UNIVERSIDADE FEDERAL DE MINAS GERAIS
Faculdade de Medicina
Programa de Pós-Graduação em Ciências da Saúde:
Infectologia e Medicina Tropical

AVALIAÇÃO DA ATIVIDADE ANTILEISHMANIAL DE CARDENOLÍDEOS PARA USO NO TRATAMENTO DA LEISHMANIOSE VISCERAL

Camila Simões de Freitas

Belo Horizonte
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Dissertação apresentada ao Programa de Pós-graduação em Ciências da Saúde: Infectologia e Medicina Tropical da Faculdade de Medicina da UFMG, como requisito parcial para a obtenção do grau de Mestre junto ao referido Programa.

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CAMILA SIMÕES DE FREITAS

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“Lembre-se sempre de que onde você está é resultado de quem você *era,* mas para onde você vai depende inteiramente de quem você escolher ser, a partir desse momento.”

Hal Elrod

CONSIDERAÇÕES INICIAIS

Esta dissertação de mestrado teve como objetivo avaliar dois derivados de cardenolídeos, digitoxigenina e β-acetil-digitoxina, por meio de experimentos *in vitro* e *in vivo* contra a espécie *Leishmania infantum*, principal espécie do parasito *Leishmania,* causador da leishmaniose visceral nas Américas. O estudo faz parte da linha de pesquisa "Leishmanioses" que venho desenvolvendo no laboratório e a escolha desse tema se deu devido à necessidade da melhoria das condições de tratamento contra a doença, uma vez que os fármacos atuais utilizados apresentam toxicidade elevada, alto custo, administração dolorosa e/ou falha terapêutica.

Outro ponto importante na escolha do tema se deve ao fato do orientador desse trabalho atuar em pesquisas a respeito das leishmanioses desde o ano de 2000 por meio do desenvolvimento de projetos nas linhas de vacina, diagnóstico e tratamentos/desenvolvimento de novos produtos terapêuticos. O trabalho possibilitou a geração de resultados que, por definição do grupo de pesquisa, foram divididos em dois artigos científicos que foram submetidos para publicação em revistas de elevado impacto internacional. Tais documentos serão apresentados nesta dissertação de mestrado.

A apresentação deste documento foi realizada de acordo com a Resolução nº02/2013, de 18 de setembro de 2013; que regulamenta o formato dos trabalhos finais de qualificação, estabelecendo condições para a marcação das defesas de teses e de dissertações do Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical da Faculdade de Medicina da UFMG.

LISTA DE ABREVIATURAS E SIGLAS

ALT	Alanina amino transferase
AmpB	Anfotericina B
AmpB/M	Anfotericina B micelar
ANOVA	Análise de variância
AST	Aspartato amino transferase
BSA	Albumina sérica bovina
B/Mic	Micela branca
b-AD	β-acetil-digitoxina
b-AD/Mic	β-acetil-digitoxina micelar
CC_{50}	Concentração da substância necessária para inviabilizar 50% de macrófagos
CEUA	Comitê de Ética no Uso de Animais
CK-MB	*Creatine kinase* fração músculo-cardíaca
dLN	Linfonodo drenante
DIGI	Digitoxigenina
DIGI/Mic	Digitoxigenina micelar
DMEM	*Dulbecco's Modified Eagle Medium*
DTNs	Doenças tropicais negligenciadas
ELISA	Análise de imunoabsorção por ligação enzimática
FBS	Soro fetal bovino
GM-CSF	Fator estimulador de colônias macrófago-granulócito
IC_{50}	Concentração da substância necessária para inibir a viabilidade de 50% de parasitos
IFN-γ	Interferon-gama
IgG	Imunoglobulina G
IL	Interleucina
IS	Índice de seletividade
LC	Leishmaniose cutânea
LCD	Leishmaniose cutâneo-difusa
LM	Leishmaniose mucosa

LT	Leishmaniose tegumentar
LV	Leishmaniose visceral
L-AmpB	Anfotericina lipossomal
MTT	3-(4,5-dimetiltiazol-2yl)-2,5-difenil brometo de tetrazolina
NO	Óxido nítrico
OD	Densidade ótica
OMS	Organização Mundial da Saúde
PBS	Tampão fosfato salina
PCR	Reação em cadeia da Polimerase
qPCR	PCR quantitativo
RBC_{50}	Concentração dos compostos necessários para causar 50% de lise em hemácias humanas
ROS	Espécies reativas de oxigênio
SLA	Extrato proteico solúvel de *Leishmania*
TGF-β	Fator de transformação de crescimento beta
Th1	T *helper* tipo 1
Th2	T *helper* tipo 2
TNF-α	Fator de necrose tumoral alfa
WHO	*World Health Organization*

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RESUMO

As leishmanioses são um complexo de doenças causadas por parasitos protozoários do gênero Leishmania, e podem ser divididas em dois grandes grupos, a leishmaniose tegumentar e a leishmaniose visceral. O tratamento atual da leishmaniose visceral (LV) apresenta limitações devido à toxicidade das drogas e/ou alto custo, juntamente com o surgimento de resistência dos parasitos. O desenvolvimento de medicamentos é um processo longo e caro e, portanto, o reposicionamento de fármacos pode representar uma alternativa. Cardenolídeos são usados no tratamento de doenças cardíacas, especialmente, aqueles obtidos em espécies do gênero *Digitalis*. No presente estudo, os cardenolídeos β-acetil-digitoxina (b-AD) e digitoxigenina (DIGI), obtidos do extrato metanólico de folhas de *Digitalis lanata*, foram avaliados *in vitro* e *in vivo* contra *Leishmania infantum*. Os resultados mostraram ação direta de b-AD e DIGI contra os parasitos, bem como eficácia no tratamento de macrófagos infectados. A investigação do mecanismo de ação mostrou que b-AD e DIGI induziram alterações no potencial da membrana mitocondrial, aumentaram os níveis de espécies reativas de oxigênio e causaram o acúmulo de corpos lipídicos nos parasitos. DIGI e b-AD foram incorporados em um sistema de micelas poliméricas à base de Pluronic® F127 (DIGI/Mic e b-AD/Mic, respectivamente) e foram usados para tratar camundongos infectados com *L. infantum*. A miltefosina foi usada como controle. Os animais tratados com miltefosina, b-AD ou b-AD/Mic e DIGI ou DIGI/Mic apresentaram reduções significativas da carga parasitária no baço, fígado, medula óssea (BM) e linfonodos drenantes (dLN), além do desenvolvimento de uma resposta específica do tipo Th1, atestada pelos altos níveis de IFN-γ, IL-12, TNF-α, GM-CSF, nitrito e anticorpos dos isotipos IgG2a, além de baixos níveis de IL-4 e IL-10, juntamente com maior frequência de células T $CD4^+$ e $CD8^+$ produtoras de IFN-γ, quando comparados aos controles. Os resultados obtidos um dia após o tratamento foram corroborados com os dados encontrados 15 dias após o mesmo. Em conclusão, os resultados sugerem que b-AD/Mic e DIGI/Mic poderiam ser considerados para a realização de novos estudos para o tratamento contra a LV.

Palavras-chave: Tratamento; β-acetil-digitoxina; digitoxigenina; reposicionamento de fármacos; leishmaniose visceral; cardenolídeos; miltefosina.

ABSTRACT

Leishmaniasis is a complex of diseases caused by protozoan parasites of the genus Leishmania, and can be divided into two major groups, cutaneous leishmaniasis and visceral leishmaniasis. The current treatment of visceral leishmaniasis (VL) has limitations due to drug toxicity and/or high cost, along with the emergence of parasite resistance. Drug development is a long and expensive process and, therefore, drug repositioning may represent an alternative. Cardenolides are used in the treatment of heart disease, especially those obtained from species of the genus Digitalis. In the present study, β-acetyl-digitoxin (b-AD) and digitoxigenin (DIGI), obtained from the methanolic extract of leaves of Digitalis lanata, were evaluated in vitro and in vivo against Leishmania infantum. The results showed direct action of b-AD and DIGI against the parasites, as well as efficacy in the treatment of infected macrophages. The investigation of the mechanism of action showed that b-AD and DIGI induced changes in the potential of the mitochondrial membrane, increased the levels of reactive oxygen species and caused the accumulation of lipid bodies in the parasites. DIGI and b-AD were incorporated into a polymeric micelle system based on Pluronic® F127 (DIGI/Mic and b-AD/Mic, respectively) and were used to treat mice infected with L. infantum. Miltefosine was used as a control. Animals treated with miltefosine, b-AD or b-AD/Mic and DIGI or DIGI/Mic showed significant reductions in parasitic load on the spleen, liver, bone marrow (BM) and draining lymph nodes (dLN), as well as the development of a specific Th1-type response, attested by the high levels of IFN-γ, IL-12, TNF-α, GM-CSF, nitrite and IgG2a isotype antibodies, in addition to low IL-4 and IL-10 contents, along with higher IFN-γ-producing $CD4^+$ and $CD8^+$ T-cell frequency, when compared to controls. The results obtained one day after the treatment were corroborated with the data found 15 days after the treatment. In conclusion, the results suggest that b-AD/Mic and DIGI/Mic could be considered for additional studies for the treatment against VL.

Keywords: Treatment; β-acetyl-digitoxin; digitoxigenin; drug repositioning; visceral leishmaniasis; cardenolides; miltefosine.

1. INTRODUÇÃO

Doenças tropicais negligenciadas (DTNs) são, em sua maioria, doenças infecciosas que afetam milhares de pessoas ao redor do mundo, atingindo principalmente, países subdesenvolvidos ou em desenvolvimento (CROFT; COOMBS, 2003). Apesar de haver um grande volume de trabalhos científicos dedicados à biologia, imunologia e genética dos parasitos causadores dessas doenças, não se consegue reverter o conhecimento adquirido em novas ferramentas terapêuticas contra as mesmas (MIRANDA-VERASTEGUI *et al.*, 2005).

As leishmanioses são doenças que integram o grupo das DTNs, uma vez que ocorrem nos países mais pobres e atingem as populações mais vulneráveis e com difícil acesso aos serviços de saúde (PAHO, 2019). São causadas por parasitos protozoários do gênero *Leishmania*, sendo consideradas endêmicas em 98 países no mundo, incluindo o Brasil (DUJARDIN, 2006; AMEEN, 2010;).

No Brasil, cerca de sete espécies do parasito podem causar a doença no homem, sendo que seis destas (*L. braziliensis, L. amazonensis, L. guyanensis, L. lansoni, L. shawii* e *L. naiffi*) são responsáveis pelos casos de leishmaniose tegumentar (LT) e a espécie *L. infantum* é responsável pelos casos de leishmaniose visceral (LV) (ALVAR *et al.*, 2012). As manifestações clínicas da doença dependem principalmente da espécie infectante do parasito e da resposta imunológica do hospedeiro infectado (NAKAMURA *et al.*, 2006).

O tratamento das leishmanioses deve ser realizado para se evitar a mortalidade causada pela LV e reduzir a morbidade provocada pelas lesões desfigurantes na LT. Normalmente, o mesmo consiste na aplicação de compostos antimoniais pentavalentes, dos quais o estibogluconato de sódio (Pentostam®) e o N-metil antimoniato de meglumina (Glucantime®) são os mais utilizados (FRANKE *et al.*, 1990; HERWALDT, 1999; CARVALHO *et al.*, 2000; MIRANDA-VERASTEGUI *et al.*, 2005).

O Glucantime® tem sido utilizado, no Brasil, como fármaco de primeira escolha, entretanto, ele é capaz de interagir com proteínas celulares, tornando a ação do produto inespecífica em relação às células infectadas e às não infectadas (CARVALHO *et al.,* 2000). Outros fármacos, tais como a anfotericina B livre e lipossomal, pentamidina e miltefosina (único fármaco de uso oral, mas ainda pouco usado no mundo), têm sido recomendados nos casos de intolerância e/ou resistência

ao tratamento convencional, porém, tais produtos apresentam também limitações devido aos efeitos colaterais causados, a necessidade de administração parenteral e/ou endovenosa, além do custo elevado dos fármacos aos pacientes (SUNDAR *et al.*, 2009; GOTO *et al.,* 2010). Em consequência, é comum a descontinuidade do tratamento dos pacientes, o que, ocasionalmente, pode levar a um aumento da resistência dos parasitos aos fármacos utilizados. Há de se destacar ainda o aumento do número de casos de recidiva à doença, fato atualmente observado em diversas regiões do Brasil e do mundo (VÉLEZ *et al.* , 2009).

Algumas das limitações ao uso de determinados fármacos incluem: resistência generalizada do paciente a medicamentos, efeitos adversos graves, duração prolongada do tratamento, perfis de toxicidade desfavoráveis e procedimentos complicados de administração do medicamento, além do custo elevado - o que pode ser um desafio nas comunidades carentes de recursos afetadas pelas DTNs. O uso de alguns fármacos também é comprometido por sua disponibilidade limitada (CHEUKA *et al.*, 2017).

Diante das limitações citadas, a identificação de novos produtos e/ou moléculas que possam ser utilizadas como fármacos, que sejam efetivos no combate ao parasito nos hospedeiros mamíferos, que não apresentem toxicidade aos pacientes, sejam economicamente viáveis e possam ser administrados por vias menos dolorosas e onerosas, torna-se necessária. Os desafios associados às atuais intervenções quimioterapêuticas para leishmaniose justificam esforços intensos de pesquisa em novas terapias antileishmaniais.

Nos últimos anos, um dos focos de pesquisa das indústrias farmacêuticas tem sido o estabelecimento de métodos eficientes para detecção de compostos bioativos, de forma a acelerar a descoberta de novas moléculas protótipos para obtenção de fármacos, bem como associando fármacos já existentes a sistemas de *delivery* para reduzir a toxicidade e aumentar a efetividade dos mesmos, mas sem perda de ação (RIBEIRO *et al.*, 2014; DUARTE *et al.*, 2016; MENDONÇA *et al.*, 2016). Tais sistemas incluem o desenvolvimento de formulações mais seguras, baseados no uso de fármacos utilizados originalmente para tratar outras doenças, chamado de reposicionamento, além de novas combinações de protocolos terapêuticos.

O desenvolvimento de metodologias que propiciem uma investigação mais racional e objetiva de produtos naturais bioativos oriundos de espécies nativas de

biomas brasileiros torna-se estratégico para uso sustentável da biodiversidade (YADAV *et al.,* 2013). A investigação de moléculas purificadas que apresentam ações biológicas pode ser realizada para avaliar seu potencial uso contra a doença, sendo considerado um campo de pesquisa promissor para identificar novos alvos farmacêuticos (CHEUKA *et al.*, 2017).

Os cardenolídeos compõem uma classe de metabólitos especiais que podem ser encontrados no reino vegetal, em famílias pertencentes aos grupos das angiospermas, e alguns deles já foram usados na clínica para o tratamento de insuficiência cardíaca congestiva (GHEORGHIADE *et al*, 2009). Além disso, algumas aplicações biológicas vêm sendo descritas para os cardenolídeos, tais como atividade antiviral (SU *et al.*, 2008; BERTOL *et al.*, 2011) e citotóxica (ELBAZ *et al.*, 2012; DIEDERICH *et al.* , 2016; MUNKERT *et al.*, 2017).

