e sua forma oxidada, o ácido vanílico, constituem os ácidos fenólicos mais comumente estudados por suas propriedades anticancerígena (Rakoczy *et al.*, 2021), antidiabética (Salau *et al.*, 2021) e antimicrobiana (Mok *et al.*, 2020), bem como ação neuroprotetora e cardioprotetora atribuídas ao seu potencial antioxidante (Yan *et al.*, 2017; Sirangelo *et al.*, 2020). Ainda, a vanilina é considerada o principal componente da baunilha natural que é obtida, tradicionalmente, a partir de extratos da fava ou vagem (Figura 5C) de uma orquídea tropical – *Vanilla planifolia* Jack ex. Andrews (Figura 5B e D). Esse composto é aromatizante, fragrância doce e agradável, e de sabor cremoso com ampla aplicação nas indústrias alimentícia, farmacêutica e cosmética (Kumar *et al.*, 2012; Zhang *et al.*, 2017).

O gênero *Vanilla*, família Orchidaceae, é composto por 110 espécies, mas apenas três são cultivadas pelo valor comercial da baunilha como *V. tahitensis* JW Moore, *V. pompona* Schiede e *V. planifolia*, sendo essa última a espécie mais expressiva (Soto Arenas & Cribb, 2013). A baunilha, nome popular dado às espécies, é originária do México e de alguns países da América Central, mas que rapidamente propagou para o mundo (Martão *et al.*, 2021).

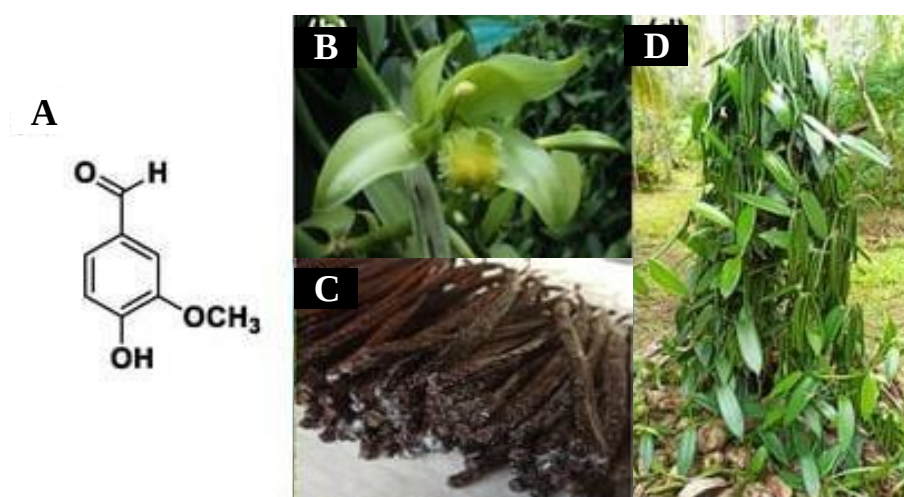


Figura 5. Estrutura química da vanilina e *Vanilla planifolia* Jack ex. Andrews.

Estrutura química da vanilina (A); detalhes da flor de Orchidacea *Vanilla planifolia* (B); fava ou vagem da orquídea, da qual se extrai a baunilha (C); espécie vegetal *V. planifolia* (D).

Fonte: Adaptado de D'Arrigo *et al.*, 2024.

Embora a vanilina possa ser extraída naturalmente dos frutos dessa planta utilizando o etanol como solvente, o longo processo de cultivo, manutenção e colheita somado ao baixo rendimento do produto, elevam o preço da vanilina natural e limitam sua aplicação na indústria (Pérez-Rodríguez *et al.*, 2016). Nesse sentido, a produção sintética é a maior fonte comercial correspondente a 95% da vanilina existente no mercado (Converti *et al.*, 2010). Geralmente, a síntese química ocorre a partir do eugenol encontrado no óleo essencial do cravo, e do guaiaacol e da lignina, componente da madeira e subproduto da indústria de papel, respectivamente (Jenkins & Erraguntla, 2014; Guerriero *et al.*, 2016; Ciriminna *et al.*, 2019; Kaur *et al.*, 2020).

Ademais, pode-se obter derivados de vanilina a partir de fragmentos 1,2,3-triazólicos. Os triazóis são compostos heterocíclicos com anéis de cinco membros com três átomos de nitrogênio e dois átomos de carbono (Bonandi *et al.*, 2017). Essa composição estrutural lhe permite mimetizar diferentes grupos funcionais, justificando sua ampla utilização na síntese de novas moléculas ativas com diversas atividades biológicas (Wang *et al.*, 2017; Avula *et al.*, 2018), além de ser componente fundamental em medicamentos (Rani *et al.*, 2020).

Para obter esses triazóis e, por conseguinte, novas moléculas como derivados de vanilina é amplamente utilizada a metodologia de reação *click* via cicloadição 1,3-dipolar de alcino-azida catalisada por cobre (I) (Gazolla *et al.*, 2023) (Figura 6). Essa reação é rápida e de fácil execução, reduz a complexidade da síntese química e oferece alto rendimento sem subprodutos

indesejáveis. Além disso, os 1,2,3-triazóis resultantes podem atuar como bioisósteres ou ligantes (Manoharan *et al.*, 2023).

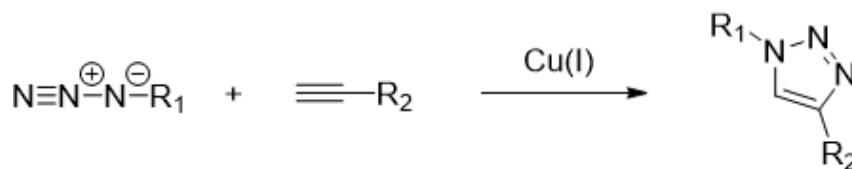


Figura 6. Representação esquemática da reação *click*.

Reação de cicloadição entre um alcino e uma azida catalizada por cobre [Cu(I)] que gera um anel 1,2,3-triazol de cinco membros 1,4-dissubstituído.

Fonte: Adaptado de Santiago, 2022.

Os compostos heterocíclicos 1,2,3-triazóis têm sido amplamente explorados com o intuito de fornecer derivados com diversas atividades biológicas. Desse modo, têm sido desenvolvidos estudos com derivados triazólicos da vanilina apontando ação em células tumorais (Ahmed *et al.*, 2023), bactérias (Elattar *et al.*, 2022), fungos (Gazolla *et al.*, 2023), vírus (Rodrigues *et al.*, 2023) e protozoários como *Entamoeba histolytica* (Singh *et al.*, 2023) e *Leishmania* spp. (Freitas *et al.*, 2023; Gazolla *et al.*, 2024), sem toxicidade significativa às células de mamíferos.

Gazolla e colaboradores (2024) analisaram 22 derivados de vanilina contendo fragmentos 1,2,3-triazólicos em ensaios *in vitro* contra *L. amazonensis*. As análises revelaram que todos os novos compostos, obtidos via reação *click*, inibiram o crescimento parasitário em diferentes graus. No entanto, dentre os 22 derivados, um deles (3-metoxi-4-(3-(1-(4-nitrobenzil)-1H-1,2,3-triazol-4-il)propoxi)benzaldeído ou “4b”) se destacou com uma concentração inibitória de 50% de parasitos (IC₅₀) de $4,2 \pm 1,0 \mu\text{mol L}^{-1}$ e índice de seletividade (SI) de 39, sendo mais eficaz que Gluncatime®.

Em outro estudo recente, desenvolvido por Freitas *et al.* (2023), dois derivados sintéticos de vanilina denominados 3s [4-(2-hidroxi-3-(4-octil-1H-1,2,3-triazol-1-il)propoxi)-3-metoxibenzaldeído] e 3t [4-(3-(4-decil-1H-1,2,3-triazol-1-il)-2-hidroxipropoxi)-3-metoxibenzaldeído], foram avaliados por sua potencial ação antileishmanial *in vitro* contra *L. infantum*, *L. amazonensis* e *L. braziliensis*. O derivado 3s demonstrou SI de 25,1; 18,2 e 22,9 contra promastigotas e de 45,2; 7,5 e 15,0 contra formas amastigotas, respectivamente, para as referidas espécies de *Leishmania*. A molécula 3t apresentou SI de 45,2; 53,0 e 80,0 contra

promastigotas e de 35,9; 46,0 e 58,4 contra amastigotas, respectivamente. Esses compostos foram mais eficazes que anfotericina B (AmpB) contra as diferentes espécies de *Leishmania*, além de apresentarem baixa toxicidade em células murinas e humanas.

Considerando que um candidato promissor às investigações de novos fármacos deve apresentar SI superior a 10 (Dardonville *et al.*, 2006), os resultados dos referidos estudos indicam a potencial ação antileishmanial de derivados triazólicos da vanilina contra *Leishmania*.

2.5. Nanotecnologia como abordagem terapêutica

O termo nanotecnologia remete à aplicação de materiais nanoparticulados e no desenvolvimento de sistemas em nanoescala, ou seja, um a 100 nanômetros. Trata-se de uma área de ampla aplicabilidade, sendo que na medicina incluem administração de fármacos, diagnósticos *in vitro* e *in vivo*, nutracêuticos e produção de materiais biocompatíveis (De Jong & Borm, 2008).

Nos últimos anos, a nanotecnologia farmacêutica recebeu considerável atenção com o propósito de direcionar e controlar a liberação de fármacos, melhorando assim sua absorção, distribuição, metabolismo e excreção (Wong *et al.*, 2012). A primeira técnica a ser desenvolvida, em meados de 1960, foi a microencapsulação, na qual uma camada de polímero atua isolando a substância ativa, evitando o efeito de sua exposição inadequada. Essa camada de agente encapsulante, sob estímulo específico (variação de pH ou temperatura), se desfaz e libera a substância em determinado local. Desse modo, a microencapsulação se torna útil para as indústrias farmacêutica e cosmética, visto que permite a proteção de substâncias sensíveis (lábeis ou voláteis) e o controle da liberação do fármaco, favorecendo a biodisponibilidade e, consequentemente, a redução da dose terapêutica e toxicidade (Kreuter, 2014).

Esses sistemas de liberação de fármacos – sistemas “*drug delivery*”, consistem em nanopartículas inorgânicas (nanopartículas de ouro e magnéticas), nanopartículas poliméricas (micelas, quitosana), nanopartículas lipídicas sólidas, lipossomas, nanotubos de carbono e outros. Além disso, essas estruturas apresentam características fundamentais à sua função, sendo biocompatíveis, não imunogênicas, atóxicas e biodegradáveis (Mendes *et al.*, 2017).

2.5.1. Micelas poliméricas baseadas em Poloxâmero 407

As micelas poliméricas são uma ampla classe de nanocarreadores composta por copolímeros anfifílicos de estrutura do tipo “núcleo-casca”, que apresentam características

físico-químicas como tamanho reduzido, fácil produção, biodegradabilidade, boa estabilidade termodinâmica sob altas temperaturas e baixa toxicidade (Bruni *et al.*, 2017). Esses copolímeros em soluções aquosas muito diluídas se encontram na forma de monômeros. No entanto, com o aumento da concentração do meio, atingem a concentração micelar crítica (CMC), na qual as micelas começam a se formar (Oerlemans *et al.*, 2010; Trivedi & Kompella, 2010).

A composição das micelas poliméricas é responsável pelas características biológicas do nanocarreador. A “casca” hidrofílica da micela (Figura 7) é responsável por uma efetiva proteção estérica e determinante na hidrofília, carga, tamanho, densidade de superfície, além de permitir a modificação da superfície por meio de grupos reativos. O “núcleo” hidrofóbico (Figura 6) indica a capacidade de armazenamento, compatibilidade e perfil de liberação do fármaco (Deshmukh *et al.*, 2017). Polietilenoglicol (PEG), poloxâmero, polivinilpirrolidona e outros polímeros hidrofílicos são utilizados como materiais de revestimento externo da micela, enquanto ácido polilático, policaprolactona, poliestireno e outros polímeros hidrofóbicos são materiais centrais (Almeida *et al.*, 2018, Hwang *et al.*, 2020). Os polímeros mais utilizados para o desenvolvimento de micelas são os copolímeros di-bloco anfifílicos, como poliestireno e polietilenoglicol, e copolímeros tribloco, como os poloxâmeros (Wang *et al.*, 2007; Neophytou *et al.*, 2014).

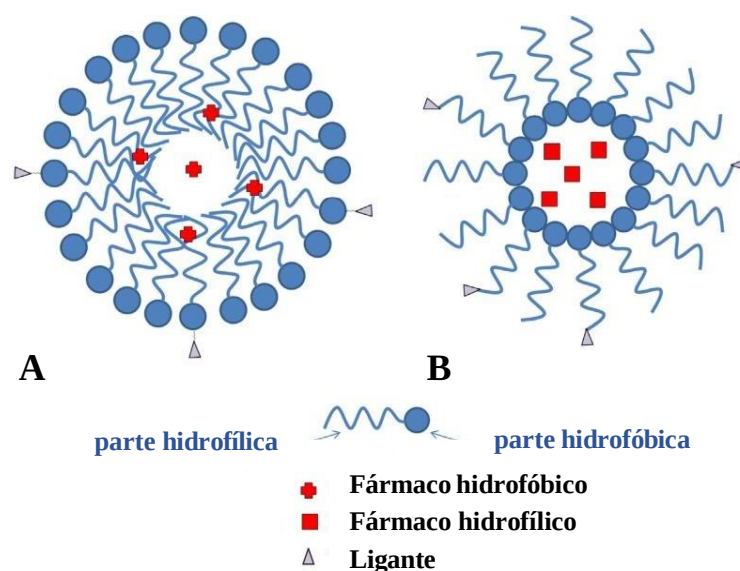


Figura 7. Representação esquemática de micelas poliméricas.

Micela reversa em solvente apolar (A) e micela típica em solvente polar (B).

Fonte: Adaptado de García *et al.*, 2017.

Com os avanços tecnológicos dos sistemas de liberação de fármacos, numerosos estudos mostraram que composições formadas por micelas poliméricas à base de Poloxâmero 407 (P407; Pluronic® F127) apresentam alto potencial como nanocarreadores devido à sua capacidade de minimizar a toxicidade das moléculas, além da sua capacidade de atingir o sistema fagocítico e da sua propriedade imunoadjuvante (Valenzuela-Oses *et al.*, 2017; Oyama *et al.*, 2019).

O Poloxâmero 407 é constituído por óxido de etileno (OE) nas extremidades e óxido de propileno (OP) na parte central da molécula (OE95-105–OP54-60–OE95- 105) (Jain *et al.*, 2003), e em concentrações de 20% ou mais em solução aquosa se apresentam em estado de gel térmico reversível (Zhang *et al.*, 2002). A natureza anfifílica desse copolímero permite a solubilização e/ou dispersão de substâncias lipofílicas; possibilita a liberação gradual de produtos agregados a ele, e, desse modo, impede que ocorram degradações hidrolíticas e enzimáticas, favorecendo a internalização celular (Ghezzi *et al.*, 2021).

Considerando todas as características das micelas poliméricas, diversos estudos foram desenvolvidos com o uso de Pluronic® com o intuito de avaliar a atividade biológica de agentes antitumorais (Wang *et al.*, 2007; Zhang *et al.*, 2011; Lamch *et al.*, 2014), bem como de compostos antileishmaniais em modelos experimentais de LT e LV. Esses compostos derivados de produtos naturais e/ou sintéticos apresentaram resultados promissores quando incorporados em um sistema de liberação controlada utilizados para o tratamento contra as leishmanioses em modelo murino. Os resultados mostraram uma redução acentuada do parasitismo nos órgãos dos animais e sem toxicidade sistêmica (Duarte *et al.*, 2016; Mendonça *et al.*, 2016; Tavares *et al.*, 2019; Freitas *et al.*, 2021), indicando que esse sistema polimérico pode ser considerado seguro e adequado para entrega de agentes antileishmaniais e, assim, garantir a eficácia do tratamento.

Alguns estudos comprovaram que AmpB incorporada em micelas poliméricas Pluronic® é liberada de forma controlada ao longo de cinco horas de teste *in vitro* (Perasoli, 2016). Além disso, essa composição micelar apresenta melhores efeitos terapêuticos em comparação a AmpB livre (Mendonça *et al.*, 2016) ou à sua formulação lipossomal com redução de carga parasitária e toxicidade em modelo experimental animal, somado ao desenvolvimento de uma resposta imune específica do tipo Th1, com elevados níveis de TNF- α e IL-12, associados à ativação de macrófagos (Singh *et al.*, 2017).

Sendo assim, as micelas poliméricas, associadas a potenciais moléculas antileishmaniais, representam uma estratégia promissora para o tratamento das leishmanioses,

podendo ser consideradas mais eficazes e menos tóxicas. Todavia, desenvolver novas estratégias terapêuticas só é possível a partir do entendimento da resposta imune do hospedeiro frente à infecção por *Leishmania* spp.

2.6. Modelo experimental murino no estudo das leishmanioses

Os processos imunológicos associados à resistência ou susceptibilidade, frente às leishmanioses, são objetos de estudos que visam desenvolver ou melhorar protocolos terapêuticos ou vacinais. Ressalta-se, ainda, a importância dos modelos animais para a realização desses estudos, considerando que devem mimetizar as alterações provocadas pelos parasitos no organismo do hospedeiro. Esses modelos animais experimentais incluem camundongos, hamsters, cães e primatas (Gupta, 2011). No entanto, alguns aspectos como susceptibilidade do modelo animal à espécie do parasito, idade, fase evolutiva do parasito e via de infecção, devem ser considerados para que a infecção experimental se estabeleça (Garg & Dube, 2006).

A manifestação clínica e a severidade das leishmanioses dependem da espécie/cepa do parasito envolvida, bem como da resposta imune do hospedeiro. A imunobiologia da infecção por *Leishmania* spp., especialmente por *L. major*, tem sido muito estudada em camundongos BALB/c e C57BL/6, representando modelos animais de susceptibilidade e resistência, respectivamente. A susceptibilidade à infecção em BALB/c está associada ao desenvolvimento de uma resposta celular Th2, caracterizada pela produção de IL-4, IL-10 e IL-13. Porém, a resistência à *L. major* em camundongos C57BL/6 ocorre devido ao perfil Th1 definido pela alta produção de IFN- γ pelas células T CD4⁺ e CD8⁺ (Sacks & Noben-Trauth, 2002; Mougneau *et al.*, 2011).

GM-CSF também desempenha um papel importante no controle da infecção em modelo experimental murino. Promastigotas e amastigotas recombinantes de *L. major* expressam e liberam maiores concentrações de GM-CSF que juntamente com IFN- γ são capazes de inibir o parasito no interior de macrófagos em camundongos BALB/c, favorecendo assim o desenvolvimento tardio de lesões cutâneas (Dumas *et al.*, 2003). Além disso, as citocinas secretadas pelas células T CD4⁺ ainda participam da diferenciação das células B e da troca de isotipos de IgG (Schroeder & Cavacini, 2010). Nessa linhagem de camundongos, a citocina IL-4 induz, preferencialmente, a produção de anticorpos IgG1 relacionada a uma resposta imune Th2, enquanto anticorpos IgG2a, isotipo estimulado pelo IFN- γ , indicam um perfil de resposta do tipo Th1 (Snapper & Paul, 1987).

Desse modo, camundongos BALB/c são um modelo experimental amplamente utilizado em estudos de novas drogas e imunobiológicos contra as leishmanioses, visto que permitem avaliar o parasitismo em órgãos distintos e, assim, analisar as respostas parasitológicas e imunológicas causadas pela infecção por *Leishmania* spp. (Oliveira *et al.*, 2012). Essa susceptibilidade advém de uma mutação no gene *Slc11a1* (carreadores de soluto da família 11 membro a1), que codifica um componente fagossômico, permitindo a intensa replicação do parasito no fígado nas primeiras semanas após o início da infecção (Crocker *et al.*, 1984; Vidal *et al.*, 1995).

A infecção experimental por *L. donovani* ou *L. infantum* em camundongos BALB/c, geralmente, não é fatal podendo evoluir para um estado crônico, tornando-os ótimos modelos para estudos sobre infecções auto resolutivas ou subclínicas da LV (Loría-Cervera & Andrade-Narváez, 2014). Com a evolução e decorrente cronicidade da infecção, o parasitismo hepático tende a diminuir com posterior imunidade à reinfecção, enquanto a carga parasitária esplênica tende a aumentar e persistir (Nieto *et al.*, 2011). A infecção com alto inóculo de *L. amazonensis* em BALB/c permite o desenvolvimento exponencial da carga parasitária no tecido infectado e nos linfonodos drenantes (infecção cutânea), e à medida que os parasitos se disseminam, no baço e no fígado (infecção sistêmica) dos animais, indicam a ocorrência da doença crônica (Oliveira *et al.*, 2012). Desse modo, mesmo utilizando um regime de tratamento eficaz, os animais infectados não são capazes de eliminar todos os parasitos nos diferentes tecidos e órgãos (Lage *et al.*, 2016b).

Apesar da dicotomia da resposta imune Th1 x Th2 ser bem evidente em modelo murino BALB/c na infecção por *L. major*, o mesmo não é observado na infecção por *L. infantum* ou *L. amazonensis* (Almeida *et al.*, 1996; Ahmed *et al.*, 2003; Abreu-Silva *et al.*, 2004). No entanto, a imunidade protetora contra as leishmanioses está associada a uma polarização da resposta imune do tipo Th1 associada, especialmente, às citocinas INF- γ e IL-12 ativadoras de macrófagos. Nesse contexto, exatamente esse perfil de resposta imune se espera de possíveis candidatos terapêuticos no tratamento das leishmanioses.

3. OBJETIVOS

3.1. Objetivo geral

Avaliar a atividade antileishmanial *in vitro* e *in vivo* de três compostos fenólicos representados por um flavonoide prenilado [CMt: (5,7,3',4'-tetrahidroxi-6,8-diprenilisoflavona)] e dois derivados sintéticos da vanilina [3s: (4- (2-hidroxi-3- (4-octil-1H-1,2,3-triazol-1-il) propoxi) -3-metoxibenzaldeído) e 3t (4- (3- (4-decil-1H-1,2,3-triazol-1-il) -2-hidroxipropoxi) -3-metoxibenzaldeído)] contra *Leishmania infantum* e *L. amazonensis*.

3.2. Objetivos específicos

- Avaliar a atividade antileishmanial do CMt contra formas promastigotas estacionárias e amastigotas axênicas de *L. infantum* e *L. amazonensis*, além da toxicidade em macrófagos peritoneais murinos e em hemácias humanas;
- Investigar a efetividade do CMt no tratamento *in vitro* de macrófagos infectados por *L. infantum* e *L. amazonensis*, e na inibição da infecção utilizando parasitos previamente incubados com essa molécula;
- Analisar o mecanismo de ação do CMt em *L. infantum*;
- Verificar a ação *in vivo* das moléculas CMt, 3s e 3t, em sua forma livre ou incorporadas em micelas poliméricas, no tratamento de camundongos infectados por *L. infantum* e *L. amazonensis*;
- Determinar a carga parasitária no tecido infectado (*L. amazonensis*) e em órgãos (*L. infantum* e *L. amazonensis*) dos animais tratados;
- Avaliar a resposta imune celular e humoral desenvolvida nos animais infectados e tratados;
- Estimar a toxicidade renal e hepática nos animais infectados e tratados com as moléculas 3s, 3t e suas composições micelares.

4. METODOLOGIA

4.1. Aspectos éticos

O acesso ao patrimônio genético da espécie vegetal *Maclura tinctoria*, para fins de pesquisa científica, foi autorizado pelo Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), credenciado pelo Conselho de Gestão do Patrimônio Genético do Ministério do Meio Ambiente (CGen/MMA; Brasil), por meio da Deliberação CGen nº 246, de 27 de agosto de 2009 (Anexo A). Os procedimentos de execução experimental do presente estudo foram realizados em conformidade com o Colégio Brasileiro de Experimentação Animal (COBEA), aprovados pela Comissão de Ética no Uso de Animais (CEUA-UFMG; Belo Horizonte, Brasil), com protocolo nº 085/2017, de 05 de junho de 2017 (Anexo B). Além disso, o uso de amostras de sangue humano foi aprovado pelo Comitê de Ética em Pesquisa (CEP-UFMG; Belo Horizonte, Brasil) nº CAAE-323431.14.9.0000.5149 e nº CAAE-94476318.8.0000.5149.

4.2. Obtenção das moléculas

4.2.1. CMt

As folhas da espécie *Maclura tinctoria* foram coletadas na biblioteca interna do Laboratório de Biodiversidade, Departamento de Bioquímica e Biologia Molecular, da UFV (Viçosa, Minas Gerais, Brasil), e submetidas à secagem em estufa com ventilação circular a 40°C por 48 horas. Em seguida, o material seco foi triturado, para aumentar a superfície de contato e otimizar o processo de extração. Esse processo foi realizado através da percolação em série exaustiva que consiste na utilização de solventes em ordem crescente de polaridade sobre o mesmo material – hexano, diclorometano, acetato de etila e etanol.

O material vegetal seco foi inserido no percolador juntamente com 1,5 L de hexano, em constante maceração, para que ocorresse a extração por aproximadamente 12 horas. Logo, o extrato hexânico foi drenado e concentrado em evaporador rotatório. Esse processo foi realizado diversas vezes até que a massa extraída tornasse irrelevante, ou seja, não se observava extração significativa no solvente utilizado. Ao término desse ciclo, um próximo foi iniciado com o solvente de polaridade superior e, assim, sucessivamente. Todos os extratos foram concentrados em evaporador rotatório e liofilizados para completa eliminação do solvente (McCloud, 2010).

A fração diclorometano concentrou uma mistura de flavonoides prenilados e, posteriormente, com o fracionamento cromatográfico em sílica gel, resultou no isolamento e na purificação do composto 5,7,3',4'-tetrahidroxi-6,8-diprenilisoflavona – denominado CMt (substância amorfa e amarelada). A elucidação estrutural do derivado flavônico foi realizada

por espectroscopia no ultravioleta (UV), espectrometria de massa por ionização *electrospray* (ESI-MS) e ressonância magnética nuclear de hidrogênio-1 (^1H NMR) e de carbono-13 (^{13}C NMR) (Uddin *et al.*, 2011; Jeon *et al.*, 2012). Após análise das informações espectroscópicas, foi confirmada a estrutura química do composto (Figura 8).

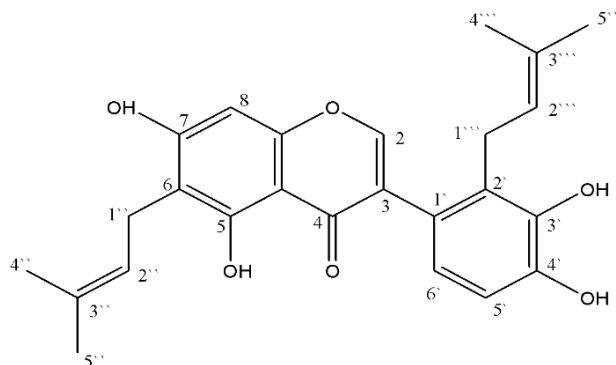


Figura 8. Estrutura química CMt.

Adaptado de: Salustiano (2019, p.65).

4.2.2. 3s e 3t

As moléculas derivadas da vanilina [4-(2-hidroxi-3-(4-octil-1H-1,2,3-triazol-1-il)propoxi)-3-metoxibenzaldeído] e [4-(3-(4-decil-1H-1,2,3-triazol-1-il)-2-hidroxipropoxi)-3-metoxibenzaldeído], denominadas 3s e 3t, respectivamente, foram sintetizadas no Laboratório de Síntese Orgânica, Departamento de Química, UFV (Viçosa, Minas Gerais, Brasil).

Em um balão de fundo redondo (10 mL) foram adicionados 4-(3-azido-2-hidroxipropoxi)-3-metoxibenzaldeído (0,154 g; 0,61 mmol), 1-Dodecene (0,102 g; 0,61 mmol), ascorbato de sódio (0,048 g; 0,24 mmol); 1 mL de diclorometano e de água destilada. Em seguida, adicionou-se sulfato de cobre pentaidratado ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$; 0,031 g; 0,12 mmol). Essa mistura reacional (reação *click*) foi mantida sob agitação magnética vigorosa a temperatura ambiente durante 19 horas. Após o término da reação, evidenciado por cromatografia em camada delgada, a mistura foi transferida para um funil de separação e a fase orgânica extraída com diclorometano (3 x 25 mL) e lavada com solução saturada de carbonato de sódio, seca com sulfato de sódio anidro, filtrada e concentrada sob pressão reduzida. Os compostos 3s e 3t foram separados por cromatografia em coluna de sílica gel, utilizando éter: acetato de etila: diclorometano (1: 2,5: 3 v/v e 1: 3,5: 3 v/v para 3s e 3t, respectivamente) e concentrados sob pressão reduzida gerando um sólido amarelado (3s) e branco prateado (3t).

A elucidação estrutural dos derivados da vanilina foi realizada através por técnicas de ressonância magnética nuclear como ^1H NMR e ^{13}C NMR, e espectroscopia no infravermelho (IV) (Santiago, 2022). Após análise das informações espectroscópicas, foi confirmada a estrutura química dos compostos 3s e 3t (Figura 9).

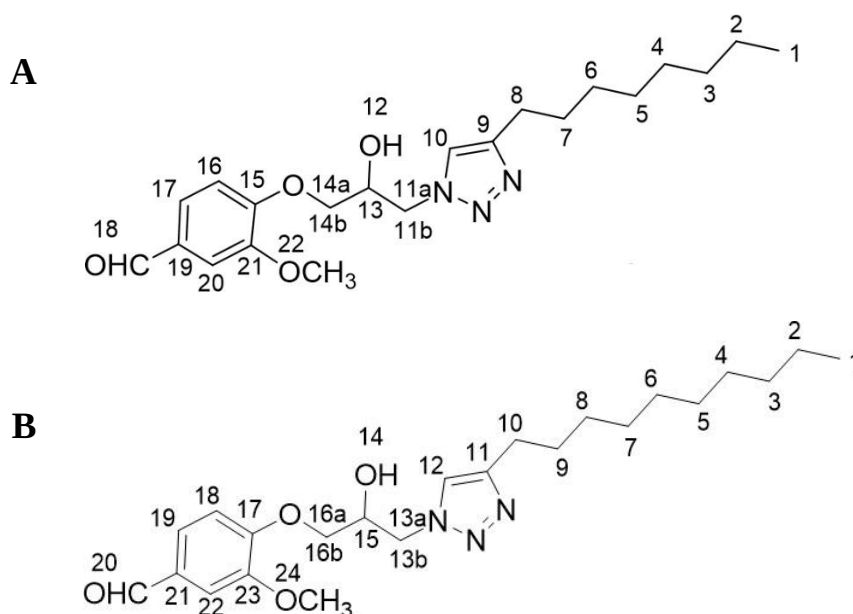


Figura 9. Estrutura química 3s (A) e 3t (B).

Adaptado de: Santiago (2022, p.50-51).

4.3. Parasitos

As cepas utilizadas foram IFLA/BR/1967/PH-8 de *Leishmania amazonensis* e MHOM/BR/1970/BH46 de *Leishmania infantum*. As formas promastigotas estacionárias foram cultivadas a 24°C, em meio de cultura *Schneider's Insect Medium* (Schneider; Sigma-Aldrich®, EUA), suplementado com 20% v/v de soro fetal bovino (SFB, Sigma-Aldrich®, EUA) inativado por calor e 20 mmol/L de L-glutamina, 200 U/mL de penicilina e 100 µg/mL de estreptomicina (pH 7,4). O extrato solúvel de *Leishmania* (SLA) foi preparado a partir de 10⁹ promastigotas centrifugadas a 4.000 x g e, posteriormente, lavadas três vezes em 5 mL de solução salina tamponada com fosfato (PBS; pH 7,4) estéril e gelado. Em seguida, cinco ciclos consecutivos de congelamento e descongelamento foram realizados, além de cinco ciclos de ultrassom a 38 MHz por 30 segundos cada. Logo após, a suspensão celular foi centrifugada a

9.000 x g durante 30 minutos a 4°C, sendo o sobrenadante coletado e armazenado a -80°C (Coelho *et al.*, 2003).

Para obter as formas amastigotas axênicas, 2×10^8 promastigotas estacionárias foram lavadas três vezes em PBS (pH 7,4), ressuspensas em 5 mL de SFB e incubadas por 48 ou 72 horas (*L. amazonensis* e *L. infantum*, respectivamente) a 37°C. Posteriormente, os parasitos foram lavados três vezes com PBS e sua morfologia foi avaliada, em microscópio óptico, através da coloração *Giemsa* (Duarte *et al.*, 2016).

4.4. Preparo das micelas poliméricas

Para a preparação das micelas contendo CMt (CMt/Mic), 3s (3s/Mic ou 3s/M) e 3t (3t/Mic ou 3t/M), utilizou-se 15 mg de Poloxamer 407 (18% m/m; Pluronic® F127, Sigma-Aldrich®, EUA) diluído em PBS (pH 7,4), sob agitação magnética, durante 18 horas a 4°C. A seguir, 10 mg de cada composto, separadamente, foram adicionados a 500 µL de diclorometano PA, sendo solubilizados em vortex. Essa mistura foi acrescentada à solução preparada anteriormente, sob vigorosa agitação e em ‘banho de gelo’, até obter uma emulsão viscosa. Através de um evaporador rotativo (Büchi®, Suíça), o diclorometano evaporou e assim, a formulação obteve uma coloração amarelada transparente em temperatura ambiente. As micelas vazias (B/Mic ou Mic; 18% m/m) também foram preparadas de acordo com o protocolo citado (Tavares *et al.*, 2019), sem incorporação do CMt, 3s ou 3t. Todas as composições micelares foram filtradas em membranas 0,45µm e armazenadas a 4°C.

A caracterização físico-química das composições micelares foi realizada pela técnica de espalhamento dinâmico de luz (DLS) para determinação do tamanho médio das partículas e do potencial zeta, utilizando o sistema Zetasizer Nano ZSP (Malvern Instruments, Reino Unido). A morfologia micelar foi avaliada através da microscopia eletrônica de transmissão (Tecnai G20, FEI Company, EUA).

4.5. Animais

Camundongos BALB/c, fêmeas, com 8 semanas de idade, foram adquiridos no Biotério Central do Instituto de Ciências Biológicas da UFMG (ICB-UFMG; Belo Horizonte, Minas Gerais, Brasil) e mantidos em condições adequadas pelo Biotério do Departamento de Patologia Clínica do COLTEC-UFMG. Para o desenvolvimento experimental *in vivo* com CMt foram utilizados 60 animais, divididos em cinco grupos (n=12 animais, cada), e com as moléculas 3s e 3t, 128 animais distribuídos em oito grupos (n=16 animais, cada).