Nesse contexto e diante das dificuldades inerentes ao tratamento das leishmanioses, o propósito deste projeto é o de avaliar a atividade de derivados de cardenolídeos, no caso, as moléculas digitoxigenina e β-acetil-digitoxina, com vistas a avaliar sua atividade antileishmanial contra a espécie *L. infantum*, além da toxicidade em macrófagos murinos e em hemácias humanas e o tratamento de macrófagos infectados. As moléculas, por apresentarem bom índice de seletividade, caracterizando ação antileishmanial e baixa toxicidade em células de mamíferos, tiveram seu mecanismo de ação avaliado e sua eficácia *in vivo* investigada em camundongos BALB/c, sendo incorporadas a sistemas de *delivery* contendo Poloxâmero P407, para o tratamento contra a LV.

2. REVISÃO DE LITERATURA

2.1. Epidemiologia das leishmanioses

As leishmanioses são doenças antropozoonóticas que causam uma série de síndromes clínicas em seres humanos que podem comprometer a pele, mucosa e vísceras, dependendo do tropismo do parasito. São causadas por diferentes espécies de protozoários do gênero *Leishmania,* pertencentes à família Trypanosomatidae e transmitidas a animais e humanos através de insetos da família Psychodidae (OPAS, 2019).

Atualmente, mais de 1 bilhão de pessoas vivem em áreas endêmicas para leishmaniose e estão sob risco de infecção. Estima-se que 30.000 novos casos de LV e mais de 1 milhão de novos casos de LT ocorram anualmente (WHO, 2018). Cerca de 90% dos casos de leishmaniose visceral no mundo estão concentrados entre Brasil, Índia, Sudão, Sudão do Sul, Etiópia e Quênia. Três quartos dos novos casos mundiais de leishmaniose cutânea (LC) ocorrem em apenas cinco países: Afeganistão, Brasil, Irã, Iraque e Síria. A leishmaniose mucosa (LM) ocorre principalmente na região das Américas, sendo a Bolívia, o Brasil e o Peru os países com os maiores registros dessa forma clínica (OPAS, 2019).

No Brasil, o cenário de transmissão de LV se encontra em expansão (**Figura 1**) (PAHO, 2019).

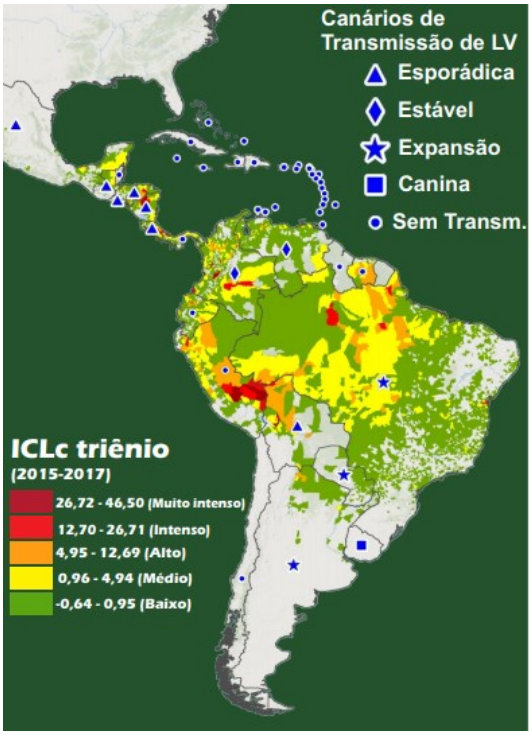

Figura 1. Cenários de transmissão de leishmaniose visceral por países e índice composto de leishmaniose cutânea triênio, Américas, 2017. Fonte: SisLeish-OPA0S/OMS: Dados reportados pelos Programas Nacionais de Leishmanioses / Serviços de vigilância. Acesso em: novembro, 2018.

A leishmaniose visceral (LV) é uma doença sistêmica grave que se não diagnosticada e tratada pode ser fatal em mais de 95% dos pacientes (WHO, 2017). Nas Américas, a LV é endêmica em 12 países e no período de 2001-2017 foram registrados 59.769 casos novos, resultando em uma média de 3.516 casos por ano. Cerca de 96% (57.582) dos casos foram reportados pelo Brasil (**Figura 2**) (PAHO, 2019).

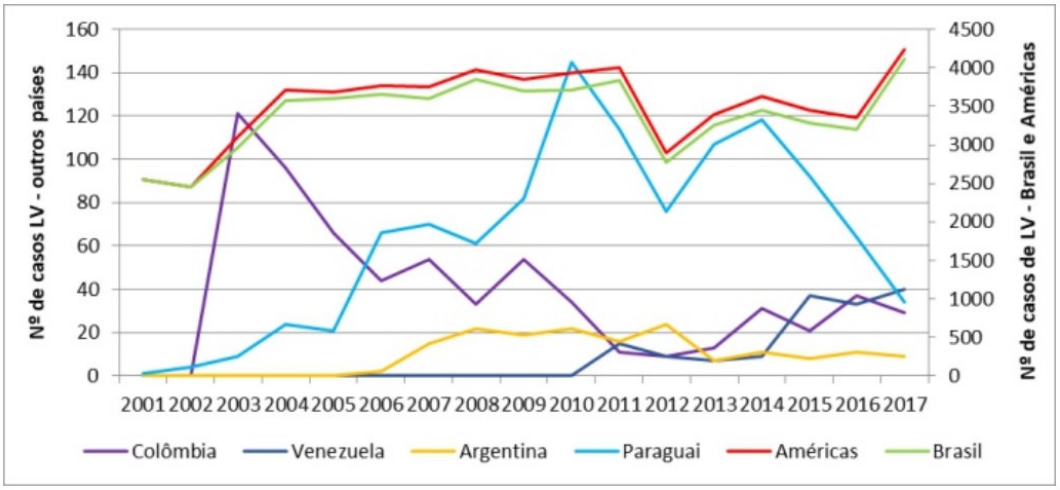

Figura 2. Casos de leishmaniose visceral nos países com maior número de casos, Américas, 2001 -2017. Fonte: SisLeish-OPAS/OMS: Dados reportados pelos Programas Nacionais de Leishmanioses / Serviços de vigilância. Acesso em: novembro, 2018.

Na série histórica de 2012 a 2019 (**Figura 3**) pode-se observar a taxa de letalidade de LV que houve um aumento a partir de 2014 na Região, e, apesar de haver uma redução no número de mortes causadas por LV entre 2017 e 2019, a taxa de letalidade se mantêm alta (PAHO, 2019).

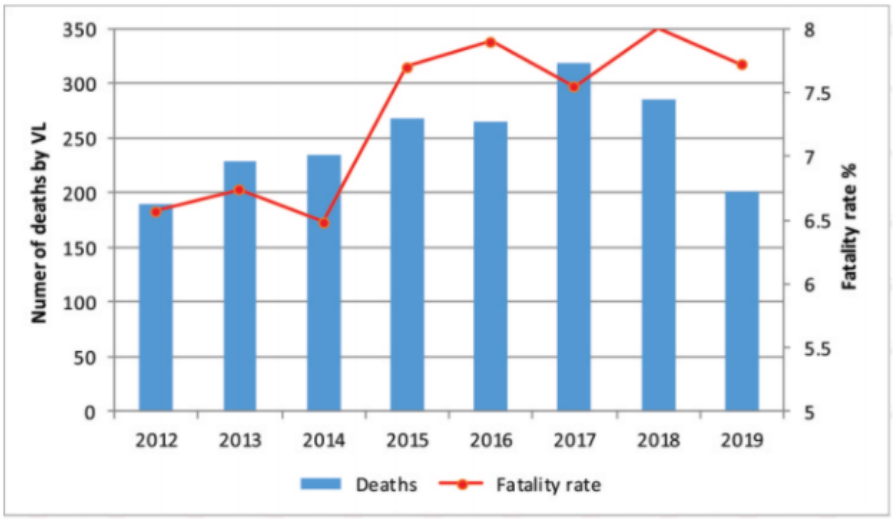

Figura 3. Número de mortes e taxa de letalidade por leishmaniose visceral, Américas, 2012 - 2019. Fonte: SisLeish - OPAS / OMS - Dados notificados pelos Programas/Serviços Nacionais de vigilância das leishmanioses. Acesso em: novembro de 2020

2.2. Etiologia e ciclo biológico do parasito *Leishmania sp.*

O gênero *Leishmania* compreende 22 espécies patogênicas para o homem. Durante seu ciclo de vida, o parasito é encontrado em duas formas ou estágios: uma forma promastigota, que é extracelular, alongada e possui um flagelo que permite a mobilidade no intestino do inseto vetor; e a forma amastigota, que é arredondado, intracelular e se multiplica nas células do sistema fagocítico mononuclear, principalmente macrófagos. Ambas as formas do parasito se reproduzem por fissão binária e possuem uma única mitocôndria modificada, conhecida como cinetoplasto (DEBRAY *et al.*, 2015).

A forma promastigota é transmitida a mamíferos suscetíveis, inclusive humanos, através da picada das fêmeas de flebotomíneos, que são pequenos dípteros da família Psychodidae que se alimentam de sangue e que são de grande importância na saúde pública devido ao seu papel como vetores dessas doenças (OPAS, 2019). Dentre esses mamíferos, nas américas, o cão é o reservatório doméstico mais importante, sendo que reservatórios são aqueles mamíferos que mantêm o parasito em estado selvagem e, portanto, permitem que os vetores sejam infectados e que o ciclo de transmissão possa persistir (OPAS, 2019).

O ciclo biológico do parasito *Leishmania* sp. compreende as etapas observadas na **Figura 4**. Durante o repasto sanguíneo do flebotomíneo em humanos ou outros animais mamíferos infectados, o parasito é ingerido e se transforma na forma paramastigota no intestino médio do vetor. Posteriormente, ocorre a diferenciação do parasito em forma promastigota metacíclica, que pode ser transferido para a derme do hospedeiro mamífero em um próximo repasto. Os parasitos, então, perdem seus flagelos à medida que se transformam em amastigotas intracelulares após internalização em macrófagos, células dendríticas ou neutrófilos. Uma vez nos fagolisossomos, as amastigotas podem persistir e se reproduzir por fissão binária. As formas amastigotas liberadas pela lise celular podem invadir ou ser fagocitadas por outros macrófagos locais e disseminar a infecção (CHEUKA *et al.*, 2017).

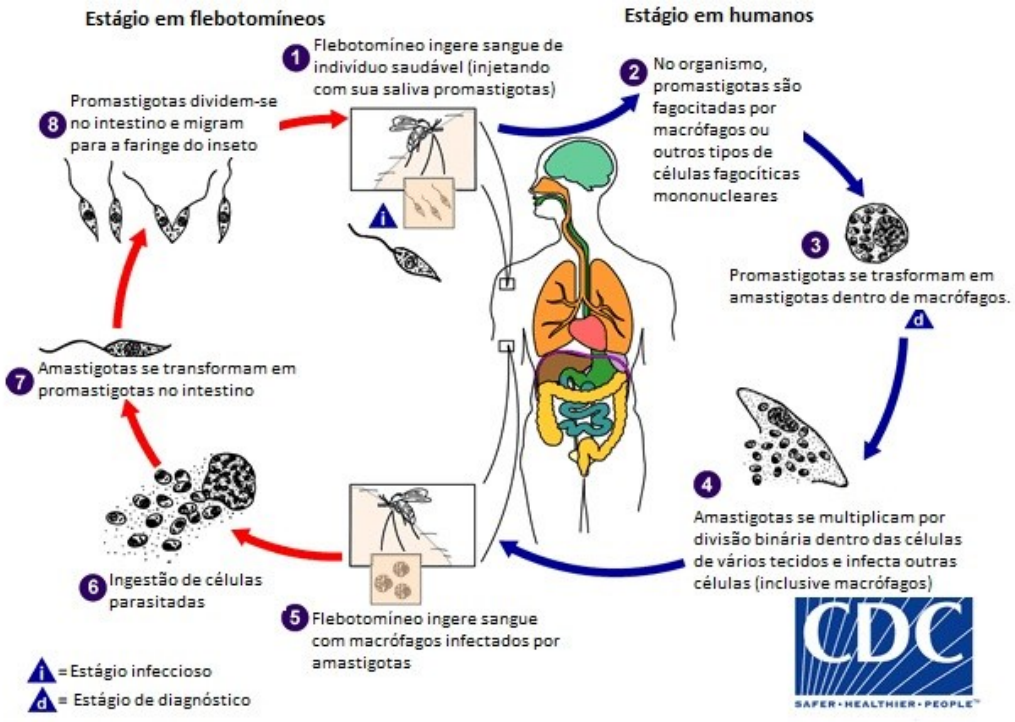

Figura 4 – Ciclo biológico do parasito *Leishmania*. Fonte: adaptado de https://www.cdc.gov/dpdx/leishmaniasis/index.html. Acesso em: 23 de julho de 2020.

2.3. Manifestações clínicas das leishmanioses

A LT pode se manifestar sob três formas clínicas principais: a leishmaniose cutânea (LC), a leishmaniose mucosa (LM) e a leishmaniose cutâneo-difusa (LCD) (WHO, 2015).

A LC é caracterizada pela presença de uma lesão cutânea única que se desenvolve no local da picada do flebotomíneo. A doença tende a evoluir à cura espontânea em alguns meses; porém, há casos em que pode permanecer ativa por vários anos, e coexistir com lesões mucosas que surgem posteriormente (GONTIJO; CARVALHO, 2003). A LC pode se apresentar sob as formas clínicas cutânea localizada, responsável por 85% dos casos e que tem tendência à cicatrização espontânea, com boa resposta terapêutica; cutânea disseminada, mais rara, caracterizada pelo aparecimento de lesões papulares múltiplas que acometem diversas partes do corpo; e a forma recidiva cútis, caracterizada por lesões nodulares em torno ou no interior da cicatriz de uma lesão prévia causada por *Leishmania spp.*

No Brasil, a LCD é causada pela espécie *L. amazonensis* e constitui-se em uma forma clínica grave da doença, além do parasito apresentar resistência ao tratamento (MENDONÇA *et al.*, 2004).

Cerca de 1 a 5% dos pacientes podem desenvolver LM, pela capacidade que algumas espécies do parasito têm em migrar para mucosas e causar extensa destruição tecidual (SCHUBACH *et al.*, 1998). A doença se caracteriza por apresentar aspectos de cronicidade e latência, além de desenvolver metástases em mucosas que conduzem a quadros clínicos desfigurantes (MARSDEN, 1986). Este quadro é de difícil diagnóstico parasitológico e apresenta resistência elevada do parasito ao tratamento. É comum que a doença se manifeste após o aparecimento das lesões cutâneas e a incidência e o diagnóstico do comprometimento da mucosa é estabelecido após meses a anos depois da cura clínica da lesão primária (MARSDEN, 1994; BOAVENTURA *et al.*, 2006.). As lesões podem ser de diversas formas e podem causar a destruição parcial ou total da região naso-oro-faringeana dos pacientes (DESJEUX, 2004).

As espécies *L. major* e *L. tropica* são as principais causas de LC no velho mundo, enquanto *L. braziliensis* e *L. mexicana* são responsáveis por infecções por LC no novo mundo. A LM é causada principalmente por *L. braziliensis*, embora espécies adicionais (*L. amazonensis*, *L. panamensis* e *L. guyanensis*) também tenham sido descritas. No subcontinente indiano, Ásia e África Oriental, a LV é causada por *L. donovani*, enquanto *L. infantum* é responsável por infecções na Europa, Norte da África e América Latina (CHEUKA *et al.*, 2017).

As principais formas clínicas de LV são as assintomáticas, oligossintomáticas e sintomáticas. A forma assintomática, também chamada de silenciosa, pode regredir em indivíduos imunocompetentes ou evoluir à oligossintomática ou subclínica e, posteriormente, à sintomática; sendo essa forma, subdividida em aguda, subaguda e crônica. A forma sintomática aguda é rara, de início abrupto, dura de 1 a 2 meses e pode levar ao óbito. A forma sintomática subaguda manifesta-se, principalmente, em crianças, é grave e, quando não tratada, pode levar a óbito no prazo de 5 meses a 1 ano. A forma sintomática crônica, também chamada de kalazar, tem evolução prolongada, de 2 a 3 anos e, em alguns casos, pode não responder ao tratamento quimioterápico e evoluir à forma clínica de maior gravidade (MARZOCHI *et al.,* 1994; SILVEIRA *et al.,* 1996; LAINSON *et al.,* 2004; DESJEUX, 2004;).

A evolução clínica da doença é influenciada pelas espécies patogênicas, bem como pelo estado de imunidade do hospedeiro. Se a resposta imune tiver a capacidade de combater a infecção, um perfil de resistência à reinfecção pode ser gerado e mantido ao longo da vida (SACKS *et al.*, 2002). No caso de falha da imunidade, a doença torna-se crônica, com progressão da infecção. É importante observar que algumas espécies de parasitos ainda podem ser responsáveis por causar lesões e infecções visceralizantes (CHEUKA *et al.*, 2017).

2.4. Imunologia das leishmanioses

A progressão da infecção pelo parasito *Leishmania* spp está classicamente associada a uma depressão das células T helper tipo 1 (Th1) e expansão preferencial das células T helper tipo 2 (Th2) e, por esse motivo, inclinar as células T helper para uma resposta Th1 pode ser considerado uma estratégia terapêutica promissora, especialmente nos casos de LV (RODRIGUES *et al.*, 2016). Na resposta Th1, as células T ativam macrófagos através da via clássica, estimulando a secreção de interferon-γ (IFN-γ) e interleucina (IL)-2. Na resposta Th2, as células T liberam citocinas IL-4, IL-5, IL-10 e fator de crescimento transformador (TGF)-β que inibem os macrófagos de matarem os parasitos (STEBUT & UDEY, 2004; RODRIGUES *et al.*, 2016).

Embora o macrófago possua mecanismos efetivos para dizimar patógenos intracelulares através de metabólitos tóxicos, como óxido nítrico e espécies reativas de oxigênio, os quais são ativados pelo IFN-γ liberado pelas células Th1, o parasito *Leishmania* é um patógeno que tende a evadir à resposta imune, atenuando seletivamente as vias de sinalização pró-inflamatórias (VÉLEZ *et al.*, 2009).

Como citado, os pacientes infectados devem desenvolver uma resposta inflamatória do tipo Th1 para eliminar o parasito. Por outro lado, a indução de um viés de IL-4 (resposta Th2) antagoniza a proteção de Th1 e inibe o controle imunológico do parasito *Leishmania* spp *in vivo*, levando a formas disseminadas graves da doença. O excesso de produção de IL-4 em hospedeiros suscetíveis provavelmente causa a falta de resposta a medicamentos específicos (VÉLEZ *et al.*, 2009).

A infecção progressiva parece mais provável de indicar uma resposta polarizada do tipo supressora (por exemplo, Th2 > Th1) em vez de uma resposta Th1 inerte (VÉLEZ *et al.*, 2009). A importância do paradigma Th1/Th2 na patogênese da leishmaniose tem sido uma área de intensa investigação. A suscetibilidade, resistência e imunopatogênese da infecção por *Leishmania* spp. depende em grande parte dos perfis de citocinas suscitados após a infecção, que varia a depender da espécie do parasito e do sistema imune do hospedeiro (VON STEBUT; UDEY, 2004).

2.5. Tratamento das leishmanioses

Os medicamentos contendo antimônio, conhecidos como antimoniais pentavalentes, são os medicamentos de primeira escolha para o tratamento das leishmanioses (CROFT; COOMBS, 2003; CHEUKA et al., 2017). O tratamento com esses fármacos possui diversas limitações que reduzem a adesão do paciente, tais como a longa duração do tratamento, as vias de aplicação dos fármacos (intramuscular ou endovenosa) e os graves efeitos colaterais provocados pelo uso diário dos produtos, como fadiga, artralgias, mialgias, além de toxicidade renal, hepática e cardíaca. Podemos citar ainda a dificuldade de transporte dos pacientes, que normalmente residem em áreas rurais, até os centros mais especializados de saúde, além do custo elevado para esse transporte e manutenção do paciente nesses centros durante todo o período de tratamento (KYLE *et al.*, 1991; TAVARES *et al.*, 2003; SUNDAR *et al.*, 2009).

Os medicamentos de segunda linha incluem a anfotericina B (AmpB), que é um produto antifúngico altamente hidrofóbico com atividade antileishmanial eficaz, mas seu uso clínico é limitado devido a alta toxicidade. Para melhorar a eficácia terapêutica da AmpB e reduzir sua citotoxicidade, foram desenvolvidas formulações baseadas em lipídios para administração parenteral, como AmBisome® (Gilead Sciences, Inc., Foster City, CA, EUA), Amphocil® (Kadmon Pharmaceuticals, New York, NY, EUA) e Abelcet® (Sigma-Tau Pharmaceuticals, Inc., Gaithersburg, MD, EUA). A Organização Mundial da Saúde recomendou o uso de AmpB lipossomal (L-AmpB) com base em sua elevada eficácia e segurança (BERN *et al.*, 2006). As formulações lipídicas apresentam vantagens em relação à formulação convencional, incluindo maior

concentração nos órgãos primários, tais como baço, fígado e pulmões; menor concentração nos rins, com marcante redução da nefrotoxicidade, além da diminuição dos efeitos adversos relacionados à infusão (VYAS *et al.*, 2006). Apesar da melhoria do índice terapêutico para as formulações lipídicas, a sua utilização permanece limitada, principalmente, pelo alto custo de venda dos produtos (EGGER *et al.*, 2010; RIBEIRO *et al.*, 2014).

Dentre outros fármacos antileishmaniais de segunda linha encontra-se a Miltefosina, originalmente desenvolvida como um agente anticâncer, sendo o primeiro medicamento administrado por via oral para o tratamento da LV. Tendo provado sua notável eficácia em ensaios clínicos, a miltefosina foi considerada um grande avanço na terapia antileishmanial. No entanto, sérias preocupações com sua teratogenicidade podem limitar seu uso, além de indícios de que sua meia-vida longa (152h) poderia incentivar o surgimento de resistência ao medicamento (SUNDAR *et al.*, 2006). A pentamidina também foi usada para tratar pacientes com LV refratários a antimoniais, embora sua eficácia em declínio tenha levado à sua retirada do mercado (DAS *et al.*, 2001).

Em adição, cerca de 10 a 25% dos pacientes com LV tratados podem apresentar resistência ao tratamento, falha terapêutica ou casos de recidiva. Para estes casos, a pentamidina e AmpB são utilizadas, apesar da toxicidade elevada e/ou do custo elevado desses fármacos (CUNNINGHAM, 2002).

2.6. Produtos naturais

Durante séculos, substâncias naturais, principalmente plantas, foram usadas para controlar e tratar doenças e isso resultou na descoberta da maioria dos agentes farmacêuticos modernos. Estima-se que, aproximadamente, metade dos produtos farmacêuticos atualmente em uso clínico sejam provenientes de produtos naturais. Alguns medicamentos derivados de produtos naturais que são muito utilizados nos cuidados farmacêuticos modernos incluem quinino, teofilina, penicilina G, morfina, paclitaxel, digoxina, vincristina, doxorrubicina, ciclosporina e vitamina A, entre muitos outros exemplos (CHEUKA *et al.*, 2017).

Embora os vegetais não sejam mais usados em sua forma bruta como preparações de medicamentos, eles continuam sendo fontes importantes de princípios ativos purificados que se tornaram essenciais na terapia moderna (NEWMAN *et al.*, 2007). As moléculas químicas de interesse que são identificadas nesses produtos são possivelmente derivadas do fenômeno da biodiversidade, no qual as interações entre organismos e seus ambientes formulam as diversas entidades químicas, permitindo sua sobrevivência e competitividade (LEE, 2010). As áreas terapêuticas das doenças infecciosas se beneficiam dessas classes de medicamentos, sendo capazes de interagir com alvos específicos dentro das células hospedeiras (MISHRA *et al.*, 2011).

2.7. Cardenolídeos

Os cardenolídeos compõem uma classe de metabólitos especiais que podem ser encontrados no reino vegetal em famílias pertencentes aos grupos das angiospermas. Mais de 100 diferentes cardenolídeos já foram isolados de espécies do gênero *Digitalis*, sendo as espécies *D. lanata* e *D. purpurea* as principais fontes de cardenolídeos usadas em terapias. Fármacos, tais como a digitoxina (glicosídeo secundário) e o lanatosídeo C (glicosídeo primário), já foram usados na clínica para o tratamento da insuficiência cardíaca congestiva (KREIS *et al.*, 1998; GHEORGHIADE *et al.*, 2009). No entanto, apenas a digoxina continua descrita na Relação Nacional de Medicamentos Essenciais (RENAME, 2020), e permanece sendo empregada no tratamento dessa enfermidade, embora não seja o fármaco de primeira escolha devido ao seu baixo índice terapêutico.

A digoxina é o medicamento cardíaco mais antigo usado na medicina contemporânea, mas apresenta um perfil farmacocinético complexo e índice terapêutico estreito. Seu uso no tratamento de pacientes com arritmias atriais ou insuficiência cardíaca pode representar um desafio para os médicos de hoje e compreender este medicamento é essencial para garantir que seja usada com segurança e eficácia na prática (EHLE *et al.*, 2011).

Apesar de alguns cardenolídeos terem sido citados como compostos tóxicos (HAUPTMAN *et al.*, 2016; ARBABIAN *et al.*, 2018) algumas aplicações biológicas vêm

sendo descritas, tais como atividade antiviral (SU *et al.*, 2008; BERTOL *et al.*, 2011;) e citotóxica (ELBAZ *et al.*, 2012; DIEDERICH *et al.*, 2016; MUNKERT *et al.*, 2017), além de ações antitumorais (SLINGERLAND *et al.*, 2013), antimalárica (CHAN *et al.*, 2016), e antioxidante (XU *et al.*, 2017), entre outras (GUREL *et al.*, 2017). Mais recentemente foi publicado um estudo que fornece novos insights sobre os efeitos farmacológicos dos cardenolídeos naturais que suprimem a replicação coronaviral e que podem constituir a base para futuros agentes antivirais (YANG *et al.*, 2020).

Visando expandir a aplicação terapêutica de derivados cardenolídeos, a avaliação biológica de suas atividades antileishmaniais são interessantes. As moléculas digitoxigenina (DIGI) e β-acetil-digitoxina (b-AD), derivadas de cardenolídeos naturais, foram selecionados para serem testados contra os parasitos no tratamento da leishmaniose visceral.

2.8. Reposicionamento de fármacos

A descoberta e o desenvolvimento de medicamentos para DTNs enfrentam uma série de desafios. Um ponto importante é que o investimento nessas áreas terapêuticas por grandes empresas farmacêuticas não é financeiramente atraente devido à perspectiva de baixos retornos financeiros (CHEUKA *et al*., 2017).

Portanto, diante de toda a problemática em torno dos fármacos disponíveis no mercado para o tratamento desse complexo de doenças, a estratégia conhecida como reposicionamento de medicamentos pode representar uma forma de desenvolvimento mais barato e rápido de novos tratamentos. O reposicionamento de fármacos consiste em averiguar e comprovar a possibilidade de novas indicações para medicamentos já existentes, ainda disponíveis ou não no mercado, que tenham indicação para outras doenças e/ou alvos terapêuticos já descobertos (ASHBURN; THOR, 2004).

O reposicionamento tornou-se uma estratégia popular para o desenvolvimento de fármacos nos últimos anos devido ao fato de, diferentemente do processo tradicional mais demorado, que envolve grandes riscos e requer altos investimentos, é eficiente, mais econômico e sem grandes riscos, oferecendo uma oportunidade para muitos países desenvolverem fármacos com menores custos (XUE *et al.*, 2018). Recentemente, existe um interesse considerável no reposicionamento de medicamentos para doenças tropicais negligenciadas. Alguns exemplos aplicados no

tratamento contra LV são conhecidos, como AmpB (antifúngico), miltefosina (anticâncer) e paramomicina (antibiótico) (CHÁVEZ-FUMAGALLI *et al.*, 2019).

Dentre os cardenolídeos naturais, o reposicionamento já vem sendo estudado em diversos casos. Novas descobertas sugerem um reposicionamento potencial da digitoxina como uma droga antiangiogênica de amplo espectro para doenças em que a angiogênese patológica está envolvida (TRENTI *et al.*, 2017). Além disso, existem evidências de que a digitoxina neutraliza as características salientes do microambiente inflamatório do câncer de ovário, indicando o reposicionamento potencial desse composto como uma droga anticâncer (TRENTI *et al.*, 2018). Em adição, os cardenolídeos foram ainda identificados como uma nova classe de agentes antitumorais para o tratamento do retinoblastoma, podendo ser reposicionado quando administrados localmente por meio de infusão intra-arterial direta (ANTCZAK *et al.*, 2009).

2.9. Sistemas de *delivery*

Em paralelo ao reposicionamento de fármacos, a veiculação de tais produtos em carreadores capazes de prolongar a sua estabilidade, promoverem sua liberação controlada e gradual e aumentarem a biodisponibilidade, têm tornado também importante objeto de estudo.

Nesse sentido, sistemas micelares à base de Polôxamero 407 (P407) têm sido usados na veiculação de diferentes produtos (CABANA *et al.,* 1997; PISAL *et al.*, 2004; CHÁVEZ-FUMAGALLI *et al.*, 2015; MENDONÇA *et al.*, 2016). Entretanto, nenhuma das composições farmacêuticas empregadas para o tratamento das leishmanioses utiliza P407 como agente formador de micelas para a veiculação e estabilização de fármacos. Ao contrário das formulações disponíveis no mercado, que são baseadas em lipídeos, o P407 é um agente formador de micelas que permite a liberação gradual de produtos a ele agregados. É formado por óxido de etileno (OE) nas extremidades e óxido de propileno (OP) na parte central da molécula (OE95-105–OP54-60–OE95-105), sendo comercializado sob os nomes Pluronic® F-127, Lutrol® F127, Synperonic™ PE/F127, Tetronic® 908, dentre outros.