4.6. Avaliação in vitro do CMt em *L. amazonensis* e *L. infantum*

4.6.1. Atividade antileishmanial

Em uma placa de cultura celular de 96 poços (Nunc, Nunclon[®], Dinamarca), 1×10^6 formas promastigotas estacionárias ou amastigotas axênicas de *L. infantum* ou *L. amazonensis*, incubadas com diferentes concentrações do composto CMt (0 a 50 µg/mL), bem como com anfotericina B (AmpB, 0 a 1,0 µg/mL; Cristália, Brasil) para controle positivo. As amostras foram diluídas em meio *Roswell Park Memory Institute* (RPMI 1640; Sigma-Aldrich[®], EUA), suplementado com 20% v/v de soro fetal bovino (SFB, Sigma-Aldrich[®], EUA) inativado por calor e 20 mmol/L de L-glutamina, 200 U/mL de penicilina e 100 µg/mL de estreptomicina (pH 7,4), em um volume final de 100 µL, e incubadas por 48 horas a 24°C. Logo após, 10 µL do reagente [brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difenil tetrazólio] (MTT; Sigma-Aldrich[®], EUA) na concentração de 5 mg/mL foram adicionados e, em seguida, a placa foi incubada por quatro horas a 24°C. As células foram analisadas em microscópio óptico composto para verificar a formação de cristais de formazan. Posteriormente, 60 µL de uma solução de dodecil sulfato de sódio a 10% de ácido clorídrico (SDS 10% HCl 0,1 M) foi acrescentado para solubilização dos cristais e, novamente, a placa foi incubada por 18 horas.

As leituras das absorbâncias foram realizadas em espectrofotômetro de microplacas (SpectraMax[®] Plus, EUA) a 570 nm. Os valores da concentração inibitória de 50% de parasitos (IC₅₀) foram calculados por análise de regressão sigmoideal das curvas dose-resposta, através do *software* Microsoft Excel (versão 10.0) (Tavares *et al.*, 2018).

4.6.2. Citotoxicidade em macrófagos

Esse ensaio foi realizado para avaliação da citotoxicidade do composto CMt sobre células de mamíferos, especificamente macrófagos peritoneais murinos. Dessa forma, camundongos BALB/c foram inoculados, via peritoneal, com 3 mL de uma solução de tioglicolato 3%. Após quatro dias, os animais foram eutanasiados e os macrófagos extraídos por lavagem peritoneal com 5 mL de PBS estéril e gelado para posterior centrifugação a $1.000 \times g$ por 10 minutos. Em seguida, foram ressuspensos em 1 mL de meio de cultura RPMI 1640 (Sigma-Aldrich[®], EUA). Logo, 5×10^5 células foram incubadas em placas de cultura de 96 poços (Nunc, Nunclon[®], Dinamarca), em meio RPMI 1640, juntamente com 20% de SFB e antibióticos, por 24 horas a 37°C em estufa 5% CO₂. Posteriormente, diferentes concentrações do CMt (0 a 100 µg/mL) ou AmpB (0 a 10 µg/mL) foram adicionadas e, novamente, incubadas

em estufa de CO₂ por 48 horas a 37°C. Logo após, foram acrescentados 50 µL de MTT para incubação por quatro horas a 37°C. As células foram solubilizadas por SDS 10% HCl 0,1M por 16 horas e as absorbâncias determinadas a 570 nm em espectrofotômetro (SpectraMax® Plus, EUA).

A concentração citotóxica de 50% das células (CC₅₀) foi determinada por análise de regressão sigmoideal das curvas dose-resposta, através do *software* Microsoft Excel (versão 10.0) (Tavares *et al.*, 2018). Em paralelo, o Índice de Seletividade (SI) foi determinado pela razão entre os valores de CC₅₀ e IC₅₀, representando o potencial efetivo do composto avaliado sobre o parasito em relação a sua citotoxicidade. Os valores de SI foram calculados para CMt e AmpB, ambos contra promastigotas estacionárias e amastigotas axênicas de *L. infantum* e *L. amazonensis*.

4.6.3. Atividade hemolítica

A atividade hemolítica foi investigada através da incubação do CMt (0 a 100 µg/mL) ou AmpB (0 a 10 µg/mL) com uma suspensão de 5% v/v de hemácias humanas, durante uma hora a 37°C em estufa 5% CO₂. Essa suspensão de hemácias foi centrifugada a 1.000 x g por 10 minutos, e a porcentagem de lise celular foi determinada por espectrofotômetro a 570 nm. A ausência (branco) ou a presença de 100% de hemólise (controle positivo) foi determinada substituindo os produtos por igual volume de PBS ou água destilada, respectivamente.

A concentração inibitória de 50% de hemácias humanas (RBC₅₀) foi estabelecida por uma curva de regressão sigmoideal das curvas dose-resposta, através do *software* Microsoft Excel (versão 10.0) (Tavares *et al.*, 2018).

4.6.4. Tratamento de macrófagos infectados

Para avaliar a eficácia do composto CMt em células infectadas, foram extraídos macrófagos peritoneais murinos, como descrito em item 4.6.2. Logo, 5 x 10⁵ células foram plaqueadas, em lâminulas redondas, em placas de cultura celular de 24 poços (Nunc, Nunclon®, Dinamarca) em meio RPMI 1640 completo, ou seja, suplementado com 20% v/v de soro fetal bovino (SFB, Sigma-Aldrich®, EUA) inativado por calor e 20 mmol/L de L-glutamina, 200 U/mL de penicilina e 100 µg/mL de estreptomicina (pH 7,4) e incubadas por 24 horas a 37°C em estufa 5% CO₂. Em seguida, as células foram lavadas em meio RPMI 1640, para a retirada de macrófagos não aderidos. As formas promastigotas estacionárias de *L. amazonensis* e *L. infantum* foram quantificadas em Câmara de Neubauer e adicionadas na concentração de 5 x

10^6 células por poço (proporção de um macrófago para 10 parasitos), seguida da incubação por 48 horas a 37°C em estufa. As células foram lavadas, mais uma vez, para retirar os parasitos não aderidos e não fagocitados, e, assim, os macrófagos infectados foram tratados com CMt (0; 1,0; 5,0 e 10 µg/mL) e AmpB (0; 0,1; 0,5 e 1,0 µg/mL), por 48 horas a 24°C.

Após a fixação com paraformaldeído 4% m/v, as células foram lavadas e coradas pelo *Giemsa*. A porcentagem de infecção e o número de amastigotas por célula infectada foram definidos pela contagem de 200 macrófagos, em triplicata, em microscópio óptico (Soyer *et al.*, 2019).

4.6.5. Inibição da infecção de macrófagos

Nesse ensaio, 5×10^6 formas promastigotas estacionárias de *L. amazonensis* e *L. infantum* foram previamente incubadas com CMt (0; 1,0; 5,0 e 10 µg/mL) e com AmpB (0; 0,1; 0,5 e 1,0 µg/mL), em lamínulas redondas, em placas de cultura de 24 poços (Nunc, Nunclon®, Dinamarca), por quatro horas a 24°C. Em seguida, os parasitos foram lavados, três vezes, com meio RPMI 1640, quantificados e incubados com macrófagos murinos (proporção de 10 parasitos para um macrófago) em estufa 5% CO₂, durante 24 horas a 37°C. Logo após a fixação com paraformaldeído 4% m/v, as células foram lavadas e coradas pelo *Giemsa*. A porcentagem de infecção, assim como o número de amastigotas por célula infectada, foram determinados pela contagem de 200 macrófagos, em triplicata, em microscópio óptico (Soyer *et al.*, 2019).

4.6.6. Mecanismos de ação em *L. infantum*

4.6.6.1. Avaliação da integridade da membrana celular

Nessa análise, 1×10^7 formas promastigotas de *L. infantum* foram incubadas na ausência ou presença de CMt (6,36 e 12,72 µg/mL, correspondentes a uma e duas vezes o valor de IC₅₀, respectivamente), por 24 horas a 25°C. Em seguida, os parasitos foram lavados com PBS e incubados com 1 µg/mL de iodeto de propídeo (PI; Sigma-Aldrich®, EUA) durante 15 minutos, no escuro e em temperatura ambiente. A intensidade de fluorescência foi mensurada em espectrofluorímetro (FLx800, BioTek Instruments®, EUA), a 540 nm e 600 nm de excitação e emissão, respectivamente. Como controle positivo do ensaio, as formas promastigotas foram pré-aquecidas a 65°C por 10 minutos (Antinarelli *et al.*, 2016).

4.6.6.2. Avaliação do potencial de membrana mitocondrial ($\Delta\psi_m$)

Inicialmente, 1×10^7 formas promastigotas estacionárias de *L. infantum* foram incubadas na ausência ou presença de CMt (6.36 e 12.72 $\mu\text{g/mL}$, conforme valor de IC_{50} e duas vezes o IC_{50} , respectivamente), por 24 horas a 25°C . Em seguida, os parasitos foram lavados em PBS e incubados com MitoTracker[®] (500 nmol/L; InvitrogenTM, EUA), durante 30 minutos, na ausência de luz e em temperatura ambiente. Após duas lavagens com PBS, as amostras foram transferidas para uma placa preta de 96 poços (SPL Life Sciences[®], Coreia) e a intensidade da fluorescência avaliada por um espectrofluorímetro (FLx800, BioTek Instruments[®], EUA) em filtro de excitação de 540 nm e de emissão de 600 nm. Além disso, os parasitos foram incubados com carbonilcianeto 4-(trifluorometoxi)fenil-hidrazona (FCCP; 5 $\mu\text{mol/L}$; Sigma-Aldrich[®], EUA), para controle positivo (Antinarelli *et al.*, 2016; Soyer *et al.*, 2019). Os valores de $\Delta\psi_m$ foram calculados através da razão entre as leituras realizadas a 600 nm e 540 nm.

4.6.6.3. Produção de espécies reativas de oxigênio

A produção de espécies reativas de oxigênio (ROS) foi avaliada a partir da incubação de 1×10^7 formas promastigotas estacionárias de *L. infantum* na ausência ou presença de CMt (6,36 e 12,72 $\mu\text{g/mL}$), durante 24 horas a 25°C . Posteriormente, as células foram lavadas com PBS e incubadas com 20 $\mu\text{mol/L}$ de diacetato de 2',7'-diclorofluoresceína (H_2DCFDA ; Sigma-Aldrich[®], EUA), por 30 minutos, no escuro e em temperatura ambiente. A intensidade de fluorescência foi determinada em espectrofluorímetro (Varioskan[®] Flash, EUA) a 485 nm e 528 nm de comprimento de onda de excitação e emissão, respectivamente. Como controle positivo, os parasitos foram tratados com 4 mmol/L de peróxido de hidrogênio (H_2O_2 ; Sigma-Aldrich[®], EUA) e parasitos não tratados foram utilizados como controle negativo (Antinarelli *et al.*, 2016).

4.7. Avaliação *in vivo* de CMt, 3s e 3t

4.7.1. Infecção, tratamento e eutanásia: CMt *versus* infecção por *L. infantum*

Devido às diferentes concentrações das moléculas utilizadas nos esquemas terapêuticos propostos no estudo, CMt será apresentada separadamente à 3s e 3t, visto que os experimentos ocorreram em diferentes intervalos de tempo.

Camundongos BALB/c (n=12 por grupo) foram infectados, via subcutânea, com 10^7 formas promastigotas estacionárias de *L. infantum*. Após 45 dias da infecção, os animais foram

distribuídos em cinco grupos experimentais e tratados durante 10 dias, a cada dois dias, com (i) 50 µL de PBS 1x (grupo controle), (ii) 50 µL de micelas vazias (B/Mic; 10 mg/kg), (iii) 50 µL de CMt (CMt; 10 mg/kg), (iv) 50 µL de CMt micelar (CMt/Mic; 5 mg/kg) ou (v) Miltefosina (Sigma-Aldrich®, EUA; 2 mg/kg). Exceto miltefosina, a qual foi administrada via oral, todos os outros tratamentos foram aplicados via subcutânea. Na eutanásia, a metade do número de camundongos, por grupo, foi sacrificada um dia e a outra metade 15 dias após o término do tratamento (n=6 por grupo, cada), para avaliação dos parâmetros parasitológicos e imunológicos.

4.7.2. Infecção, tratamento e eutanásia: 3s e 3t versus infecção por *L. infantum*

Camundongos BALB/c (n=16 por grupo) foram infectados, via subcutânea, com 10^7 formas promastigotas estacionárias de *L. infantum*. Após 60 dias da infecção, os animais foram distribuídos em oito grupos experimentais e tratados com um dos seguintes esquemas: (i) 50 µL de PBS 1x (grupo salina); (ii) 50 µL de micelas vazias (B/Mic; 5 mg/kg); (iii) 50 µL de anfotericina B (AmpB; 1 mg/kg); (iv) 8 µL de AmBisome® (AmBisome®; 1 mg/kg); (v) 50 µL de 3s (3s; 5 mg/kg); (vi) 50 µL 3s micelar (3s/M; 2,5 mg/kg); (vii) 50 µL de 3t (3t; 5 mg/kg) e (viii) 50 µL de 3t micelar (3t/M; 2,5 mg/kg). Com exceção dos grupos AmpB e AmBisome®, os quais foram administrados via intraperitoneal e intravenosa respectivamente, os demais esquemas terapêuticos foram aplicados nos animais, via subcutânea, quatro vezes a cada cinco dias. Os camundongos foram eutanasiados um dia e 30 dias após o término do tratamento (n=8 por grupo, cada), coletando amostras de sangue e órgãos para análises bioquímicas, imunológicas e parasitológicas.

4.7.3. Infecção, tratamento e eutanásia: 3s e 3t versus infecção por *L. amazonensis*

Camundongos BALB/c foram infectados via subcutânea, na base da cauda após tricotomia, com 10^7 formas promastigotas estacionárias de *L. amazonensis*. Após o desenvolvimento da doença, aproximadamente 60 dias pós-infecção, os animais apresentaram diâmetro médio de lesão cerca de 4 mm, sendo agrupados (n=16 por grupo). O esquema de tratamento constituiu-se em: (i) 50 µL de PBS 1x; (ii) 50 µL de anfotericina B (AmpB; 1 mg/kg); (iii) 8 µL de AmBisome® (AmBisome®; 1 mg/kg); (iv) 50 µL de micelas vazias (Mic; 5 mg/kg); (v) 50 µL de 3s (3s; 5 mg/kg); (vi) 50 µL de 3s micelar (3s/Mic; 2,5 mg/kg); (vii) 50 µL de 3t (3t; 5 mg/kg) e (viii) 50 µL de 3t micelar (3t/Mic; 2,5 mg/kg).

Os animais foram tratados, via subcutânea, com sete doses em intervalo de sete dias entre eles, exceto anfotericina B (via intraperitoneal) e AmBisome® (via intravenosa) com quatro doses a cada cinco dias. Medições semanais foram realizadas com auxílio de paquímetro eletrônico (modelo 799-6/150, Starrett®, Brasil) para avaliar o desenvolvimento das lesões durante e após o término do tratamento. Na eutanásia, a metade do número de camundongos foi sacrificada um dia e a outra metade 30 dias pós-tratamento (n=8 por grupo, cada), coletando amostras de sangue, órgãos e fragmentos de lesão para avaliação dos parâmetros bioquímicos, imunológicos e parasitológicos.

4.7.4. Determinação da carga parasitária por diluição limitante

Para avaliar o parasitismo pela técnica de diluição limitante, foram retirados e, em seguida, pesados fragmentos de lesão (na avaliação da LT experimental), baço, fígado, medula óssea e linfonodos dos animais infectados e tratados nos períodos de um e 15 e/ou 30 dias pós-tratamento. Os órgãos foram macerados em PBS estéril. Logo após, os fragmentos dos tecidos foram removidos por centrifugação a 150 x g por 10 minutos a 4°C, e as células foram concentradas a 2.000 x g por 15 minutos a 4°C. Os *pellets* formados foram ressuspensos em 1 mL de meio Schneider completo. Em placas de 96 poços (Nunc, Nunclon®, Dinamarca), foram realizadas diluições seriadas no mesmo meio de cultura, partindo de diluição de 10⁻¹ até a máxima diluição de 10⁻¹², sendo posteriormente incubadas em estufa a 24°C durante sete dias.

Com o auxílio de um microscópio trinocular invertido (Axiovert 25, Zeiss, Alemanha), foi determinada a maior diluição, na qual se pôde visualizar parasitos (Coelho *et al.*, 2003). Os resultados foram apresentados como log negativo do título (diluição correspondente ao último poço positivo), ajustado por miligrama do órgão.

4.7.5. Determinação da carga parasitária por qPCR

O parasitismo esplênico também foi avaliado pela técnica de Reação em Cadeia da Polimerase quantitativa em tempo real (qPCR) (Tavares *et al.*, 2019). O DNA do baço foi extraído através do *Kit Wizard® Genomic DNA Purification* (Promega®, Brasil), seguindo as instruções do fabricante. Ao final, o DNA foi ressuspensionado em 100 µL de água milli-Q para amplificação, utilizando os iniciadores 5'-CCTATTTTACACCAACCCCAGT-3' e 5'-GGGTAGGGGCCTTCTGCGAAA-3' (*Forward* e *Reverse*, respectivamente). Além disso, foi utilizado o gene da β-actina de camundongo (*Forward* 5'-CAGAGCAAGAGAGGTATCC-3' e *Reverse* 5'-TCATTGTAGAAGGTGTGGTGC-3') como controle endógeno, para verificar a

integridade da amostra. As curvas padrão foram obtidas a partir do DNA extraído de 10^8 parasitos, para o kDNA, e de 10^8 macrófagos peritoneais, para o gene da β -actina (ACTB), sob as mesmas condições de extração das outras amostras.

As reações foram processadas e analisadas em um sistema de detecção ABI Prism 7500 (placa de 96 poços; Applied Biosystems®, EUA), utilizando uma mistura de PowerUp™ SYBR™ Green PCR Master Mix (5 μ L; Thermo Fisher, EUA), 2 mM de cada *primer* (1 μ L) e 4 μ L de DNA (25 ng/ μ L). As amostras foram incubadas a 95°C por 10 minutos e submetidas a 40 ciclos de 95°C por 15 segundos, e de 60°C por um minuto, sendo que a cada período foram coletados os dados de fluorescência. A quantificação da carga parasitária, por amostra, foi calculada por interpolação a partir da curva padrão, realizada em duplicata e convertida em número de parasitos pelo total de DNA.

4.7.6. Avaliação da resposta celular

O baço dos animais infectados e tratados foi coletado para cultura de esplenócitos e, conseqüentemente, para avaliação da resposta celular através da produção de citocinas e de nitrito. Os órgãos foram macerados em meio de cultura RPMI 1640 e centrifugados a 1.200 x g durante 10 minutos. As células esplênicas foram ressuspensas em 3 mL de tampão lise de hemácias (17 mmol/L Tris-HCl e 144 mmol/L NH₄Cl; pH 7,4) e incubadas por quatro minutos. A reação de lise foi interrompida pela adição de 7 mL de meio RPMI 1640 incompleto e, em seguida, as células foram centrifugadas a 1.200 x g por 10 minutos. O sobrenadante foi descartado e, o precipitado, foi ressuspendido em 1 mL de RPMI 1640 (Sigma-Aldrich®, EUA) completo. Os esplenócitos foram ajustados para a concentração final de 5×10^6 células/mL e incubados em placas de 24 poços (Nunc, Nunclon®, Dinamarca) em meio RPMI completo na presença, ou não (controle negativo), de 25 μ g/mL SLA de *L. infantum* ou *L. amazonensis* em estufa 5% CO₂, durante 48 horas a 37°C. Logo após, os sobrenadantes foram coletados para dosagem de citocinas e de nitrito (Martins *et al.*, 2013).

As citocinas IFN- γ , IL-4, IL-10, IL-12 e GM-CSF foram avaliadas no sobrenadante celular através do ensaio de imunoabsorção enzimática de captura (ELISA; BD Pharmingen®, EUA), nos períodos de um dia e 15 e/ou 30 dias após tratamento, seguindo as instruções do fabricante. Os resultados foram obtidos em pg/mL. A análise de nitrito foi realizada pelo método de Griess (Green *et al.*, 1982), nos períodos de um dia e 15 e/ou 30 dias pós-tratamento. Em uma placa de 96 poços, foram adicionados 50 μ L do reagente de Griess (0.1% N-(1-naftiletilenodiamina, 1% sulfanilamida, 5% ácido fosfórico; Sigma-Aldrich®, EUA) ao mesmo

volume dos sobrenadantes da cultura de esplenócitos, para incubação ao abrigo da luz, por 10 minutos a temperatura ambiente. Logo após, a absorbância foi avaliada em espectrofotômetro (SpectraMax® Plus, EUA) a 540 nm e comparada a uma curva padrão. Os dados foram apresentados em μM de nitrito.

4.7.7. Avaliação da frequência de células T produtoras de citocinas

Para avaliar a frequência de células T CD4^+ e CD8^+ produtoras de $\text{INF-}\gamma$, $\text{TNF-}\alpha$, IL-2 e IL-10, foi realizado um ensaio de citometria de fluxo (Moreira *et al.*, 2012). Primeiramente, 5×10^6 células/mL foram incubadas em meio de cultura RPMI 1640 completo em placas de cultura de 96 poços de fundo redondo (Nunc™, Nunclon, EUA), estimuladas ou não com SLA (25 $\mu\text{g/mL}$), durante 48 horas a 37°C , em estufa 5% CO_2 . Quatro horas antes do término da incubação, foi adicionada às culturas Brefeldina A (10 $\mu\text{g/mL}$; Sigma-Aldrich®, EUA). Os poços de controle positivo foram estimulados com forbol 12-miristato 13-acetato (PMA; 5,0 ng/mL) e ionomicina (1,0 $\mu\text{g/mL}$), diluídos em meio RPMI 1640 completo. As células foram marcadas por *Fixable Viability Stain* 450 (FVS450; BD Biosciences, EUA) por 15 minutos em temperatura ambiente e ao abrigo da luz, seguida pela marcação com anticorpos anti- CD3^+ (BV650 anti-camundongo, clone 145.2C11), CD4^+ (BV605 anti-camundongo, clone RM4-5) e CD8^+ (BV786 anti-camundongo, clone 53-6.7) por 30 minutos em temperatura ambiente. As células foram fixadas com solução *Fluorescence Assisted Cell Sorter* (FACS; BD Biosciences, EUA), lavadas e permeabilizadas com PBS 5% saponina. Em seguida, após centrifugação, o sobrenadante foi descartado, a placa homogeneizada e adicionados os anticorpos anti-IL-12 (PE anti-camundongo, clone JES6-5H4), $\text{INF-}\gamma$ (AF700 anti-camundongo, clone XMG1.2), $\text{TNF-}\alpha$ (PE-Cy7 anti-camundongo, clone LG.3A10) e IL-10 (APC anti-camundongo, clone JES5-16E), durante 30 minutos a temperatura ambiente.

As células (100.000 eventos) foram adquiridas no citômetro de fluxo LSR Fortessa (BD Biosciences, EUA), utilizando o *software* FACSDiva. Para análise no *software* FlowJo, as células mortas foram excluídas após marcação com FVS450, e as células vivas foram selecionadas nos gráficos relativos ao tamanho (*Forward Scatter*- FSC) e granularidade (*Side Scatter* - SSC). Com a seleção da região de interesse (R1), contendo as células do fenótipo FSC^{Low} e SSC^{Low} , foram construídos gráficos de distribuição de CD4/FL1 ou CD8/FL1 *versus* $\text{INF-}\gamma/\text{FL2}$, $\text{TNF-}\alpha/\text{FL2}$, IL-2/FL2 e IL-10/FL2 para determinar a frequência das células T produtoras de $\text{INF-}\gamma$, $\text{TNF-}\alpha$, IL-2 e IL-10. Os resultados foram expressos pela razão entre a porcentagem de células T CD4^+ e CD8^+ em culturas estimuladas *versus* não estimuladas.

4.7.8. Avaliação da resposta humoral

A resposta humoral foi avaliada, nos períodos de um dia e 15 e/ou 30 dias, pós-tratamento através da produção de anticorpos IgG, IgG1 e IgG2a (Lage *et al.*, 2023). Amostras de sangue do plexo braquial dos animais infectados e tratados foram coletadas no momento da eutanásia e, posteriormente, centrifugadas a 5.000 x g durante 10 minutos para obtenção do soro. Os níveis de anticorpos foram determinados ao sensibilizar placas de 96 poços (JetBiofil®, Brasil) com SLA de *L. infantum* ou *L. amazonensis* (1 µg/poço) diluídos em 50 mM de tampão carbonato pH 9,6, por 18 horas a 4°C. Após a sensibilização, fez-se o bloqueio utilizando PBS-T (PBS 1x 0,05% Tween 20) acrescido de 5% (m/v) albumina sérica bovina (BSA) durante uma hora a 37°C. Em seguida, as placas foram lavadas com PBS-T e incubadas com os soros coletados dos animais (1:100 em PBS-T) por uma hora a 37°C. As placas foram novamente lavadas e aplicados os conjugados anti-IgG [anti-camungondo IgG (molécula inteira) conjugada com peroxidase; Sigma-Aldrich®, EUA], IgG1 e IgG2a (anti-camundongo IgG1 e IgG2a conjugada com peroxidase; Invitrogen, EUA) diluídos em PBS-T e incubadas por uma hora a 37°C. Logo após a lavagem das placas, as reações foram reveladas com uma solução composta de 4 µL de H₂O₂, 2 mg de orto-fenilenodiamina e 10 mL de tampão fosfato-citrato (pH 5,0), durante 30 minutos no escuro. Essa reação foi interrompida pela adição de 20 µL de 2N H₂SO₄ e os valores de absorbância foram determinados em espectrofotômetro a 492 nm (Molecular Devices, SpectraMax® Plus, EUA).

4.8. Análises complementares na avaliação *in vivo* de 3s e 3t

4.8.1. RT-qPCR para detecção da expressão de IFN-γ

A expressão de IFN-γ em cultura de esplenócitos, estimuladas com SLA dos animais infectados e tratados (n=8 por grupo, cada), um e 30 dias após o término do tratamento, também foi avaliada por Reação em Cadeia da Polimerase com Transcrição Reversa quantitativa (RT-qPCR). Para isso, o ácido ribonucleico (RNA) foi extraído da cultura celular utilizando o reagente TRIzol (Invitrogen, EUA), seguindo as instruções do fabricante. O material foi, posteriormente, ressuspensionado em água destilada UltraPure™ DNase/RNase-Free (Invitrogen, EUA), e a concentração determinada por NanoDrop LITE (Thermo Fisher Scientific, EUA), com razão de absorbância de 260/280 nm. O RNA extraído foi tratado por 15 minutos, em temperatura ambiente, com DNase I (Invitrogen, EUA) e a enzima desativada com ácido etilenodiamino tetra-acético (EDTA; 25 mM) durante 10 minutos a 65°C. O RNA total (2 µg) foi transcrito reversamente utilizando o kit Applied Biosystems (Thermo Fisher Scientific,

EUA), formando o DNA complementar (cDNA), e utilizando os parâmetros: 25°C por 10 minutos, 37°C por 120 minutos e 85°C por cinco minutos.

O ensaio de RT-qPCR foi realizado utilizando *master mix* Applied Biosystems PowerUp™ SYBR™ Green PCR (Thermo Fisher Scientific, EUA) e *primers* específicos do gene para IFN- γ : 5'-TCAAGTGGCATAGATGTGGAAGAA-3' (*forward*) e 5'-TGGCTCTGCAGGATTTTCATG-3' (*reverse*) em um termociclador 7900HT Fast Real-Time PCR System (Applied Biosystems®, EUA). As transcrições foram normalizadas utilizando genes de referência gliceraldeído-3-fosfato desidrogenase (GAPDH) e ACTB (Giulietti *et al.*, 2001). Os parâmetros do ciclo foram desnaturação inicial a 95°C por 10 minutos, 40 ciclos a 95°C por 15 segundos e extensão a 60°C por um minuto, seguido de um estágio de dissociação para registro da curva de *melting*. Os resultados foram expressos graficamente como alterações na expressão gênica utilizando a média $\pm$ desvio padrão do gene alvo (Hellemans *et al.*, 2007). Os dados foram analisados de acordo com a expressão relativa pelo método $2^{-\Delta\Delta CT}$.

4.8.2. Toxicidade orgânica

A toxicidade orgânica nos animais infectados e tratados foi avaliada em soro, no primeiro e no trigésimo dia pós-tratamento (n=8 por grupo, cada). Para isso, foram mensurados os níveis das enzimas alanina aminotransferase (ALT) e aspartato aminotransferase (AST) como marcadores de dano hepático, e ureia e creatina como marcadores de dano renal, utilizando *kits* bioquímicos comerciais (Labtest Diagnostica®, Brasil), de acordo com as instruções do fabricante. Como controle das análises foram aplicadas amostras de soro de camundongos não infectados e não tratados (n=5).

4.9. Análise estatística

As análises estatísticas foram realizadas através do GraphPad Prism™ (GraphPad Software®, EUA; versão 10.0.2 para Windows). Os valores de IC₅₀, CC₅₀ e RBC₅₀ foram inseridos no Microsoft Excel (versão 10.0) e calculados utilizando-se curvas dose-resposta. Os resultados foram avaliados por análise de variância (ANOVA) seguida do pós-teste *Bonferroni* para comparação entre os grupos. Os dados foram apresentados por média $\pm$ desvio padrão. Todos os dados foram submetidos a testes de normalidade e as diferenças consideradas significativas para valores de *p* menores que 0,05 (*p* < 0,05).

5. RESULTADOS E DISCUSSÃO

Conforme Resolução nº 02/2013, de 18 de setembro de 2013, que regulamenta o formato dos trabalhos finais do Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical da Faculdade de Medicina da UFMG, especificamente, Anexo 1 – Tese de doutorado, Item nº 6, os resultados e a discussão do presente estudo estão representados em artigos científicos já publicados e divididos em três capítulos. Ademais, ao final de cada capítulo, também são apresentadas as considerações finais da publicação.

- **Capítulo 1:** “Parasitological and immunological evaluation of a novel chemotherapeutic agente against visceral leishmaniasis”;
- **Capítulo 2:** “New synthetic molecules incorporated into polymeric micelles used for treatment against visceral leishmaniasis”;
- **Capítulo 3:** “Treatment using vanillin-derived synthetic molecules incorporated into polymeric micelles is effective against infection caused by *Leishmania amazonensis* species”.

5.1. CAPÍTULO 1 – CMt

Neste primeiro capítulo são apresentados os resultados e a discussão sobre a ação antileishmanial *in vitro* da molécula CMt contra diferentes espécies de *Leishmania*, bem como na LV experimental.

5.1.1. Artigo

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TITLE

Parasitological and immunological evaluation of a novel chemotherapeutic agent against visceral leishmaniasis

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ABSTRACT

Aims: Treatment for visceral leishmaniasis (VL) is hampered by the toxicity and/or high cost of drugs, as well as by emergence of parasite resistance. Therefore, there is an urgent need for new antileishmanial agents. **Methods and Results:** In this study, the antileishmanial activity of a diprenylated flavonoid called 5,7,3,4'-tetrahydroxy-6,8-diprenylisoflavone (CMt) was tested against *Leishmania infantum* and *L. amazonensis* species. Results showed that CMt presented selectivity index (SI) of 70.0 and 165.0 against *L. infantum* and *L. amazonensis* promastigotes, respectively, and of 181.9 and 397.8 against respective axenic amastigotes. Amphotericin B (AmpB) showed lower SI values of 9.1 and 11.1 against *L. infantum* and *L. amazonensis* promastigotes, respectively, and of 12.5 and 14.3 against amastigotes, respectively. CMt was effective in the treatment of infected macrophages and caused alterations in the parasite mitochondria. *L. infantum*-infected mice treated with miltefosine, CMt alone or incorporated in polymeric micelles (CMt/Mic) presented significant reductions in the parasite load in distinct organs, when compared to the control groups. An antileishmanial Th1-type cellular and humoral immune response were developed one and 15 days after treatment, with CMt/Mic-treated mice presenting a better protective response. **Conclusion:** Our data suggest that CMt/Mic could be evaluated as a chemotherapeutic agent against VL.

Keywords: Delivery systems, flavonoids, immune response, leishmaniasis, miltefosine, treatment.