Já foi demonstrado que micelas contendo AmpB à base de P407 são eficazes no tratamento de camundongos BALB/c infectados com *L. amazonensis* (MARTINS *et al.*, 2016; MENDONÇA *et al.*, 2016; TAVARES *et al.*, 2019). Portanto, a associação de sistemas de administração de medicamentos para o tratamento das leishmanioses pode ser considerada relevante. A este respeito, micelas compostas por P407 podem ser consideradas como uma opção interessante (RIBEIRO *et al.*, 2014).

3. OBJETIVOS

3.1. Objetivo geral

Avaliar a atividade antileishmanial de dois derivados de cardenolídeos, digitoxigenina e β-acetil-digitoxina, contra a espécie *L. infantum in vitro* e *in vivo*, tanto na sua forma livre quanto associados a sistema de *delivery*.

3.2. Objetivos específicos

- Avaliar a atividade antileishmanial dos cardenolídeos contra as formas promastigotas e amastigotas-like de *L. infantum*.
- Avaliar a toxicidade em macrófagos murinos e hemácias humanas.
- Realizar o tratamento *in vitro* de macrófagos infectados com *L. infantum* utilizando as moléculas e verificar a inibição da infecção com parasitos pré-expostos.
- Avaliar o mecanismo de ação das moléculas frente a *L. infantum*.
- Desenvolver formulações micelares incorporando as moléculas DIGI e b-AD.
- Avaliar o uso, tanto das formulações quanto de suas formas livres, para o tratamento da infecção experimental em camundongos BALB/c causada pela espécie *L. infantum*.
- Quantificar carga parasitária no baço, fígado, medula óssea (BM) e linfonodos drenantes (dLN) dos animais infectados e tratados.
- Avaliar a resposta imune celular pela produção das citocinas IFN-γ, IL-4, IL-10, IL-12 e GM-CSF.
- Avaliar a resposta imune humoral pela determinação dos níveis de IgG1 e IgG2a específicos aos parasitos após protocolo de tratamento.

4. METODOLOGIA

4.1. Animais de experimentação e parasitos

O projeto foi aprovado pelo Comitê de Ética em Pesquisa Animal da UFMG (protocolo 085/2017). Foram utilizados camundongos BALB/c fêmeas, de 8 semanas de idade, adquiridos junto ao Biotério Central do Instituto de Ciências Biológicas e mantidos no Biotério do Departamento de Patologia Clínica do COLTEC, sob as devidas condições de manejo. A cepa MHOM/BR/1970/BH46 de *L. infantum* foi utilizada nos ensaios *in vitro* e para a infecção *in vivo*. Os parasitos foram cultivados em meio Schneider's completo (Sigma-Aldrich, EUA), composto por meio mais 20% de soro fetal bovino inativado pelo calor (FBS; Sigma-Aldrich, EUA) e L-glutamina 20 mM pH 7,4, a 24 ° C, de acordo com o protocolo descrito previamente (COELHO *et al.*, 2003).

4.2. Obtenção dos cardenolídeos

Os cardenolídeos DIGI e b-AD foram extraídos do extrato metanólico de folhas de *Digitalis lanata*. As moléculas foram entregues já purificadas (grau de pureza maior que 98%) pelo grupo coordenado pelos professores Rodrigo Maia de Pádua e Fernão Braga, ambos da Faculdade de Farmácia da UFMG, em quantidade suficiente para a realização de todos os experimentos *in vitro* e *in vivo*.

4.3. Ensaios de atividade leishmanicida

O protocolo utilizado é baseado naquele descrito previamente (VALADARES *et al.*, 2011), com modificações de acordo com Mendonça e colaboradores (MENDONÇA *et al.*, 2016). Para tal, em uma placa de cultura celular de 96 poços (Nunc, Nunclon®), $1x10^6$ formas promastigotas estacionárias de *Leishmania* foram incubadas com diferentes concentrações de DIGI e b-AD (0 a 50.0 µg/mL). Amp B (0 a 1.0 µg/mL) foi usada como controle. As amostras foram diluídas em meio RPMI-PR$^-$ em volume final de 100 µL, por 48h a 24^{o}C. Após, 10 µL do reagente MTT (brometo de [3-(4,5-dimetiltiazol-2yl)-2,5-difenil tetrazolium]) (5 mg/mL) foram adicionados e incubados por

4h a 24ºC. As células foram analisadas em microscópio ótico para a verificação da formação de cristais de formazan. Logo após, 60 µL de SDS 10%-HCL 0.1M foram adicionadas para a solubilização dos cristais de formazan, e a placa foi incubada por 18h. As leituras das absorbâncias foram realizadas em comprimento de onda de 570 nanômetros(nm). A determinação dos valores de IC_{50}, ou seja, a concentração da amostra necessária para inviabilizar 50% dos parasitos, foi obtida por análise de regressão não-linear dos pontos plotados graficamente.

4.4. Ensaios de citotoxicidade

Um ensaio foi realizado para a avaliação da citotoxicidade em macrófagos murinos. Para tal, camundongos foram inoculados por via peritoneal com 3 mL de uma solução de tioglicolato a 3%. Após cinco dias, foi realizada a eutanásia dos animais com a posterior extração dos macrófagos, que foram centrifugados a 1.200x rpm por 20 min a 4ºC, sendo ressuspendidos em 1 mL de meio RPMI completo (meio RPMI acrescido com 20% de soro fetal bovino inativado). Os macrófagos foram quantificados em câmara de Neubauer e 5 x 10^5 células foram plaqueadas em placas de cultura celular de 96 poços por 2h. Após esse tempo, foram incubados com diferentes concentrações das moléculas (0 a 100.0 µg/mL), por 48h e a 37ºC. Amp B (0 a 10.0 µg/mL) foi usada como controle. Posteriormente, 50 µL de MTT foram acrescentados e uma nova incubação foi realizada por 4 h e a 37ºC. As células foram solubilizadas em solução de SDS 10%/HCl 0.1M por 18h, e as absorbâncias foram determinadas a 570nm.

A determinação da CC_{50}, ou seja, a concentração da amostra necessária para inviabilizar 50% dos macrófagos foi obtida por análise de regressão não-linear dos pontos plotados graficamente. O índice de seletividade (IS) foi calculado pela razão entre os valores de CC_{50} e IC_{50}. Esse índice demonstra a relação entre citotoxicidade e atividade biológica, ou seja, representa o potencial efetivo das substâncias sobre o parasito em relação a sua citotoxicidade. Quanto maior o valor, mais tóxico para os parasitos e menos tóxico para as células de mamíferos, então, demonstrando maior viabilidade do produto.

4.5. Determinação da atividade hemolítica

Como parâmetro adicional de citotoxicidade, a atividade hemolítica foi investigada pela incubação das duas moléculas (0 a 100.0 µg/mL) com uma suspensão de 5% de hemácias humanas de indivíduos do grupo sanguíneo O por 1h e a 37ºC. Amp B (0 a 10.0 µg/mL) foi usada como controle. A suspensão foi centrifugada a 1.000 x g por 10min e a lise celular foi determinada espectrofotometricamente (540nm), conforme descrito previamente (LÖFGREN *et al.*, 2008). A ausência (branco) ou a presença de 100% (controle positivo) de hemólise foi determinada substituindo os produtos por igual volume de PBS 1x ou água destilada, respectivamente. A concentração dos compostos necessários para causar 50% de lise em hemácias humanas (RBC_{50}) foi determinada aplicando uma curva de regressão sigmoidal utilizando as diferentes concentrações dos produtos.

4.6. Tratamento de macrófagos infectados

Macrófagos peritoneais foram extraídos, quantificados e plaqueados na concentração de 5 x 10^5 células por poço em placas de cultura celular de 24 poços (Nunc). As células foram incubadas por 2h a 37ºC e 5% de CO_2. Em seguida, as mesmas foram lavadas em meio RPMI completo para a retirada daquelas não-aderidas. Então, formas promastigotas estacionárias de *Leishmania* foram incubadas com os macrófagos (proporção de 10 parasitos para cada macrófago), durante 24h a 37ºC e 5% de CO_2. As células foram lavadas para a retirada dos parasitos não-aderidos e não-fagocitados e, em seguida, os macrófagos infectados foram tratados com DIGI e b-AD (0, 2.5, 5.0 e 10.0 µg/mL) por 48h a 37ºC. AmpB foi utilizada como controle do experimento (0, 0.25, 0.5 e 1.0 µg/mL). Após as 48h de incubação, as células foram coradas pelo método panótico e, posteriormente, quantificadas em microscópio ótico, quando a porcentagem de macrófagos infectados e o número de amastigotas intra-macrófagos nos grupos experimentais foram determinados por meio da contagem de 200 células, em triplicata.

4.7. Inibição de infecção usando parasitos pré-tratados

As promastigotas (5 x 10^6 células) foram incubados com cada um dos compostos por 1h a 24°C. As células foram lavadas e adicionadas com macrófagos (proporção de 10 parasitos por célula), durante 24h a 37°C em 5% de CO2. As células infectadas foram lavadas três vezes em meio RPMI e coradas com Giemsa. A porcentagem de infecção por parasitos pré-tratados e o número de amastigotas recuperadas foram determinadas pela contagem de 200 células, em triplicata, em um microscópio óptico (MENDONÇA *et al.*, 2018).

4.8. Avaliação dos mecanismos de ação das moléculas

Para avaliar o potencial de membrana mitocondrial, promastigotas estacionárias (10^7 células) foram cultivadas na ausência ou presença de b-AD (33,80 µg/mL, correspondendo a 2 vezes o valor de seu IC_{50}) ou DIGI (13,8 µg/mL, correspondendo a 2 vezes o valor de seu IC_{50}) por 24 horas e a 25°C. Os parasitos foram lavados em PBS e incubados com 500 nM de MitoTracker Red CM-H2XROS (Invitrogen, EUA) por 30 min no escuro e em temperatura ambiente. Depois de lavar duas vezes com PBS, as amostras foram adicionadas a uma placa preta de 96 poços e a intensidade da fluorescência foi medida usando um fluorômetro (FLx800, BioTek Instruments, Inc., Winooski, VT, EUA) com comprimentos de onda de excitação e emissão de 528 nm e 600 nm, respectivamente. Parasitos incubados com carbonil cianeto-4-(trifluorometoxi) fenil-hidrazon (FCCP, 5,0 µM; Sigma-Aldrich, EUA) por 10 min foram usados como controle (SOUSA *et al.*, 2019).

A fim de avaliar a integridade da membrana mitocondrial, promastigotas estacionários (10^7 células) foram cultivadas na ausência ou presença de DIGI (13,8 µg/mL) durante 24 horas a 25°C. Os parasitas foram lavados com PBS e incubados com iodeto de propídio (1,0 µg/mL; Sigma-Aldrich) por 15 min, no escuro e em temperatura ambiente. Após duas lavagens com PBS, as amostras foram adicionadas a uma placa preta de 96 poços e a intensidade da fluorescência foi medida usando em fluorômetro, com comprimentos de onda de excitação e emissão de 540 nm e 600 nm, respectivamente. Parasitos pré-aquecidos por 10 min a 65ºC foram usados como controle (SOUSA *et al.*, 2019).

Para analisar o produção de espécies reativas de oxigênio (ROS), promastigotas estacionárias (10^7 células) foram cultivadas na ausência ou presença de b-AD (33,80 µg/mL) ou DIGI (13,8 µg/mL) por 24 h a 25°C. Os parasitos foram lavados com PBS e incubados com 20 µM de permeante celular 2',7'-diacetato de diclorodihidrofluoresceína (H2DCFDA; Sigma-Aldrich, EUA) por 30 min no escuro e em temperatura ambiente. A intensidade da fluorescência foi medida em espectrofluorômetro (Varioskan® Flash, Thermo Scientific, EUA), com comprimentos de onda de excitação e emissão de 485 nm e 528 nm, respectivamente. Parasitos tratados com H_2O_2 (4,0 mM; Sigma-Aldrich, EUA) foram usados como controle nos experimentos (SOUSA *et al.*, 2019).

4.9. Desenvolvimento das formulações micelares

O processo para a obtenção do sistema micelar foi desenvolvido conforme descrito previamente (BARICHELLO *et al.*, 1999), compreendendo as seguintes etapas:

(a) adicionar o P407, para uma concentração final de 10 a 20% p/p, a uma solução tampão pH 7.4, sob agitação e em temperatura entre 4 a 8°C;

(b) manter a solução a 4ºC por um período de 12 a 24 horas;

(c) solubilizar a molécula de interesse (1 mg/mL) a ser incorporada nas micelas usando diclorometano PA;

(d) adicionar essa solução diluída àquela preparada na etapa “b” e manter sob agitação moderada até a formação de um gel límpido e em temperatura entre 4 a 8°C;

(e) eliminar o solvente orgânico da mistura preparada na etapa “d” por evaporação rotatória sob vácuo, utilizando banho de água em temperatura entre 40 e 45°C.

4.10. Avaliação da eficácia *in vivo* contra a infecção experimental

Camundongos BALB/c foram infectados pela via subcutânea no dorso com 10^7 formas promastigotas estacionárias de *L. infantum*. Após o desenvolvimento da infecção (cerca de 60 dias), o tratamento foi iniciado. Os animais receberam o tratamento uma vez ao dia a cada 2 dias, durante 10 dias, recebendo injeções na base da cauda ou via oral (miltefosina) utilizando:

i) Grupo salina - 50 μL de PBS.

ii) Grupo controle de tratamento (miltefosina) – 2mg de miltefosina por kg de peso corporal.

iii) Grupo digitoxigenina (DIGI) - 50μL de DIGI (5 mg/kg de peso corporal).

iv) Grupo digitoxigenina micelar (DIGI/Mic) - 50μL de micelas contendo DIGI (5 mg/kg de peso corporal).

v) Grupo β-acetil-digitoxina (b-AD) - 50μL de DIGI (5 mg/kg de peso corporal).

vi) Grupo β-acetil-digitoxina micelar (b-AD/Mic) - 50μL de micelas contendo b-AD (5 mg/kg de peso corporal).

vii) Grupo micelas vazias (B/Mic) - 50μL de micelas vazias (10mg/kg de peso corporal).

Os animais foram sacrificados um e quinze dias após o fim do tratamento.

4.11. Avaliação da carga parasitária

Uma técnica de diluição-limitante desenvolvida previamente (TITUS *et al.*, 1985) e modificada por Martins e colaboradores (MARTINS *et al.*, 2013) foi utilizada para avaliar a carga parasitária nos animais infectados e tratados. Para isso, os fragmentos de baço, fígado e dLN dos animais foram recolhidos, pesados e homogeneizados em PBS 1x estéril utilizando um macerador de tecido. Os detritos de tecido foram removidos por centrifugação a 150 x g e as células foram concentradas por centrifugação a 2.000 x g. O sedimento foi ressuspenso em 1 mL de solução completa de Schneider e 200 μL da ressuspensão foram plaqueados em placas de microtitulação de fundo plano de 96 poços (Nunc). As diluições foram realizadas de

forma seriada em meio Schneider completo começando de 10^{-1} até 10^{-12}. As pontas das pipetas foram descartadas após cada diluição para evitar o transporte de parasitos aderidos de um poço para outro. Cada amostra foi plaqueada em triplicata e incubada a 24°C, sendo analisadas 7 dias após o início da cultura. Os resultados foram expressos como o log negativo do título (i.e., a diluição correspondente ao último poço positivo) ajustado por miligrama de tecido ou órgão infectado.

O parasitismo no baço dos animais foi também avaliado pela técnica de PCR quantitativo (qPCR), conforme descrito (DUARTE *et al.*, 2016). Resumidamente, o DNA do baço foi extraído usando o kit Wizard Genomic DNA Purification (Promega Corporation) e ressuspenso em água milli-Q. A carga parasitária foi estimada utilizando os primers para amplificar o kDNA de *L. infantum*: Forward (CCTATTTTACACCAACCCCCAGT) e Reverse (GGGTAGGGGCGTTCTGCGAAA). O gene da β-actina de camundongo (Forward: CAGAGCAAGAGAGGTATCC; Reverse: TCATTGTAGAAGGTGTGGTGC) foi usado como controle. Curvas-padrão foram obtidas a partir de DNA extraído de 10^8 parasitos para kDNA e 10^8 macrófagos peritoneais para β-actina, nas mesmas condições utilizadas para extrair as amostras do presente estudo. As reações foram processadas e analisadas em um ABI Prism 7500 Sequence Detection System (placa de 96 poços; Applied Biosystems) usando 2x SYBRTM Select Master Mix (5 µL; Applied Biosystems), com 2 mM de cada primer (1 µL) e 4µL de DNA (25 ng/µL). As amostras foram incubadas a 95°C por 10min, e submetidas a 40 ciclos de 95°C por 15 s e 60°C por 1min. Durante cada tempo, os dados de fluorescência foram coletados. Os resultados foram calculados por interpolação de uma curva padrão incluída na mesma execução, que foi realizada em duplicata e expressa como o número de organismos *L. infantum* por DNA total.

4.12. Avaliação da resposta celular e humoral

O perfil da resposta celular foi avaliado pela produção das citocinas IFN-γ, IL-4, IL-10, IL-12, TNF-α (Fator de necrose tumoral α) e GM-CSF nos sobrenadantes de cultura dos esplenócitos, após estímulo específico. As dosagens foram realizadas utilizando os kits *Intertest Mouse (Pharmingen®)*, de acordo com as instruções do fabricante e por citometria de fluxo. Foi também realizada a dosagem de óxido nítrico

(NO) nos sobrenadantes da cultura celular, no momento da coleta dos sobrenadantes, utilizando-se, para tal, o método de Griess (GREEN *et al.,* 1982). O perfil da resposta humoral nos animais infectados e tratados foi avaliado por meio de técnica descrita previamente (COELHO *et al.*, 2003).

Para avaliar a resposta celular desenvolvida no final do tratamento, culturas de esplenócitos e ensaios de dosagem de citocinas foram realizados como descrito previamente (MARTINS *et al.*, 2015). Para isto, suspensões de células esplênicas ($5x10^6$ células por poço) foram plaqueadas em duplicata em placas de 24 poços (Nunc) e incubadas em DMEM (*Dulbecco's Modified Eagle Medium, Sigma-Aldrich*) suplementado com FBS a 20% e L-glutamina 20 µM, pH 7,4, ou estimuladas com SLA de *L. infantum* (25 µg / mL), durante 48 h a 37°C e 5% de CO_2. Os níveis de IFN-γ, IL-4, IL-10, IL-12p70 e GM-CSF foram avaliados nos sobrenadantes das culturas pela metodologia de análise de imunoabsorção por ligação enzimática (ELISA) sanduíche fornecido em kits comerciais (BD OptEIA TM set mouse o IFN-γ, IL-4, IL-10 e IL-12p70 e GM-CSF, Pharmingen®, San Diego, CA, EUA), seguindo as instruções do fabricante.

O envolvimento das células T $CD4^+$ e $CD8^+$ na produção de IFN-γ nos esplenócitos dos camundongos infectados e tratados foi avaliado após a estimulação in vitro com SLA (25 µg/mL), na presença ou ausência de anticorpos monoclonais anti-IL-12 murino (C17,8), anti-CD4 (GK 1) ou anti-CD8 (53-6.7), a uma concentração de 5 µg/mL. Foram utilizados controles adequados para avaliar a integridade dos testes [IgG_{2a} de rato (R35-95) e IgG_{2b} de rato (95-1)]. Todos os anticorpos utilizados (sem azida/endotoxina baixa) foram adquiridos da BD (Pharmingen).

A produção de nitrito foi também avaliada usando os sobrenadantes celulares, por meio do método de Griess. Em adição, a técnica de citometria de fluxo foi desenvolvida para avaliar a frequência de células T CD4+ e CD8+ produtoras de IFN-γ, TNF-α e IL-10 nos grupos experimentais (salina, B/Mic, miltefosina, b-AD, b-AD/Mic, DIGI e DIGI/Mic), quando as células esplênicas foram coletadas 15 dias após o tratamento. Para tal, as análises foram realizadas com base em gráficos de tamanho de citometria de fluxo relativo (dispersão direta de laser) e granularidade (dispersão de laser lateral). Após a seleção da região de interesse R1 contendo células de fenótipo FSCLow e SSCLow, gráficos de distribuição de densidade de células CD4/FL1 ou CD8/FL1 versus IFN-γ/FL2+, TNF-α/FL2+ e células IL-10/FL2+ foram construídos, em ordem para determinar a frequência de células T produtoras de IFN-

γ, TNFα e IL-10. Os resultados foram expressos como índices, que foram calculados pelas razões entre a porcentagem de células T CD4+ e CD8+ nas culturas estimuladas versus não estimuladas (controle).

A produção de anticorpos foi avaliada um dia após o término dos tratamentos. Para isso, os níveis de anticorpos de isotipo IgG1 ou IgG2a específicos contra o parasito foram medidos pela técnica de ELISA direta, como descrito previamente (COELHO *et al.*, 2003). Resumidamente, após titulação, utilizou-se SLA de *L. infantum* a uma concentração de 1,0 µg por por poço, como antígeno de sensibilização, enquanto as amostras de soro foram diluídas a 1: 100 em tampão PBS-T (PBS 1x mais 0,05% Tween 20). Anticorpos anti-mouse IgG_1 e IgG_{2a} marcados com peroxidase (Sigma-Aldrich) foram todos utilizados numa diluição de 1: 10.000 (também diluída em PBS-T). As reações colorimétricas foram desenvolvidas por incubação com uma solução composta por 2 µL de H_2O_2, 2 mg de orto-fenilenodiamina e 10 mL de tampão citrato-fosfato a pH 5,0, durante 30 min no escuro e paradas pela adição de 20 µL de H_2SO_4 2 N. A densidade ótica foi determinada utilizando-se um espectrofotómetro (*Molecular Devices, Spectra Max Plus,* Canadá), a 492 nm.

4.13. Avaliação da toxicidade *in vivo*

Foram colhidas amostras de soro dos animais infectados e tratados para realização de dosagens bioquímicas. Utilizou-se amostras de camundongos *naive* (não tratados e não infectados) como controle. A função hepática foi analisada por dosagem de aspartato aminotransferase (AST) e alanina aminotransferase (ALT), enquanto que a cardiotoxicidade foi avaliada através do exame dos níveis do marcador cardíaco *creatine kinase-MB* (CK-MB). As análises bioquímicas foram desenvolvidas por meio de kits comerciais (Labtest Diagnostica®, Belo Horizonte, Minas Gerais, Brasil) e um aparelho auto-analisador (analisador Thermo Plate TP), de acordo com as instruções do fabricante.

4.14. Análise estatística

As análises estatísticas foram realizadas por meio da análise de variância (ANOVA), seguida pelo teste de comparação múltipla utilizando-se o método de Dunn ou teste de Tukey. Valores de $P < 0.05$ foram considerados como indicativos de significância.

5. RESULTADOS E DISCUSSÃO

Os tópicos de Resultados e Discussão dessa dissertação serão apresentados sob a forma de dois artigos científicos, que foram submetidos para publicação, sendo que um deles já foi aceito para publicação. Cabe ressaltar que a divisão do trabalho avaliando dois cardenolídeos em dois artigos ocorreu por decisão do grupo de pesquisa e de seus colaboradores. As legendas das figuras e tabelas dos artigos foram inseridas nas respectivas listas no presente documento, bem como a metodologia descrita anteriormente se enquadra naquela que foi desenvolvida e é apresentada nos dois artigos, sendo o primeiro tratando do uso da b-AD e o segundo sobre a DIGI contra a infecção por *L. infantum*.

5.1. Artigo 1: estudo envolvendo a β-acetil-digitoxina contra *L. infantum*

Este artigo foi submetido para publicação no periódico *Parasite* na data de 20 de agosto de 2020 e aceito para publicação em 01 de abril de 2021.

In vitro and *in vivo* antileishmanial activity of β-acetyl-digitoxin, a cardenolide of *Digitalis lanata* potentially useful to treat visceral leishmaniasis

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Abstract

Current treatments of visceral leishmaniasis (VL) face limitations due to drug side effects and/or high cost, along with the emergence of parasite resistance. Novel and low-cost antileishmanial agents are therefore demanded. We herein report the antileishmanial activity of β-acetyl-digitoxin (b-AD), a cardenolide isolated from *Digitalis lanata* leaves, assayed *in vitro* and *in vivo* against *Leishmania infantum*. Results showed direct action of b-AD against parasites, as well as an efficacy for the treatment of *Leishmania*-infected macrophages. *In vivo* experiments using b-AD-containing Pluronic® F127 polymeric micelles (b-AD/Mic) to treat *L. infantum*-infected mice showed that this composition reduced in more significant levels the parasite load in distinct organs, as well as induced to the development of anti-parasite Th1-type immunity, attested by the high levels of IFN-γ, IL-12, TNF-α, GM-CSF, nitrite and specific IgG2a antibodies, in addition to low IL-4 and IL-10 contents, along with higher IFN-γ-producing $CD4^+$ and $CD8^+$ T-cell frequency. Furthermore, low toxicity was found in the organs of the treated animals. Comparing the therapeutic effect between the treatments, b-AD/Mic was the most effective in protect animals against infection, when compared to the other groups including miltefosine used as a drug control. Data found 15 days after treatment were similar to those obtained one day post-therapy. In conclusion, the obtained results suggest that b-AD/Mic is a promising antileishmanial agent and deserve further studies to investigate its potential to treat VL.

Keywords: Treatment; β-acetyl-digitoxin; visceral leishmaniasis; drug repositioning; toxicity; miltefosine.

Introduction

Leishmaniases are vector-borne diseases caused by distinct species of protozoan parasites of the genus *Leishmania*. This disease complex is endemic in several countries in the world, where approximately 380 million people are at risk and 2.0 million new cases are registered per year [67]. Leishmaniases comprise distinct clinical manifestations, including tegumentary leishmaniasis (TL), which involves the cutaneous, mucosal and diffuse-cutaneous clinical forms, and visceral leishmaniasis (VL), which can be fatal if acute and untreated [33]. Disease control methods are not effective; the current treatment of the human cases causes toxicity and/or presents high cost, in addition to the emergence of resistant parasite strains [62].

VL is caused by the *Leishmania infantum* species in Latin America, Central Asia and Mediterranean region [3]. In the symptomatic disease, splenomegaly, fever, anemia, weight loss and weakness are commonly observed in the patients [66]. Ideally, a suitable treatment should be safe and non-toxic, effective against parasites and presents shorten time. Since the late 1940s, treatment is based on the use of pentavalent antimonials; however, these compounds are toxic, demand endovenous or intramuscular administration, which is uncomfortable for the patients, and parasite resistance has increased [13, 49]. Amphotericin B (AmpB) has been also used for such therapeutic purpose. Although effective against *Leishmania* parasites, its toxicity is high. Lipid formulations have reduced AmpB toxicity and showed high efficacy; however, the high cost is still an impeditive factor [44]. Miltefosine has been also used as a therapeutic option in several countries in the world, and it was the first oral drug administered against human VL. However, miltefosine causes teratogenicity and parasite resistance has been also registered [19]. Within this context, there is a current demand for the research and development of novel and low-cost antileishmanial agents.

The drug discovery is a long and expensive process. In this aspect, drug repositioning could be considered and tests using compounds with other known biological applications could be evaluated as antileishmanial agents [4]. Cardenolides are glycosides clinically used for over 200 years, with the mechanism of action based on the inhibition of Na^+/K^+-ATPase, involved in the Na^+/K^+ pump mechanism dependent on these ions [41, 46]. Cardenolides have been used for the treatment

against congestive heart failure [26], besides present antitumor [56], anti-inflammatory [29], antimalarial [14], anti-oxidant and anti-aging [68] activities.

The chemical investigation of *Digitalis* species resulted in the isolation of over 80 cardenolides, ascribed as the main bioactive constituents of the genus [21, 27]. Aiming to further explore new antileishmanial candidates, in the present work, the *in vitro* and *in vivo* activity of β-acetyl-digitoxin (b-AD) cardenolide, which was isolated from the leaves of *D. lanata*, was evaluated against *L. infantum* species. *In vitro* assays showed that b-AD was effective against *L. infantum* promastigotes and amastigotes, besides present low toxicity in murine and human cells. Preliminary data showed that this cardenolide derivative present its mechanism of action based on the parasite mitochondria causing their cell death. Additionally, *in vivo* treatment performed in *L. infantum*-infected mice showed that both free b-AD and a composition formed by the molecule incorporated in polymeric micelles (b-AD/Mic) resulted in significant reductions in the parasite load in the spleen, liver, bone marrow (BM) and draining lymph nodes (dLN) of the animals, when analyses were performed one and 15 days post-treatment. Results obtained using miltefosine were similar to those found using the free molecule, but lower as compared to those found using b-AD/Mic. In addition, an anti-parasite Th1-type immunity was observed in the treated and infected animals; suggesting then the possibility to test this molecule as a therapeutic target against VL.

Materials and methods

Drugs and chemicals

Miltefosine, AmpB and Poloxamer 407 (Pluronic® F127) were commercially acquired and present catalog number 58066-85-6, 1397-89-3 and 16758, respectively (Sigma-Aldrich, St. Louis, USA). β-acetyl-digitoxin ($C_{43}H_{66}O_{14}$; molecular weight 807 g/mol) was extracted and purified from leaves of *Digitalis lanata* species. After isolated and purified, the structural elucidation of the compound was carried out using ultraviolet spectrophotometry, mass spectrometry and nuclear magnetic resonance. Data obtained were analyzed and the chemical structure was clarified and is shown *(Figura 5)*.

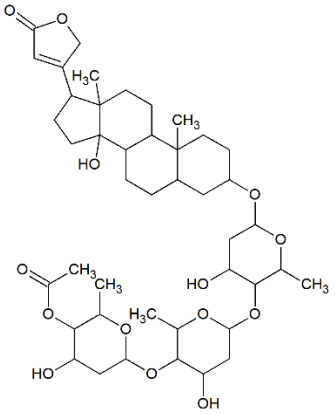

Figura 5. Chemical structure of the β-acetyl-digitoxin.