INTRODUCTION

Leishmaniasis is a vector-borne disease complex caused by distinct species of *Leishmania* parasites, with an estimated incidence of 12 million cases worldwide. According to the World Health Organization, the disease represents a major public health concern, with 2.4 million disability-adjusted life years and 380 million individuals exposed to risks of infection.¹

The main clinical manifestations are tegumentary leishmaniasis (TL), composed by cutaneous, mucosal and cutaneous-diffuse disease, and visceral leishmaniasis (VL), a fatal disease if acute and untreated. The clinical manifestations of leishmaniasis depends on several factors, such as the parasite species, genetic background and host's immune response.² Some *Leishmania* species, such as *Leishmania major*, *L. tropica*, *L. aethiopica*, *L. braziliensis*, *L. amazonensis* and *L. mexicana* cause tegumentary leishmaniasis (TL), while others, such as *L. donovani* and *L. infantum* cause visceral leishmaniasis (VL).³

Treatment against VL mainly depends on the use of pentavalent antimonials, which are unpleasant to the patient due to their endovenous or intramuscular administration.⁴ Most importantly, antimonials are toxic, since they can cause chemical pancreatitis, increase in serum aminotransferase levels and electrocardiographic abnormalities. In addition, development of parasitic resistance to pentavalent antimonials has been reported.⁵ Amphotericin B (AmpB) is another effective antileishmanial drug but it is similarly toxic to mammalian hosts.^{6,7} Liposomal formulations of these compounds can reduce their toxicity while maintaining their efficacy against *Leishmania*, but at the expense of significantly higher costs.⁸ Alternative therapeutic agents include miltefosine, which was originally developed as an anticancer drug⁹ and it was the first orally administered drug in patients with VL.^{10,11} However, miltefosine can cause teratogenicity and parasitic resistance to this compound has also been reported, limiting its clinical use to treat VL.^{12,13}

Therefore, there is an urgent need to identify novel and low-cost antileishmanial agents to improve treatment quality and effectiveness against this disease. In the recent decades, attention has been given to secondary compounds extracted from plants as substitute antileishmanial medicines.^{14,15} Several studies evaluating semi-purified fractions and/ or purified molecules have been performed, some of which showing promising results. However,

no effectively formulated product to treat *Leishmania* infection has been made available to date.¹⁵⁻¹⁷

Flavonoids are secondary metabolites extracted from plants which present diverse biological functions, such as antimicrobial, larvicidal, antiallergic and anti-inflammatory activity. Studies have shown that these molecules can act also as antileishmanial agents.¹⁷⁻¹⁹ *Maclura tinctoria* is a plant popularly known as mulberry, taiúva or amarelinho, which belongs to the Moraceae family and is found in tropical countries worldwide.^{20,21} Numerous studies have reported that some flavonoids isolated from *M. tinctoria* leaves extracts,^{22,23} such as 5,7,3',4'-tetrahydroxy-6,8-diprenylisoflavone (CMt), have neuroprotective activity and anti-Trypanosoma brucei action.^{24,25}

The diversity of clinical manifestations of leishmaniasis is influenced by the host's immune response,²⁶ generally characterized by a CD4⁺ T-cell subtype expansion with resulting production of Th1- or Th2-type cytokines.²⁷ Interferon-gamma (IFN- γ), tumour necrosis factor-alpha (TNF- α) and Interleukin-12 (IL-12), among other cytokines, stimulate the development of Th1-type cellular responses, usually associated with resistance against infection since parasitized macrophages are stimulated to kill parasites.²⁸ On the other hand, Th2-type cytokines, such as IL-4, IL-5, IL-6, IL-10, among others, are associated with susceptibility to infection, since macrophages are deactivated and hence parasites are not eliminated.^{29,30} In leishmaniasis, IL-10 fulfils a wider role, since it is not only related to parasite survival, but also to the immunological counterbalance necessary for resolution of infection, avoiding an otherwise exacerbated inflammatory response. What is more, Th1- and Th2-type response profiles cannot be generalized for all forms of leishmaniasis. In fact, exacerbated Th1-type response to TL can cause tissue damage and contribute to lesion progression.^{26,31} In addition, other T-cell populations, such as regulatory T cells and Th17 cells, are associated with immunity to leishmaniasis, since they are recruited to the infection site^{32,33} and contribute to balance the production of Th1- and Th2-type cytokines during disease.³⁴

In this context, effective therapeutic candidates would be those capable of inducing a specific Th1-type response in treated hosts, sufficient to combat *Leishmania* parasites while avoiding an exacerbated inflammatory response. In the present study, CMt was evaluated for its in vitro antileishmanial action against *L. infantum* and *L. amazonensis* species, as well as for its efficacy and toxicity in the treatment of infected macrophages. Results showed that CMt was effective against both parasite species, without presenting toxicity to murine cells. Higher selectivity indexes were found against promastigotes and axenic amastigotes when compared to values obtained using AmpB, suggesting higher in vitro CMt-specific therapeutic efficacy.

Furthermore, treatment of *L. infantum*-infected mice with miltefosine (control), CMt alone or incorporated in polymeric micelles resulted in significant reductions of the parasite load in their spleens, livers, bone marrows (BM) and draining lymph nodes (dLN) one and 15-day post-treatment, in comparison to the untreated and infected control mice. In addition, *in vitro* stimulation of murine spleens induced an antileishmanial Th1-type immune response, suggesting potential therapeutic efficacy *in vivo* against *L. infantum* infection.

MATERIALS AND METHODS

CMt and CMt-containing micelles preparation

5,7,3',4'-tetrahydroxy-6,8-diprenylisoflavone (CMt) was acquired from the in-house compound library belonging to the Biodiversity Laboratory at Federal University of Viçosa (Viçosa, Minas Gerais, Brazil).²¹ CMt was obtained from *Maclura tinctoria* leaves extracts and identified by spectroscopic assays such as ESI-MS, UV, ¹H NMR and ¹³C NMR.^{23,35} CMt-containing micelles (CMt/Mic) were prepared as previously described.³⁶ Briefly, Poloxamer 407 (18% w/w; catalog number 16758, Sigma-Aldrich, USA) was diluted in Phosphate Buffered Saline (PBS) pH 7.4 under magnetic agitation for 18 hours at 4°C. CMt (10 mg) was dissolved in 500 µL dichloromethane by vortexing and added to the previously prepared solution under vigorous magnetic agitation in an ice bath, until a viscous emulsion was obtained. The dichloromethane was then evaporated using a rotary evaporator (Buchi, Flawil, Switzerland), resulting in a transparent, yellow gel at room temperature. Empty micelles (called B-Mic; 18% w/w Poloxamer) were prepared following the same protocol, without the incorporation of CMt.

Mice

Female BALB/c mice (8 weeks old) were purchased from the Institute of Biological Sciences of Federal University of Minas Gerais (UFMG; Belo Horizonte, Minas Gerais, Brazil) and were kept in pathogen-free conditions. The study was approved by the Committee for the Ethical Handling of Research Animals of UFMG (protocol number 085/2017).

Parasites

L. infantum (MHOM/BR/1970/BH46) and *L. amazonensis* (IFLA/ BR/1967/PH-8) strains were used. Stationary promastigotes were grown in Schneider's medium (Sigma-Aldrich) supplemented with 20% v/v heat-inactivated foetal bovine serum (FBS; Sigma-

Aldrich) and 20 mmol/L L-glutamine (pH 7.4) at 24°C. Soluble *Leishmania* antigenic extract (SLA) was prepared from stationary promastigotes. Briefly, parasites (10^9 cells) were washed three times in cold, sterile PBS pH 7.4, subjected to five freeze-thaw cycles followed by five cycles of ultrasonication of 30 seconds each at 38 MHz. The suspension was centrifuged at $9,000\times g$ for 30 minutes at 4°C, and supernatant containing SLA was collected and stored at -80°C until use.³⁷ To obtain axenic amastigotes, 2×10^8 stationary promastigotes were washed three times in PBS pH 7.4, resuspended in 5 mL FBS and incubated for 48 or 72 hours (*L. amazonensis* and *L. infantum*, respectively) at 37°C. Parasites were then washed three times in PBS and their morphology was evaluated by Giemsa staining and visualization using an optical microscope.

***In vitro* antileishmanial activity**

50% *Leishmania* inhibitory concentration (IC_{50}) was determined by incubating *L. infantum* or *L. amazonensis* stationary promastigotes or axenic amastigotes (1×10^6 cells, each) with CMt (0 to 50.0 $\mu\text{g/mL}$) or Amphotericin B (AmpB, 0 to 1.0 $\mu\text{g/mL}$; Cristália, São Paulo, São Paulo, Brazil) in 96-well culture plates (Nunc, Nunclon, Roskilde, Denmark) for 48 hours at 24°C. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich) method. Optical density (OD) values were measured using a microplate spectrophotometer (Molecular Devices, Spectra Max Plus, San Jose, CA, USA) at 570 nm. IC_{50} values were calculated by sigmoidal regression of dose-response curves with Microsoft Excel software (version 10.0).³⁸

Cytotoxicity assay

CMt cytotoxicity to murine macrophages (CC_{50}) and red blood cells (RBC_{50}) was evaluated by determining the compound's concentration required for 50% reduction of cell viability. Macrophages were obtained by peritoneal lavage with 5 mL cold PBS pH 7.4. Peritoneal exudate cells were centrifuged at $1000\times g$ for 10 minutes and resuspended in RPMI 1640 medium. Thus, macrophages (5×10^5 cells/mL) or a 5% v/v red blood cells suspension were incubated with CMt (0 to 100.0 $\mu\text{g/mL}$) or AmpB (0 to 10.0 $\mu\text{g/mL}$) in RPMI 1640 medium in 96-well plates (Nunc) for 48 hours (macrophages) or 1 hours (red blood cells) at 37°C in 5% CO_2 . Macrophage viability was assessed by MTT method. The red blood cells suspension was centrifuged for 10 minutes at $1000\times g$ and lysis percentage was determined spectrophotometrically at 570 nm. The absence of (blank) or 100% haemolysis was determined

by replacing CMt for an equal volume of PBS or distilled water, respectively. CC₅₀ and RBC₅₀ values were calculated by sigmoidal regression of dose-response curves with Microsoft Excel software (version 10.0).³⁸

Treatment of infected macrophages

CMt efficacy for treatment of infected cells was evaluated *in vitro* using murine macrophages. Cells (5×10^5 cells/mL) were seeded in a microwell plate in RPMI 1640 medium supplemented with 20% v/v heat-inactivated FBS and 20 mmol/L L-glutamine, pH 7.4. They were then incubated for 24 hours at 37°C in 5% CO₂. Next, cells were infected with stationary promastigotes at a ratio of 10 parasites per macrophage, followed by further 48 hours incubation at 37°C in 5% CO₂. Free parasites were removed by washing with RPMI 1640 medium and infected macrophages were then treated with CMt (0, 1.0, 5.0 and 10.0 µg/mL) or AmpB (0, 0.1, 0.5 and 1.0 µg/mL), for 48 hours at 24°C in 5% CO₂. After fixation with 4% w/v paraformaldehyde, cells were washed and stained with Giemsa. Percentage of infection and the number of amastigotes per infected cell were determined by counting 200 macrophages, in triplicate, using an optical microscope.³⁹

Inhibition of infection by pretreatment of parasites

Stationary promastigotes (5×10^6 cells) were pre-incubated with CMt (0, 1.0, 5.0 and 10.0 µg/mL) or AmpB (0, 0.1, 0.5 and 1.0 µg/mL), for 4 hours at 24°C. Parasites were washed three times in RPMI 1640 medium, quantified and used to infect macrophages at a ratio of 10 parasites per macrophage for 24 hours at 37°C in 5% CO₂. After fixation with 4% w/v paraformaldehyde, cells were washed and stained with Giemsa. Percentage of infection and the number of amastigotes per infected cell were determined by counting 200 macrophages, in triplicate, using an optical microscope.³⁹

Evaluation of the mitochondrial membrane integrity and potential

Stationary promastigotes (1×10^7 cells) were cultured in the absence or presence of CMt (6.36 and 12.72 µg/mL, corresponding to one and two times its IC₅₀ value, respectively) for 24 hours at 25°C. Parasites were washed in PBS and incubated with propidium iodide (1.0 µg/mL; Sigma-Aldrich) or MitoTracker (500 nmol/L; Invitrogen, USA) for 15 and 30 minutes, respectively, in the dark and at room temperature. After washing twice with PBS, samples were added to a black 96-well plate and fluorescence intensity was measured using a fluorometer

(FLx800, BioTek Instruments, Inc, Winooski, VT, USA), with excitation and emission wavelengths of 540 and 600 nm, respectively. Promastigotes pre-heated for 10 minutes at 65°C or carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazon (FCCP, 5.0 $\mu\text{mol/L}$; Sigma-Aldrich) were used as controls, respectively.^{39,40}

Reactive oxygen species (ROS) production

Stationary promastigotes (1×10^7 cells) were cultured in the absence or presence of CMt (6.36 and 12.72 $\mu\text{g/mL}$) for 24 hours at 25°C. Parasites were incubated with 20 $\mu\text{mol/L}$ cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA; Sigma-Aldrich) for 30 minutes in the dark and at room temperature. Fluorescence intensity was measured in a spectrofluorometer (Varioskan® Flash, Thermo Scientific, USA), with excitation and emission wavelengths of 485 and 528 nm, respectively. H_2O_2 -treated promastigotes (4.0 mmol/L ; Sigma-Aldrich) were used as control.⁴⁰

Infection and treatment

BALB/c mice ($n = 12$ per group) were injected subcutaneously with 10^7 *L. infantum* stationary promastigotes. 45-day post-challenge, infected mice were administered saline (control group), empty micelles (B/Mic; 10 mg/kg body weight), CMt (10 mg/kg body weight), CMt-containing micelles (CMt/Mic, 5 mg/kg body weight) or miltefosine (2 mg/kg body weight; catalog 58066-85-6, Sigma-Aldrich) every two days for a period of 10 days. Except for miltefosine which was administered by oral route, all other compounds were administered by subcutaneous injection. Six mice of each group were euthanized one and 15-day post-treatment.

Evaluation of parasite load

One and 15-day post-treatment, spleens, livers, bone marrows (BM) and draining lymph nodes (dLN) were collected from the infected and treated animals and the corresponding parasite load was estimated by limiting dilution technique. Briefly, organs were macerated in a glass tissue grinder with sterile PBS, tissue debris was removed by centrifugation at $150 \times g$ for 10 minutes at 4°C, and cells were concentrated by centrifugation at $2000 \times g$ for 15 minutes at 4°C. Pellets were resuspended in 1 mL complete Schneider's medium and a log-fold serial dilution (10^{-1} to 10^{-12} dilutions) was performed in Schneider's medium. Each sample was plated in triplicate and incubated for 7 days at 24°C, and results were expressed as the negative log of the titre (the dilution corresponding to the last positive well) adjusted per milligram of organ.

The splenic parasite load was also determined by quantitative polymerase chain reaction (qPCR).³⁶ Briefly, spleen DNA was extracted using Wizard® Genomic DNA Purification Kit (Promega Corporation) and *L. infantum* kDNA was amplified by qPCR using the following primers: Forward (CCTATTTTACACCAACCCCCAGT) and Reverse (GGGTAGGGGCGTTCTGCGAAA). Mouse β -actin gene was used as an endogenous control. Standard curves for kDNA and β -actin quantification were obtained from DNA extracted from 10^8 parasites or 10^8 peritoneal macrophages, respectively, in the same conditions used to quantify the spleen samples.

Reactions were developed in an ABI Prism 7500 Sequence Detection System (96 wellsplate; Applied Biosystems) using 2x SYBRTM Select Master Mix (5 μ L; Applied Biosystems), with 2 mmol/L of each primer (1 μ L) and 4 μ L DNA (25 ng/ μ L). Samples were incubated at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minutes, with fluorescence data being recorded after each cycle. All experiments were performed at the real-time PCR facility - RPT09D PDTIS/René Rachou Institute – FIOCRUZ/MG. Parasite load was quantified using the standard curve as a reference and converted to ‘number of parasites per nucleated cell’ by multiplying by 1,000 to facilitate visualization. The experiment was performed in duplicate.

Cellular response

Cellular response was evaluated using the spleens of infected and treated animals, one and 15-day post-treatment. In brief, splenocytes (5×10^6 cells/mL) were incubated in DMEM (medium) supplemented with 20% v/v heat-inactivated FBS and 20 mmol/L L-glutamine at pH 7.4, or stimulated with *L. infantum* SLA (25.0 μ g/mL), for 48 h at 37°C in 5% CO₂. IFN- γ , IL-4, IL-10, IL-12p70 and GM-CSF levels were measured in the cell supernatant by capture ELISA (BD Pharmingen®, San Diego, CA, USA), according to the manufacturer's instructions. Nitrite production in the cellular supernatant was evaluated by Griess reaction.⁴¹ A flow cytometry assay was also performed to evaluate the IFN- γ , TNF- α and IL-10-producing CD4⁺ and CD8⁺ T-cell prevalence.⁴² Briefly, spleen cells (5×10^6 per mL) were incubated in complete RPMI 1640 medium only (control) or stimulated with *L. infantum* SLA (25 μ g/mL) for 48 hours at 37°C in 5% CO₂. Intracytoplasmic cytokine-producing T-cell frequency was determined based on their relative flow cytometry size (forward laser scatter – FSC) and granularity (side laser scatter – SSC). After selection of FSCLow and SSCLow phenotype cells, density plots for CD4/FL1 or CD8/FL1 versus IFN- γ /FL2, TNF- α /FL2, or IL-10/FL2 were created to determine

IFN- γ , TNF- α and IL-10-producing T-cell frequency. Results were expressed as indexes, which were calculated by the ratio between CD4⁺ and CD8⁺ T-cell percentages in the stimulated versus unstimulated cultures.

Humoral response

Antibody production was evaluated in sera samples from infected and treated mice, one and 15-day post-treatment, by ELISA assay. Anti-parasite IgG1 and IgG2a isotype antibody levels were measured using *L. infantum* SLA as coating antigen (1.0 μ g/well). Samples were diluted 1:100 in PBS-T (PBS plus 0.05% v/v Tween 20) and anti-mouse IgG1 and IgG2a horseradish-peroxidase conjugated antibodies (Sigma-Aldrich) were used both at 1:10,000 dilution (in PBS-T). Reactions were developed in the presence of H₂O₂, ortho-phenylenediamine and citrate-phosphate buffer pH 5.0 for 30 minutes in the dark. Next, reactions were stopped by addition of 2 NH₂SO₄, after which OD values were measured at 492 nm using an ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Canada).

Statistical analysis

IC₅₀, CC₅₀ and RBC₅₀ values were calculated by dose-response curves using Microsoft Excel software (version 10.0) and plotted in GraphPad Prism 5.03. Results were analysed by one-way analysis of variance (ANOVA) followed by Bonferroni's post-test for comparison between the groups. Results were expressed as mean $\pm$ standard deviation for each group. Two independent experiments were performed. Differences were considered significant when $P < 0.05$.

RESULTS

Analysis of biological activity *in vitro*

Antileishmanial activity of CMt was evaluated *in vitro* against *L. infantum* and *L. amazonensis* species. Results showed higher IC₅₀ values against *L. infantum* and *L. amazonensis* promastigotes and amastigotes for CMt, when compared to AmpB (**Table 1**).

Table 1. *In vitro* antileishmanial activity.

Compound	IC ₅₀ (µg/mL)			
	Stationary promastigotes		Axenic amastigotes	
	<i>L. infantum</i>	<i>L. amazonensis</i>	<i>L. infantum</i>	<i>L. amazonensis</i>
CMt	6.36 ± 0.75	2.70 ± 0.67	2.45 ± 0.66	1.12 ± 0.24
Amphotericin B	0.11 ± 0.02	0.09 ± 0.04	0.08 ± 0.04	0.07 ± 0.02

Note: *L. infantum* and *L. amazonensis* stationary promastigotes and axenic amastigotes (1×10^6 cells, each) were incubated with CMt (0 to 50 µg/mL) or AmpB (0 to 1.0 µg/mL) for 48 h at 24°C. Cell viability was analysed by MTT method, and 50% Leishmania inhibitory concentration factor (IC₅₀) was calculated for each compound by sigmoidal regression of their corresponding dose-response curves. Results are expressed as mean ± standard deviation.

Toxicity assays showed CC₅₀ and RBC₅₀ values for CMt of 445.5 ± 21.5 µg/mL and 302.6 ± 19.8 µg/mL, respectively, while for AmpB, values were of 1.0 ± 0.2 µg/mL and 12.2 ± 1.6 µg/mL, respectively. Thus, selectivity index (SI) values showed higher antileishmanial activity and lower toxicity in mammalian cells for CMt, when compared to AmpB (**Table 2**).

Table 2. Determination of selectivity index.

Compound	Selectivity index			
	Stationary promastigotes		Axenic amastigotes	
	<i>L. infantum</i>	<i>L. amazonensis</i>	<i>L. infantum</i>	<i>L. amazonensis</i>
CMt	70.0	165.0	181.9	397.8
Amphotericin B	9.1	11.1	12.5	14.3

Note: Selectivity index (SI) values were calculated as the ratio of the minimum concentration of the agent required for 50% inhibition of macrophages (CC₅₀) to the 50% Leishmania inhibitory concentration (IC₅₀). SI values were calculated for CMt and AmpB, both against *L. infantum* and *L. amazonensis* stationary promastigotes and axenic amastigotes, using murine macrophages as the infection model. Results are expressed as mean ± standard deviation.

Treatment of *L. infantum*-infected macrophages with CMt (10.0 µg/mL) and AmpB (1.0 µg/mL) resulted in infection percentages of $39.2 \pm 3.6\%$ and $31.2 \pm 3.7\%$, respectively, with corresponding number of recovered amastigotes of 0.4 ± 0.1 and 0.9 ± 0.4 (**Table 3**). Control of infected and untreated macrophages presented instead $88.7 \pm 5.5\%$ percentage of infection and 4.8 ± 0.5 recovered amastigotes (**Table 3**). On the other hand, treatment of *L. amazonensis*-

infected macrophages with CMt (10.0 µg/mL) and AmpB (1.0 µg/mL) resulted in infection percentages of $10.5 \pm 2.3\%$ and $16.7 \pm 2.2\%$, respectively, with corresponding number of recovered amastigotes of 0.6 ± 0.2 and 1.3 ± 0.5 . Control of infected and untreated cells presented an infection percentage and number of recovered amastigotes of $78.9 \pm 5.5\%$ and 5.8 ± 0.4 , respectively.

Table 3. Treatment of infected macrophages.

Products	Concentration (µg/mL)	Percentage of infected macrophages after treatment		Number of amastigotes per treated and infected macrophage	
		<i>L. amazonensis</i>	<i>L. infantum</i>	<i>L. amazonensis</i>	<i>L. infantum</i>
CMt	10.0	10.5 ± 2.3	39.2 ± 3.6	0.6 ± 0.2	0.4 ± 0.1
	5.0	19.9 ± 3.2	48.8 ± 6.2	1.5 ± 0.4	0.8 ± 0.2
	1.0	36.6 ± 5.1	60.2 ± 4.4	3.0 ± 0.5	1.2 ± 0.3
	0	78.9 ± 5.5	88.7 ± 5.5	5.8 ± 0.4	4.8 ± 0.5
Amphotericin B	1.0	16.7 ± 2.2	31.2 ± 3.7	1.3 ± 0.5	0.9 ± 0.4
	0.5	23.3 ± 4.0	47.7 ± 4.4	1.8 ± 0.8	2.1 ± 0.5
	0.1	39.9 ± 2.6	59.1 ± 7.7	3.8 ± 0.5	3.0 ± 0.4
	0	78.9 ± 5.5	88.7 ± 5.5	5.8 ± 0.4	4.8 ± 0.5

Note: Murine macrophages (5×10^5 cells) were infected with *L. amazonensis* or *L. infantum* promastigotes (at a ratio of 10 parasites per macrophage), followed by treatment with CMt (0, 1.0, 5.0 and 10.0 µg/mL) or AmpB (0, 0.1, 0.5 and 1.0 µg/mL) for 48 h at 24°C in 5% CO₂. Infection percentage values and the number of amastigotes per infected cell were calculated by counting 200 macrophages in triplicate. Results are expressed as mean $\pm$ standard deviation.

The efficacy of CMt to reduce infection was evaluated also by pre-treating the parasites with this drug before infection. With *L. infantum* species, infection percentages of $30.4 \pm 4.5\%$ and $35.5 \pm 3.7\%$ were observed, when parasites were pre-treated with CMt (10.0 µg/mL) or AmpB (1.0 µg/mL), respectively. The number of recovered amastigotes for pre-treatment with CMt or AmpB was 0.7 ± 0.3 and 1.6 ± 0.4 , respectively (**Table 4**). With pre-treated *L. amazonensis*, infection percentages of $20.0 \pm 3.5\%$ and $29.9 \pm 3.7\%$ were observed for CMt (10.0 µg/mL) and AmpB (1.0 µg/mL), respectively, with a corresponding number of recovered amastigotes of 0.8 ± 0.3 and 1.7 ± 0.6 .

Table 4. Inhibition of infection by pre-treatment of parasites.

Products	Concentration n (µg/mL)	Percentage of infection using pre-treated parasites		Number of recovered amastigotes per macrophage	
		<i>L. amazonensis</i>	<i>L. infantum</i>	<i>L. amazonensis</i>	<i>L. infantum</i>
CMt	10.0	20.0 ± 3.5	30.4 ± 4.5	0.8 ± 0.3	0.7 ± 0.3
	5.0	33.2 ± 2.5	55.4 ± 5.0	1.6 ± 0.5	1.6 ± 0.7
	1.0	48.0 ± 4.4	58.8 ± 4.3	2.4 ± 0.5	2.4 ± 0.6
	0	94.8 ± 3.7	96.6 ± 4.5	4.9 ± 0.9	4.9 ± 0.7
Amphotericin B	1.0	29.9 ± 3.7	35.5 ± 3.7	1.7 ± 0.6	1.6 ± 0.4
	0.5	44.2 ± 3.7	56.7 ± 5.0	2.5 ± 0.6	2.7 ± 0.9
	0.1	59.9 ± 5.0	66.7 ± 6.7	3.9 ± 0.4	3.2 ± 0.7
	0	94.8 ± 3.7	96.6 ± 4.5	4.9 ± 0.9	4.9 ± 0.7

Note: *L. amazonensis* and *L. infantum* promastigotes (5×10^6 cells) were pre-incubated with CMt (0, 1.0, 5.0 and 10.0 µg/mL) or AmpB (0, 0.1, 0.5 and 1.0 µg/mL) for 4 h at 24°C. Parasites were washed three times in RPMI 1640 medium, quantified and used to infect murine macrophages (at a ratio of 10 parasites per macrophage) for 24 h at 37°C in 5% CO₂. Infection percentage values and the number of amastigotes per infected cell were calculated by counting 200 macrophages in triplicate. Results are expressed as mean ± standard deviation.

Mechanism of action in *L. infantum*

The proof of concept for the mechanism of action of CMt on *L. infantum* was evaluated as follows. Parasite membrane integrity was analysed by incubating the parasites with propidium iodide, a non-permeant nucleic acid stain which can cross parasites' permeabilized membranes only. Results showed that the CMt, when used at a concentration of 6.36 µg/mL (IC₅₀ value calculated for this parasite species), caused alterations in the parasite membrane integrity in the order of 56.8% (**Figure 1**).

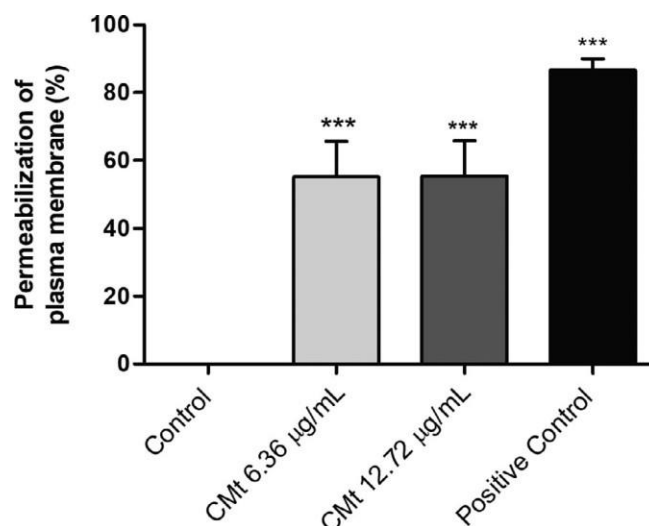


Figure 1. Evaluation of the parasite membrane integrity.

L. infantum stationary promastigotes (10^7 cells) were cultured in the absence (control) or presence of CMt (6.36 and 12.72 µg/mL, corresponding to one and two times its IC_{50} value, respectively) for 24 h at 25°C. Parasites were washed in PBS and incubated with 1.0 µg/mL propidium iodide for 15 min in the dark at room temperature. Fluorescence intensity was measured using a fluorometer. Promastigotes pre-heated at 65°C for 10 min were used as a positive control. Bars indicate the mean plus standard deviation of the groups. (***) indicates statistically significant difference in relation to the control ($P < .0001$).

In comparison, pre-heated *L. infantum* used as control showed 86.6% membrane permeabilization. Mitochondrial dysfunction after CMt treatment was also evaluated using MitoTracker fluorescent marker. Results showed that CMt used at a concentration of 6.36 µg/mL induced an alteration of 63.5% in *L. infantum* membrane potential (**Figure 2**). Control with FCCP showed values in the order of 68.8%. Furthermore, ROS production was increased by 182.4% and 383.0% after treatment with CMt (6.36 µg/mL) or H_2O_2 (control), respectively (**Figure 3**).

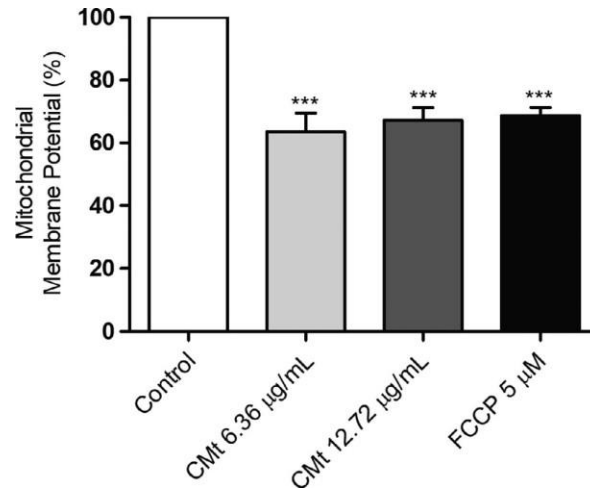


Figure 2. Analysis of *Leishmania* mitochondrial potential.

L. infantum stationary promastigotes (10^7 cells) were cultured in the absence (control) or presence of CMt (6.36 and 12.72 µg/mL, corresponding to one and two times its IC_{50} value, respectively) for 24 h at 25°C. Cells were incubated for 30 min in the dark with 500 nmol/L MitoTracker. After washing twice with PBS, treated promastigotes were transferred to a black 96-well plate and fluorescence intensity was measured using a fluorometer. FCCP (5.0 µmol/L; Sigma-Aldrich) was used as a positive control. Bars indicate the mean plus standard deviation of the groups. (***) indicates statistically significant difference in relation to the control ($P < .0001$).

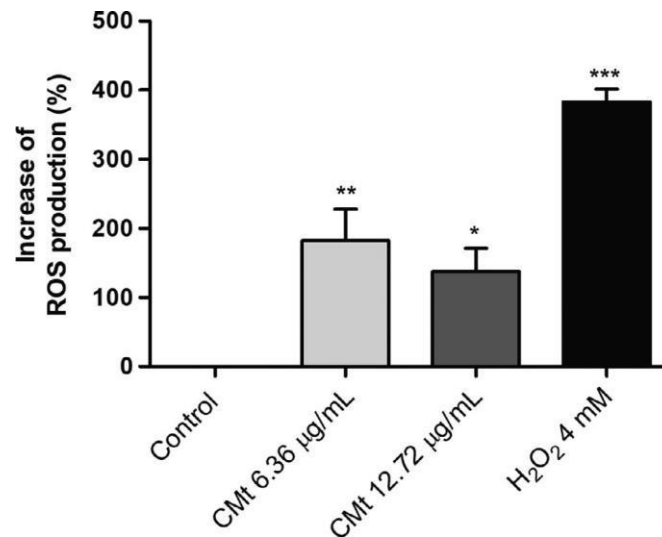


Figure 3. Production of reactive oxygen species.

L. infantum stationary promastigotes (10^7 cells) were cultured in the absence (control) or presence of CMt (6.36 and 12.72 µg/mL, corresponding to one and two times its IC_{50} value, respectively) for 24 h at 25°C, after which the parasites were probed with H₂DCFDA for 30 min in the dark at room temperature. Fluorescence intensity was measured using a fluorometer. Promastigotes treated with 4.0 mmol/L H₂O₂ were used as a positive control. Bars indicate the mean plus standard deviation of the groups. (*), (**) and (***) indicate statistically significant differences in relation to the control ($P < .01$, $P < .001$ and $P < .0001$, respectively).

Evaluation of the parasite load

Parasite load was evaluated in infected and treated mice, one and 15-day post-treatment, using limiting dilution technique. Results showed that miltefosine, CMt or CMt/Mic-treated mice all presented significant reductions in the levels of parasitism in their liver, spleen, BM and dLNs, when compared to the control (saline and B/Mic) groups, both at one and 15-day post-treatment (**Figure 4**). However, CMt/Mic-treated mice were those presenting the most significant reduction in parasitism, in comparison to mice treated with miltefosine or CMt only. In addition, analysis by qPCR assay showed that miltefosine-, CMt- and CMt/Mic-treated mice all presented significant reductions in their splenic parasitism levels, when compared to the controls groups (**Figure 5**). Likewise, CMt/Mic treatment presented the best therapeutic action when compared to all other groups.

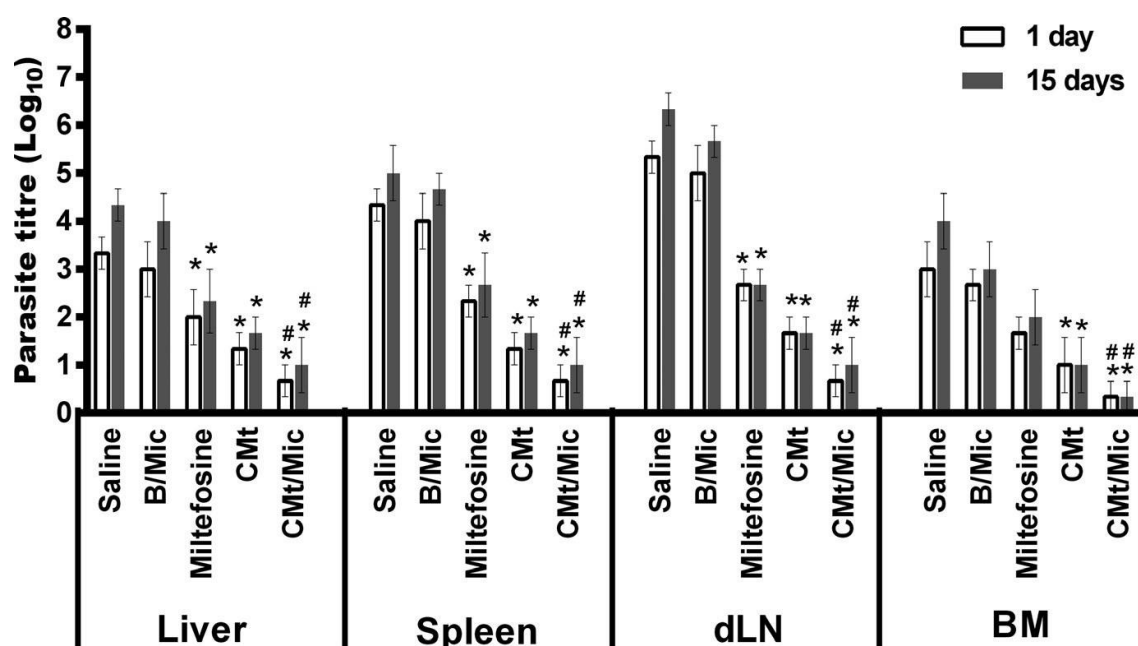


Figure 4. Parasite load evaluation by limiting dilution technique.