Ethics statement, experimental animals and parasites

The work was submitted and approved by the Ethical Committee in Animal Research (CEUA) from Federal University of Minas Gerais (UFMG; Belo Horizonte, Minas Gerais, Brazil), with protocol number 085/2017. Female BALB/c mice (6 to 8 weeks old) were acquired from Institute of Biological Sciences (ICB) of UFMG and were kept under pathogen-free conditions. *Leishmania infantum* (MHOM/BR/1970/BH46) was grown in Schneider's medium (Sigma-Aldrich) added with 20% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich) and 20 mM L-glutamine at pH 7.4, 24°C [15].

In vitro antileishmanial activity

The 50% *Leishmania* inhibitory concentration (IC_{50}) was evaluated *in vitro* by incubating logarithmic phase promastigotes in the presence of b-AD (0 to 61.96 µM) or AmpB (0 to 1.08 µM) in 96-well culture plates (Nunclon, Roskilde, Denmark) for 48 h at 24°C. Cell viability was assessed by MTT [3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide; Sigma-Aldrich] method. The optical density (OD) values were read in microplate spectrophotometer (Molecular Devices, Spectra Max Plus, CA, USA), at 570 nm. Results were entered into Microsoft Excel (version 10.0) spreadsheets and IC_{50} values were calculated by sigmoidal regression of the dose-response curve [63].

Cytotoxicity assay

The cytotoxicity was evaluated in murine macrophages and human red cells, when the inhibition 50% of macrophage (CC_{50}) and red cells (RBC_{50}) viability was calculated. For this, macrophages were obtained by peritoneal lavage of female BALB/c mice (n=5) using 5 mL cold phosphate buffered saline (PBS 1x pH 7.4). Peritoneal exudate cells were centrifuged at 1,000 x *g* for 10 min and resuspended in RPMI 1640 medium. Macrophages (5×10^5 cells/mL) were then incubated with (0 to 123.92 µM) or AmpB (0 to 10.82 µM) in RPMI 1640 medium for 48 h at 37°C in 5% CO_2. The cell viability was assayed by MTT method. To evaluate the hemolytic activity, a 5% human red cell suspension was incubated with b-AD (0 to 123.92 µM) or AmpB (0 to 10.82 µM) for 1 h at 37°C in 5% CO_2. The suspension was centrifuged by 1,000 × *g* for 10 min and the lyses percentage was read in spectrophotometer, at 570 nm. The absence or presence of hemolysis were evaluated by replacing b-AD for PBS or distilled water, respectively. Results were entered into Microsoft Excel spreadsheets and CC_{50} and RBC_{50} values were calculated using sigmoidal regression by means of dose-response curves [63].

Treatment of infected macrophages and inhibition of the infection

Stationary phase promastigotes (5×10^5) were cultured in RPMI 1640 medium added with 20% FBS and 20 mM L-glutamine pH 7.4, for 24 h at 37 °C in 5% CO_2. Parasites were then included in the cultures, at a ratio of 10 parasites per macrophage, and the incubation were developed for 48 h at 37°C in 5% CO_2. Free parasites were removed by washing with medium and *Leishmania*-infected macrophages were incubated with b-AD (0, 3.09, 6.19 and 12.39 µM) or AmpB (0, 0.27, 0.54 and 1.08 µM) for 48 h at 24 °C in 5% CO_2. In another experiment, stationary phase promastigotes (5×10^6 cells) were incubated with b-AD (0, 3.09, 6.19 and 12.39 µM) or AmpB (0, 0.27, 0.54 and 1.08 µM) for 4 h at 24°C. Parasites were washed in RPMI 1640, quantified and added in cultures to infect murine macrophages, at a ratio of 10 parasites per macrophage, for 48 h at 37°C in 5% CO_2. After cell fixation using 4% paraformaldehyde, cells were washed and stained with Giemsa, when the infection percentage, the number of amastigotes per treated macrophage, and the reduction in

the infection percentage were determined by counting 200 cells, in triplicate, using optical microscope [39].

Evaluation of the mitochondrial membrane potential

Leishmania infantum promastigotes (10^7 cells) were cultured in the absence or presence of b-AD (41.93 µM, corresponding to 2× the IC_{50} value) for 24 h at 25°C. Parasites were washed in PBS and incubated with 500 nM MitoTracker Red CM-H2XROS (Invitrogen, USA), for 30 min in the dark and at room temperature. After washing twice with PBS, cells were added to a black 96-well plate and fluorescence intensity was measured in a fluorometer (FLx800, BioTek Instruments, Inc., VT, USA), with excitation and emission wavelengths of 528 nm and 600 nm, respectively. Parasites treated with carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazon (FCCP; 5.0 µM) for 10 min were used as positive control, while untreated parasites were used as negative control [25].

Reactive oxygen species (ROS) production

Leishmania infantum promastigotes (10^7 cells) were cultured in the absence or presence of b-AD (41.93 µM, corresponding to two times the IC_{50} value) for 24 h at 25°C. After, parasites were incubated with 20 µM cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Sigma-Aldrich, USA) for 30 min 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 parasites (4000 µM; Sigma-Aldrich, USA) were used as positive control, while untreated parasites were used as negative control [25].

Prepare of b-AD-containing micelles

The b-AD-containing micelles (b-AD/Mic) were prepared according described elsewhere [54]. Briefly, Poloxamer 407 (18% w/w) was diluted in PBS under magnetic agitation for 18 h at 4°C. Eight milligrams of b-AD were added to dichloromethane (500

μL) and solubilized using vortex. The mixture was added to the prepared solution under vigorous agitation and in ice bath, until a viscous emulsion has been obtained. The alcohol was evaporated using a rotary evaporate (Buchi, Flawil, Switzerland), and the b-AD/Mic composition was obtained as a transparent yellow gel at 22ºC. Empty micelles were prepared (18% w/w) using the protocol described above but without addition of b-AD.

Mice infection and treatment regimens

Mice (n=12 per group) were infected subcutaneously with *L. infantum* stationary-phase promastigotes (10^7 parasites) and, 60 days after, they were grouped and received one of the following regimens, which was administered by subcutaneous route: saline group: mice received PBS (50 μL); empty micelles (B/Mic) group: mice received empty micelles (10 mg/kg body weight diluted in 50 μL of PBS); miltefosine group: mice received miltefosine by oral route (2 mg/kg body weight); b-AD group: mice received b-AD (5 mg/kg body weight diluted in 50 μL of PBS) and b-AD/micelle (b-AD/Mic) group: mice received b-AD-containing micelles (5 mg/kg body weight diluted in 50 μL of PBS). Treatments were performed each two days and during 10 days. One and 15 days after treatment, respectively, half of the animals of each group were euthanized, when parasitological and immunological analyses were developed.

Evaluation of parasite load

The organ parasitism was evaluated in spleen, liver, BM and dLN of the treated and infected animals, which were collected one and 15 days post-treatment, by a limiting dilution technique [64]. For this, organs were macerated in a glass tissue grinder using sterile PBS, and tissue debris were removed by centrifugation at 150 × *g*. Cells were centrifuged at 2,000 x *g* and pellets were resuspended in 1 mL of complete Schneider's medium, when a log-fold serial dilution was performed in complete Schneider's medium. Each sample was plated in triplicate and read 7 days after the beginning of the cultures at 24°C. Results were expressed as the negative log of the titer (the dilution corresponding to the last positive well) adjusted per milligram of organ. The spleen parasitism was also evaluated by a quantitative PCR

(qPCR) [5, 20]. For this, DNA was extracted using commercial kit (Wizard Genomic DNA Purification Kit, Promega Corporation, USA) and resuspended in milli-Q water. The following primers were used to amplify *L. infantum* kDNA: *Forward* (CCTATTTTACACCAACCCCCAGT) and *Reverse* (GGGTAGGGGCGTTCTGCGAAA), while β-actin gene (*Forward*: CAGAGCAAGAGAGGTATCC; *Reverse*: TCATTGTAGAAGGTGTGGTGC) was used as a control. Standard curves were obtained from DNA extracted from 10^8 parasites for kDNA and 10^8 macrophages for β-actin, using the same technical conditions. Reactions were developed and analyzed in an ABI Prism 7500 Sequence Detection System (96 wells-plate; Applied Biosystems), using 2x SYBR™ Select Master Mix (Applied Biosystems), 2 mM of each primer and 25 ng/μL DNA. 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. Fluorescence data were obtained in each time and results were determined by interpolation from standard curve used in the same run, in duplicate, and expressed as the parasite number per total DNA.

Cellular response

Mice spleens were collected one and 15 days after treatment, when they (5×10^6 cells per mL) were incubated in 24-well plates (Nunc) cultured in DMEM plus 20% FBS and 20 mM L-glutamine at pH 7.4. Cells were unstimulated (medium) or stimulated with *L. infantum* SLA (50.0 μg/mL) for 48 h at 37°C in 5% CO_2. Levels of IFN-γ, IL-12, GM-CSF, IL-4 and IL-10 were measured in the culture supernatant by commercial kits obtained from BD Pharmingen® (San Diego, CA, USA), according to the manufacturer's instructions. The nitrite production was also evaluated in the cell supernatant by Griess reaction. In addition, in some culture wells, anti-CD4 (GK 1.5) and anti-CD8 (53-6.7) monoclonal antibodies (5.0 μg each; Pharmingen®, USA) were also added and processed according described above, aiming to evaluate the IFN-γ-producing T-cell profile in the miltefosine, b-AD or b-AD/Mic-treated mice groups. For this experiment, appropriate isotype-matched controls [rat IgG2a (R35-95) and rat IgG2b (95-1)] were also used. A flow cytometry assay was developed in the stimulated cultures to evaluate the IFN-γ and IL-10-producing $CD4^+$ and $CD8^+$ T cell frequency. Experiments were based on the cell relative flow cytometry size (forward laser scatter

– FSC) and granularity (side laser scatter – SSC) graphs. After the selection of the interest region R1 containing FSCLow and SSCLow phenotype cells, graphs of density plot distribution of CD4/FL1 or CD8/FL1 versus IFN-γ/FL2$^+$, TNF-α/FL2$^+$ and IL-10/FL2$^+$ cells were constructed to determine the IFN-γ$^+$, TNF-α and IL-10$^+$-producing T cell frequency. Values were calculated by ratio between the CD4$^+$ and CD8$^+$ T-cell percentage in the SLA-stimulated versus unstimulated cultures, and they were expressed as indexes in the graphs [64].

Antibody production

Levels of anti-*Leishmania* IgG1 and IgG2a isotype antibodies were measured in sera samples of the treated animals, which were obtained one and 15 days after treatment, according described [25]. Briefly, SLA was added as an antigen in the plates, at a concentration of 1.0 µg per well, for 16 h at 4ºC. Free binding sites were then blocked with PBS and Tween 20 0.05% (PBS-T) plus 5% casein, for 1 h at 37°C, when plates were washed five times with PBS-T and incubated with mouse sera (1:100 diluted in PBS-T), for 1 h at 37°C. After, plates were washed five times and incubated with anti-mouse IgG1 and IgG2a horseradish-peroxidase conjugated antibodies (both 1:10,000 diluted in PBS-T), for 1 h at 37°C. They were again washed and reactions were developed using a solution composed by H_2O_2, ortho-phenylenediamine and citrate-phosphate buffer pH 5.0, for 30 min in the dark. After, reactions were stopped by adding 2 N H_2SO_4 and OD values were read in a spectrophotometer, at 492 nm.

***In vivo* toxicity**

The *in vivo* cytotoxicity was evaluated in serum samples of the treated and infected animals, one and 15 days post-treatment. For this, levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatine kinase muscle brain fraction (CK-MB) were evaluated in the samples, by using commercial kits (Labtest Diagnostica®, Belo Horizonte) according to the manufacturer´ instructions.

Statistical analysis

IC_{50}, CC_{50} and RBC_{50} values were entered into Microsoft Excel (version 10.0) spreadsheets and calculated by dose-response curves, which were plotted in GraphPad Prism 5.03. Results were evaluated by the one-way analysis of variance (ANOVA) followed by Bonferroni´s post-test. Data were expressed as mean ± standard deviation of the groups. Two independent experiments presenting similar results were performed and differences were significant when $P < 0.05$.

Results

In vitro biological assays

The *in vitro* antileishmanial effect of the compounds was evaluated against *L. infantum* species, where the IC_{50} values of 20.94±2.60 and 0.11±0.03 μM were, respectively, obtained for b-AD and AmpB *(Tabela 1)*. In its turn, the CC_{50} and RBC_{50} values determined for b-AD were of 453.04±23.29 and 334.95±24.90 μM, respectively, and from 0.86±0.11 and 12.66±1.52 μM for AmpB, respectively. The calculated selectivity indexes were 21.64 and 7.82 for b-AD and AmpB, respectively *(Tabela 1)*. Treatment of infected macrophages reduced the infection percentage by 89.0% and 71.2%, when b-AD and AmpB were tested at 12.39 and 1.08 μM, respectively *(Tabela 2)*. The inhibition of infection using pre-treated parasites was also evaluated, and results showed reductions in the infection percentage by 84.8% and 68.2%, when b-AD and AmpB were assayed at 12.39 and 1.08 μM, respectively *(Tabela 3).* Preliminary investigations of the effects of b-AD in *L. infantum* suggested that the molecule cause alterations in the parasites´ mitochondria, by altering their membrane potential *(Figura 6)* and causing increase in the ROS production *(Figura 7).*

***Tabela 1*. *In vitro* biological activity.** *L. infantum* logarithmic phase promastigotes were incubated with b-AD (0 to 61.96 µM) or AmpB (0 to 1.08 µM) for 48 h at 24ºC. Cell viability was analyzed by MTT method and the 50% *Leishmania* inhibitory concentration (IC_{50}) was calculated by applying sigmoidal regression of dose-response curve. Murine macrophages were also incubated with b-AD (0 to 123.92 µM) or AmpB (0 to 10.82 µM) for 48 h at 37°C in 5% CO_2, and the 50% cell inhibitory concentration (CC_{50}) was also evaluated by a dose-response curve. The selectivity index (SI) was calculated by ratio between the CC_{50} and IC_{50} values. The 50% human red cells inhibitory concentration (RBC_{50}) was determined by incubating the 5% red cell suspension with b-AD (0 to 123.92 µM) or AmpB (0 to 10.82 µM) for 1 h at 37°C in 5% CO_2. The lyse percentage was evaluated spectrophotometrically, and the absence or presence of hemolysis were determined by replacing b-AD for PBS or distilled water, respectively. Results are showed as mean ± standard deviation of the groups.

Compound	IC_{50} (µM)	CC_{50} (µM)	SI	RBC_{50} (µM)
β-acetyl-digitoxin	20.94±2.60	453.04±23.29	21.6	334.95±24.90
Amphotericin B	0.11±0.03	0.86±0.11	8.0	12.66±1.52

***Tabela 2*. Treatment of infected macrophages**. Murine macrophages (5 x 10^5 cells) were cultured in complete RPMI 1640 medium for 24 h at 37°C in 5% CO_2. After, cells were washed twice with PBS and *L. infantum* stationary promastigotes were added in the cultures at a ratio of 10 parasites per macrophage, for 48 h at 37°C in 5% CO_2. Free parasites were removed by washing with RPMI 1640 medium, and infected macrophages were treated with b-AD (0, 3.09, 6.19 and 12.39 µM) or AmpB (0, 0.27, 0.54 and 1.08 µM) for 48 h at 24˚C in 5% CO_2. The percentage of infected cells, the infectiveness reduction and the number of recovered amastigotes per cell were determined by counting 200 macrophages in triplicate. Results are showed as mean ± standard deviation of the groups.