BALB/c mice (n = 12 per group) were infected with 10^7 *L. infantum* promastigotes and, 45-day post-challenge, subcutaneously injected with saline, empty micelle (B/Mic), free CMt, CMt/Mic or miltefosine, every two days for a period of 10 days. Half of the animals were euthanized one and 15 days after treatment, when their liver, spleen, bone marrow (BM) and draining lymph nodes (dLN) were collected for determination of the corresponding parasite loads by limiting dilution technique. Bars indicate the mean $\pm$ standard deviation of the groups, one (white bars) and 15-day (grey bars) post-treatment. (*) indicates statistically significant difference in relation to the saline and B/Mic groups ($P < 0.001$). (#) indicates statistically significant difference in relation to the miltefosine and CMt groups ($P < 0.001$).

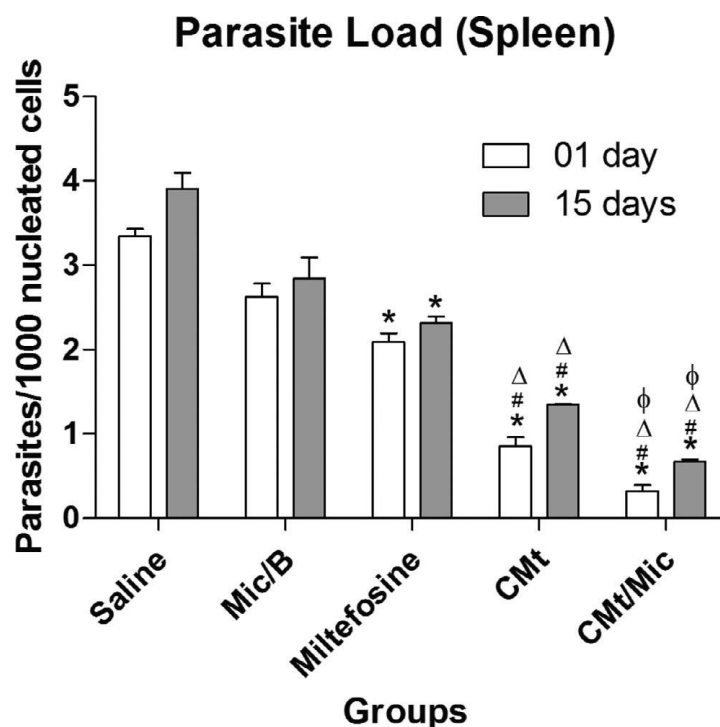


Figure 5. Parasite load evaluation by quantitative PCR (qPCR) technique.

The splenic parasitism was also evaluated by qPCR technique, one and 15 days after treatment. Results were converted to the number of parasites (log) per nucleated cell (multiplied by 1,000 to facilitate visualization). Bars indicate the mean plus standard deviation of the groups. (*), (#), (Δ) and (φ) indicate significant statistical differences in comparison to saline, Mic/B, miltefosine and CMt groups, respectively ($P < 0.05$).

Analysis of murine immune responses developed after infection and treatment

The immune response of infected and treated mice was analysed one and 15-day post-therapy. Results showed that miltefosine, CMt or CMt/Mic-treated mice presented significantly higher levels of IFN- γ , IL-12 and GM-CSF, with corresponding lower IL-4 and IL-10 levels, both one (**Figure 6A**) and 15 (**Figure 6B**)-day post-treatment, in comparison to the control groups. Moreover, CMt/Mic-treated animals presented a more polarized Th1-type response, in comparison to those groups of mice treated with miltefosine and CMt.

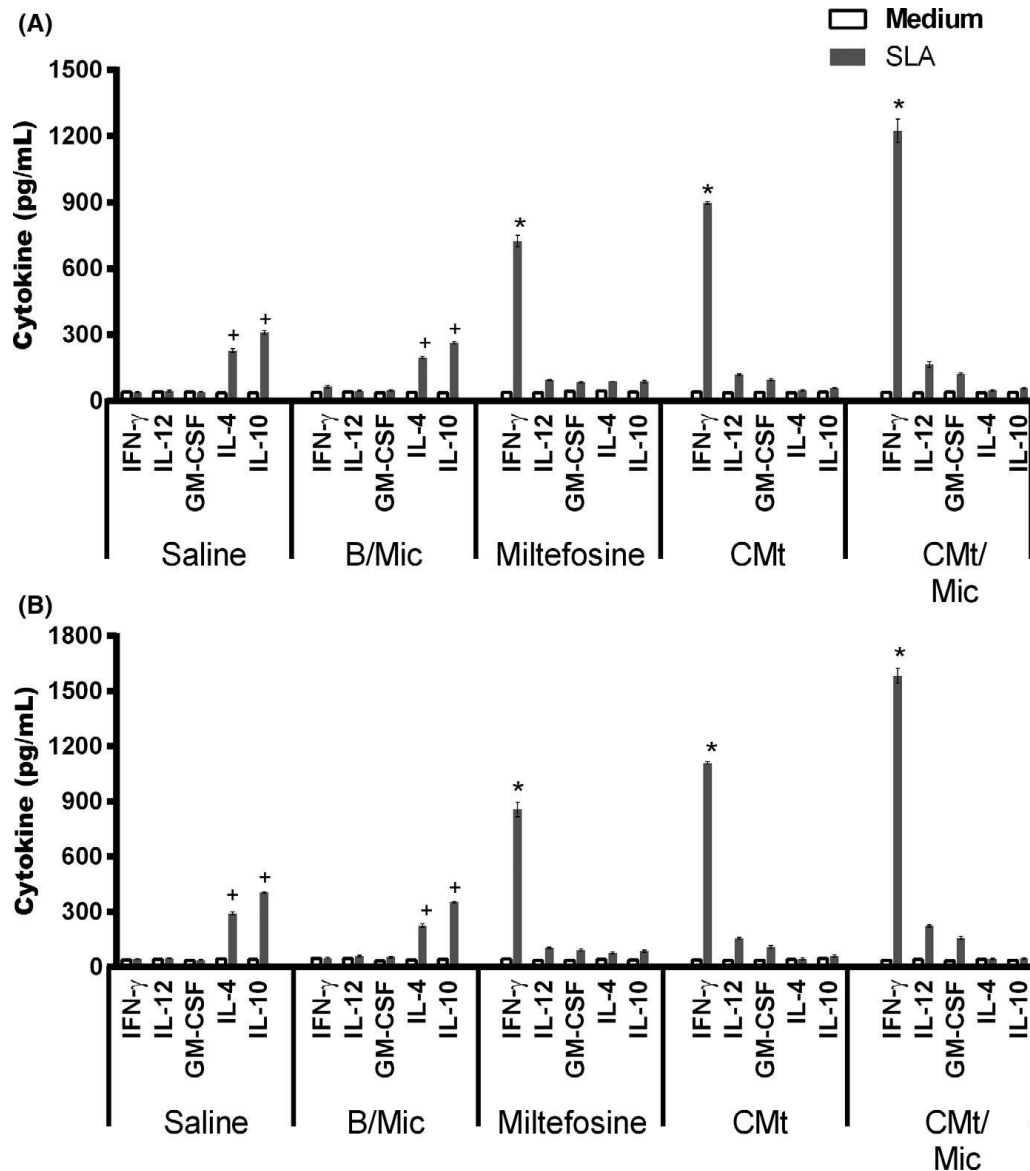


Figure 6. Cellular response analysis in infected and treated mice.

Spleen cells of infected and treated animals were collected one and 15 days after treatment. Cells were stimulated with *L. infantum* SLA (25 μ g/mL) for 48 h at 37°C in 5% CO₂, or left untreated (medium) as controls. IFN- γ , IL-4, IL-10, IL-12p70 and GM-CSF levels were measured in the cell supernatant, one (A) and 15 (B) days after treatment, using commercial kits. Bars indicate the mean $\pm$ standard deviation of the groups. (*) indicates statistically significant difference in relation to the saline and B/Mic groups ($P < .001$). (+) indicates statistically significant difference in relation to the miltefosine, CMt and CMt/Mic groups ($P < 0.001$).

Additionally, flow cytometry assay showed significantly higher IFN- γ and TNF- α -producing CD4⁺ and CD8⁺ T-cell frequency in the miltefosine-, CMt- and CMt/Mic-treated mice when compared to the controls, with CMt/Mic-receiving mice presenting slightly higher levels than the miltefosine-treated group (**Figure 7**). As expected, saline and B/Mic control groups presented higher IL-10- producing T-cell frequency than treated mice.

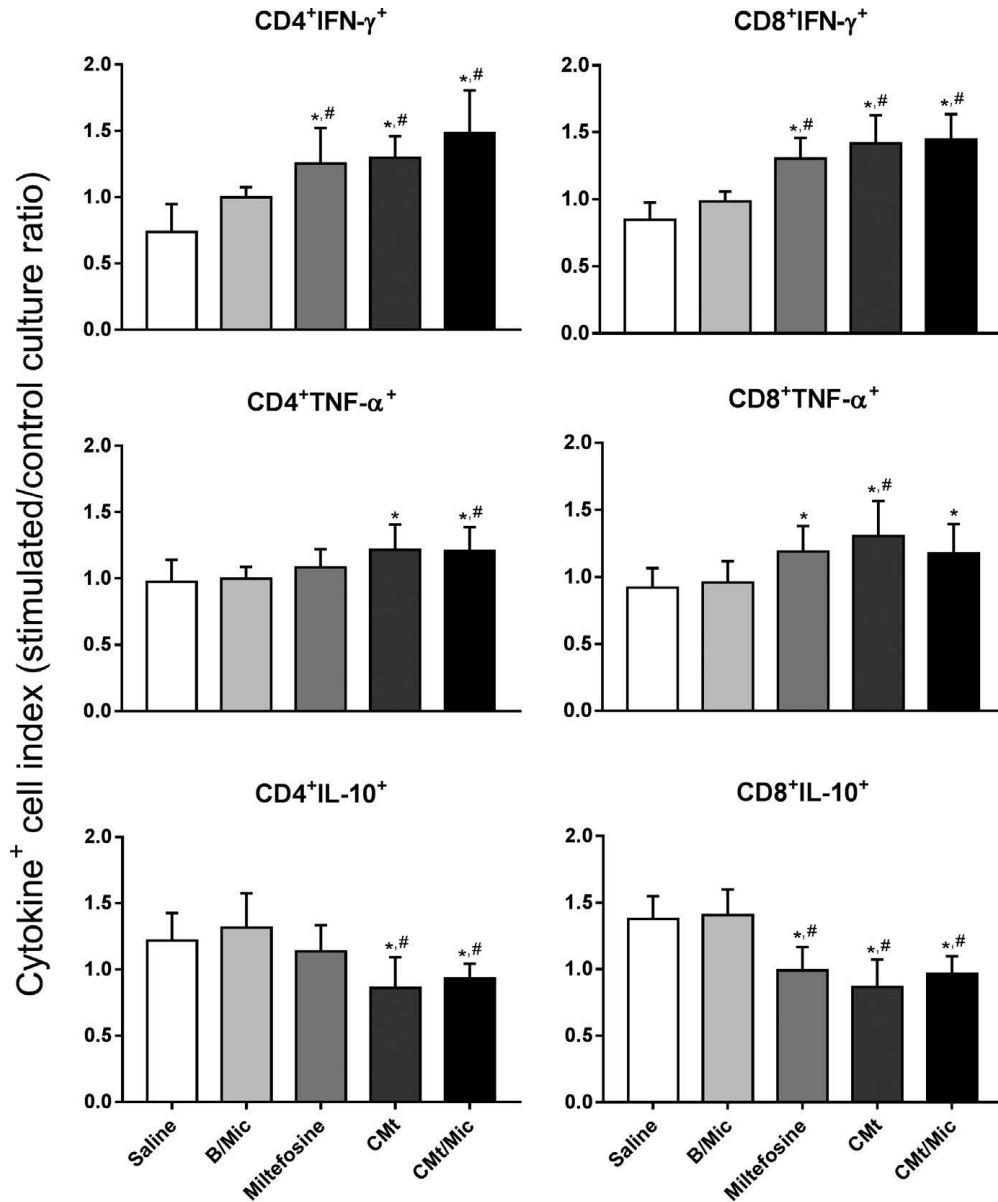


Figure 7. Intracytoplasmic cytokineproducing T-cell frequency in treated mice.

L. infantum-infected mice and treated with saline, empty micelle (B/ Mic), free CMt, CMt/Mic or miltefosine were euthanized 15 days after treatment, when their spleen cells were collected for stimulation with *L. infantum* SLA, or left unstimulated as control. Results are expressed as cytokine indexes (Stimulated culture/Control culture ratio) for IFN-γ, TNF-α, or IL-10-producing T-cell frequency in CD4⁺ and CD8⁺ T cells. Bars indicate the mean ± standard deviation of the groups. (*) and (#) indicate significant difference in relation to the saline and B/ Mic groups, respectively ($P < 0.05$).

Nitrite production was also evaluated one and 15-day post-treatment. Results showed that nitrite levels were significantly higher in the miltefosine, CMt and CMt/Mic groups, when compared to the controls (**Figure 8**). Moreover, CMt/Mic-treated mice presented the highest

nitrite levels, when compared to all other mice groups. In addition, evaluation of the humoral response showed that CMt- and CMt/Mic-treated mice presented significantly higher anti-parasite IgG2a isotype levels than IgG1 levels, both one and 15-day post-treatment (**Figure 9**). Unsurprisingly, control mice groups presented higher IgG1 levels as compared to IgG2a levels.

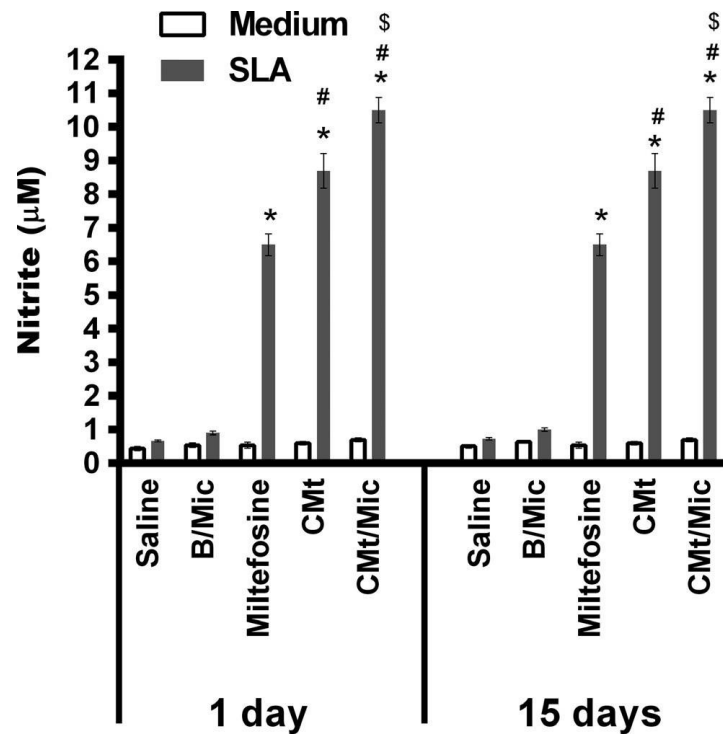


Figure 8. Nitrite production.

Cell supernatants used to evaluate cytokine production was also utilized to evaluate nitrite production. The values correspond to unstimulated or SLA stimulated cell cultures (white and grey bars, respectively). Bars indicate the mean $\pm$ standard deviation of the groups. (*) indicates statistically significant difference in relation to the saline and B/Mic groups ($P < 0.0001$). (#) indicates statistically significant difference in relation to the saline, B/Mic and miltefosine groups ($P < 0.0001$). (\$) indicate statistically significant difference in relation to the saline, B/Mic, miltefosine and CMt groups ($P < 0.001$).

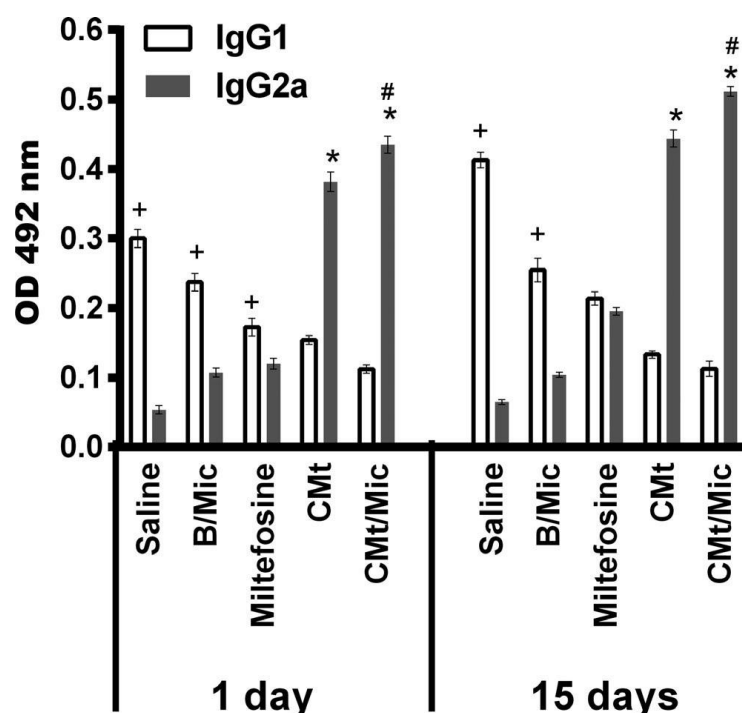


Figure 9. Humoral response analysis in infected and treated mice.

Sera samples were collected from infected and treated animals, one and 15 days after treatment, and anti-parasite IgG1 (white bars) and IgG2a (grey bars) antibody levels were measured. Bars indicate the mean $\pm$ standard deviation of the groups. (*) indicates statistically significant difference in relation to the saline, B/Mic and miltefosine groups ($P < 0.0001$). (+) indicates statistically significant difference in relation to the CMt and CMt/Mic groups ($P < 0.0001$). (#) indicates statistically significant difference in relation to the saline, B/Mic, miltefosine and CMt groups ($P < 0.001$).

DISCUSSION

Treatment against leishmaniasis is currently hampered by drugs' toxicity, their high cost and/or the emergence of parasite resistance.^{1,5} In this scenario, an urgent need to identify novel antileishmanial agents has led to an alternative line search focused on the study of plant-extracted molecules, such as flavonoids.^{18,43-45} Thus, in the present study, the antileishmanial activity of a non-described diprenylated flavonoid known as CMt, obtained from *M. tinctoria* leaves extracts, was evaluated against *Leishmania* species capable of causing TL and VL worldwide. Results showed that the flavonoid presented an effective antileishmanial action in vitro, low toxicity to murine cells and, most importantly, the ability to control *L. infantum* infection in vivo. Miltefosine was used as a well-characterized control drug.^{46,47} All in all, results suggested that CMt displayed similar immunological and parasitological effects to those

obtained when miltefosine was used, mainly when the flavonoid was incorporated into micelles as its delivery system.

Initially, antileishmanial action of CMt against *L. amazonensis* and *L. infantum* species was analysed in vitro. Results showed that the flavonoid presented higher selectivity index than AmpB, a well-known and widely used antileishmanial agent,^{5,6} against both promastigotes and amastigotes stages of the parasite. CMt treatment was also effective in reducing the parasite burden of infected macrophages, as well as in inhibiting infection of phagocytic cells when parasites pre-treated with the molecule were used to infect. AmpB was similarly efficient in killing parasites in vitro. However, its higher toxicity to murine macrophages and red blood cells suggested CMt as a better alternative for treatment with diminished adverse effects to mammalian hosts.^{36,39,45}

A mechanism of action for the flavonoid was evaluated in *L. infantum*. Results showed that CMt altered the parasite mitochondrial membrane integrity and potential, as well as stimulated parasitic ROS production. These observations imply that CMt is potentially leading to parasite death by disturbing *Leishmania* mitochondria stability. Future studies, however, will need to analyse further the mechanism of action of CMt on intra-macrophage or axenic amastigotes, and use ROS-chelating substances to evaluate ROS contribution to the effectiveness of CMt.

Regardless, other studies investigating plant-extracted flavonoids as antileishmanial agents have shown also that these molecules function in the parasite mitochondria.^{17,48} For instance, a study on the in vitro leishmanicidal activity of strychnobiflavone, a flavonoid isolated from *Strychnos pseudoquina* St. Hil., showed its effective action against *L. infantum* promastigotes and amastigotes, as well as alterations in the parasite mitochondrial membrane potential when treated with this compound.⁴⁸ The authors therefore suggested that strychnobiflavone could be considered as an antileishmanial agent which exerts its action in the *Leishmania* mitochondria. On the whole, this and other studies acknowledge the fact that the parasite mitochondria seems to be a preferred target of purified antileishmanial agents.

The immune response developed after treatment of infected mice was evaluated by means of dosage of Th1- or Th2-type cytokines levels, which can be usually correlated to the outcome of leishmanial infection. In general, IFN- γ plays a role in the pathogen control, since it activates infected macrophages to kill internalized parasites.⁴⁹ On the other hand, anti-inflammatory cytokines, such as IL-4 and IL-10, are associated with deactivation of macrophages, maintenance of parasite integrity and consequential disease progression.⁵⁰ In our study, miltefosine, CMt and CMt/Mic-treated mice produced significantly higher levels of IFN-

γ , IL-12 and GM-CSF, with corresponding low IL-4 and IL-10 levels, both one and 15-day post-treatment, suggesting induction of specific antileishmanial Th1-type response. This observation was also reported elsewhere. For instance, antileishmanial activity of a flavonoid extracted from *Bergenia ligulata* (Wall.) Engl. was analysed, and the immune response of *L. donovani*-infected mice was evaluated after treatment.

Results showed that the flavonoid reduced in significant levels the parasite load in the treated animals, as well as increased IFN- γ and IL-12 production, which was associated with low levels of IL-4 and IL-10. In addition, a predominance of antileishmanial IgG2a isotype antibody levels, when compared to IgG1 levels, and an increased delayed-type hypersensitivity (DTH) response were also observed after treatment.⁵¹ In this context, development of a specific Th1-type response by *Leishmania*-infected mammalian hosts seems to be relevant for the effective control of the disease.

Distinct delivery systems, such as liposomes, nanoemulsions, polymeric micelles, among others, have been used extensively in the field of drug development, since they can increase drug efficacy and thus allow lower drug concentration in their formulations.⁵²⁻⁵⁵ In a similar fashion, the use of polymeric micelles for delivery of antileishmanial agents has led also to increased drug efficacy against *Leishmania*.^{56,57} Espuelas et al demonstrated that an AmpB-incorporated Poloxamer-based micelle system activated infected macrophages to kill *L. donovani* parasites. The authors suggested a synergic action between AmpB and the micelles, what might facilitate interaction with the parasite membrane and insertion of AmpB.⁵⁸ In a more recent study, Singh et al demonstrated that AmpB-loaded Pluronic micelles were also effective for the treatment of *L. donovani*-infected hamsters. In that study, the authors showed that the formulation effectively stimulated the development of an antileishmanial Th1 immune response and significantly reduced the parasite load in infected and treated animals, without causing toxicity to the host.⁵⁹ In the present work, the CMt-containing micelle system had similar therapeutic efficacy in the treatment of *L. infantum*-infected mice, judged by significant reductions in the parasite load in spleen, liver, BM and dLN, analysed by both limiting dilution technique and qPCR assay.

It has been described previously that, in the early stages of murine infection with *L. infantum*, parasites multiply in large numbers in the liver and, once the infection becomes chronic, the hepatic parasite burden tends to decrease, while the splenic parasitism tends to increase.^{60,61} In this light, presence of parasites in the liver is a marker of early infection, while parasitic load in the spleen is a marker of chronic disease.^{62,63} In the present study, splenic parasitism was quantified by qPCR assay and corroborated by limiting dilution technique.

Future studies will need to address a similar analysis of parasitism in the liver, BM and dLN. Despite the lack of these analyses in the current study, the therapeutic efficacy observed for CMt, one and 15-day post-treatment, strongly suggests the feasibility to test further the CMt/Mic formulation as a candidate chemotherapeutic agent against VL.

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CONFLICT OF INTEREST

The authors hereby declare that there is no conflict of interest.

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5.1.2. Considerações finais

O CMt apresentou atividade antileishmanial *in vitro* contra duas importantes espécies de *Leishmania* com baixa toxicidade em células murinas, além de provocar distúrbios na estabilidade mitocondrial de *L. infantum* ao alterar a integridade e o potencial de membrana mitocondrial, e estimular a produção de ROS. O derivado flavônico mostrou-se também eficaz

na redução da carga parasitária em macrófagos murinos, bem como na inibição da infecção nessas células utilizando parasitos previamente tratados com a molécula.

Nos ensaios *in vivo*, em camundongos BALB/c infectados com *L. infantum*, CMt livre, mas em especial sua formulação micelar (CMt/Mic), apresentaram expressiva ação antileishmanial associada ao desenvolvimento de uma resposta imune celular e humoral do tipo Th1 com altos níveis de IFN- γ , nitrito, IL-12, GM-CSF e IgG2a, e à redução significativa do parasitismo tecidual. Sendo assim, o presente trabalho sugere que especialmente CMt/Mic seja considerada, em estudos futuros, como uma estratégia terapêutica para as leishmanioses.

5.2. CAPÍTULO 2 – 3s e 3t na LV experimental

Neste capítulo são apresentados os resultados e a discussão sobre a ação antileishmanial das moléculas 3s e 3t, em sua forma livre e incorporados em micelas poliméricas, na infecção experimental por *L. infantum* em camundongos BALB/c.

5.2.1. Artigo

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TITLE

New synthetic molecules incorporated into polymeric micelles used for treatment against visceral leishmaniasis.

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ABSTRACT

Treatment against visceral leishmaniasis (VL) presents problems, mainly related to drug toxicity, high cost and/or by emergence of resistant strains. In the present study, two vanillin synthetic derivatives, **3s** [4-(2-hydroxy-3-(4-octyl-1H-1,2,3-triazol-1-yl) propoxy)-3-methoxybenzaldehyde] and **3t** [4-(3-(4-decyl-1H-1,2,3-triazol-1-yl)-2-hydroxypropoxy)-3-methoxybenzaldehyde], were evaluated as therapeutic candidates in a murine model against *Leishmania infantum* infection. Molecules were used pure (**3s** and **3t**) or incorporated into Poloxamer 407-based micelles (**3s/M** and **3t/M**) in the infected animals, which also received amphotericin B (AmpB) or Ambisome® as control. Results showed that **3s/M** and **3t/M** compositions induced a Th1-type immune response in treated animals, with higher levels of IFN- γ , IL-2, TNF- α , IL-12, nitrite, and IgG2a antibodies. Animals presented also low toxicity and significant reductions in the parasite load in their spleens, livers, bone marrows and draining lymph nodes, as compared as control groups mice, with the evaluations performed one and 30 days after the application of the therapeutics. In conclusion, preliminary data suggest that **3s/M** and **3t/M** could be considered for future studies as therapeutic agents against VL.

Keywords: Treatment, visceral leishmaniasis, synthetic molecules, vanillin, toxicity, polymeric micelles.

INTRODUCTION

Leishmaniasis are tropical diseases endemic in 98 countries, with 12 million people clinically affected.⁵⁵ Visceral leishmaniasis (VL) is the more severe clinic form of this disease complex, and it has a 95% fatality rate in acute and untreated cases, with an estimated incidence of about 0.5 million new cases annually.⁴ The causative agents of the VL are the *Leishmania donovani* and *L. infantum* species.²⁹ *L. donovani* is found in East-Africa, India and parts of the Middle East. *L. infantum* is the main etiological agent of VL in the New World, causing the disease in populations from South America, Mediterranean

basin, and West and Central Asia.⁵⁵ This parasite species is also responsible by cases of canine leishmaniasis in such regions, and it can be found in cats, rabbits, hares, and wild rodents³⁴, demonstrating its importance and the need of adoption of effective control measures against disease.

Treatment against disease is based on use of pentavalent antimonials, amphotericin B (AmpB) deoxycholate and their lipid-based formulations, pentamidine, miltefosine, amongst others.⁵³ These products are administered intramuscularly causing local pain, present high cost, and induce toxicity in the patients. Additionally, the increase of parasite resistant strains has been also registered, demonstrating the necessity to identify new antileishmanial agents.^{37,22,50,54} In this context, the search to identify new compounds, either of natural or synthetic origin should be recommended, and natural products have emerged with such biological purposes.^{16,1}

Vanillin is a natural organoleptic compound derived from vanilla, which has been tested in biological applications, such as anticancer⁴⁵, antibacterial³³, antidiabetics⁴⁶ and antileishmanial¹⁵ activity. In a recent study, two vanillin synthetic derivatives, called “3s” [4-(2-hydroxy-3-(4-octyl-1H-1,2,3-triazol-1-yl)propoxy)-3-methoxybenzaldehyde] and “3t” [4-(3-(4-decyl-1H-1,2,3-triazol-1-yl)-2-hydroxypropoxy)-3-methoxybenzaldehyde], were evaluated regarding their in vitro antileishmanial activity against *L. infantum*, *L. amazonensis* and *L. braziliensis* species.¹⁵ Results showed selectivity index (SI) of 25.1, 18.2 and 22.9, respectively, when 3s was used against promastigotes, and of 45.2, 7.5 and 15.0, respectively, against amastigotes. 3t showed SI of 45.2, 53.0 and 80.0, respectively, against promastigotes, and of 35.9, 46.0 and 58.4, respectively, against amastigotes. Otherwise, AmpB presented SI

of 5.0, 7.5 and 15.0, respectively, against promastigotes, and of 3.8, 5.0 and 7.5, respectively, against amastigotes.¹⁵ The authors concluded their study suggesting a promising antileishmanial action of these vanillin synthetic derivatives against distinct parasite species.¹⁵

In this context, in the present study, the *in vivo* antileishmanial activity of 3s and 3t was evaluated against *L. infantum* infection in a murine model. BALB/c mice were infected with parasite stationary promastigotes and, 60 days post-infection, they were treated with 3s or 3t, which were used pure or incorporated into Poloxamer P407-based micelles, used with delivery and adjuvant purposes.^{52,51,32} As drug control, AmpB and Ambisome[®] were also used to treat infected animals. Immunological, parasitological and biochemical evaluations were performed one and 30 days after therapeutics, by employing techniques, such as RT-qPCR, qPCR, capture and indirect ELISA, Griess reaction, and limiting dilution assay, among others; in order to evaluate the possible therapeutic efficacy of the compositions.

MATERIALS AND METHODS

Compounds and preparation of 3s and 3t-containing micelles

3s and 3t compounds were prepared according described.¹⁵ The compositions were prepared using 15 mg of Poloxamer 407 (18% w/w; Pluronic[®] F127, Sigma-Aldrich, USA), which was diluted in sterile phosphate-buffered saline (PBS 1x) at pH 7.4 under magnetic stirring for 18 h at 4°C. Ten milligrams of molecules were added to 500 µL of PA dichloromethane and solubilized by vortexing. The mixture was added to the previously prepared solution under vigorous stirring and in na ‘ice bath, until a viscous emulsion was obtained. The dichloromethane solvent was evaporated using a rotary evaporator (Büchi[®], Switzerland), and the formulations were obtained with transparent yellow color, at room temperature. Empty micelles were prepared according to the aforementioned protocol, but without the incorporation of the molecules. All preparations were filtered through a 0.45 µm microfiltration membrane and stored at 4°C.⁵²

Characterization of 3s- and 3t-containing micelles

A physical-chemical characterization of 3s/M and 3t/M compositions was performed, with dynamic light scattering (DLS) being employed to measure the average particle size and zeta potential using the Zetasizer Nano ZSP system (Malvern Instruments, UK). The micelle morphology was evaluated by transmission electron microscopy (TEM; Tecnai G20, FEI

Company, USA) and the stability of the preparation was evaluated by measuring the particle size and zeta potential, during seven days.²⁷

Mice and parasites

BALB/c mice (female, 8 weeks of age) were maintained under specific pathogen-free conditions and received ad libitum water. The study was approved by Committee on the Ethical Handling of Research Animals of Federal University of Minas Gerais (UFMG; Belo Horizonte, Minas Gerais, Brazil), with protocol number 056/2022. *L. infantum* (MHOM/BR/1970/BH46) promastigotes were cultured at 24°C in complete Schneider's medium (Sigma-Aldrich, USA), which was composed by medium added with 20% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich, USA), 20 mM L-glutamine, 200 U/mL penicillin, and 100 µg/mL streptomycin, at pH 7.4. Soluble *L. infantum* antigenic extract (SLA) was prepared as described elsewhere.²⁷

Infection and therapeutics regimens

Mice (n=16 per group) were infected subcutaneously with 10^7 *L. infantum* stationary-phase promastigotes and, 60 days after infection, they were grouped and treated with one of the following schedules: (i) 50 µL of PBS (saline group); (ii) 50 µL of empty micelles (5 mg/kg body weight; B/Mic group); (iii) 50 µL of AmpB (1 mg/kg of weight; AmpB group); (iv) 8 µL of AmBisome® (1 mg/kg of weight; AmBisome® group); (v) 50 µL of 3s (5 mg/kg of weight; 3s group); (vi) 50 µL of 3s/Mic (2.5 mg of 3s per kg; 3s/M group); (vii) 50 µL of 3t (5 mg/kg of weight; 3t group); and (viii) 50 µL of 3t/Mic (2.5 mg of 3t per kg; 3t/M group). Except for AmpB, which was administered by intraperitoneal route and AmBisome® that was applied by intravenous route, the others were subcutaneously administered four times every five days. Animals were euthanized one and 30 days after treatments (n=8 per group, each), when organs and blood samples were collected to perform biochemical, immunological and parasitological analyses.

Organ toxicity

Hepatic [alanine transaminase (ALT) and aspartate transaminase (AST)] and renal [urea and creatinine] damage markers were measured in sera samples obtained from treated animals, one and 30 days after therapeutics (n=8 per group, each). Sera of uninfected and untreated mice (n=5) were used as control. Laboratorial analyzes were performed using commercial kits (Labtest Diagnostica®, Belo Horizonte), following manufacturer instruction.

Antibody production

Humoral response was evaluated in sera samples of animals, one and 30 days after therapeutics (n=8 per group, each). For this, anti-SLA IgG1 and IgG2a isotypes antibodies were measured by indirect ELISA according described (Lage et al., 2023). Briefly, microtiter plates (Jetbiofil®, Belo Horizonte) were coated with SLA (1.0 µg per well), which was diluted in 50 mM carbonate buffer pH 9.6, for 18 h at 4°C. Free binding sites were blocked with PBS-T/BSA [PBS 1x, Tween 20 0.05% and 5% (w/v) bovine serum albumin] for 1 h at 37°C. Plates were washed in PBS-T and incubated with individual sera (1:100 diluted in PBS-T) for 1 h at 37°C. Plates were again washed in PBS-T, and incubated with anti-mouse IgG1 (anti-mouse IgG1 secondary antibody, peroxidase-conjugated antibody produced in rat, catalog SA1- 35640, Invitrogen, USA) or IgG2a (anti-mouse IgG2a secondary antibody, peroxidase- conjugated antibody produced in rat, catalog SA1-35646, Invitrogen, USA) antibodies. A new incubation was performed during 1 h at 37°C. Plates were washed with PBS-T, and reactions were developed using H₂O₂, ortho-phenylenediamine and citrate-phosphate buffer pH 5.0, for 30 min and in the dark. Reactions were stopped by adding 2N H₂SO₄ and optical density (OD) values were read in a spectrophotometer (Molecular Devices, Spectra Max Plus, Concord, Canada), at 492 nm.

Cytokine response

Mice were euthanized one and 30 days after therapeutics (n=8 per group, each), when their spleens were collected to culture splenocytes, which were plated in complete RPMI 1640 medium in duplicate in 24-well plates (Nunc). Cells (5 x 10⁶ per well) were unstimulated (medium, control) or stimulated with SLA (25 µg/mL), for 48 h at 37°C in 5% CO₂.²⁵ Culture supernatant was collected and used to measure levels of IFN-γ, IL-4, IL-10, and IL-12, by using commercial kits (catalogs 555138, 555232, 555252, and 555256, respectively; OptEIA™ set mouse Kits; BD Pharmingen, USA), following manufacturer instruction.

Nitrite secretion

Culture supernatant used to qualify cytokines was also used to measure the nitrite secretion through Griess reaction.⁵² For this, 50 µL of culture supernatant were mixed with an equal volume of Griess reagent, and the nitrite concentration was estimated using a standard curve with absorbances measured at 540 nm.

Flow cytometry

Immunostaining for cell surface markers and intracellular cytokine assay were performed as described.³ Briefly, splenocytes were incubated in the presence of 200 μ L of complete RPMI medium in 96-well round-bottom culture plates, at a concentration of 5×10^5 cells per well. Cells were unstimulated (medium, control) or stimulated with SLA (20 μ g/mL), when na incubated for 48 h at 37°C in 5% CO₂ was performed. Brefeldin A (Sigma-Aldrich, USA) (10 mg/mL) was added in the cultures, 4 h before the end of the period of incubation. Some wells were also stimulated with Phorbol 12-myristate 13-acetate (PMA; 5 ng/mL) and ionomycin (1 mg/mL), which were used as positive control. Afterwards, cells were stained by Fixable Viability Stain 450 (FVS450, BD Biosciences) for 15 minutes at room temperature, followed by labeling with antibodies against CD3⁺ (BV650 anti-mouse, clone 145.2C11), CD4⁺ (BV605 anti-mouse, clone RM4-5), and CD8⁺ (BV786 anti-mouse, clone 53-6.7) at room temperature for 30 min. Cells were fixed with FACS fixing solution, washed and permeabilized with PBS buffer plus 0.5% saponin and stained with antibodies against IL-2 (PE anti-mouse, clone JES6-5H4), IFN- γ (AF700 anti-mouse, clone XMG1.2), TNF- α (PE-Cy7 anti-mouse, clone LG.3A10), and IL-10 (APC anti-mouse, clone JES5-16E) at room temperature for 30 min. All antibodies were purchased from BD Biosciences, USA.

Cells were acquired (100,000 events) on LSR Fortessa cytometer (BD Biosciences, USA) using FACSDiva software. For analysis in FlowJo software, dead cells were excluded after FVS450 stain and alive cells were gated for stained CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells and intracellular cytokine production. Data were expressed as indexes, which were calculated by dividing the percentage of positive cells found in the stimulated cultures versus the unstimulated cultures.

Real-Time-qPCR (RT-qPCR)

IFN- γ expression in the SLA-stimulated cell cultures was evaluated using animals' spleens, one and 30 days after therapeutics (n=8 per group, each) according described.²⁸ For this, RNA content was extracted from stimulated cell cultures by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and resuspended in UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, Carlsbad, CA, USA). RNA concentration was estimated in a NanoDrop LITE (Thermo Scientific, Waltham, MA, USA), with absorbance ratios of 260/280 nm. RT-qPCR assay was performed using the Applied Biosystems PowerUp™ SYBR™ Green PCR master mix (Thermo Fisher, USA), and the following gene-specific primers for IFN- γ were

used: 5'-TCAAGTGGCATAGATGTGGAAGAA-3' (Forward) and 5'-TGGCTCTGCAGGATTTTCATG-3' (Reverse) in a 7900HT Fast Real-Time PCR System (Applied Biosystems, USA). Results were shown graphically as fold changes in gene expression by using the mean $\pm$ standard deviation of target gene.¹⁸ Data were analyzed according to the relative expression using the $2^{-\Delta\Delta CT}$ method and are representative of two independent experiments.

Limiting dilution assay

The parasite load was evaluated in the livers, spleens, bone marrows (BMs), and draining lymph nodes (dLNs) from treated animals, one and 30 days after therapeutics (n=8 per group, each), according described.⁵¹ Briefly, organs were collected, macerated in a glass tissue grinder using sterile PBS, and tissue debris was removed by centrifugation at $150 \times g$. Cells were concentrated by centrifugation at $2,000 \times g$. Cells were resuspended in 1 mL of complete Schneider's medium and serially diluted 10^{-1} to 10^{-12} in the same medium. Each sample was plated in triplicate and cultured at 24°C for 7 days. Results were expressed as the negative log of the titer (the dilution corresponding to the last positive well) adjusted per milligram of organ.

Quantitative polymerase chain reaction (qPCR)

Parasitism was also evaluated by qPCR in the spleens of infected and treated mice (n=8 per group), which were collected 30 days after therapeutics.²⁸ Briefly, DNA content was extracted using the Wizard Genomic DNA Purification Kit (Promega Corporation), and suspended with UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, Carlsbad, CA, USA). Parasite load was estimated using primers to amplify *L. infantum* kDNA: 5'-CCTATTTTACACCAACCCCCAGT-3' and 5'-GGGTAGGGGCGTTCTGCGAAA-3' (Forward and Reverse, respectively). These primers are used to amplify a specific and conserved region of the *Leishmania* parasite, which is used as an indicative of the presence of parasite DNA in the organs evaluated. In addition, primers (Forward 5'-CAGAGCAAGAGAGGTATCC-3' and Reverse 5'-TCATTGTAGAAGGTGTGGTGC-3') to amplify the mouse β -actin gene were used as endogenous control.

Reactions were processed in 7500 HT (96-wells plate; Applied Biosystems), using the Applied Biosystems PowerUp™ SYBR™ Green PCR master mix (5 μ L; Thermo Fisher, Waltham, MA, USA), added with 2 mM of each primer (1 μ L) and 4 μ L of DNA (25 ng/ μ L). Samples were incubated at 95°C for 10 min and submitted to 40 cycles of 95°C for 15 s and

60°C for 1 min. At each time point, fluorescence data were collected and results were calculated by interpolation from a standard curve included in the same run, which was prepared in duplicate and expressed as the number of *L. infantum* organisms per total DNA.²⁸

Statistical analysis

Results were entered into Microsoft Excel (version 10.0) spreadsheets and analyzed using GraphPad Prism™ (version 10.0.2 for Windows). Two independent experiments, which showed similar results, were performed. The data shown are representative of one of the experiments. Data were expressed as mean $\pm$ standard deviation of the groups. In the toxicity assay, a paired t-test was used to compare differences between the time-points. For the other assays, the one-way analysis of variance (ANOVA) followed by Bonferroni's post-test were used for comparisons. Differences were considered significant with $P < 0.05$.

RESULTS

Characterization of the 3s and 3t-containing micelles

3s/M and 3t/M compositions showed mean particle size of 90.34 ± 6.45 and 91.72 ± 5.98 nm, respectively, and narrow distribution of 0.203 ± 0.035 and 0.215 ± 0.027 by DLS, respectively. Micelles presented negative zeta potential of -8.96 ± 1.25 and -9.05 ± 1.13 mV, respectively, and morphology assay with homogeneous spherical shapes and smooth surfaces, making possible to suggest that they can be taken by host phagocytes. The zeta potential evaluation showed that 3s/M and 3t/M were stable by seven days tested. The polydispersity index was 0.14 and 0.17, respectively, after examining the size distribution by DLS.

Evaluation of organic toxicity after treatment from infected animals

We evaluated the organ toxicity caused by infection and therapeutics used, and results showed that mice receiving saline, AmpB and empty micelles presented higher levels of urea, creatinine, ALT and AST, when compared to the other groups, at one (**Figure 1A**) and 30 (**Figure 1B**) days after therapeutics. Otherwise, animals receiving 3s/M or 3t/M presented lower levels of these enzymatic markers, suggesting that these compositions were less toxic for the animals, as well as reduced the toxicity caused by own *Leishmania* infection.

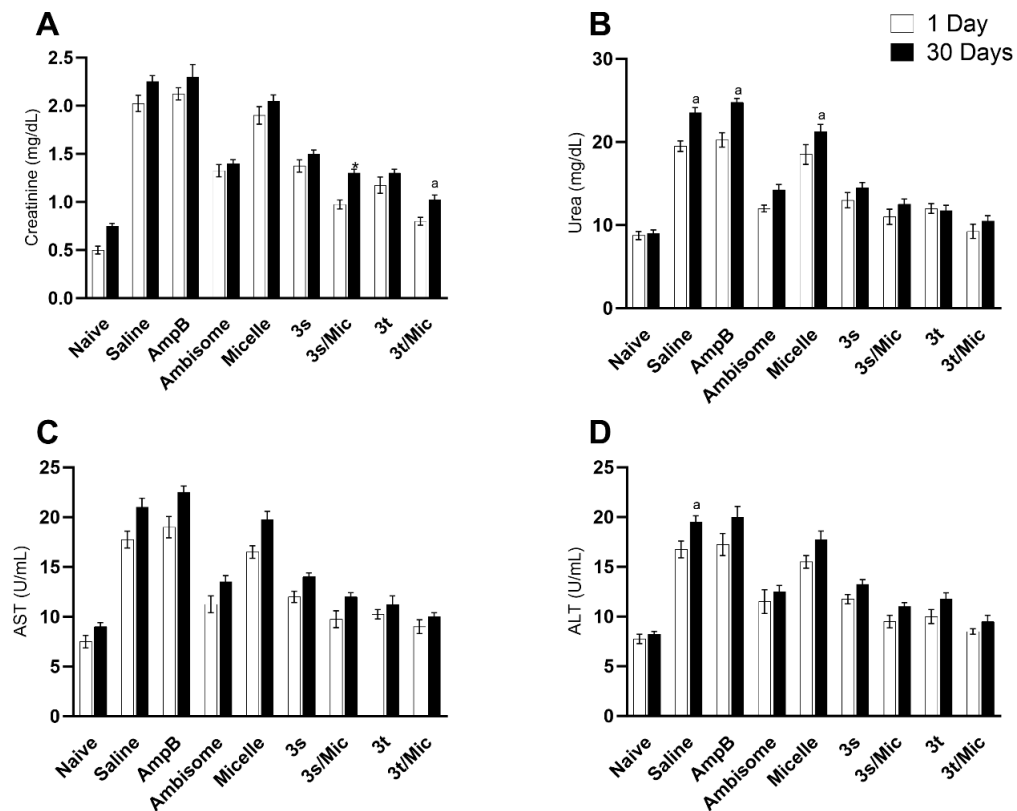


Figure 1. Evaluation of the *in vivo* toxicity after treatments.

BALB/c mice ($n = 16$ per group) were infected with *L. infantum* promastigotes and, 60 days after, they received saline, amphotericin B (AmpB), micelles, Ambisome®, 3 s, 3 t, 3 s/M or 3 t/M. One and 30 days after therapeutics, sera samples were collected and levels of urea, creatinine, alanine aminotransferase and aspartate aminotransferase were evaluated in each time ($n = 8$ mice per group) using commercial kits. Sera samples obtained from non-infected and non-treated mice (naive) were used as control. Results obtained one (A) and 30 (B) days after the therapeutics are shown as bars, which indicate the mean $\pm$ standard deviation of the groups. “a” indicates a significant difference concerning data obtained with evaluations performed one day after therapeutics ($P < 0.05$).

Immunological response developed in the infected and treated animals

The immune response was evaluated in the treated and infected animals. Results obtained one day after therapeutics showed that, when animals received 3s/M or 3t/M, their spleen cell cultures produced significantly higher levels of anti-SLA IgG2a antibodies, as compared to the other groups. On the other hand, control groups produced higher levels of antileishmanial IgG1 antibody (**Figure 2A**). Ratios between IgG2a and IgG1 levels were then calculated and results are shown (**Figure 2B**), where is possible to observe that mice receiving 3s/M or 3t/M presented higher IgG2a and lower IgG1 levels, as compared to the others, suggesting the development of a more polarized Th1-type humoral response in these groups.

Cellular response was also evaluated in SLA- stimulated cell cultures, and results showed that mice treated with 3s/M or 3t/M presented significantly higher levels of IFN- γ and IL-12, which were associated with significantly lower production of IL-4 and IL-10, as compared to the others. On the other hand, mice receiving saline, AmpB or empty micelles produced significantly higher levels of antileishmanial IL-4 and IL-10 (**Figure 2B**).

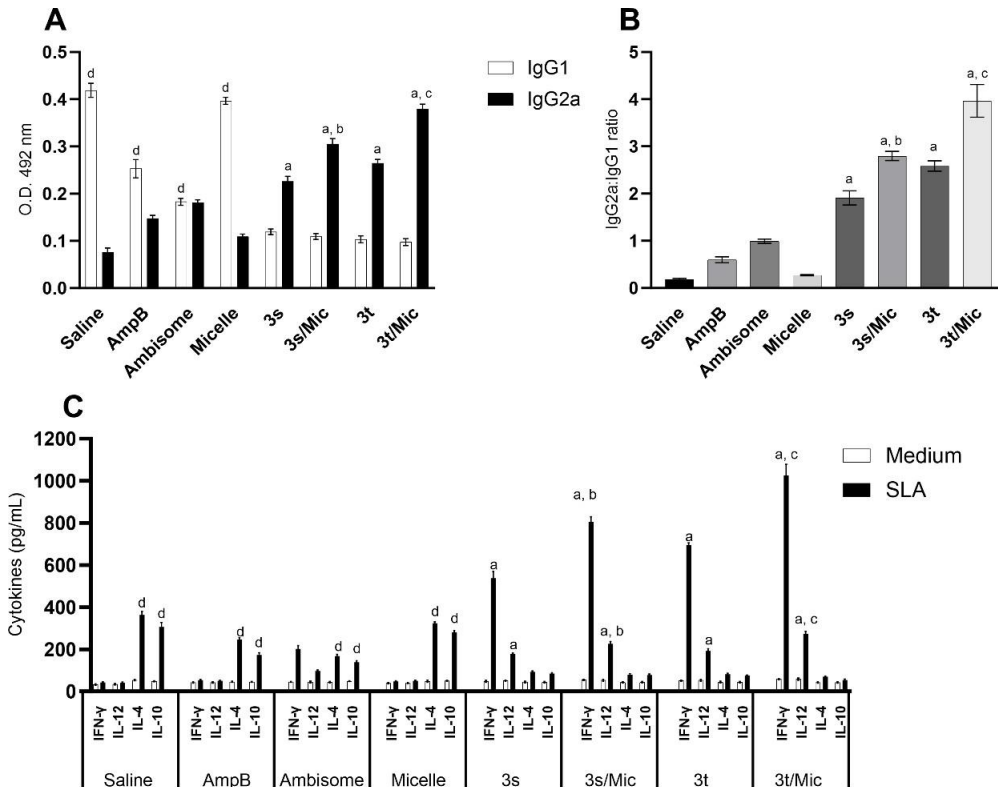


Figure 2. Immune response generated one day after therapeutics.

Mice ($n = 8$ per group) were infected with *L. infantum* promastigotes and, 60 days after they received saline, amphotericin B (AmpB), micelles, Ambisome[®], 3 s, 3 t, 3 s/M or 3 t/M. One day after therapeutics, animals were euthanized and their sera samples were collected to evaluate the therapeutics of anti-SLA IgG1 and IgG2a antibodies through an indirect ELISA. Results of optical density (OD) values are shown (A), as well as the ratios between IgG2a/IgG1 levels (B). In addition, mice spleens were collected and splenocytes (5×10^6 per well) were cultured in complete RPMI 1640 medium, at which time cells were non-stimulated (medium, control) or stimulated with SLA (25 μ g/mL) for 48 h at 37 °C in 5 % CO₂. The culture supernatant was collected and levels of IFN- γ , IL-12, IL-4 and IL-10 were measured using commercial kits. Results are also shown (C). In all cases, bars indicate the mean $\pm$ standard deviation of the groups. “a” indicates significant difference in relation to the saline, amphotericin B (AmpB), Ambisome, and micelle groups ($P < 0.0001$). “b” indicates significant difference in relation to the 3 s group ($P < 0.001$). “c” indicates significant difference in relation to the 3 t group ($P < 0.001$). “d” indicates significant difference in relation to the 3 s, 3 s/Mic, 3 t, and 3 t/Mic groups ($P < 0.0001$).

The immune response was also evaluated 30 days after therapeutics, and results corroborated with those described one day after therapy. In this context, mice receiving 3s/M or 3t/M produced significantly higher levels of anti-SLA IgG2a antibody, as compared to the other groups (**Figure 3A**), which reflected in higher IgG2a/IgG1 ratios (**Figure 3B**), signaling the maintenance of Th1-type humoral response. The cytokine profile was also maintained, since animals receiving 3s/M or 3t/M produced significantly higher levels of IFN- γ and IL-12, with correspondent lower production of IL-4 and IL-10, as compared to the others (**Figure 3B**). Otherwise, mice receiving saline, AmpB and empty micelles produced higher levels of IL-4 and IL-10 cytokines. Slightly higher IFN- γ and IL-12 and lower IL-4 and IL-10 production was observed at 30 days after therapeutics, as compared to values obtained one day after therapy, suggesting the long-lasting response of the antileishmanial agents in the treated animals.

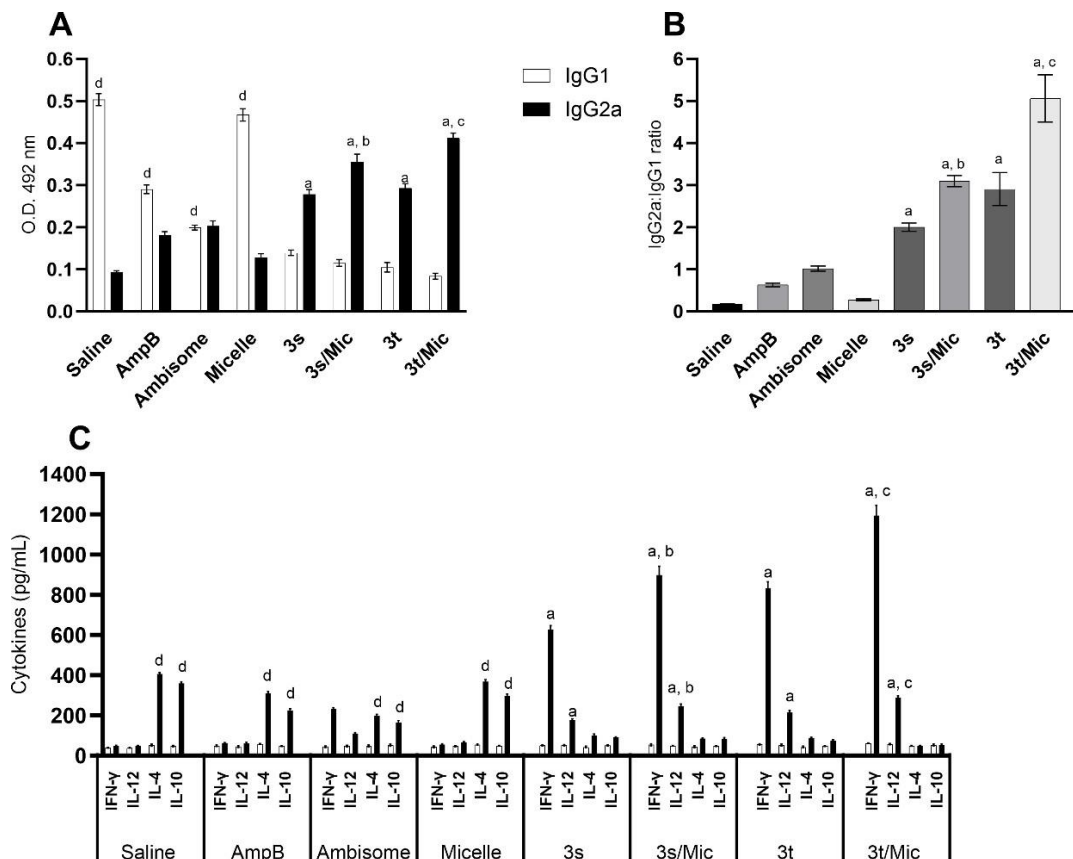


Figure 3. Antibody and cytokine production developed 30 days after therapeutics.

Mice (n = 8 per group) were infected and 60 days after they received saline, amphotericin B (AmpB), micelles, Ambisome®, 3 s, 3 t, 3 s/M or 3 t/M. Thirty days after therapeutics, they were euthanized and their sera samples were collected to evaluate the presence of anti-SLA IgG1 and IgG2a antibodies through an indirect ELISA. Results

of optical density (OD) values are shown (A), as well as the ratios between IgG2a/IgG1 levels (B). In addition, mice splenocytes were cultured (5×10^6 cells per well) in complete RPMI 1640 medium, at which time cells were non-stimulated (medium, control) or stimulated with SLA (25 $\mu\text{g/mL}$) for 48 h at 37 °C in 5 % CO_2 . The culture supernatant was collected and levels of IFN- γ , IL-12, IL-4 and IL-10 were measured. Results are shown (C). In all cases, bars indicate the mean $\pm$ standard deviation of the groups. “a” indicates significant difference in relation to the saline, amphotericin B (AmpB), Ambisome, and micelle groups ($P < 0.0001$). “b” indicates significant difference in relation to the 3 s group ($P < 0.001$). “c” indicates significant difference in relation to the 3 t group ($P < 0.001$). “d” indicates significant difference in relation to the 3 s, 3 s/Mic, 3 t, and 3 t/Mic groups ($P < 0.0001$).

The nitrite production was measured in the culture supernatants, and results showed significantly higher production of this antileishmanial molecule in mice receiving 3s/M or 3t/M, as compared to the other groups, in both one (**Figure 4A**) and 30 (**Figure 4B**) days after therapeutics.

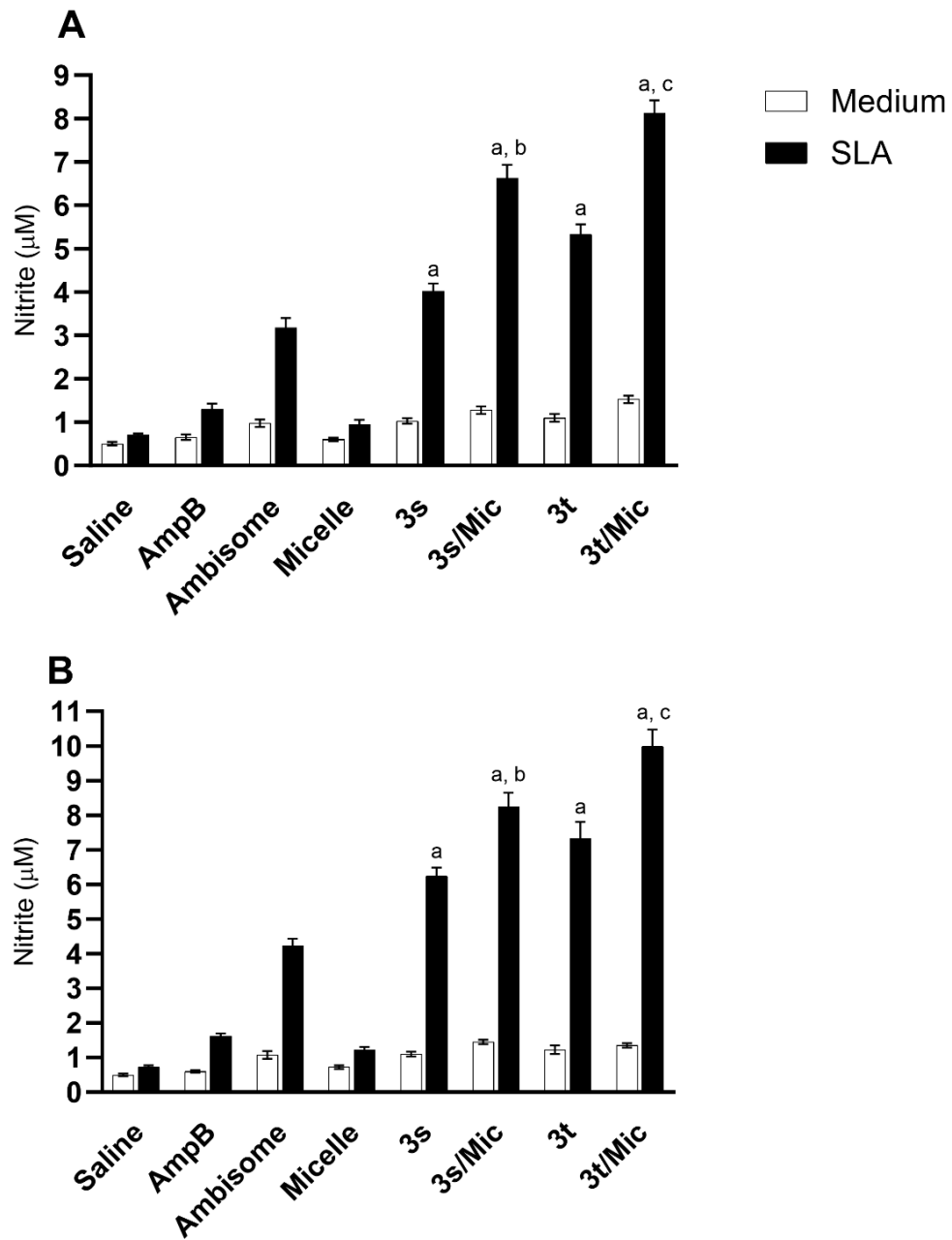


Figure 4. Nitrite production one and 30 days after therapies.

Culture supernatant used to quantify cytokines was also employed to evaluate the nitrite presence through Griess reaction. Results obtained one (A) and 30 (B) days after the therapeutics are shown. Bars indicate the mean $\pm$ standard deviation of the groups. “a” indicates significant difference in relation to the saline, amphotericin B (AmpB), Ambisome, and micelle groups ($P < 0.0001$). “b” indicates significant difference in relation to the 3 s group ($P < 0.001$). “c” indicates significant difference in relation to the 3 t group ($P < 0.001$).

A flow cytometry assay performed with SLA-stimulated cell cultures, which were collected from animals 30 days after therapeutics, showed that mice receiving 3s/M or 3t/M presented higher percentage of both IFN- γ and TNF- α -producing T cells, when compared to values obtained in the controls, which showed higher percentage of IL-10-producing T cells (**Figure 5**). IFN- γ expression in SLA-stimulated spleen cell cultures showed also that mice treated with 3s/Mic or 3t/Mic presented higher IFN- γ mRNA expression, as compared to the other groups, at both one (**Figure 6A**) and 30 (**Figure 6B**) days after therapeutics. Higher IFN- γ expression was also found in mice receiving 3s, 3t or Ambisome[®], when compared to saline, AmpB and empty micelle groups, although the values have being lower in comparison to those from 3s/M and 3t/M groups.

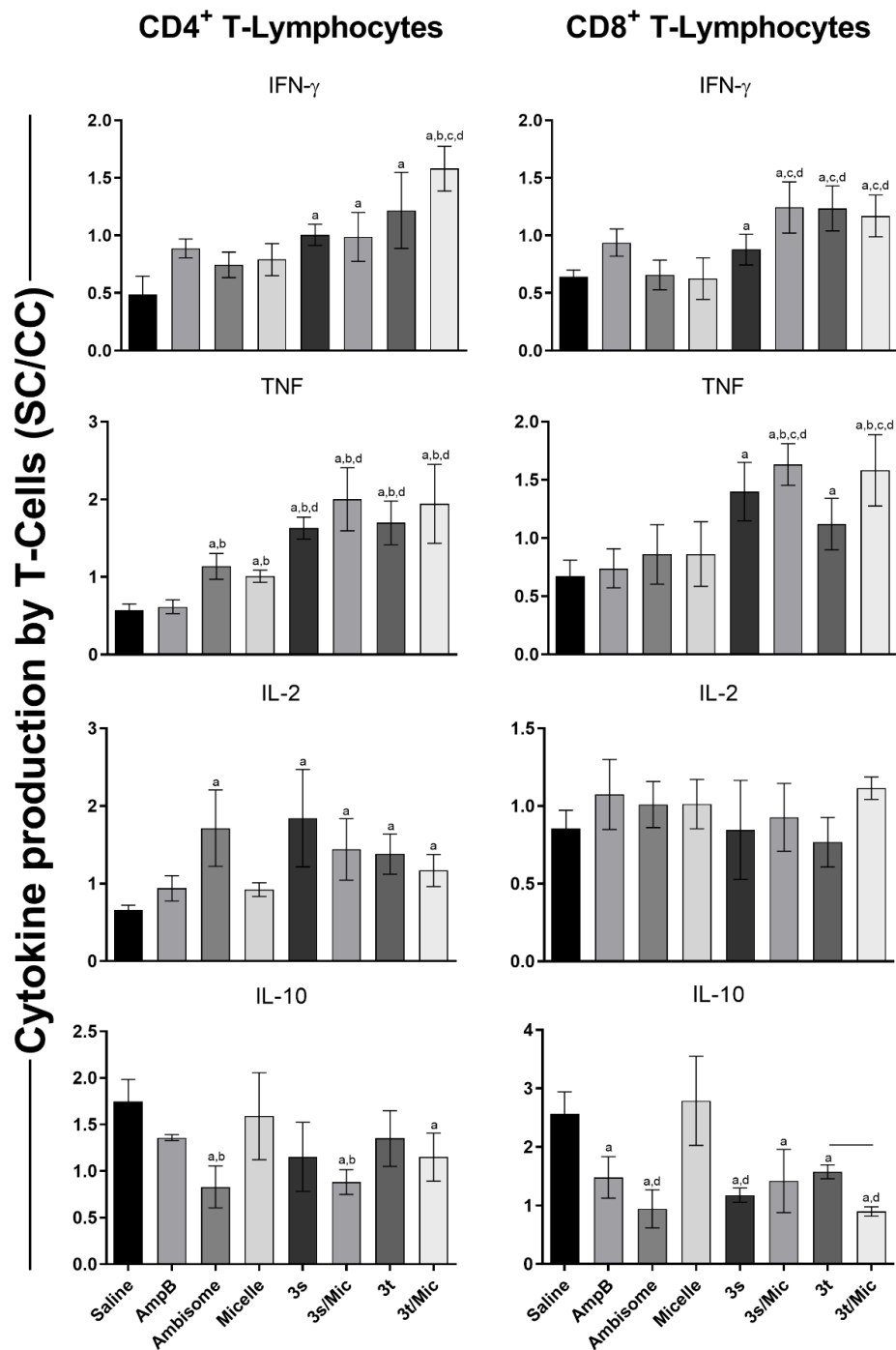


Figure 5. Profile of intracytoplasmic cytokines produced by splenocytes after *in vitro* stimulation with soluble *Leishmania* antigen (SLA).

Plots represent the cytokines IFN- γ , TNF- α , IL-2, and IL-10 produced by CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells. Results are expressed by the ratio of cytokine produced by SLA-stimulated (SC) cultures divided by the control culture (CC). Bars indicate the mean $\pm$ standard deviation of the groups. “a”, “b”, “c”, and “d” indicate significant difference in relation to the saline, amphotericin B (AmpB), Ambisome®, and micelle groups, respectively ($P < 0.05$). The connecting line indicates significant difference in relation to the 3 t/Mic group ($P < 0.05$).

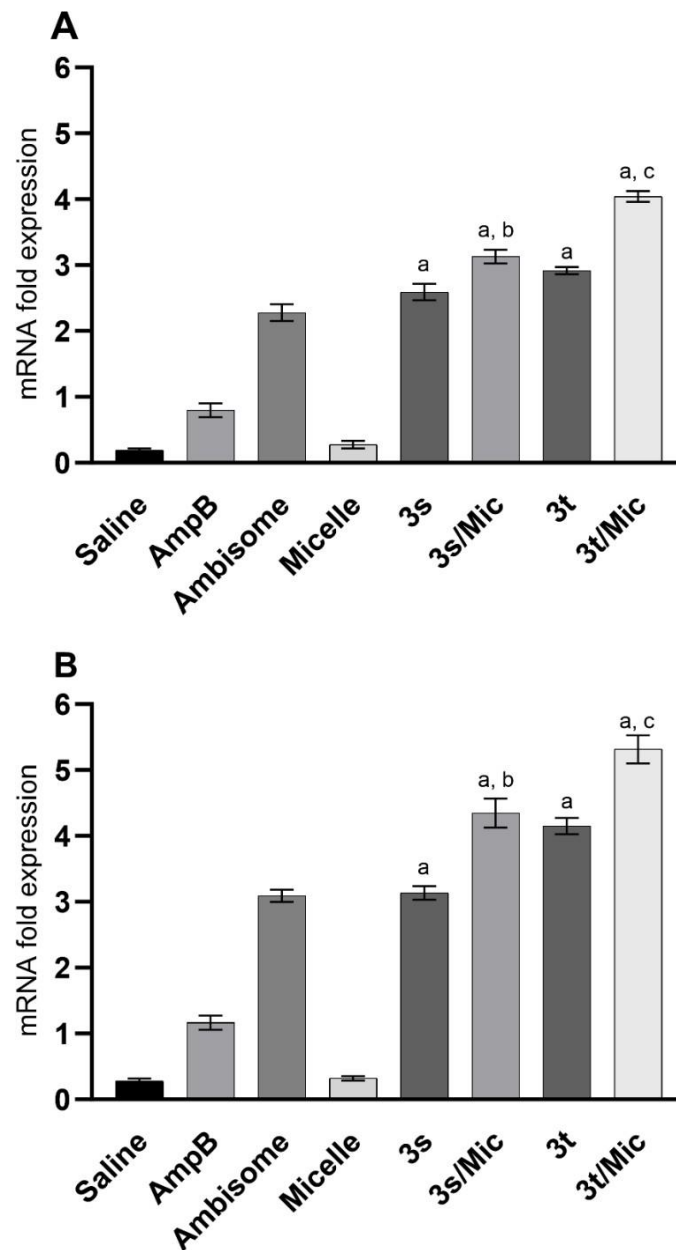


Figure 6. IFN- γ mRNA expression in the stimulated spleen cell cultures.