Compound	Concentration (µM)	Percentage of infected macrophages after treatment	Infectiveness reduction (%)	Number of amastigotes per macrophage
β-acetyl-digitoxin	12.39	7.6±1.2	89.0	0.1±0
	6.19	14.3±2.5	79.2	0.4±0.2
	3.09	24.3±3.3	64.6	1.2±0.4
	0	68.7±4.0	(-)	3.9±0.5
Amphotericin B	1.08	19.8±2.4	71.2	0.8±0.4
	0.54	32.1±3.8	53.3	1.3±0.3
	0.27	42.5±5.2	38.1	2.1±0.4
	0	68.7±4.0	(-)	3.9±0.5

Tabela 3. **Inhibition of infection using pre-treated parasites.** The inhibition of infection of murine macrophages using pre-treated parasites was performed by incubating *L. infantum* stationary promastigotes (5 x 10^6 cells) with b-AD (0, 3.09, 6.19 and 12.39 µM) or AmpB (0, 0.27, 0.54 and 1.08 µM) for 4 h at 24°C. Parasites were then washed in RPMI 1640 and used to infect murine cells at a ratio of 10 parasites per macrophage, for 48 h at 37°C in 5% CO_2. The percentage of infected macrophages, the infectiveness reduction and the number of amastigotes per treated macrophage were evaluated by counting 200 macrophages in triplicate. Results are showed as mean ± standard deviation of the groups.

Compound	Concentration (µM)	Infection percentage using pre-treated parasites	Infectiveness reduction (%)	Number of amastigotes per macrophage
β-acetyl-digitoxin	12.39	8.8±2.0	84.8	0.2±0.1
	6.19	13.3±2.4	77.0	0.7±0.3
	3.09	27.4±4.0	52.6	1.6±0.5
	0	57.8±5.2	(-)	3.3±0.6
Amphotericin B	1.08	18.4±2.6	68.2	0.7±0.3
	0.54	26.5±2.7	54.2	1.2±0.3
	0.27	38.7±4.3	33.0	2.2±0.4
	0	57.8±5.2	(-)	3.3±0.6

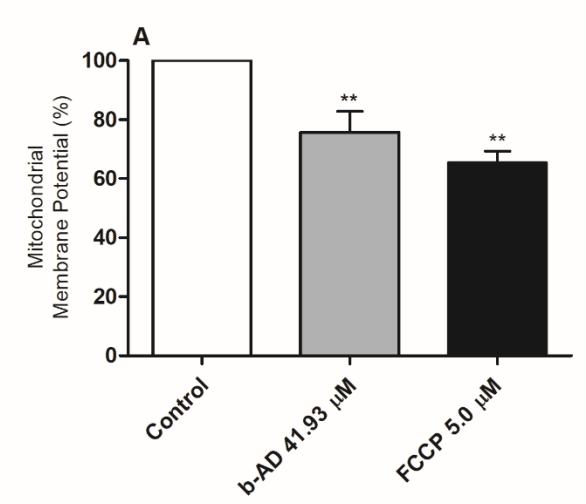

Figura 6. **Evaluation of the mitochondrial membrane potential.** *L. infantum* stationary promastigotes (10^7 cells) were cultured in the absence (control) or presence of b-AD (41,93 µM, corresponding to two times the IC_{50} value) for 24 h at 25°C. Cells were incubated for 30 min in the dark with 500 nM MitoTracker Red CM-H2XROS. After washing twice with PBS, treated promastigotes were transferred to a black 96-well plate and fluorescence intensity was measured

using a fluorometer, with excitation and emission wavelengths of 540 and 600 nm, respectively. Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazon (FCCP, 5.0 μM)-treated parasites were used as positive control, while non-treated parasites were used as negative control (control). Bars indicate the mean plus standard deviation of the groups. (**) indicates statistically significant difference in relation to the control ($P < 0.0001$).

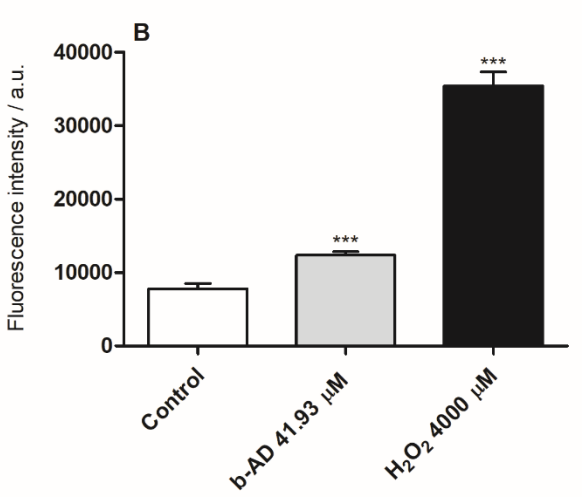

***Figura 7*. Production of the reactive oxygen species.** *L. infantum* (10^7 cells) were incubated alone (control) or in the presence of b-AD (41.93 μM) for 24 h at 25°C. After, parasites were washed twice in PBS and incubated with 20 μM cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), for 30 min and in the dark. The fluorescence was measured in a fluorometer, with excitation and emission wavelengths of 485 and 528 nm, respectively. H_2O_2 (4000 μM; Sigma-Aldrich, USA)-treated parasites were used as positive control, while untreated parasites were used as negative control (control). Bars represent the mean plus standard deviation of the groups. (***) indicate statistically significant difference as compared to the control ($P < 0.0001$).

Estimation of the parasite load

The organ parasitism was evaluated one and 15 days post-treatment in liver, spleen, BM and dLNs of the treated animals. Results showed that miltefosine, free b-AD or b-AD/Mic groups mice presented significant reductions in the parasite load as compared to values encountered in the saline and B/Mic groups *(Figura 8)*. One day post-therapy, miltefosine, b-AD and b-AD/Mic groups mice presented lower parasitism in their spleens (3.0, 3.7 and 4.6-log reductions, respectively), livers (2.3, 2.7 and 4.0-log reductions, respectively), dLNs (3.7, 4.3 and 5.3-log reductions, respectively), and BMs (1.7, 2.0 and 2.7-log reductions, respectively), when compared to the saline group. Fifteen days after treatment, reductions in the parasite load in the miltefosine,

b-AD and b-AD/Mic groups were in the order of 3.3, 4.0 and 5.7-log reductions, respectively, in their spleens; of 2.7, 3.0 and 4.3-log reductions, respectively, in their livers; of 4.0, 4.7 and 6.0-log reductions, respectively, in their dLNs; and of 2.0, 2.3 and 3.7-log reductions, respectively, in their BM; when compared to the saline group. Comparison between the treated groups revealed that mice receiving b-AD/Mic showed the highest reductions in the organ parasitism. The spleens of the animals were also used to determine the parasite load by qPCR technique, and results showed also that miltefosine, free b-AD or b-AD/Mic-treated mice presented significant reductions in the splenic parasitism, when compared to values found in the control groups. Additionally, b-AD/Mic induced also the highest reductions in the splenic parasitism as compared to the others *(Figura 9)*.

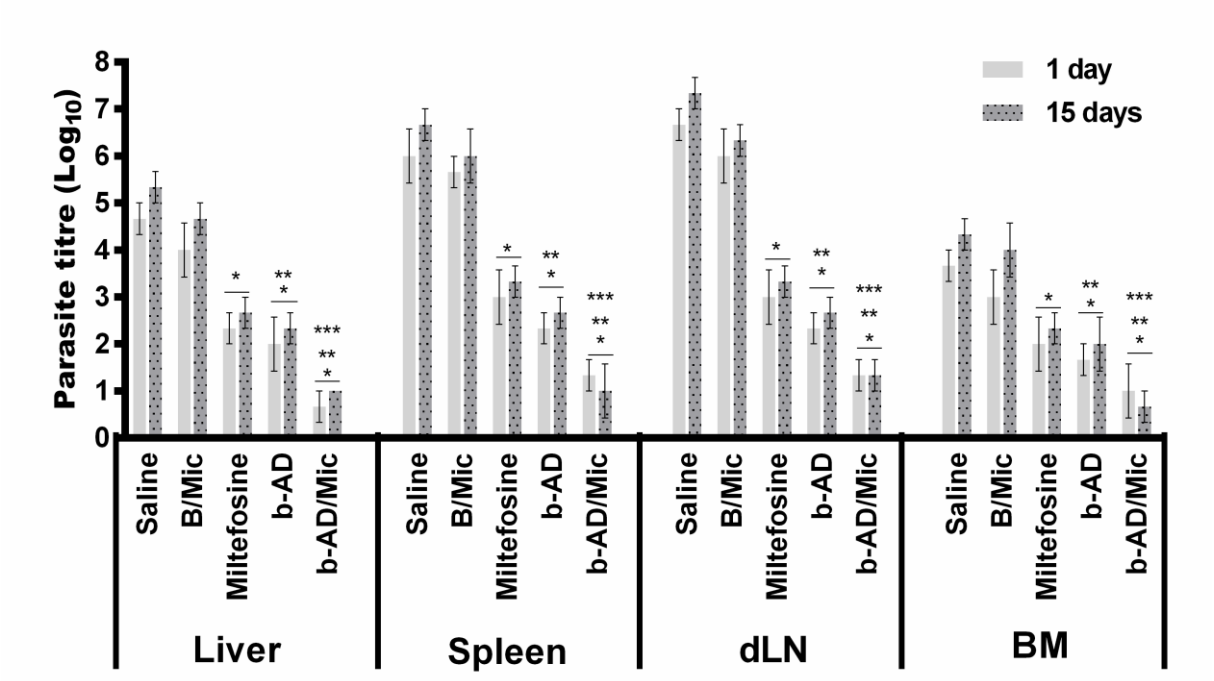

Figura 8. **Parasite load evaluated by limiting dilution technique.** *L. infantum*-infected BALB/c mice (n=12 per group) were treated and, one and 15 days post-therapy, animals (n=6 per group, in each case) were euthanized, and their livers, spleens, bone marrows (BM) and draining lymph nodes (dLN) were collected to evaluate the parasite load by limiting dilution technique. Bars indicate the mean ± standard deviation of the groups, one and 15 days post-treatment. (*) indicate significant difference in relation to the saline and B/Mic groups ($P < 0.05$). (**) indicate statistically significant difference in relation to the miltefosine group ($P < 0.05$). (***) indicate statistically significant difference in relation to the b-AD group ($P < 0.05$).

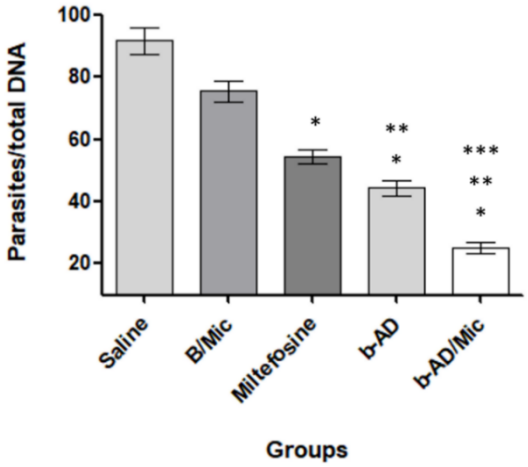

***Figura 9*. Splenic parasite load evaluated by quantitative PCR technique.** *L. infantum*-infected BALB/c mice (n=12 per group) were treated and, 15 days post-treatment, animals (n=6 per group) were euthanized, their spleen collected and the parasite load was estimated by qPCR technique. Results were expressed as the number of parasites per total DNA. Bars indicate the mean ± standard deviation of the groups. (*) indicate significant difference in relation to the saline and B/Mic groups ($P < 0.05$). (**) indicate statistically significant difference in relation to the miltefosine group ($P < 0.05$). (***) indicate statistically significant difference in relation to the b-AD group ($P < 0.05$).

Investigating the immune response in the treated animals

Anti-parasite Th1 and Th2-type cytokines were evaluated in the cell supernatant of splenocytes cultures of the treated animals. Results showed that spleen cells of miltefosine, b-AD or b-AD/Mic groups mice produced significantly higher levels of IFN-γ, IL-12 and GM-CSF, as well as low levels of IL-4 and IL-10, when compared to data found in the saline and B/Mic groups, when both periods of time were evaluated *(Figura 10)*. Aiming to investigate the T cell subtype responsible by IFN-γ production in the treated and infected animals, anti-CD4 and anti-CD8 monoclonal antibodies were added into the *in vitro* cultures, and results showed that both antibody subtypes reduced in significant levels the production of this cytokine; suggesting then that $CD4^+$ and $CD8^+$ T cells were important for immunological response against *L. infantum* infection *(Figura 11).* A flow cytometry assay showed also that miltefosine, b-AD and b-AD/Mic-treated mice presented higher IFN-γ and TNF-α-producing T-cell subtype frequency as compared to data obtained in the saline and B/Mic groups, which showed higher IL-10-producing $CD4^+$ and $CD8^+$ T-cell levels *(Figura 12)*. Between the

therapeutics, b-AD/Mic induced higher presence of IFN-γ and TNF-α-producing $CD4^+$ and $CD8^+$ T cells. The nitrite production was evaluated by Griess reaction, and results showed that miltefosine, b-AD or b-AD/Mic groups mice produced higher levels of this cell activation marker, when compared to values found in the saline and B/Mic groups *(Figura 13)*. The evaluation of humoral response showed also that miltefosine, b-AD or b-AD/Mic groups mice produced significantly higher levels of anti-*Leishmania* IgG2a antibody, as compared to IgG1 levels. Otherwise, saline and B/Mic groups mice produced higher anti-parasite IgG1 isotype levels than IgG2a isotype, in both periods of time post-treatment *(Figura 14)*. The treatment using b-AD/Mic induced to the highest levels of anti-parasite IgG2a antibodies as compared to the other groups.