Spleen cells were collected from mice, one and 30 days after therapeutics ($n = 8$ per group in each step), and they (5×10^6 cells per well) were non-stimulated (medium, control) or stimulated with SLA ($25 \mu\text{g/mL}$) for 48 h at 37°C in 5 % CO_2 . RNA content was then extracted and used to evaluate the IFN- γ expression through RT-qPCR technique. Results obtained one (A) and 30 (B) days after the therapeutics are shown. Bars indicate the mean $\pm$ standard deviation of the groups. “a” indicates significant difference in relation to the saline, amphotericin B (AmpB), Ambisome, and micelle groups ($P < 0.0001$). “b” indicates significant difference in relation to the 3 s group ($P < 0.001$). “c” indicates significant difference in relation to the 3 t group ($P < 0.001$).

Estimation of parasite load in infected and treated BALB/c mice

The organic parasitism was evaluated in the spleens, livers, BMs and dLNs from treated animals, one and 30 days after therapeutics. Results showed that mice receiving 3s/M or 3t/M presented significant reductions in the parasite load in all organs evaluated, when compared to the other groups (**Figure 7A-7D**). One day after therapeutics, such animals showed reductions in the parasite load in the order of 2.8 and 3.0-log in their livers (**Figure 7A**), of 3.5 and 4.5-log in their spleens (**Figure 7B**), of 4.5 and 4.8-log in their dLNs (**Figure 7C**), and of 1.8 and 2.0-log in their BMs (**Figure 7D**), respectively, as compared to the saline group. Thirty days after therapeutics, results were kept, since animals receiving 3s/M or 3t/M showed reductions in the parasite load in the order of 3.0 and 3.5-log in their livers (**Figure 8A**), of 3.8 and 4.8-log in their spleens (**Figure 8B**), of 4.5 and 5.0-log in their dLNs (**Figure 8C**), and of 1.8 and 2.8-log in their BMs (**Figure 8D**), respectively, when compared to the saline group.

A qPCR assay to evaluate the splenic parasitism was also performed, and results showed that mice receiving 3s/M or 3t/M presented lower parasite number in their spleens, with values of 153.3 ± 30.6 and 115.3 ± 14.9 , respectively; whereas animals receiving saline, AmpB and empty micelles presented values of 460.5 ± 39.8 , 216.5 ± 21.5 , and 396.9 ± 15.6 , respectively, at one day after therapeutics. Mice receiving Ambisome[®], 3s or 3t presented values of 173.9 ± 20.4 , 208.9 ± 10.9 , and 188.8 ± 17.9 , respectively. With analyzes performed 30 days after therapeutics, results also showed that mice treated with 3s/M or 3t/M presented values of 196.5 ± 16.2 and 105.8 ± 11.3 , respectively; while animals receiving saline, AmpB, empty micelles, Ambisome[®], 3s or 3t presented values of 535.8 ± 41.6 , 269.8 ± 30.6 , 452.3 ± 32.9 , 198.5 ± 16.1 , 218.9 ± 19.0 , and 183.3 ± 13.7 , respectively. In general, a slightly increase in the parasite load was observed in control groups mice between one and 30 days after therapeutics, although groups treated with 3s/M or 3t/M have showed similar results, suggesting also a long-lasting parasitological protective response.

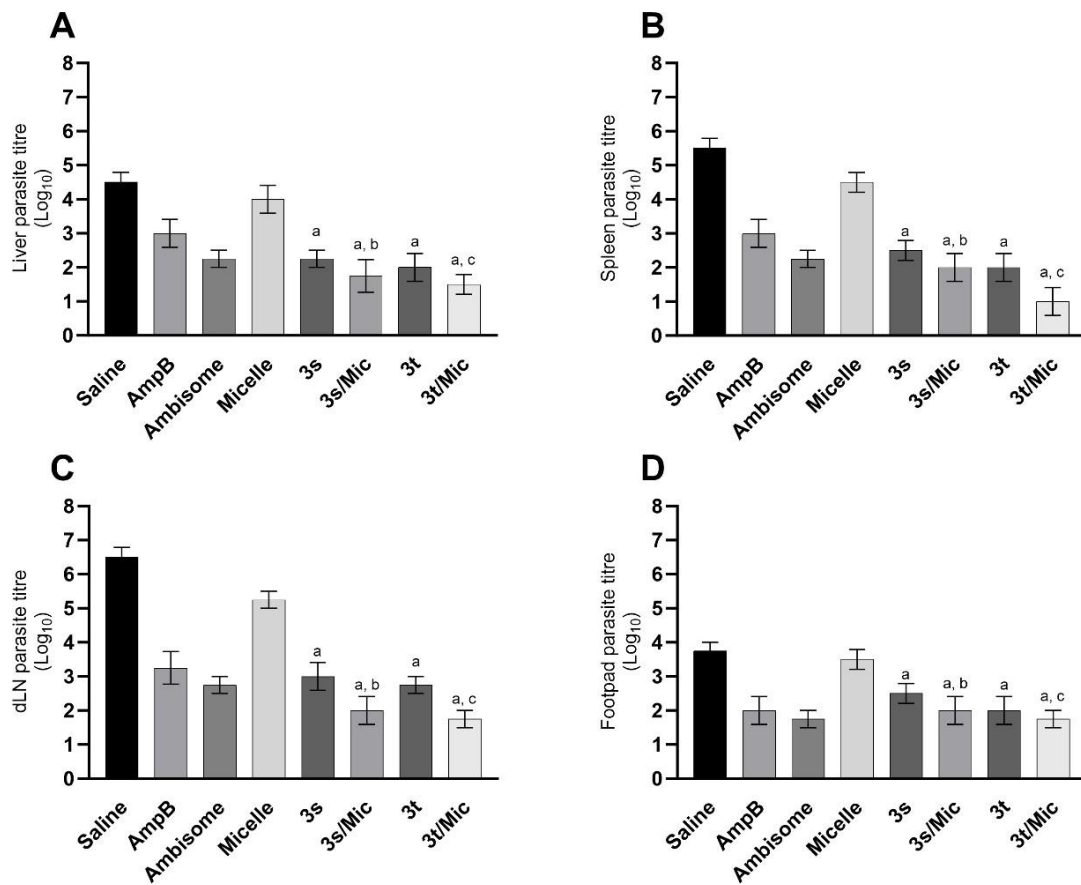


Figure 7. Parasite burden estimated one day after treatment.

Mice ($n = 8$ per group) were infected with *L. infantum* promastigotes and, 60 days after, they received saline, amphotericin B (AmpB), micelles, Ambisome®, 3 s, 3 t, 3 s/M or 3 t/M. One day after therapeutics, their livers (A), spleens (B), BMs (C), and dLNs (D) were collected and used to evaluate the parasite load through limiting dilution technique. Bars indicate the mean $\pm$ standard deviation of the groups. “a” indicates significant difference in relation to the saline, amphotericin B (AmpB), Ambisome, and micelle groups ($P < 0.0001$). “b” indicates significant difference in relation to the 3 s group ($P < 0.001$). “c” indicates significant difference in relation to the 3t group ($P < 0.001$).

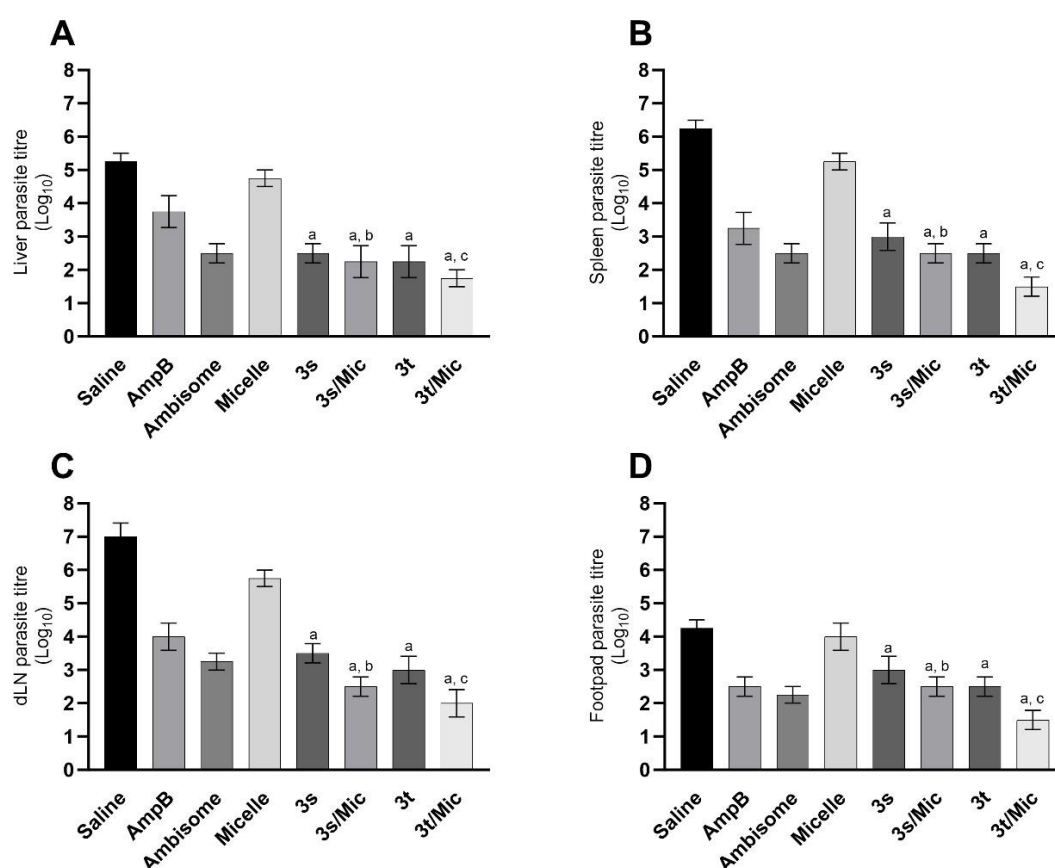


Figure 8. Parasite burden evaluated 30 days after therapies.

Mice ($n = 8$ per group) were infected with *L. infantum* promastigotes and later treated with one of the therapeutic regimens. Thirty days after therapeutics, they were euthanized and their livers (A), spleens (B), BMs (C), and dLNs (D) were collected to evaluate the organ parasitism through limiting dilution assay. Bars indicate the mean $\pm$ standard deviation of the groups. “a” indicates significant difference in relation to the saline, amphotericin B (AmpB), Ambisome, and micelle groups ($P < 0.0001$). “b” indicates significant difference in relation to the 3 s group ($P < 0.001$). “c” indicates significant difference in relation to the 3 t group ($P < 0.001$).

DISCUSSION

Treatment against VL presents problems mainly related to drug toxicity, high cost and/or by emergence of parasite resistant strains; indicating the necessity to identify new and safe antileishmanial agents.⁴³ New compounds presenting higher efficacy, lower toxicity, and better affordability could improve the therapeutic arsenal against this neglected diseases complex.¹ Natural compounds, such as flavonoids^{37,14,16}, quinones^{40,42}, alkaloids^{9,49}, and terpenes^{35,38}, amongst others; have been evaluated as antileishmanial candidates, with promising results being reached. Synthetic molecules can be easily produced, present higher yield and lower cost; making possible their use instead of current toxic chemotherapy.^{48,13,19}

In this context, in the present study, two vanillin synthetic derivatives were evaluated as antileishmanial candidates against VL, being administered in *L. infantum*-infected mice in a purê form or incorporated into polymeric micelles as delivery and adjuvant. Results showed that 3s and 3t induced some degree of parasitological and immunological effectivity, but that was significantly higher when the 3s/M and 3t/M compositions were administered in the infected animals, being the therapeutic effect kept 30 days after the application of therapeutics. An antileishmanial Th1-type cellular and humoral response was found in these animals, corroborating with significant reductions in the parasite load; suggesting then that 3s/M and 3t/M could induce long-lasting therapeutic efficacy and protect against VL.

In *L. infantum*-infected BALB/c mice, the control of disease is linked to the development of an antileishmanial Th1-type immune response, with the production of pro- inflammatory cytokines that activate macrophages to kill parasites.^{36,23,8} On the other hand, anti-inflammatory cytokines, such as IL-4, IL-5, and IL-10, among others, deactivate parasitized macrophages contributing to the development of disease.^{31,20,47} In the present study, SLA-stimulated spleen cell cultures from mice treated with 3s/M or 3t/M produced significantly higher levels of IFN- γ , TNF- α , and IL-12, when animals were evaluated one and 30 days after therapeutics. These animals produced also very low levels of IL-4 and IL-10, suggesting the development of a polarized Th-1 type immune response. Similar results have been also obtained evaluating other synthetic molecules in murine models against VL, such as 8- hydroxyquinoline¹¹, Flau-A³², eugenol⁴⁴, amongst others; suggesting that this immunological profile is desirable against infection and participate of the cure of the infected animals.

VL is a neglected disease and, as a consequence, investment in the development of new therapeutics is scarce, since it is a long and very expensive task.⁵ Considering these aspects, the optimization of treatment through delivery systems for known therapeutic agents or the use of new candidates is an interesting alternative. Delivery systems employing liposomes, polymeric micelles, among others, can improve the absorption, distribution, metabolism, and/or excretion of the products; improving the therapeutic efficacy and/or reducing the toxicity of drugs. In this context, AmpB, which is a known antifungal agent and proved to present an effective action against distinct *Leishmania* species; is also toxic for mammals, due to its affinity to cell membranes containing ergosterol. The toxicity caused by drug includes nephrotoxicity, cardiac alterations, hemolysis and liver damage from patients. Liposomal formulation containing AmpB, AmBisome[®], presents reduced toxicity causing less nephrotoxic and heart damage; however, the high cost is an important factor limiting the widespread use of this product.

Here, we tested Poloxamer P407-based micelles to incorporate 3s and 3t, and to evaluate the antileishmanial activity against VL. We observed that both compositions were more effective and less toxic than the pure molecules, suggesting then the potential of use these formulations to treat VL. In fact, polymeric micelles have hydrophilic and hydrophobic properties, size average varying from 10 to 100 nm, and high stability. In some studies, they also increased the immunological and parasitological efficacy of AmpB (Mendonça et al., 2016), and of molecules with antileishmanial action tested, such as 8-hydroxyquinoline¹¹, clioquinol⁵², Flau-A (Mendonça et al., 2019), acarbose⁷; suggesting then a potential as delivery and adjuvant role of such compounds. In these studies, these polymers increased also the effectivity of the tested molecules, suggesting also their potential as carriers of antileishmanial candidates for mammals.

Additionally, Poloxamer 407 is an amphiphilic synthetic triblock copolymer consisting of a hydrophobic poly(oxypropylene) (POP) block between two hydrophilic poly(oxyethylene) (POE) blocks^{30,21}, and has a molecular weight of approximately 12,600 Da. This polymer has been used in the cosmetic and pharmaceutical fields.^{17,39} The compounds 3s and 3t exhibit a combination of hydrophilic elements (such as a hydroxyl (OH), a formyl (CHO), and a 1,2,3-triazole ring) and hydrophobic features (alkyl chains and aromatic ring). These attributes enable the establishment of intermolecular interactions with the polymer. In addition, this characteristic significantly facilitates the formulation of 3s and 3t compounds in conjunction with Poloxamer 407-based micelles.

We evaluated the parasite burden in distinct organs of the animals, through a limiting dilution technique; since the reduction of the parasite load is considered an important parameter from therapeutic response against VL.^{5,41} Results showed that both 3s/M and 3t/M reduced by more than 70% the parasite load in the livers, spleens, BMs and dLNs of infected and treated animals, when compared to data found in control mice. The splenic parasitism was also evaluated by a qPCR technique, and results corroborated with those obtained by limiting dilution technique, in which animals receiving 3s/M and 3t/M reduced the parasitism in significant levels as compared to the other groups.

Regarding the qPCR technique used to evaluate the parasite burden, Dymond¹⁰ indicated that the use of fluorescent dyes that specifically label dsDNA or a target DNA species lead to the analysis of product accumulation in qPCR, in which the DNA content present in the sample is proportional to the amount of fluorescence produced. Kralik and Ricchi²⁴ suggested that qPCR allows the identification, by inference, of the relative content of the template DNA present in the sample evaluated, and that this technique can be considered as a suitable method

for the detection, quantification and typing of microbial agents in biological samples. In this context, we can infer in our study that both limiting dilution technique and qPCR indicated that the use of 3s/M and 3t/M reduced the parasite load in distinct organs of the treated animals, contributing to the reduction of infection in such animals.

We used a dose concentration of 10 mg/kg/day of 3s and 3t, and of 5 mg/kg/day of 3s/M and 3t/M to treat infected animals. In fact, we have used concentrations based on previous studies developed by the group, when other antileishmanial candidates based on synthetic molecules were also evaluated in murine models, such as 8- HQN¹¹, clioquinol⁵¹, Flau-A³², ivermectin (Reis et al., 2022), amongst others. In these cases, molecules and their compositions were also administered by subcutaneous route, and parasitological and immunological responses were benefic for the infected hosts.

However, others have used lower concentrations of the tested products and/or different routes of administration, such as Emiliano and Almeida-Amaral¹², which administered apigenin to treat *L. infantum*-infected mice in concentrations of 2 mg/kg/day orally receiving the product daily. They found reductions higher than 90% in the liver parasite load of the animals, suggesting that apigenin could be a potential candidate for VL chemotherapy by oral administration. In other study, lapachol was tested as therapeutic agent to treat *L. infantum*-infected mice, and it was administered orally for 10 days at concentration of 25 mg/kg/day. Results showed that the application significantly reduced the parasitic load in the livers and spleens of the animals, with similar values to AmpB. The authors concluded their study suggesting that lapachol could be considered as a promising antileishmanial agent.²

In this context, a dose-response curve using different concentrations of the 3s/M and 3t/M should be performed, in order to define the minimal concentration of the compounds able to induce the best therapeutic response, as well as to define the best route of administration of these compositions. In this context, additional studies should be performed in order to solve these questions, and data can be considered as preliminary of the efficacy of these products against VL.

This study evaluated the therapeutic efficacy of the 3s/M and 3t/M at two endpoints, one and 30 days after the immunotherapeutics procedures. Immunological, biochemical, and parasitological analyses performed in both periods showed that data were similar between the prior and later analyses, although a more polarized Th1-type response and lower parasitism have been found with experiments performed 30 days after therapeutics. This fact suggests an improvement, even if discrete, of the cell response profile and parasitological protection against infection, in a higher period of time after treatment. However, additional assays should be also

performed between one and 30 days, or more, in the treated animals, aiming to define the long-term protection and to evaluate a possible sterile cure of the treated animals.

In addition, the compositions could be also administered by different routes in the infected animals, aiming to improve the quality of the therapeutic response, and such fact can be also considered as a variable to interfere in the results obtained in the present work. Additionally, the absence of a dose-response curve to define the best concentration of the compositions that could cause minimal toxicity and maximum antileishmanial effect in the treated animals is a limitation of the study. In this context, preliminary data can be considered as a proof-of-concept of therapeutic response of 3s/M and 3t/M against VL.

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CONFLICT OF INTEREST

The authors confirm that they have no conflicts of interest in relation to this work.

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5.2.2. Considerações finais

O tratamento de camundongos BALB/c infectados com *L. infantum* com as moléculas 3s e 3t, especialmente sua composição micelar (3s/M e 3t/M), induziram uma resposta imune do tipo Th1 com altos níveis de IFN- γ , IL-2, TNF- α , IL-12, nitrito e anticorpos IgG2a. Além disso, os animais infectados e tratados com 3s/M e 3t/M apresentaram uma redução significativa da carga parasitária no baço, fígado, medula óssea e linfonodos drenantes em relação aos grupos controles em ambos os períodos de tempo avaliados, demonstrando assim a manutenção do efeito terapêutico das moléculas até 30 dias após o término do tratamento.

Ademais, o tratamento com 3s e 3t micelar mostrou-se de baixa toxicidade com diminuição na concentração sérica de AST, ALT, ureia e creatinina em comparação aos grupos tratados com salina, AmpB e micela vazia. Esses dados sugerem, portanto, que os derivados de vanilina são menos tóxicos para os animais do que os tratamentos usuais, bem como reduz a toxicidade causada pela própria infecção por *Leishmania*. Assim, 3s/M e 3t/M se mostraram efetivas no tratamento da leishmaniose visceral experimental.

5.3. CAPÍTULO 3 – 3s e 3t na LT experimental

Neste capítulo são apresentados os resultados e a discussão sobre a ação antileishmanial das moléculas 3s e 3t, em sua forma livre, e incorporados em micelas poliméricas, na infecção experimental por *L. amazonensis*.

5.3.1. Artigo

Adaptado de: Pereira, I. A. G.; Freitas, C. S., Câmara, R. S. B. *et al.* Treatment using vanillin-derived synthetic molecules incorporated into polymeric micelles is effective against infection caused by *Leishmania amazonensis* species. *Experimental Parasitology*, v.260, e108743, 2024. doi: 10.1016/j.exppara.2024.108743.

TITLE

Treatment using vanillin-derived synthetic molecules incorporated into polymeric micelles is effective against infection caused by *Leishmania amazonensis* species.

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ABSTRACT

Treatment against leishmaniasis presents problems, mainly due to the toxicity of the drugs, high cost, and the emergence of resistant strains. A previous study showed that two vanillin-derived synthetic molecules, 3s [4-(2-hydroxy-3-(4-octyl-1H-1,2,3-triazol-1-yl) propoxy)-3-methoxybenzaldehyde] and 3t [4-(3-(4-decyl-1H-1,2,3-triazol-1-yl)-2-hydroxypropoxy)-3-methoxybenzaldehyde], presented antileishmanial activity against *Leishmania infantum*, *L. amazonensis*, and *L. braziliensis* species. In the present work, 3s and 3t were evaluated to treat *L. amazonensis*-infected mice. Molecules were used pure or incorporated into Poloxamer 407-based micelles. In addition, amphotericin B (AmpB) and its liposomal formulation, Ambisome[®], were used as control. Animals received the treatment and, one and 30 days after, they were euthanized to evaluate immunological, parasitological, and biochemical parameters. Results showed that the micellar compositions (3s/Mic and 3t/Mic) induced significant reductions in the lesion mean diameter and parasite load in the infected tissue and distinct organs, as well as a specific and significant antileishmanial Th1-type immune response, which was based on significantly higher levels of IFN- γ , IL-12, nitrite, and IgG2a isotype antibodies.

Drug controls showed also antileishmanial action; although 3s/Mic and 3t/Mic have presented better and more significant parasitological and immunological data, which were based on significantly higher IFN- γ production and lower parasite burden in treated animals. In addition, significantly lower levels of urea, creatinine, alanine transaminase, and aspartate transaminase were found in mice treated with 3s/Mic and 3t/Mic, when compared to the others. In conclusion, results suggest that 3s/Mic and 3t/Mic could be considered as therapeutic candidates to treat against *L. amazonensis* infection.

Keywords: Tegumentary leishmaniasis, treatment, vanillin, amphotericin B, *Leishmania amazonensis*, polymeric micelles.

INTRODUCTION

Leishmaniasis are parasitic diseases caused by more than 20 different *Leishmania* species, with approximately 0.7 to 1.0 million new cases each year occurring in 98 countries around the world.¹ This disease complex presents two main clinical manifestations: visceral leishmaniasis (VL), which is caused by the *Leishmania donovani* and *L. infantum* species and can cause fever, weight loss, hepatosplenomegaly, anemia, among others. This disease can be fatal if acute and untreated; and tegumentary leishmaniasis (TL), which is the most common clinical form of leishmaniasis and is caused by distinct parasite species.² TL present three main clinic forms: cutaneous leishmaniasis (CL), mucosal leishmaniasis (ML), and diffuse-cutaneous leishmaniasis (DCL). These diseases cause since self-limiting lesions until scars in mucous membranes, leading to the patient's morbidity.^{3,4} TL can be evoked by infection with several *Leishmania* species, such as *Leishmania braziliensis*, *L. guyanensis*, *L. amazonensis*, among others; and parasitological and molecular evaluations have shown that *L. braziliensis* is the most important etiological agent of the disease in the Americas, and responsible by severe clinic manifestations in the patients.⁵

Parasitological diagnosis is considered to be the gold standard for TL, in which the visualization of the parasite in skin or mucosal lesion samples, its isolation in cultures, and/or its inoculation in animals are all considered to be valid diagnostic techniques.⁶ Although these methods present high specificity, the sensitivity is variable, between 60% and 95% in cultures and smears.^{7,8} The molecular parasitological diagnosis has also been performed and presents

specific advantages, such as higher sensitivity than conventional parasitological techniques; however, it is expensive and requires a specialized laboratory structure.^{9,10}

Treatment against TL is hampered due to the toxicity of the drugs, high cost, and/or the emergence of resistant strains.^{11,12} Pentavalent antimonials are considered the first-choice treatment; however, they can cause severe adverse effects in patients.¹³ Other compounds, such as deoxycholate amphotericin B and its liposomal formulations, pentamidine, paramomycin, among others, may also be used, but they all cause adverse effects, which limit their use and adherence to treatment.¹⁴ In addition, in endemic regions of TL, the treatment failure rate can reach 50% of all cases¹⁵, which indicate the requirement for the development of new, safer, and more effective antileishmanial drugs.

Therefore, the development of new strategies to treat leishmaniasis has become a priority. In this light, natural products and synthetic molecules have traditionally played an important role in drug discovery and were the basis of most early medicines.^{16,17} Vanillin is an organoleptic compound of natural vanilla acquired from the pods of an orchid named *Vanilla planifolia*. It presents a chemical structure that allows for its use in distinct biological applications, since vanillin and its derivatives have shown anticancer¹⁸, antibacterial¹⁹, antidiabetic²⁰, and antileishmanial²¹ activities.

In a recent study developed by our research group, two novel synthetic derivatives from vanillin, called 3s [4-(2-hydroxy-3-(4-octyl-1H-1,2,3-triazol-1-yl) propoxy)-3-methoxybenzaldehyde] and 3t [4-(3-(4-decyl-1H-1,2,3-triazol-1-yl)-2-hydroxypropoxy)-3-methoxybenzaldehyde], were evaluated regarding their *in vitro* antileishmanial activity against *L. infantum*, *L. amazonensis*, and *L. braziliensis* promastigotes and intra-amastigotes.²¹ Results showed that both compounds were effective and selective against parasites, and they reduced the parasite load in treated and infected murine macrophages. The mechanism of action evaluated in *L. infantum* revealed that 3s and 3t altered the parasite mitochondrial membrane potential, leading to reactive oxygen species production, an increase in lipid bodies, and changes in the cell cycle, causing the parasite's death.

In this context, in the present study, we evaluated the *in vivo* antileishmanial activity of 3s and 3t against *L. amazonensis* infection. For this, BALB/c mice were infected with parasite promastigotes and later received 3s and 3t, which were administered pure or incorporated into Poloxamer P407-based micelles, which were used with delivery and adjuvant purposes.^{22,24} Other animals were treated with AmpB or its liposomal formulation (Ambisome®), and the lesion development, parasite burden, cellular and humoral responses, and biochemical markers were evaluated one and 30 days after treatment.

MATERIALS AND METHODS

Parasites

The *L. amazonensis* (IFLA/BR/1967/PH-8) strain was used. Stationary promastigotes were cultured at 24°C in complete Schneider's medium (Sigma-Aldrich, USA), which consisted of the medium plus 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich, USA), 20 mM L-glutamine, 200 U/mL penicillin, and 100 µg/mL streptomycin, at pH 7.4. Before infection, promastigotes were viewed under optical light microscopy, and the cell density was estimated through a Newbauer chamber count, with their morphology being evaluated after staining with Giemsa. In addition, the soluble *Leishmania* antigenic extract (SLA) was prepared as described elsewhere.²⁵

Mice

Female BALB/c mice (8 weeks of age) were used. These were maintained under specific pathogen-free conditions and received water ad libitum. The Committee on the Ethical Guidelines for the Use of Animals in Research from the Federal University of Minas Gerais (UFMG; Belo Horizonte, Minas Gerais, Brazil) approved this study, logged under protocol number 056/2022.

Molecules and preparation of 3s and 3t-containing micelles

The 3s and 3t compounds were previously prepared and characterized by our group according to that described elsewhere.²¹ Briefly, vanillin was alkylated with epichlorohydrin. Next, an epoxide ring opening reaction was carried out with sodium azide, followed by copper (I)-catalyzed alkyne-azide cycloaddition (CuAAC) reaction with appropriate terminal alkynes, in which the compounds 3s and 3t were obtained.

To prepare the micelle compositions, 15 mg of Poloxamer 407 (18% w/w; Pluronic® F127, Sigma-Aldrich, USA) were diluted in sterile phosphate-buffered saline (PBS 1x) at pH 7.4, under magnetic stirring for 18 h, at 4°C. Next, 10 mg of 3s or 3t were added to 500 µL of PA dichloromethane and solubilized by vortexing. The mixture was added to the previously prepared solution under vigorous stirring and placed in an ice bath until a viscous emulsion had been obtained. The dichloromethane solvent was evaporated using a rotary evaporator (Büchi®, Switzerland), and the formulations were obtained with a transparent yellow color, at room temperature.

Empty micelles were prepared according to the aforementioned protocol, but without the incorporation of 3s or 3t. All preparations were filtered through a 0.45 µm microfiltration membrane and stored at 4°C.²⁶ The physical-chemical characterization of the micelle compositions was performed by dynamic light scattering (DLS) to measure the average particle size and zeta potential, using the Zetasizer Nano ZSP system (Malvern Instruments, UK). Micelle morphology was evaluated through transmission electron microscopy (Tecnai G20, FEI Company, USA).

Infection and treatment regimens

BALB/c mice were infected subcutaneously at the base of the tail after trichotomy, by using 10^7 *L. amazonensis* stationary-phase promastigotes. After the development of disease, which occurred at approximately 60 days post-infection, animals presented a lesion mean diameter of around 4 millimeters (mm), and they were grouped (n=16 animals per group). At this point, mice received one of the following schedules: (a) untreated group: mice received only 50 µL of PBS 1x; (b) AmpB group: mice received 50 µL of AmpB (1 mg/kg of weight); (c) AmBisome[®] group: mice received 8 µL of AmBisome[®] (1 mg/kg of weight); (d) micelle group (Mic): mice received 50 µL of empty micelles (5 mg/kg weight); (e) 3s group: mice received 50 µL of 3s (5 mg/kg of weight); (f) 3s/Mic group: mice received 50 µL of micellar composition (2.5 mg of 3s/kg weight); (g) 3t group: mice received 50 µL of 3t (5 mg/kg of weight), and (h) 3t/Mic group: mice received 50 µL of micellar composition (2.5 mg of 3t/kg weight).

The doses were administered seven times by subcutaneous route, with a seven days interval between them in the untreated mice or in these receiving empty micelles, 3s or 3t. AmpB group were treated by intraperitoneal route, while AmBisome[®] group was treated by intravenous route. Both drugs were administered four times, with a five days interval between them. Treatment schedules used here were similar and/or adapted from previous studies described.^{27,28} Weekly measurements were performed using an electronic caliper (model 799-6/150, Starrett, São Paulo, Brazil) to evaluate the lesion development during and after treatment. Mice were euthanized one and 30 days after treatment (n=8 per group, each step), when organs and blood samples were collected to perform the biochemical, immunological and parasitological evaluations.

Cytokine and nitrite production

Spleen cells (5×10^6 per well) were collected from euthanized animals, in both periods of time ($n=8$ per group in each step), and cells were plated in complete RPMI 1640 medium in duplicate in 24-well plates (Nunc). These were non-stimulated (w/o SLA, control) or stimulated with SLA (25 $\mu\text{g/mL}$) for 48 h at 37°C in 5% CO_2 . IFN- γ , IL-4, IL-10, and IL-12 were measured in the culture supernatants by capture ELISA (catalogs 555138, 555232, 555252, and 555256, respectively; OptEIA™ set mouse Kits; all acquired from BD Pharmingen®, USA). In addition, a culture supernatant was used to evaluate the nitrite secretion by Griess reaction.²⁶ Briefly, 50 μL of cell supernatant were mixed with an equal volume of Griess reagent, and the nitrite concentration was estimated by using a standard curve, with absorbances measured at 540 nm.

Reverse Transcription-qPCR (RT-qPCR) to evaluate the mRNA IFN- γ expression

Cell response was also evaluated in the animals' spleens by means of extraction of mRNA content and RT-qPCR assay, according described elsewhere.²⁹ Briefly, RNA was extracted from the animals' spleens by using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). It was resuspended in UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, Carlsbad, CA, USA), and concentration was estimated in a NanoDrop LITE (Thermo Scientific, Waltham, MA, USA), with absorbance ratios of 260/280 nm. RT-qPCR was performed using the GoTaq® 1-Step RT-qPCR System (Promega Corporation) and gene-specific primers for IFN- γ : 5'-TCAAGTGGCATAGATGTGGAAGAA-3' (Forward) and 5'-TGGCTCTGCAGGATTTTCATG-3' (Reverse). In addition, 5'-TCACCACCATGGAGAAGCC-3' (Forward) and 5'-GCTAAGCAGTTGGTGTGCA-3' (Reverse) primers for endogenous control GAPDH were also used. Reactions were processed in a 7900HT Fast Real-Time PCR system (Applied Biosystems, USA). Results were shown graphically as fold changes in gene expression using the mean $\pm$ standard deviation of the target gene.³⁰ Data were analyzed according to the relative expression using the $2^{-\Delta\Delta\text{CT}}$ method, representative of two independent experiments.

Flow cytometry to evaluate cytokine-producing polyfunctional T cells

The stimulated cultures were used to evaluate cell surface markers and intracellular cytokine (IFN- γ , TNF- α , IL-2, and IL-10), as described in³¹. Briefly, splenocytes (5×10^6 cells per well) were incubated in complete RPMI 1640 medium in 96-well round-bottom culture

plates (Nunc™, Nunclon, USA), and were non-stimulated (medium, control) or stimulated with SLA (25 µg/mL) for 48 h at 37°C in 5% CO₂. Brefeldin A (10 mg/mL; Sigma-Aldrich, USA) was added to the cultures 4 h before the end of incubation. Some wells were stimulated with Phorbol 12-myristate 13-acetate (5.0 ng/mL) and Ionomycin (1.0 mg/mL), as positive controls. Cells were then stained by Fixable Viability Stain 450 (FVS450, BD Biosciences) for 15 min at room temperature, followed by labeling with antibodies against CD3⁺ (BV650 anti-mouse, clone 145.2C11), CD4⁺ (BV605 anti-mouse, clone RM4-5), and CD8⁺ (BV786 anti-mouse, clone 53-6.7) for 30 min at room temperature. Cells were fixed with FACS fixing solution, washed and permeabilized with PBS plus 0.5% saponin, and stained with antibodies against IL-2 (PE anti-mouse, clone JES6-5H4), IFN-γ (AF700 anti-mouse, clone XMG1.2), TNF-α (PE-Cy7 anti-mouse, clone LG.3A10), and IL-10 (APC anti-mouse, clone JES5-16E), for 30 min at room temperature. All antibodies were purchased from BD Biosciences (USA).

Cells were acquired (100,000 events) on an LSR Fortessa cytometer (BD Biosciences, USA), using FACSDiva software. For analysis in FlowJo software, dead cells were excluded after the FVS450 stain and live cells were gated for stained CD3⁺ CD4⁺ and CD3⁺CD8⁺ cells and intracytoplasmic cytokine production. Data were expressed as the percentage of cells producing the respective cytokines in the stimulated cultures.

Antibody production

Levels of anti-SLA IgG, IgG1, and IgG2a antibodies were measured in sera samples collected in both times after treatment (n=8 mice per group in each step), as described.³² Briefly, microtiter plates (Jetbiofil®, Belo Horizonte) were coated with *L. amazonensis* SLA (1.0 µg per well) diluted in 50 mM carbonate buffer at pH 9.6 for 18 h at 4°C. Free binding sites were blocked using PBS-T (PBS 1x and Tween 20 0.05% plus 5% (w/v) bovine serum albumin) for 1 h at 37°C. Plates were washed in PBS-T and incubated with sera (1:100 diluted in PBS-T) for 1 h at 37°C. They were again washed in PBS-T and incubated with anti-mouse IgG (anti-mouse IgG whole molecule, peroxidase-conjugated antibody produced in rabbit, catalog A9044, Sigma-Aldrich, USA), IgG1 (anti-mouse IgG1 secondary antibody, peroxidase-conjugated antibody produced in rat, catalog SA1-35640, Invitrogen, USA), or IgG2a (anti-mouse IgG2a secondary antibody, peroxidase-conjugated antibody produced in rat, catalog SA1-35646, Invitrogen, USA). A new incubation was performed for 1 h at 37°C, when plates were washed with PBS-T, and reactions were developed using a solution comprised of H₂O₂, ortho-phenylenediamine and citrate-phosphate buffer at pH 5.0 for 30 min and in the dark. Reactions

were stopped by adding 2N H₂SO₄, and optical density (OD) values were read in a spectrophotometer (Molecular Devices, Spectra Max Plus, Concord, Canada) at 492 nm.

Toxicity markers evaluated in sera samples from animals

Levels of alanine transaminase (ALT), aspartate transaminase (AST), urea and creatinine were measured in sera collected from animals (n=8 per group, each step), on the 1st and 30th days after treatment, as described.³² Sera from non-infected and non-treated mice (naive; n=5) were used as controls. Analyses were developed using commercial kits (Labtest Diagnostica®, Belo Horizonte) following manufacturer instructions.

Parasite burden estimated by limiting dilution technique

The parasite load was evaluated in the infected tissue and in the livers, spleens, and draining lymph nodes (dLNs) of the animals, one and 30 days after treatment (n=8 per group in each step), through a limiting dilution technique.²⁶ Briefly, tissue and organs were macerated in a glass tissue grinder using sterile PBS, and tissue debris was removed by centrifugation at 150 × g. Cells were concentrated by centrifugation at 2,000 × g. Cells were resuspended in 1 mL of complete Schneider's medium and serially diluted 10⁻¹ to 10⁻¹² in the same medium. Each sample was plated in triplicate and cultured at 24°C for 7 days. Results were expressed as the negative log of the titer (the dilution corresponding to the last positive well) adjusted per milligram of infected tissue or organ.

Quantitative polymerase chain reaction to estimates the splenic parasitism

The parasite load was also evaluated in the animals' spleens by a qPCR technique, one and 30 days after treatment (n=8 per group, each step), as described.²⁹ Briefly, DNA content was extracted using the Wizard Genomic DNA Purification Kit (Promega Corporation), and suspended with UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, Carlsbad, CA, USA). Parasite load was estimated using primers to amplify *L. infantum* kDNA: 5'-CCTATTTTACACCAACCCCCAGT-3' and 5'-GGGTAGGGGCGTTCTGCGAAA-3' (Forward and Reverse, respectively). These primers are used to amplify a specific and conserved region of the *Leishmania* parasite, which is used as an indicative of the presence of parasite DNA in the organs evaluated. In addition, primers (Forward 5'-CAGAGCAAGAGAGGTATCC-3' and Reverse 5'-TCATTGTAGAAGGTGTGGTGC-3') to amplify the mouse β-actin gene were used as endogenous control.

Reactions were processed in 7500 HT (96-wells plate; Applied Biosystems), using the Applied Biosystems PowerUp™ SYBR™ Green PCR master mix (5 µL; Thermo Fisher, Waltham, MA, USA), added with 2 mM of each primer (1 µL) and 4 µL of DNA (25 ng/µL). Samples were incubated at 95°C for 10 min and submitted to 40 cycles of 95°C for 15 s and 60°C for 1 min. At each time point, fluorescence data were collected and results were calculated by interpolation from a standard curve included in the same run, which was prepared in duplicate and expressed as the number of *L. amazonensis* organisms per total DNA.

Statistical analysis

Microsoft Excel (version 10.0) spreadsheets and GraphPad Prism™ were employed. Results were analyzed using one-way analysis of variance (ANOVA), followed by Bonferroni's post-test for comparison between the groups. Data were expressed as mean ± standard deviation. Two independent experiments, which showed similar results, were performed. Data shown are as representative of one single experiment. Differences were considered significant with $P < 0.05$.

RESULTS

Cellular response generated after treatment

The cellular response was evaluated in chronically *L. amazonensis*-infected BALB/c mice, on 1st and 30th days after treatment, and results showed that animals from the control (untreated and micelle) groups produced significantly higher levels of antileishmanial IL-4 and IL-10 cytokines, on both the 1st (**Figure 1A**) and 30th (**Figure 1B**) days after the applications. Otherwise, mice receiving 3s/Mic or 3t/Mic developed an antileishmanial Th1-type cellular response, with significantly higher levels of IFN-γ and IL-12 present in the cell supernatants, which were associated with significantly lower production of IL-4 and IL-10. In addition, animals treated with 3s, 3t, and Ambisome® produced also higher levels of IFN-γ, albeit this production has been less pronounced than that observed in 3s/Mic and 3t/Mic groups mice.

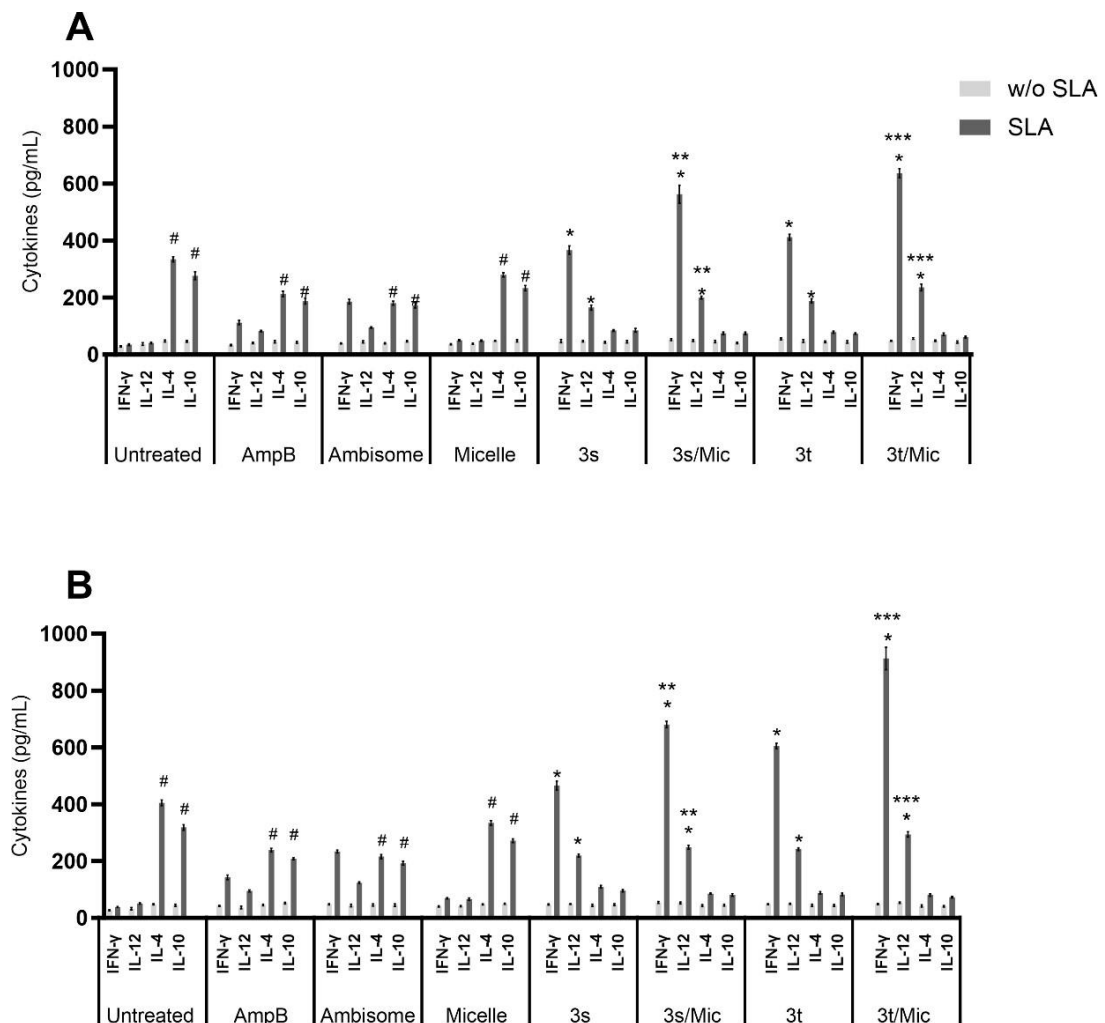


Figure 1. Cytokine response developed on the 1st and 30th days after treatment.

BALB/c mice (n=16 per group) were infected with *L. amazonensis* promastigotes and, 60 days later, they were untreated or received amphotericin B (AmpB), micelles, Ambisome®, 3s, 3t, 3s/Mic, or 3t/Mic. In both periods of time, the animals' splenocytes (n=8 mice per group in each step) were collected and cultured (5×10^6 cells per well) in complete RPMI 1640 medium, at which time they were unstimulated (w/o SLA, medium) or stimulated with SLA (25 µg/mL) for 48 h at 37°C in 5% CO₂. IFN-γ, IL-12, IL-4, and IL-10 levels were measured in the culture supernatants by capture ELISA, with experiments performed on the 1st (A) and 30th (B) days after treatment. Bars indicate the mean ± standard deviation of the groups. * indicates significant difference in relation to the untreated, amphotericin B (AmpB), Ambisome®, and micelle groups ($P < 0.0001$). ** indicates significant difference in relation to the 3s group ($P < 0.001$). *** indicates significant difference in relation to the 3t group ($P < 0.001$). # indicates significant difference in relation to the 3s, 3s/Mic, 3t, and 3t/Mic groups ($P < 0.0001$).

The nitrite presence was evaluated in the culture supernatants, and results showed significantly higher production levels of this anti-parasite molecule in the 3s/Mic and 3t/Mic

groups, when compared to the others, on both the 1st (**Figure 2A**) and 30th (**Figure 2B**) days after treatment. Similar to the IFN- γ production, nitrite secretion proved to be higher in mice receiving 3s, 3t, and Ambisome[®], when compared to values obtained in untreated and micelle groups mice.

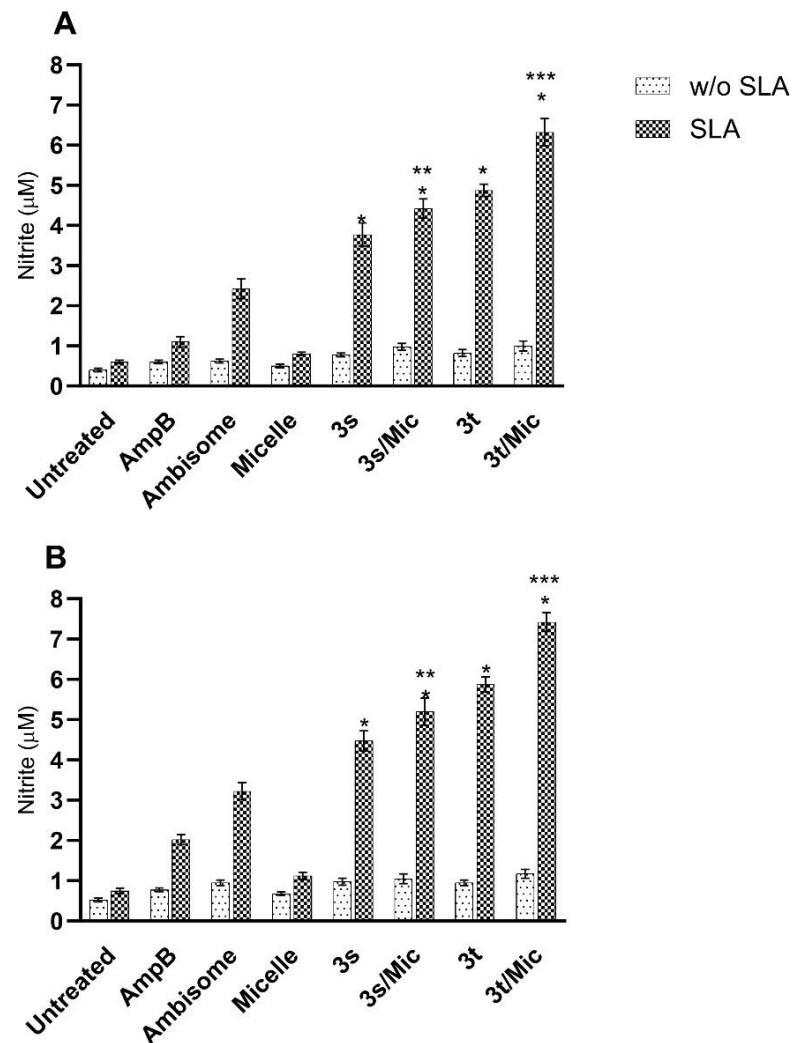


Figure 2. Nitrite secretion evaluated on the 1st and 30th days after treatment.

The supernatants used to evaluate the cytokine production in the unstimulated (w/o SLA, medium) or SLA-stimulated cell cultures by capture ELISA were also used to quantify nitrite levels, through the Griess reaction. Results obtained on the 1st (A) and 30th (B) days after treatment are shown as bars, which represent the mean $\pm$ standard deviation of the groups. * indicates significant difference in relation to the untreated, amphotericin B (AmpB), Ambisome[®], and micelle groups ($P < 0.0001$). ** indicates significant difference in relation to the 3s group ($P < 0.001$). *** indicates significant difference in relation to the 3t group ($P < 0.001$).

The IFN- γ mRNA expression was evaluated in the stimulated splenic cultures, and results showed that cells from the 3s/Mic and 3t/Mic groups mice expressed higher levels of IFN- γ mRNA, as compared to data found in the other groups (**Figure 3**). Animals receiving 3s, 3t, and Ambisome[®] showed also higher IFN- γ mRNA expression, as compared to data found in the controls, although such values have been lower as compared to data found in animals receiving 3s/Mic or 3t/Mic.

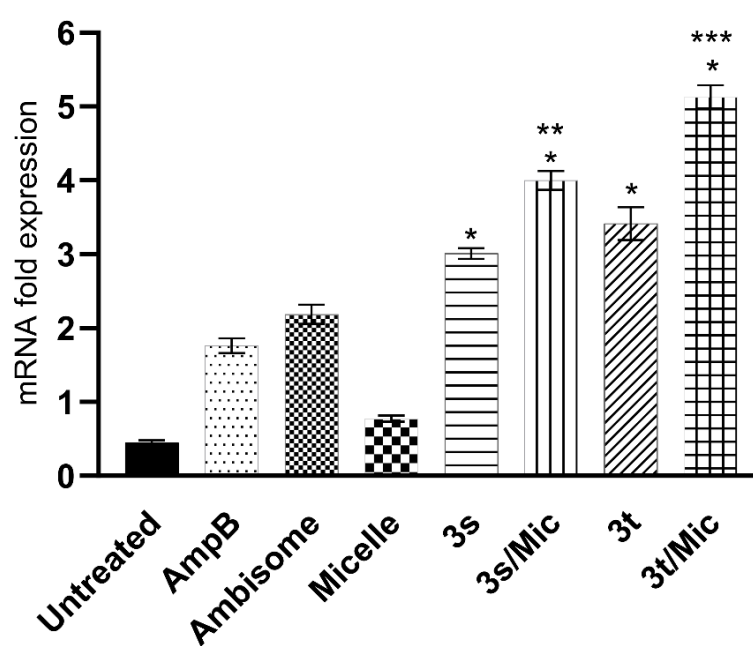


Figure 3. IFN- γ mRNA expression in the stimulated cell cultures.

Mice' spleens (n=8 per group) collected on the 30th day after treatment were cultured and cells (5×10^6 per well) were stimulated with SLA (25 μ g/mL) for 48 h at 37°C in 5% CO₂. The RNA content was extracted to evaluate the IFN- γ mRNA expression in the stimulated cultures, through a RT-qPCR technique. Results are shown as bars, which indicate the mean $\pm$ standard deviation of the groups. * indicates significant difference in relation to the untreated, amphotericin B (AmpB), Ambisome[®], and micelle groups ($P < 0.0001$). ** indicates significant difference in relation to the 3s group ($P < 0.001$). *** indicates significant difference in relation to the 3t group ($P < 0.001$).

A flow cytometry assay showed also that these treated mice presented higher percentage of IFN- γ and IL-2-producing T cells, when compared to values found in the controls, with a similar profile observed when both T-cell subtypes were evaluated. Otherwise, higher

percentage of IL-10- producing T cells was found in untreated mice, when compared to the others (**Figure 4**).

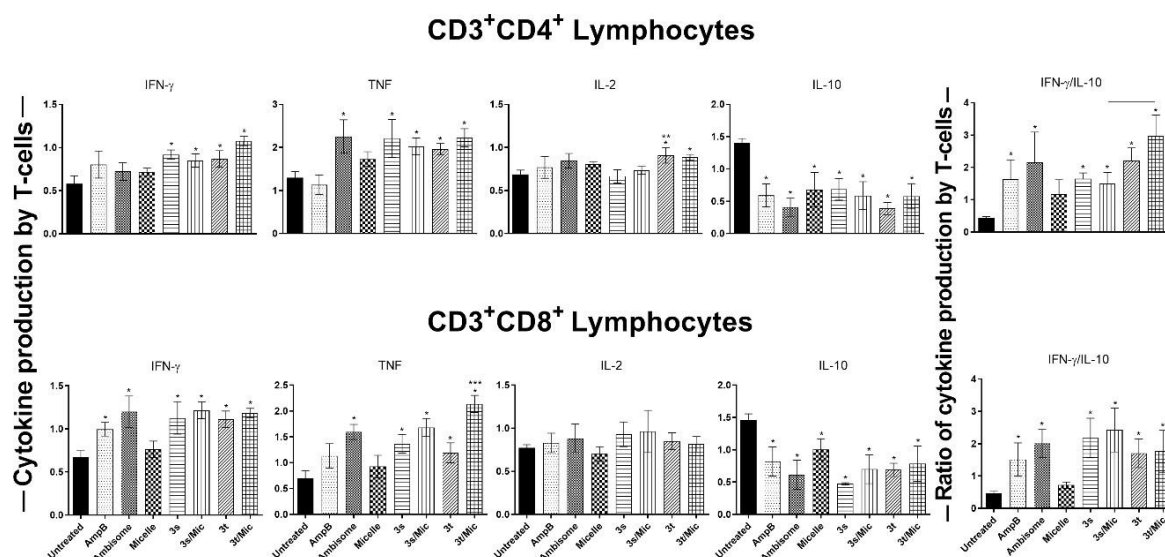


Figure 4. Profile of intracytoplasmic cytokines produced by splenocytes after *in vitro* stimulation with a *Leishmania* antigenic extract.

Plots represent the percentage of CD3⁺ CD4⁺ and CD3⁺ CD8⁺ T cells producing the IFN- γ , TNF- α , IL-2, and IL-10 cytokines. IFN- γ /IL-10 ratios were also calculated in the T-cells subpopulations. Results are shown as bars, which indicate the mean $\pm$ standard deviation of the groups. * indicates significant difference in relation to the untreated group ($P < 0.05$). ** indicates significant difference in relation to the 3s group ($P < 0.05$). *** indicates significant difference in relation to the 3t group ($P < 0.05$).

Antibody production generated after treatment

The humoral response was evaluated in the 1st and 30th days after treatment, by means of the production of anti-parasite IgG, IgG1, and IgG2a antibodies. Results showed that sera from mice receiving 3s/Mic and 3t/Mic presented significantly higher levels of IgG2a isotype, when compared to the others, on both the 1st (**Figure 5**, panels A, C and E) and 30th (**Figure 5**, panels B, D and H) days after treatment. Otherwise, control groups mice produced significantly higher levels of anti-SLA IgG and IgG1 antibodies, in addition to lower IgG2a levels, on both periods of time after treatment. Animals receiving 3s, 3t, and Ambisome[®] produced also higher levels of IgG antibodies, as compared to the 3s/Mic and 3t/Mic groups; however, significantly higher IgG2a and lower IgG1 levels were found in mice receiving the micellar compositions, when compared to those treated with 3s, 3t, or Ambisome[®].

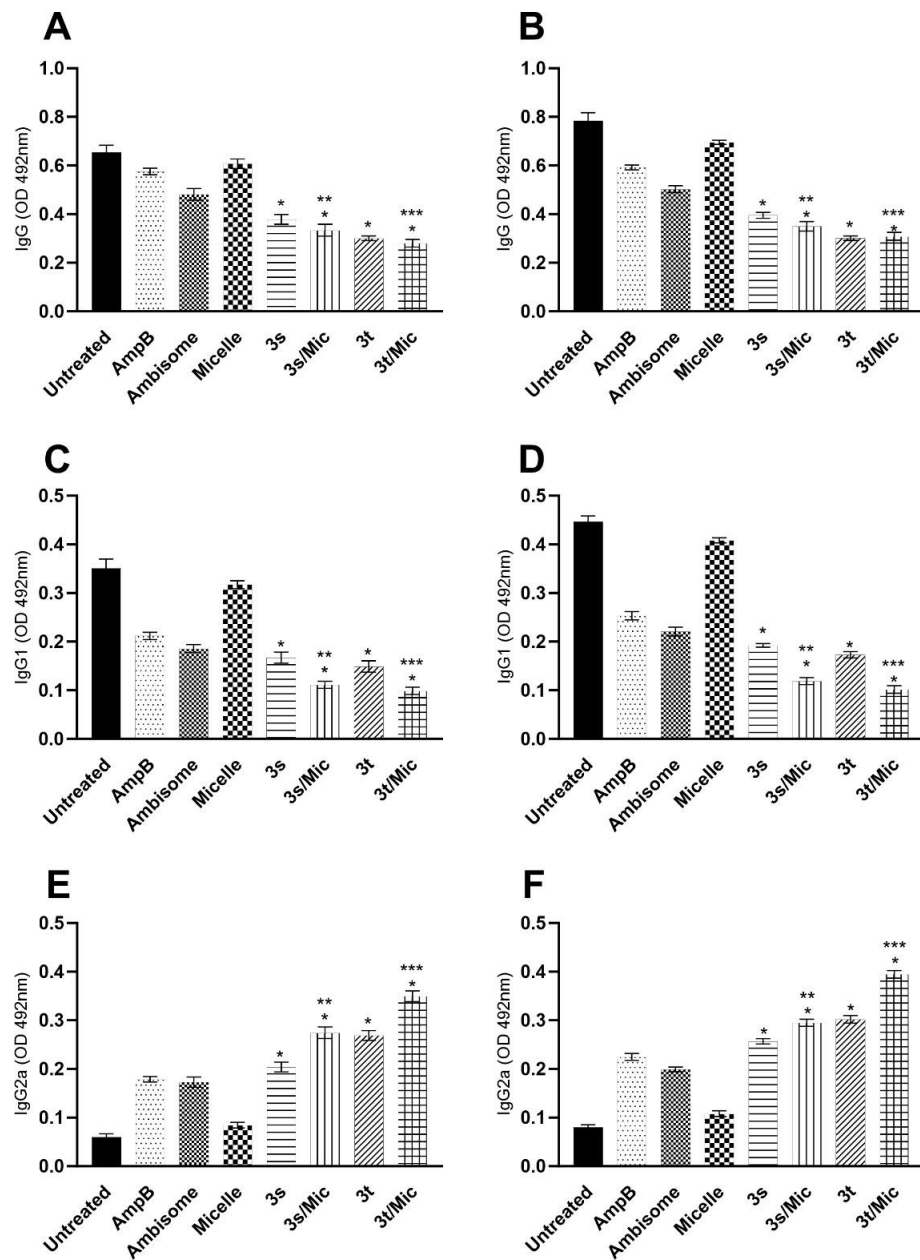


Figure 5. Humoral response generated after treatment.

Mice (n=16 per group) were infected with *L. amazonensis* promastigotes, and 60 days later, they were untreated or received amphotericin B (AmpB), micelles, Ambisome®, 3s, 3t, 3s/Mic, or 3t/Mic. On the 1st and 30th days after treatment, sera samples were collected (n=8 per group, each step) and levels of anti-SLA IgG, IgG1, and IgG2a antibodies were measured through an indirect ELISA. Results of the optical density (OD) values for IgG, IgG1, and IgG2a antibodies are shown on the 1st (A, C and E, respectively) and 30th (B, D and G, respectively) days after treatment. Bars indicate the mean $\pm$ standard deviation of the groups. * indicates significant difference in relation to the untreated, amphotericin B (AmpB), Ambisome®, and micelle groups ($P < 0.0001$). ** indicates significant difference in relation to the 3s group ($P < 0.001$). *** indicates significant difference in relation to the 3t group ($P < 0.001$).

Organic toxicity evaluated in the treated and infected animals

Enzymatic markers of renal and hepatic damages were evaluated in sera from treated mice, at both the 1st and 30th days after treatment. Results showed that the use of 3s/Mic and 3t/Mic not only reduced parasitism, but also influenced the improvement of the renal and hepatic conditions from infected and treated animals, since values of creatinine (**Figure 6A**), urea (**Figure 6B**), AST (**Figure 6C**), and ALT (**Figure 6D**) were quite similar to those found in naive mice, in both periods of time. By contrast, control groups mice produced significantly higher levels of these organic damage markers, which could reflect a parasitic action during the active infection. In addition, mice receiving AmpB presented significantly higher levels of urea, creatinine, AST, and ALT (**Figure 6**, panels A-D); suggesting then the occurrence of well-known toxicity caused by this drug in mammalian hosts.

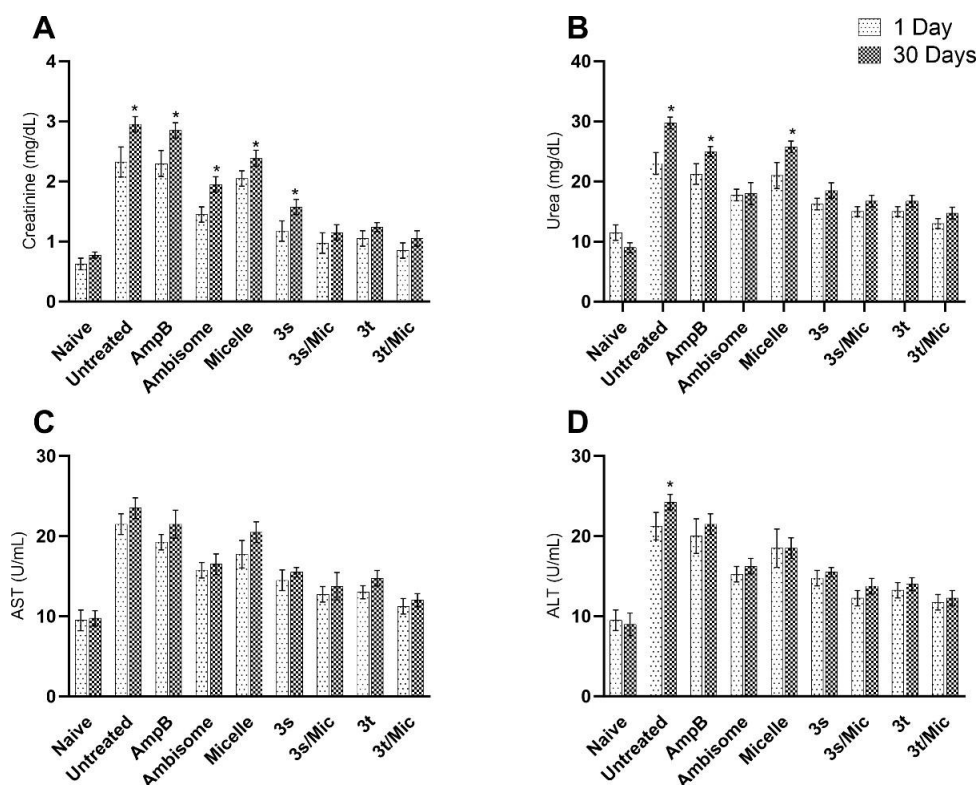


Figure 6. Analysis of *in vivo* toxicity.

The organic toxicity was evaluated in the treated animals, on the 1st (A) and 30th (B) days after treatment (n=8 per group, each step). For this, sera samples were collected and levels of urea, creatinine, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were measured, by using commercial kits. Sera from uninfected and untreated mice (naive) were used as control. Results are shown as bars, which indicate the mean $\pm$ standard deviation of the groups. * indicates significant difference in relation to the untreated, amphotericin B (AmpB), Ambisome®, and micelle groups (P < 0.0001). ** indicates significant difference in relation to the 3s group (P < 0.001). *** indicates significant difference in relation to the 3t group (P < 0.001).

Estimation of the parasite load after the treatment

The lesion's average diameter and parasite load were evaluated in the infected animals, during and after the course of treatment. Regarding the lesion's diameter, results showed that the mice from the 3s/Mic and 3t/Mic groups presented the significantly lower values, as compared to data found in the other groups (**Figure 7**). In addition, mice receiving 3s, 3t, and Ambisome[®] showed also a decrease in the lesion's diameter, when compared to data obtained in untreated and micelle groups mice; however, such values were significantly higher when compared to data obtained in the 3s/Mic and 3t/Mic groups.

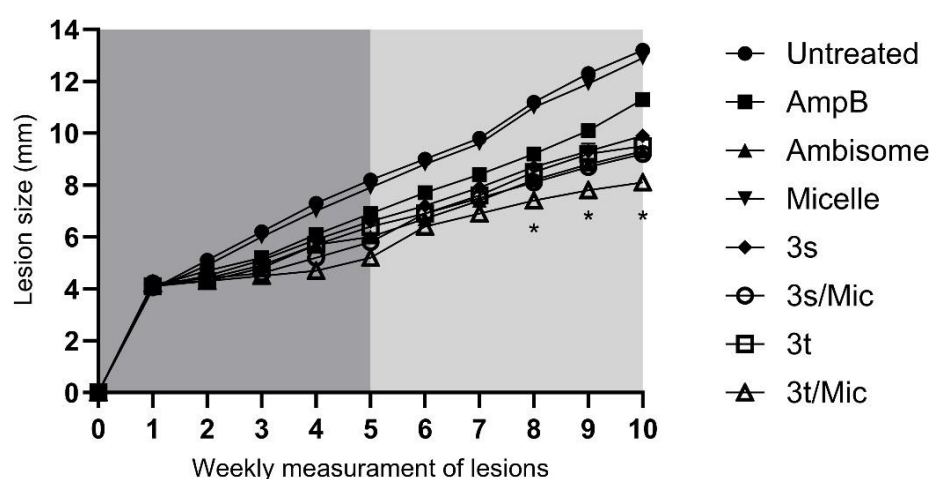


Figure 7. Lesion mean diameter during and after treatment.

Mice were infected with *L. amazonensis* promastigotes, and 60 days after infection (when they presented a lesion mean diameter of around 4 millimeters (mm), represented by week “1” in the x-axis), they were grouped (n=16 mice per group) and were untreated or received amphotericin B (AmpB), micelles, Ambisome[®], 3s, 3t, 3s/Mic, or 3t/Mic. During and after treatment, the lesion average diameter was measured weekly through an electronic caliper. Results are shown as lines, which indicate the mean of the groups. * indicates significant difference in relation to the untreated, amphotericin B (AmpB), Ambisome[®], and micelle groups ($P < 0.01$).

In the evaluation of the parasite load, animals treated with the 3s/Mic and 3t/Mic compositions presented significantly lower levels of parasitism in the infected tissue and organs, when compared to data found in the other groups, on both the 1st (**Figure 8**, panels **A**, **C**, **E**, and **G**) and 30th (**Figure 8**, panels **B**, **D**, **F**, and **H**) days after treatment. Additionally, animals receiving 3s, 3t, and Ambisome[®] presented also significant reductions in the parasite burden, when compared to the control mice, although such values have been significantly

higher in comparison to data from 3s/Mic and 3t/Mic groups (**Figure 8**). These results corroborate with the qPCR assay, since significantly lower levels of splenic parasitism were found in 3s/Mic and 3t/Mic groups mice, when compared to the other groups, on both the 1st (**Figure 9A**) and 30th (**Figure 9B**) days after treatment.

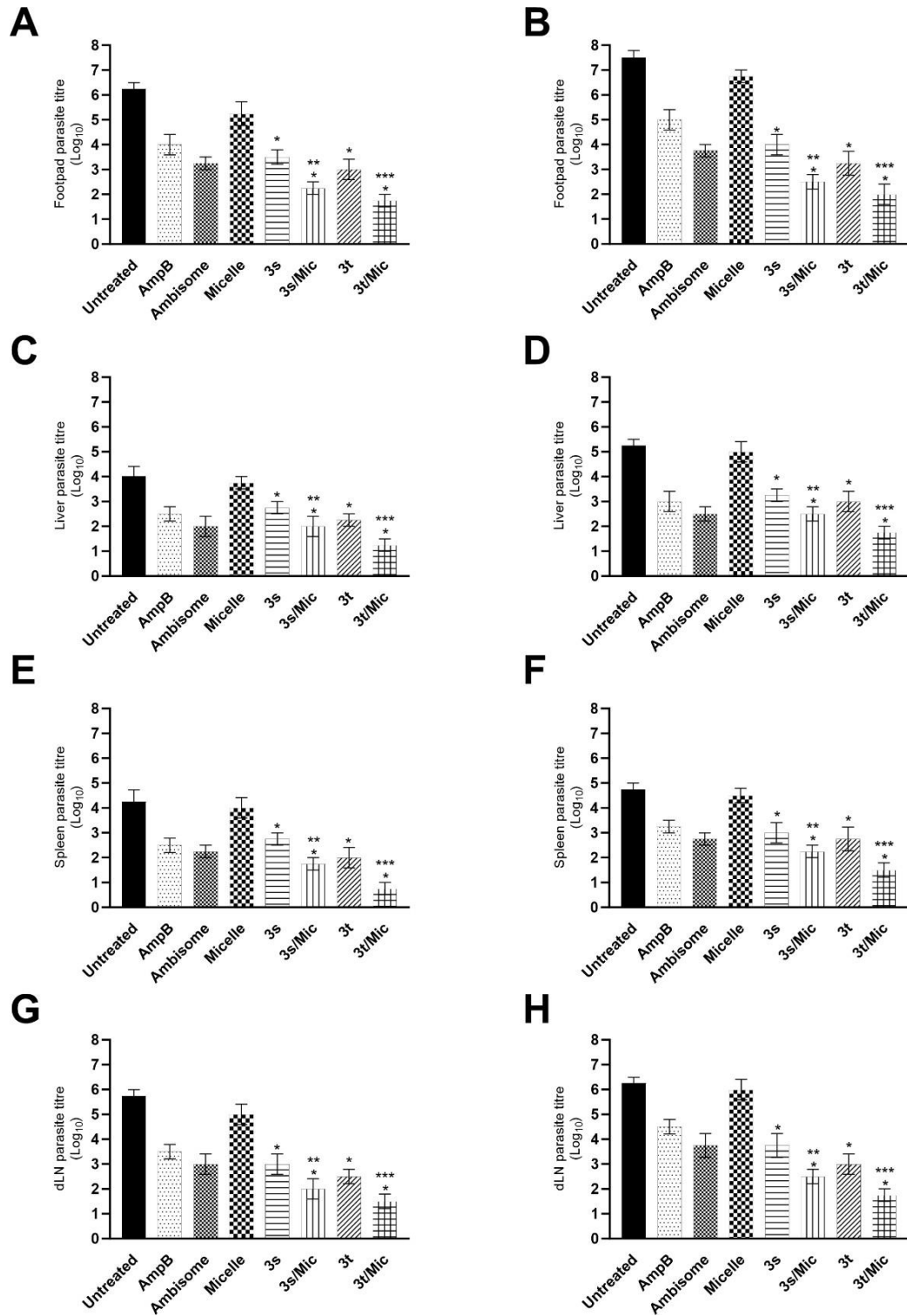


Figure 8. Parasite burden evaluated through a limiting dilution technique.

Mice (n=16 per group) were infected with *L. amazonensis* promastigotes, and 60 days after infection, they were untreated or received amphotericin B (AmpB), micelles, Ambisome®, 3s, 3t, 3s/Mic, or 3t/Mic. On the 1st and 30th (n=8 per group in each step) days after treatment; infected tissue and organs [livers, spleens, and draining lymph nodes (dLNs)] from animals were collected, and the parasite load was estimated through a limiting dilution technique. Results obtained on the 1st (A, C, E, and G) and 30th (B, D, F, and H) days after treatment are shown as bars, which represent the mean $\pm$ standard deviation of the groups. * indicates significant difference in relation to the untreated, amphotericin B (AmpB), Ambisome®, and micelle groups ($P < 0.0001$). ** indicates significant difference in relation to the 3s group ($P < 0.001$). *** indicates significant difference in relation to the 3t group ($P < 0.001$).

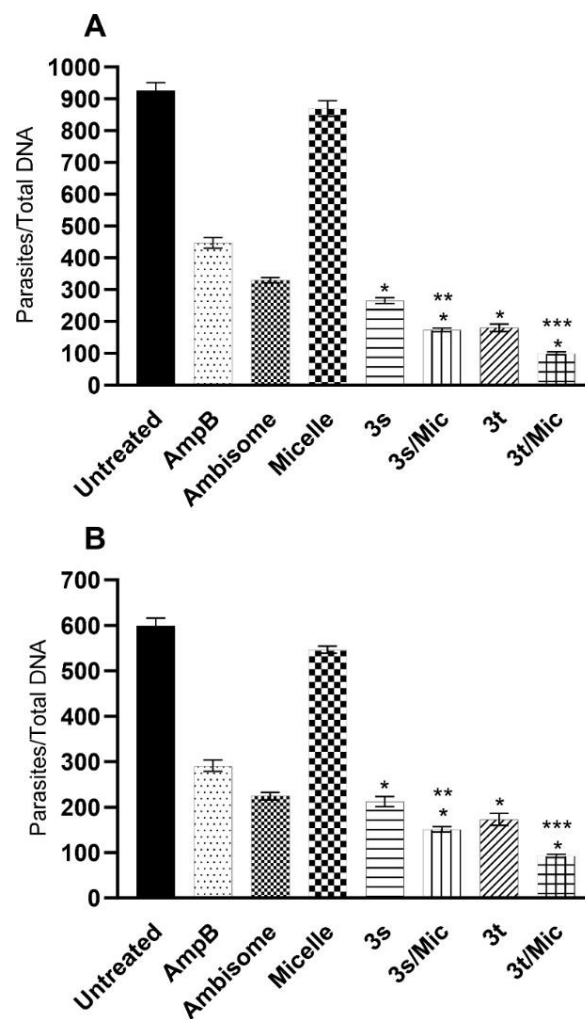


Figure 9. Parasite load evaluated through a qPCR technique.

The splenic parasite load was also evaluated in the treated animals (n=8 mice per group, each step) on the 1st (A) and 30th (B) days after treatment. Results are shown as bars, which indicate the mean $\pm$ standard deviation of the groups. * indicates significant difference in relation to the untreated, amphotericin B (AmpB), Ambisome®, and micelle groups ($P < 0.0001$). ** indicates significant difference in relation to the 3s group ($P < 0.001$). *** indicates significant difference in relation to the 3t group ($P < 0.001$).

DISCUSSION

Treatment against TL is based on the administration of pentavalent antimonials, even though the toxicity of the drugs, high costs, and parasite resistance have represented hindrances that limit the treatment success and highlight the need to obtain new and less toxic compounds.^{12,13} In addition, drug discovery is a long and expensive process, requiring an average of about 20 years and an investment of more than \$1.0 billion before a new biological product is developed and commercially approved for clinical applications.³³⁻³⁵ In this context, the search for new products that are active against *Leishmania* parasites, less toxic, and more effective is desirable, and natural compounds and their synthetic derivatives have gained considerable attention.^{16,17}

In previous work, our research group showed the antileishmanial activity of two synthetic derivatives from vanillin, called 3s and 3t, against promastigotes and intramacrophage amastigotes from *Leishmania* species able to cause VL and TL around the world. Both molecules were active against both parasite stages; they presented a selectivity index that was better than AmpB and were able to treat *Leishmania*-infected macrophages, thereby reducing the parasite load. In addition, a preliminary assay using cell culture supernatant from treated and infected macrophages showed that 3s and 3t induced high IL-12 and low IL-10 production, thus suggesting the development of an *in vitro* Th1-type immune response in the treated cells.²¹

In this context, this study was conducted to assess the *in vivo* antileishmanial activity of two vanillin derivatives for the treatment of *L. amazonensis*-infected BALB/c mice. To verify the adjuvant and delivery effect on the activity of the compounds, polymeric micelles were prepared and used to incorporate both molecules. In addition, AmpB and its liposomal formulation, Ambisome[®], were used as control, and results showed that the 3s/Mic and 3t/Mic compositions were more effective in combatting the infection in the animals, when analyses were performed on the 1st and 30th days after treatment. Experiments showed that, in addition to the decrease in lesion size, a reduced parasite load in the infected tissues, liver, spleen, and dLNs of the infected animals was observed, as well as the development of a polarized Th1-type cellular and humoral response. Additionally, treatment using 3s/Mic and 3t/Mic reduced the hepatic and renal damage markers to values near of those obtained using sera from non-infected animals.

Regarding the immunology on leishmaniasis, the development of specific T-cell responses are relevant to protection against disease, in which the production of pro-

inflammatory cytokines, such as IFN- γ , IL-2, IL-12, GM-CSF, TNF- α , among others, is critical for parasite clearance.³⁶⁻³⁸ Otherwise, other immunoregulatory cytokines, such as IL-4, IL-5, IL-10, among others, are activated following infection and can contribute to the suppression of anti-parasitic T-cell functions.^{39,40} Here, we found that animals treated with 3s, 3t, AmpB, and Ambisome[®], but especially 3s/Mic or 3t/Mic, developed a Th1-type cellular response, based on high levels of IFN- γ , IL-2, IL-12, and TNF- α , which corroborated with the reduction in lesion size and parasite load. On the other hand, the low IFN- γ and high IL-10 dosages found in the culture supernatants from the control groups showed that the occurrence of a Th2-type response was associated with a higher lesion diameter and parasitism in these animals, revealing a severe diagnosis of the disease.⁴⁰

Studies showing the association between regulatory cytokines and TL progression have been performed, and corroborated with data found in this work.^{41,42} The treatment of infected mice with 3s/Mic and 3t/Mic also led to an increase in the IFN- γ mRNA levels in the animals' splenocytes, constituting an important driving effect towards a Th1 response.⁴³ This effect was demonstrated at both endpoints after treatment, suggesting its association with the protective effects on mice infected with *L. amazonensis* parasites.⁴⁴ The compositions reduced the infection degree through the induction of NO production, as observed through the Griess reaction using the stimulated culture supernatants, indicating that both compositions showed *in vivo* leishmanicidal properties and action as potent inducers of IFN- γ production in the treated and infected mice, which possibly resulted in the parasite load reduction in these animals.^{26,45,46}

We also evaluated the humoral response induced by infection and the treatment of the animals, and results showed that the 3s/Mic and 3t/Mic compositions led to the development of high levels of anti-parasite IgG2a antibodies. By contrast, control mice produced higher levels of IgG and IgG1 isotype antibodies. Studies have shown that antileishmanial antibodies can play a critical role in the disease pathogenesis, leading to the development of more severe lesions in *L. amazonensis*-infected mice.^{47,48} Kima *et al.*⁴⁹ showed that the establishment of infection by this parasite species was impaired by the absence of circulating antibodies in the BALB/c mice or in XID mice, which displayed smaller lesions and a decrease in the IL-10 and antibody production, when compared to wild-type mice, thus suggesting a pathogenic role of B cells in *L. amazonensis* infection.^{50,51} Here, we found that treatment with both 3s/Mic and 3t/Mic induced higher IgG2a and lower IgG1 levels, corroborating with the higher IFN- γ and lower IL-4 production, respectively, which are associated with the switch of antibody isotypes described in mammals.⁵²

L. amazonensis-infected BALB/c mice are a non-cure model of *Leishmania* infection, where a progressive disease is found to be associated with parasitic migration to internal organs and consequent organic and tissue injury.^{53,54} Studies have showed that *L. amazonensis* may cause the localized, diffuse, or mucosal clinical forms of leishmaniasis, in addition to the capacity of dissemination in organs, causing the visceral form in this susceptible mammalian lineage.⁵⁵ In this context, although it is not the perfect model for testing the effectiveness of antileishmanial compounds, it is important to note that only the most active and promising drugs can reduce parasitism in BALB/c mice; therefore, any clinical improvement in these treated animals should be attributed to the effects of tested compounds.

Here, we used two evaluations regarding the parasitological response in treated and infected animals: the measuring of lesion size and parasite load. We found that the treatment using 3s/Mic and 3t/Mic were those in which more significant inhibitions were viewed in both lesion growth and parasite burden, as compared to data found in control group mice. The association of the molecules into polymeric micelles increased the treatment responses induced by these synthetic derivatives. Such a fact could be attributed to the delivery action of these products, since they interact with antigen-presenting cells, favoring the molecule's processing and uptake, stimulating the activation of a Th1-type immune response, increasing the retention of the molecule within the mouse's body and, consequently, allowing for a better response against the parasites in the treated animals.⁵⁶

Like described above, *L. amazonensis* infection in murine models can cause a progressive disease, in which parasite migration leads to organic and tissue injury, in turn causing internal damages.⁵⁷ In fact, studies have shown that renal and hepatic alterations represented by high levels of enzymatic markers, such as urea, creatinine, ALT, and AST, are found in the chronic infection by *Leishmania* parasites. In this context, an antileishmanial candidate should not only reduce parasitism, but also induce a clinic improvement in the treated hosts, as represented by the reduction of organic toxicity caused by infection. AmpB presents known antileishmanial activity, but it also causes severe adverse effects in the hosts, represented mainly by nephrotoxicity and hepatotoxicity, characterized by an increase in the urea, creatinine, ALT, and AST levels in the blood. AmBisome® is a lipid formulation of AmpB that is also effective against leishmaniasis.⁵⁸ In our study, it was also effective for the treatment of infected animals, when compared to data obtained in untreated mice or those receiving AmpB. However, treatment with 3s/Mic and 3t/Mic induced significantly lower values of hepatic and renal damage markers, when compared to those found in the others, being similar to data found in sera from naive mice. Such fact highlights the biological action of the micelle-based

compositions, and reflects the possibility to use such products as therapeutic targets against *L. amazonensis* infection.

In the present study, we have used a dose concentration of 5 mg/kg/day of pure 3s and 3t to treat the infected animals, besides a concentration of 2.5 mg/kg/day of the micellar compositions in the therapeutic regimens. Other studies have also used similar concentrations of synthetic molecules in their therapeutic schedules to treat Leishmania-infected mice, such as described in⁵⁹⁻⁶¹. Results obtained here could be considered as satisfactory, since significant reductions in the parasite burden and a specific antileishmanial Th1-type immune response were found in the animals after the treatments. However, a dose-response curve using different concentrations of 3s and 3t, mainly when incorporated to polymeric micelles, should be performed, aiming to define a minimal concentration of the micellar compositions able to induce the best therapeutic response, as well as to define the best route of administration of these products.

In this context, such facts can be considered as limitation of the study, and additional works are necessary to be performed aiming to solve such questions. Other limitations also include the absence of the evaluation of immunological, parasitological, and biochemical responses for longer periods of time; as well as the use of other drugs and delivery systems to treat infected animals. In this context, data presented here are preliminary and should be considered a proof-of-concept of the use of 3s/Mic and 3t/Mic as drug candidates against *L. amazonensis* infection.

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CONFLICT OF INTEREST

The authors confirm that they have no conflicts of interest in relation to this work.

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5.3.2. Considerações finais

O tratamento de camundongos BALB/c infectados com *L. amazonensis* com os derivados de vanilina 3s e 3t, em sua forma livre, mas principalmente incorporados em micelas poliméricas, foram mais efetivos no combate à infecção em relação aos grupos controles tratados com anfotericina B e AmBisome®, com efeito prolongado até 30 dias após o término da terapia. Os animais tratados com 3s/Mic e 3t/Mic desenvolveram uma resposta imune celular e humoral do tipo Th1 baseada nos altos níveis de IFN- γ , IL-2, IL-12, TNF- α , nitrito e IgG2a, corroborando com os achados de redução do tamanho das lesões e da carga parasitária nos tecidos infectado, baço, fígado e linfonodos drenantes desses grupos.

Além disso, o tratamento com 3s/Mic e 3t/Mic reduziram os valores dos marcadores de danos hepático e renal a níveis próximos aos obtidos no soro de animais não infectados. Desse modo, tendo em vista a efetiva atividade biológica dessas composições micelares, 3s e 3t podem ser consideradas como candidatas, em estudos futuros, no tratamento da leishmaniose tegumentar.

6. CONSIDERAÇÕES FINAIS

O presente estudo, de caráter básico e exploratório, objetivou identificar novos compostos capazes de controlar e/ou proteger contra as leishmanioses visceral e tegumentar, considerando as limitações dos fármacos atuais disponíveis como alta toxicidade e alto custo, bem como o surgimento de cepas resistentes. Aqui, foram descritos pela primeira vez o flavonoide CMt extraído da planta *M. tinctoria* e duas moléculas sintéticas derivadas da vanilina (3s e 3t) que demonstram ação antileishmanial contra *L. infantum* e *L. amazonensis*.

A avaliação da efetividade desses compostos fenólicos ampliou o conhecimento a respeito dos produtos naturais e de seus derivados sintéticos, assim como propiciou a descoberta de sua potencial atividade biológica, manifestando-se como uma alternativa mais eficaz, segura e economicamente viável para o tratamento das leishmanioses.

Os resultados apresentados demonstraram que CMt, 3s e 3t reduziram a carga parasitária e induziram uma resposta imune celular e humoral específica do tipo Th1 nos animais, concordante com o perfil de controle da infecção, além de baixa toxicidade orgânica. A efetividade desses compostos foi ainda maior ao serem incorporados em micelas poliméricas, visto que favorecem o processamento e a captação dos produtos pelas células, e aumentam a sua retenção no organismo. Apesar da ausência de avaliações imunológicas, parasitológicas e bioquímicas por longos períodos de tempo, os esquemas terapêuticos propostos no estudo, especialmente formulações micelares dos compostos fenólicos, sugerem efeito prolongado da ação antileishmanial ao apresentarem perfis de resposta igual ou melhor após 30 (3s e 3t) ou 15 (CMt) dias do término do tratamento.

Desse modo, além da prova de conceito, o referido trabalho contribui de maneira considerável para pesquisas futuras com vistas a melhora das condições de tratamento das leishmanioses e, por conseguinte, estimula a comunidade científica a atentar-se para a descoberta de novos fármacos. Vale ressaltar, ainda, a biodiversidade vegetal do Brasil e a potencialidade de moléculas bioativas encontradas em plantas, tal qual os derivados sintéticos análogos aos naturais que, quimicamente modificados, permitem atingir máxima eficácia terapêutica e baixa toxicidade.

Assim sendo, os compostos CMt, 3s e 3t são facilmente extraídos/produzidos com alto rendimento, baixo custo e, juntamente à eficaz atividade antileishmanial, mostram-se como alternativa à utilização das terapias vigentes.

7. CONCLUSÃO

O presente estudo evidenciou o uso de novos compostos fenólicos como potencial estratégia terapêutica para as leishmanioses visceral e tegumentar, bem como demonstrou maior eficácia ao serem incorporados em sistemas poliméricos, potencializando a sua ação antileishmanial. Desse modo, CMt, 3s e 3t micelares podem ser consideradas em estudos futuros para o tratamento das leishmanioses.

8. PERSPECTIVAS

Pretende-se dar continuidade ao presente trabalho com a realização de:

- Ensaios para avaliar a efetividade das composições micelares contendo CMt, 3s e 3t em hamster como abordagem terapêutica, para as leishmanioses tegumentar e visceral;
- Estudos de associação desses compostos com outros fármacos já empregados no tratamento das leishmanioses, para verificar a potencialização da atividade antileishmanial.

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APÊNDICE A – Artigos publicados e apresentados na tese

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WILEY

Parasitological and immunological evaluation of a novel chemotherapeutic agent against visceral leishmaniasis

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Abstract

Aims: Treatment for visceral leishmaniasis (VL) is hampered by the toxicity and/or high cost of drugs, as well as by emergence of parasite resistance. Therefore, there is an urgent need for new antileishmanial agents.

Methods and Results: In this study, the antileishmanial activity of a diprenylated flavonoid called 5,7,3,4'-tetrahydroxy-6,8-diprenylisoflavone (CMT) was tested against *Leishmania infantum* and *L. amazonensis* species. Results showed that CMT presented selectivity index (SI) of 70.0 and 165.0 against *L. infantum* and *L. amazonensis* promastigotes, respectively, and of 181.9 and 397.8 against respective axenic amastigotes. Amphotericin B (AmB) showed lower SI values of 9.1 and 11.1 against *L. infantum* and *L. amazonensis* promastigotes, respectively, and of 12.5 and 14.3 against amastigotes, respectively. CMT was effective in the treatment of infected macrophages and caused alterations in the parasite mitochondria. *L. infantum*-infected mice treated with miltefosine, CMT alone or incorporated in polymeric micelles (CMT/Mic) presented significant reductions in the parasite load in distinct organs, when compared to the control groups. An antileishmanial Th1-type cellular and humoral immune



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New synthetic molecules incorporated into polymeric micelles used for treatment against visceral leishmaniasis

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Vanillin
Polymeric micelles

ABSTRACT

Treatment against visceral leishmaniasis (VL) presents problems, mainly related to drug toxicity, high cost and/or by emergence of resistant strains. In the present study, two vanillin synthetic derivatives, 3 s [4-(2-hydroxy-3-(4-octyl-1H-1,2,3-triazol-1-yl)propoxy)-3-methoxybenzaldehyde] and 3 t [4-(3-(4-decyl-1H-1,2,3-triazol-1-yl)-2-hydroxypropoxy)-3-methoxybenzaldehyde], were evaluated as therapeutic candidates in a murine model against *Leishmania infantum* infection. Molecules were used pure (3 s and 3 t) or incorporated into Poloxamer 407-based micelles (3 s/M and 3 t/M) in the infected animals, which also received amphotericin B (AmpB) or Ambisome® as control. Results showed that 3 s/M and 3 t/M compositions induced a Th1-type immune response in treated animals, with higher levels of IFN- γ , IL-2, TNF- α , IL-12, nitrite, and IgG2a antibodies. Animals presented also low toxicity and significant reductions in the parasite load in their spleens, livers, bone marrows and draining lymph nodes, as compared as control groups mice, with the evaluations performed one and 30 days after the application of the therapeutics. In conclusion, preliminary data suggest that 3 s/M and 3 t/M could be considered for future studies as therapeutic agents against VL.

1. Introduction

Leishmaniases are tropical diseases endemic in 98 countries, with 12 million people clinically affected [55]. Visceral leishmaniasis (VL) is the more severe clinic form of this disease complex, and it has a 95 % fatality

rate in acute and untreated cases, with an estimated incidence of about 0.5 million new cases annually [4]. The causative agents of the VL are the *Leishmania donovani* and *L. infantum* species [29]. *L. donovani* is found in East-Africa, India and parts of the Middle East. *L. infantum* is the main etiologic agent of VL in the New World, causing the disease in

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Treatment using vanillin-derived synthetic molecules incorporated into polymeric micelles is effective against infection caused by *Leishmania amazonensis* species

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ABSTRACT

Treatment against leishmaniasis presents problems, mainly due to the toxicity of the drugs, high cost, and the emergence of resistant strains. A previous study showed that two vanillin-derived synthetic molecules, 3s [4-(2-hydroxy-3-(4-octyl-1H-1,2,3-triazol-1-yl)propoxy)-3-methoxybenzaldehyde] and 3t [4-(3-(4-decyl-1H-1,2,3-triazol-1-yl)-2-hydroxypropoxy)-3-methoxybenzaldehyde], presented antileishmanial activity against *Leishmania infantum*, *L. amazonensis*, and *L. braziliensis* species. In the present work, 3s and 3t were evaluated to treat *L. amazonensis*-infected mice. Molecules were used pure or incorporated into Poloxamer 407-based micelles. In addition, amphotericin B (AmpB) and its liposomal formulation, Ambisome®, were used as control. Animals received the treatment and, one and 30 days after, they were euthanized to evaluate immunological, parasitological, and biochemical parameters. Results showed that the micellar compositions (3s/Mic and 3t/Mic) induced significant reductions in the lesion mean diameter and parasite load in the infected tissue and distinct organs, as well as a specific and significant antileishmanial Th1-type immune response, which was based on significantly higher levels of IFN- γ , IL-12, nitrite, and IgG2a isotype antibodies. Drug controls showed also antileishmanial action; although 3s/Mic and 3t/Mic have presented better and more significant parasitological and immunological data, which were based on significantly higher IFN- γ production and lower parasite burden in treated animals. In addition, significantly lower levels of urea, creatinine, alanine transaminase, and aspartate transaminase were found in mice treated with 3s/Mic and 3t/Mic, when compared to the others. In conclusion, results suggest that 3s/Mic and 3t/Mic could be considered as therapeutic candidates to treat against *L. amazonensis* infection.

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APÊNDICE B – Produções científicas durante o doutoramento

Artigos

1. FREITAS, Camila S.; **PEREIRA, Isabela A.G.**; LAGE, Daniela P.; VALE, Danniele L.; PIMENTA, Breno L. ; SOARES, Nícia P.; SANTIAGO, Samira S.; MARTINS, Vívian T.; CÂMARA, Raquel S.B.; JESUS, Marcelo M.; TAVARES, Grasielle S.V.; RAMOS, Fernanda F.; LUDOLF, Fernanda; MAGALHÃES, Lícia N.D.; OLIVEIRA, Fabrício M.; DUARTE, Mariana C.; CHÁVEZ-FUMAGALLI, Miguel A.; COSTA, Adilson V.; ROATT, Bruno M.; COELHO, Eduardo A. F. New synthetic molecules incorporated into polymeric micelles used for treatment against visceral leishmaniasis. **Cytokine**, v.177, p.156543, 2024.
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2. COELHO, E. A. F.; RAMOS, F. F.; RIBEIRO, F. L.; SANTOS, T. T. O.; MACHADO, A. S.; TAVARES, G. S. V.; **PEREIRA, I. A. G.**; GOULART, L. R.; VAZ, E. R.; SOUZA, T. A. (PATENTE NACIONAL) Peptídeos recombinantes, kit e método para diagnóstico da leishmaniose visceral e coinfeção com vírus da imunodeficiência humana. 2021, Brasil. Patente: Privilégio de Inovação. Número do registro: BR1020210152095, título: "(PATENTE NACIONAL) Peptídeos recombinantes, kit e método para diagnóstico da leishmaniose visceral e coinfeção com vírus da imunodeficiência humana", Instituição de registro: INPI - Instituto Nacional da Propriedade Industrial. Depósito: 02/08/2021.

3. COELHO, E. A. F.; OLIVEIRA-SANTOS, T. T.; **PEREIRA, I. A. G.**; TAVARES, G. S. V.; RAMOS, F. F.; RIBEIRO, F. L.; OLIVEIRA-DA-SILVA, J. A.; REIS, T. A. (PATENTE NACIONAL) Kit, método para diagnóstico da leishmaniose visceral e coinfeção com vírus da imunodeficiência humana, e usos. 2021, Brasil. Patente: Privilégio de Inovação. Número do registro: BR1020210260424, título: "(PATENTE NACIONAL) Kit, método para diagnóstico da leishmaniose visceral e coinfeção com vírus da imunodeficiência humana, e usos", Instituição de registro: INPI - Instituto Nacional da Propriedade Industrial. Depósito: 22/12/2021.

4. GALDINO, A. S.; CHAVES, A. T.; COELHO, E. A. F.; GALDINO, M. C. P. L.; MAIA, A. C. G.; GIUNCHETTI, R. C.; FRANCISCO, M. F. C.; TAVARES, G. S. V.; **PEREIRA, I. A. G.**; RIBEIRO, A. J.; GANDRA, I. B.; NOGUEIRA, L. M.; MACHADO, J. M. (PATENTE NACIONAL) Peptídeo sintético, método para diagnóstico da Doença de Chagas e uso. 2022, Brasil. Patente: Privilégio de Inovação. Número do registro: BR1020220180261, título: "(PATENTE NACIONAL) Peptídeo sintético, método para diagnóstico da Doença de Chagas e uso", Instituição de registro: INPI - Instituto Nacional da Propriedade Industrial. Depósito: 09/09/2022.

5. COELHO, E. A. F.; RAMOS, F. F.; RIBEIRO, F. L. ; MACHADO-DE-AVILA, R. A.; CARDOSO, M. M.; BANDEIRA, R. S.; **PEREIRA, I. A. G.**; ANASTACIO, R. S.; LUIZ, G. P. (PATENTE NACIONAL) Proteína quimérica recombinante, kit e método para diagnóstico de infecção pelo SARS-COV-2. 2023, Brasil. Patente: Privilégio de Inovação. Número do registro: BR1020230161120, título: "(PATENTE NACIONAL) Proteína quimérica recombinante, kit e método para diagnóstico de infecção pelo SARS-COV-2", Instituição de registro: INPI - Instituto Nacional da Propriedade Industrial. Depósito: 10/08/2023.

ANEXO A – Conselho de Gestão do Patrimônio Genético (CGen/MMA)**MINISTÉRIO DO MEIO AMBIENTE
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO****DELIBERAÇÃO Nº 246, DE 27 DE AGOSTO DE 2009**

Credencia o Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq para autorizar outras instituições a realizar as atividades que especifica, e dá outras providências.

O MINISTRO DE ESTADO DO MEIO AMBIENTE faz saber que o CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO, no uso das competências que lhe foram conferidas pela Medida Provisória nº 2.186-16, de 23 de agosto de 2001, e pelo Decreto nº 3.945, de 28 de setembro de 2001, tendo em vista o disposto no art. 13, inciso III, e no art. 14 do seu Regimento Interno, publicado por meio da Portaria nº 316, de 25 de junho de 2002, resolve:

Art. 1º Credenciar o Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq para autorizar instituições nacionais, públicas ou privadas, que exerçam atividades de pesquisa e desenvolvimento nas áreas biológicas e afins a:

I - acessar amostra de componente do patrimônio genético para fins de pesquisa científica; e

II - remeter amostra de componente do patrimônio genético a instituição sediada no exterior, para fins de pesquisa científica.

§ 1º No exercício das competências a que se refere este artigo, o CNPq poderá dispensar a anuência prévia formal, nos termos da Resolução no 8, de 24 de setembro de 2003.

§ 2º O credenciamento a que se refere este artigo não inclui a competência para autorizar o acesso ao conhecimento tradicional associado, ainda que este envolva o acesso ao patrimônio genético.

§ 3º O credenciamento de que trata este artigo não prejudica o disposto na Deliberação no 40, de 24 de setembro de 2003.

Art. 2º No exercício das prerrogativas que lhe são conferidas por meio do credenciamento de que trata esta Deliberação, o CNPq obriga-se a:

I - observar as Resoluções e Orientações Técnicas aprovadas pelo Conselho de Gestão do Patrimônio Genético-CGEN;

II - encaminhar ao CGEN as solicitações de autorização de acesso e remessa de patrimônio genético para fins de bioprospecção e desenvolvimento tecnológico e as que envolverem acesso a conhecimento tradicional associado;

III - manter e disponibilizar ao CGEN as bases de dados previstas no art. 10, inciso III, alínea "c", itens 2 e 3, do Decreto no 3.945, de 28 de setembro de 2001;

IV - encaminhar ao CGEN relatório anual das atividades realizadas.

Art. 3º Fica criado, no âmbito do CGEN, grupo de trabalho permanente composto por representantes da Secretaria-Executiva do CGEN e dos órgãos e entidades credenciadas, indicados pelos representantes dessas instituições junto ao CGEN, com as seguintes atribuições:

I - proceder à harmonização de procedimentos e normas para concessão de autorizações de que trata esta Deliberação entre os órgãos e entidades credenciadas; e

II - avaliar periodicamente o desempenho das atividades e procedimentos adotados na concessão de autorização pelos órgãos e entidades credenciadas e submeter relatório sobre estas atividades ao CGEN anualmente e quando entender necessário.

Parágrafo único. O grupo de trabalho a que se refere este artigo será coordenado por representante da Secretaria-Executiva do CGEN.

Art. 4º O CNPq implementará as atividades para as quais foi credenciado até o dia 1º de março de 2010.

Art. 5º Esta Deliberação entra em vigor na data de sua publicação.

CARLOS MINC

Ministro de Estado do Meio Ambiente

ANEXO B – Certificado da Comissão de Ética no Uso de Animais (CEUA/UFMG)



UNIVERSIDADE FEDERAL DE MINAS GERAIS

CEUA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o projeto intitulado "Emprego de moléculas sintéticas no tratamento in vitro e in vivo das leishmanioses.", protocolo do CEUA: 85/2017 sob a responsabilidade de Eduardo Antonio Ferraz Coelho que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem) para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899 de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DA UNIVERSIDADE FEDERAL DE MINAS GERAIS, em reunião de 05/06/2017.

Vigência do Projeto (requerida pelo pesquisador)	20/05/2017 a 10/01/2021
Finalidade	Pesquisa
*Espécie/linhagem	Camundongo isogênico / BALB/c
Nº de animais	8
Peso/Idade	20g / 8(semanas)
Sexo	feminino
Origem	Biotério do ICB/UFMG
*Espécie/linhagem	Camundongo isogênico / BALB/c
Nº de animais	8
Peso/Idade	20g / 8(semanas)
Sexo	feminino
Origem	Biotério do ICB/UFMG
*Espécie/linhagem	Camundongo isogênico / BALB/c
Nº de animais	8
Peso/Idade	20g / 8(semanas)
Sexo	feminino
Origem	Biotério do ICB/UFMG
*Espécie/linhagem	Camundongo isogênico / BALB/c
Nº de animais	8
Peso/Idade	20g / 8(semanas)
Sexo	feminino
Origem	Biotério do ICB/UFMG
*Espécie/linhagem	Camundongo isogênico / BALB/c
Nº de animais	8
Peso/Idade	20g / 8(semanas)

Sexo	feminino
Origem	Biotério do ICB/UFMG
*Espécie/linhagem	Camundongo isogênico / BALB/c
Nº de animais	8
Peso/Idade	20g / 8(semanas)
Sexo	feminino
Origem	Biotério do ICB/UFMG
*Espécie/linhagem	Camundongo isogênico / BALB/c
Nº de animais	8
Peso/Idade	20g / 8(semanas)
Sexo	feminino
Origem	Biotério do ICB/UFMG

Considerações posteriores:

05/06/2017	Aprovado na reunião do dia 05/06/2017. Validade: 05/06/2017 à 04/06/2022
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Belo Horizonte, 23/09/2018.

Atenciosamente,

Sistema Solicite CEUA UFMG
https://aplicativos.ufmg.br/solicite_ceua/

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