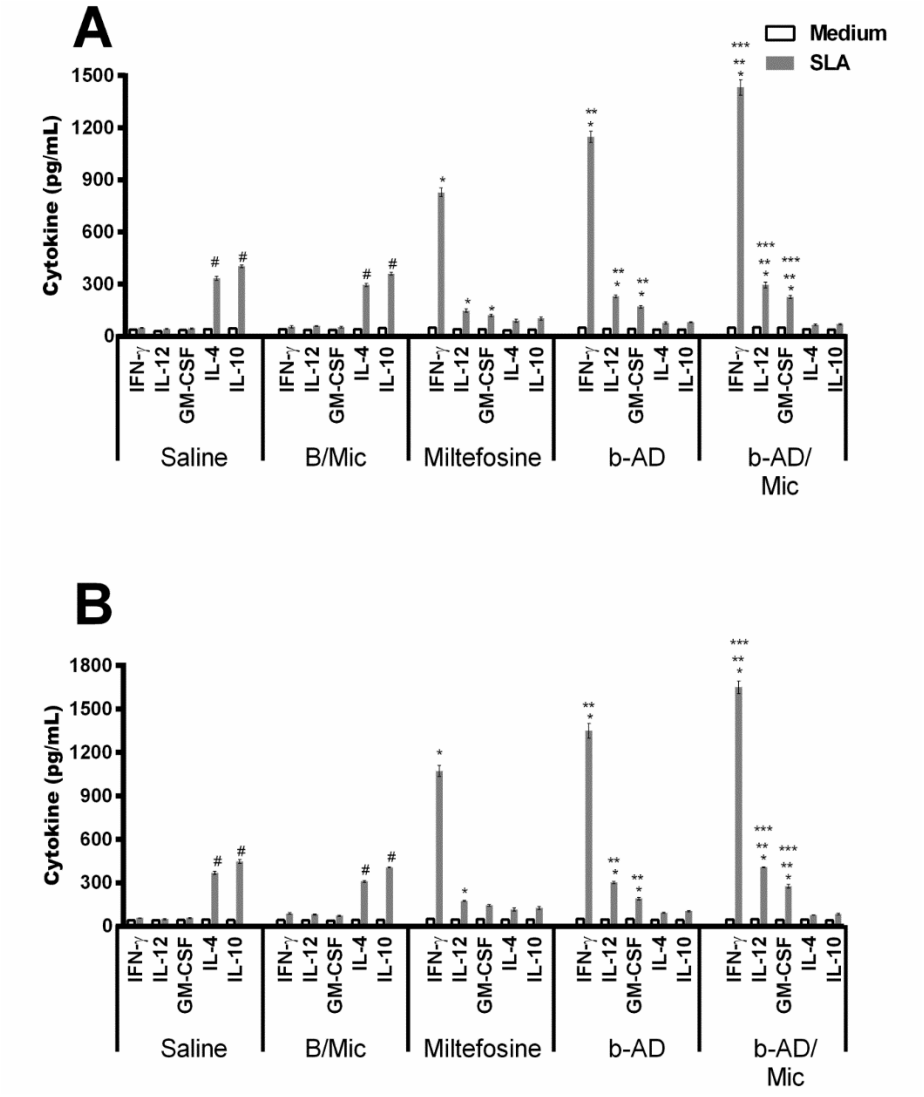

Figura 10. **Cellular response developed in the treated and infected animals.** *L. infantum*-infected BALB/c mice (n=12 per group) were treated and, one and 15 days post-treatment, their spleens were collected, and spleen cells were non-stimulated (medium) or stimulated with *L. infantum* SLA (50 µg/mL) for 48 h at 37°C in 5% CO_2. IFN-γ, IL-4, IL-10, IL-12p70 and GM-CSF

levels were measured in the cell supernatant, one (A) and 15 (B) days post-treatment, by using commercial kits. Bars indicate the mean ± standard deviation of the groups. (*) indicate significant difference in relation to the saline and B/Mic groups ($P < 0.05$). (**) indicate statistically significant difference in relation to the miltefosine group ($P < 0.05$). (***) indicate statistically significant difference in relation to the b-AD group ($P < 0.05$). (#) indicate statistically significant difference in relation to the miltefosine, b-AD and b-AD/Mic groups ($P < 0.05$).

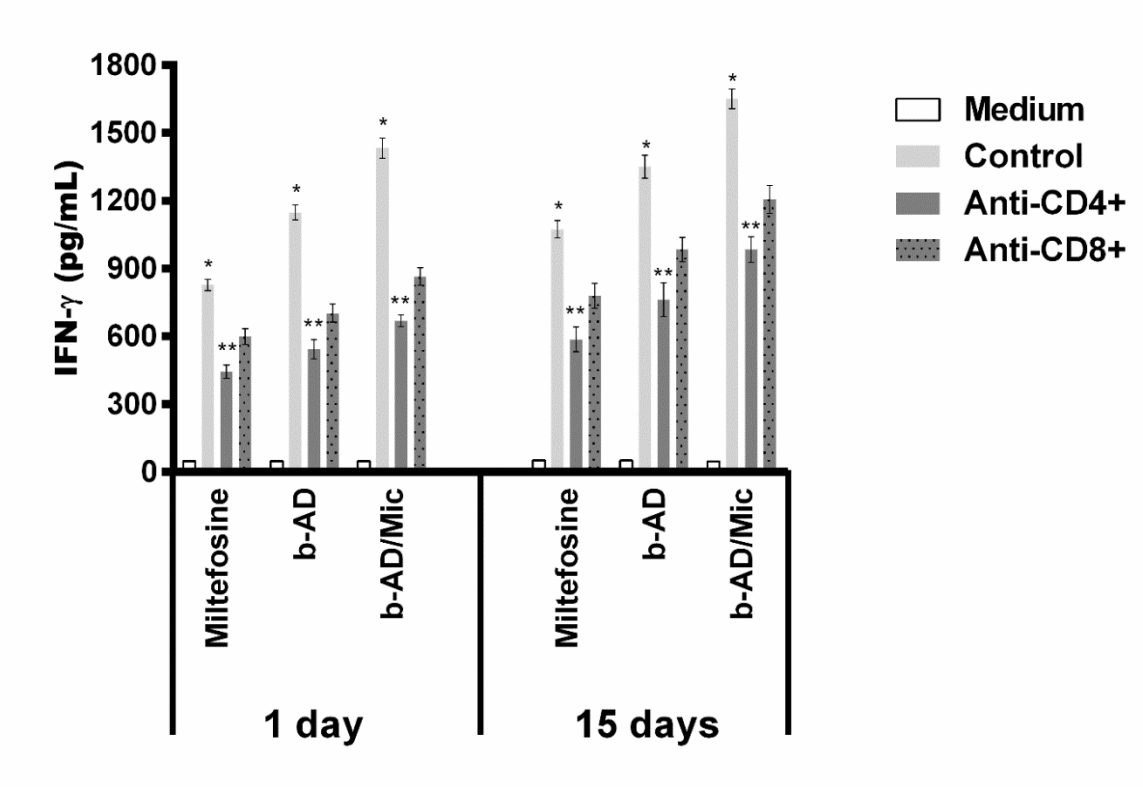

Figura 11. **Involvement of T-cell subtypes in the IFN-γ production.** Splenocytes of the miltefosine, b-AD or b-AD/Mic-treated and infected mice (n=6 per group) were cultured in DMEM (medium) or stimulated with SLA (50 μg/mL) in the presence of anti-CD4 or anti-CD8 antibody, for 48 h at 37°C in 5% CO_2. IFN-γ levels were then measured in the cell supernatant. Bars indicate the mean ± standard deviation of the groups. (*) indicate statistically significant difference in relation to the use of anti-CD4 and anti-CD8 monoclonal antibodies ($P < 0.05$). (**) indicate statistically significant difference in relation to the use of anti-CD8 monoclonal antibody ($P < 0.05$).

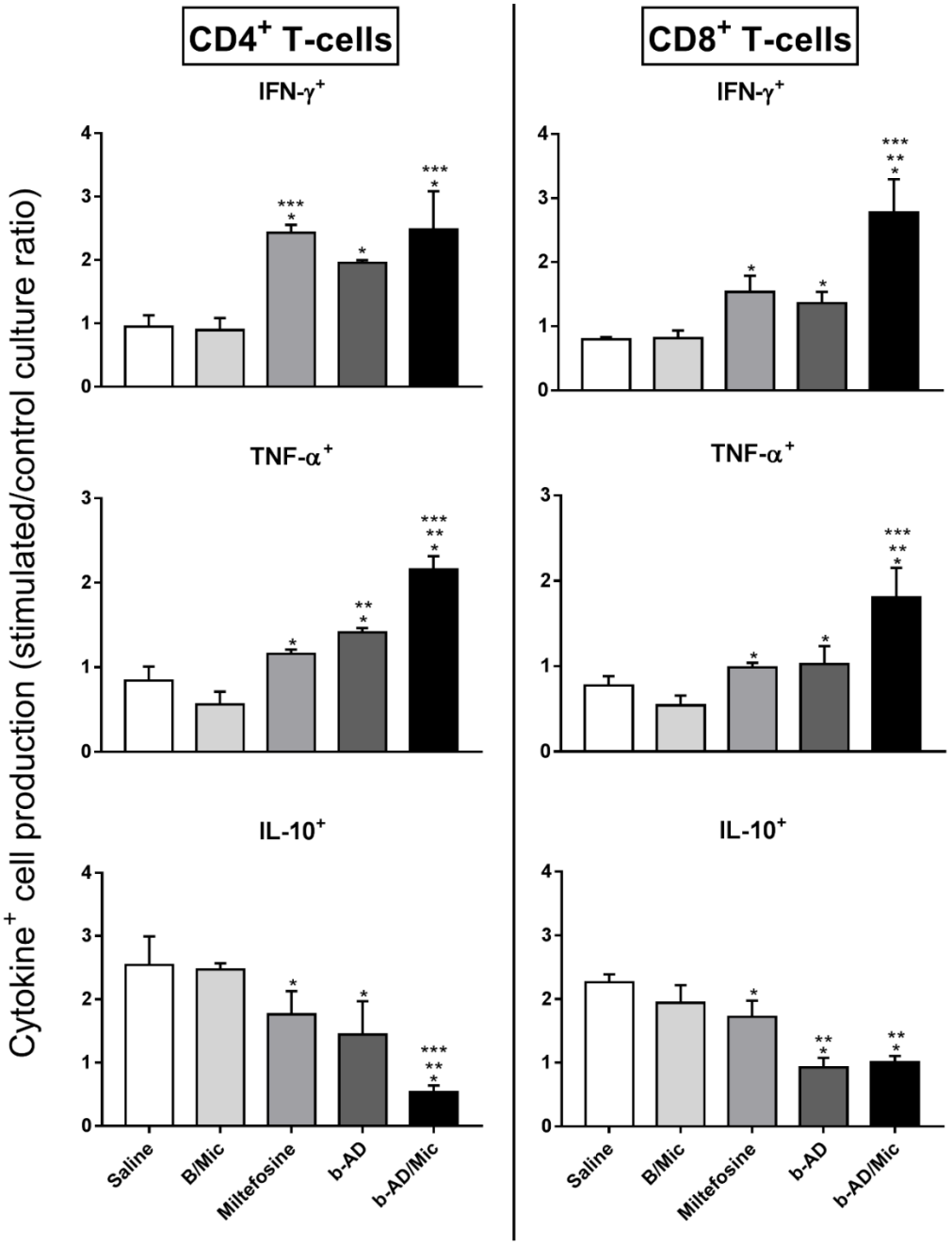

Figura 12. **Flow cytometry assay to investigate the intracytoplasmic cytokine-producing T cell frequency**. The IFN-γ, TNF-α and IL-10-producing T $CD4^+$ and $CD8^+$ cell frequency in the spleen cells of the treated and infected mice was evaluated 15 days post-treatment, by using a flow cytometry technique. Results were calculated by the ratio between positive cell cultures after SLA-stimulation divided by the control (unstimulated) cultures (SLA/CC ratio). Results were reported as cytokine indexes (SLA/CC ratio) for $CD4^+$ and $CD8^+$ T cell subtypes. Bars represent the mean plus standard deviation of the groups. (*) indicate significant difference in relation to the saline and B/Mic groups ($P < 0.05$). (**) indicate statistically significant difference in relation to the miltefosine group ($P < 0.05$). (***) indicate statistically significant difference in relation to the b-AD group ($P < 0.05$).

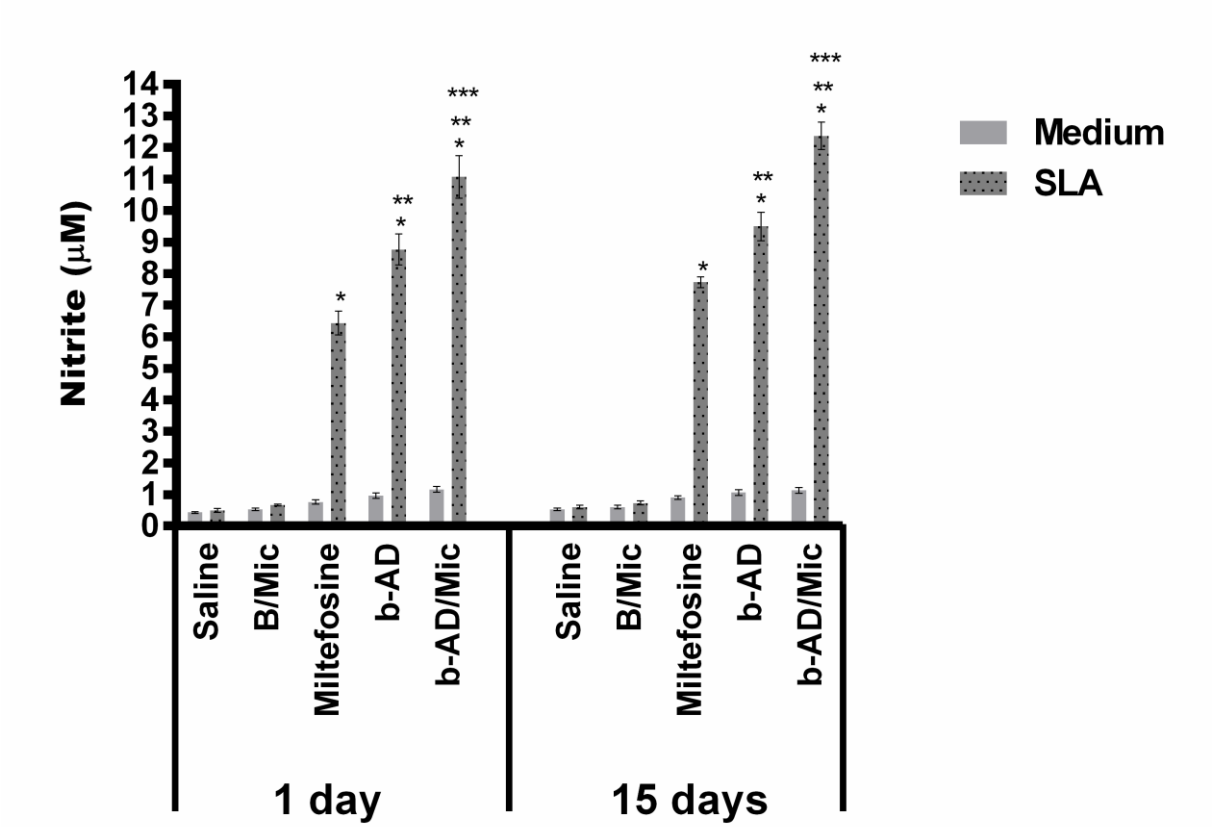

Figura 13. **Nitrite secretion.** The cell supernatant used to quantify cytokines was also employed to evaluate the nitrite production. Bars indicate the mean ± standard deviation of the groups. (*) indicate significant difference in relation to the saline and B/Mic groups ($P < 0.05$). (**) indicate statistically significant difference in relation to the miltefosine group ($P < 0.05$). (***) indicate statistically significant difference in relation to the b-AD group ($P < 0.05$). ($^{\#}$) indicate statistically significant difference in relation to the miltefosine, b-AD and b-AD/Mic groups ($P < 0.05$).

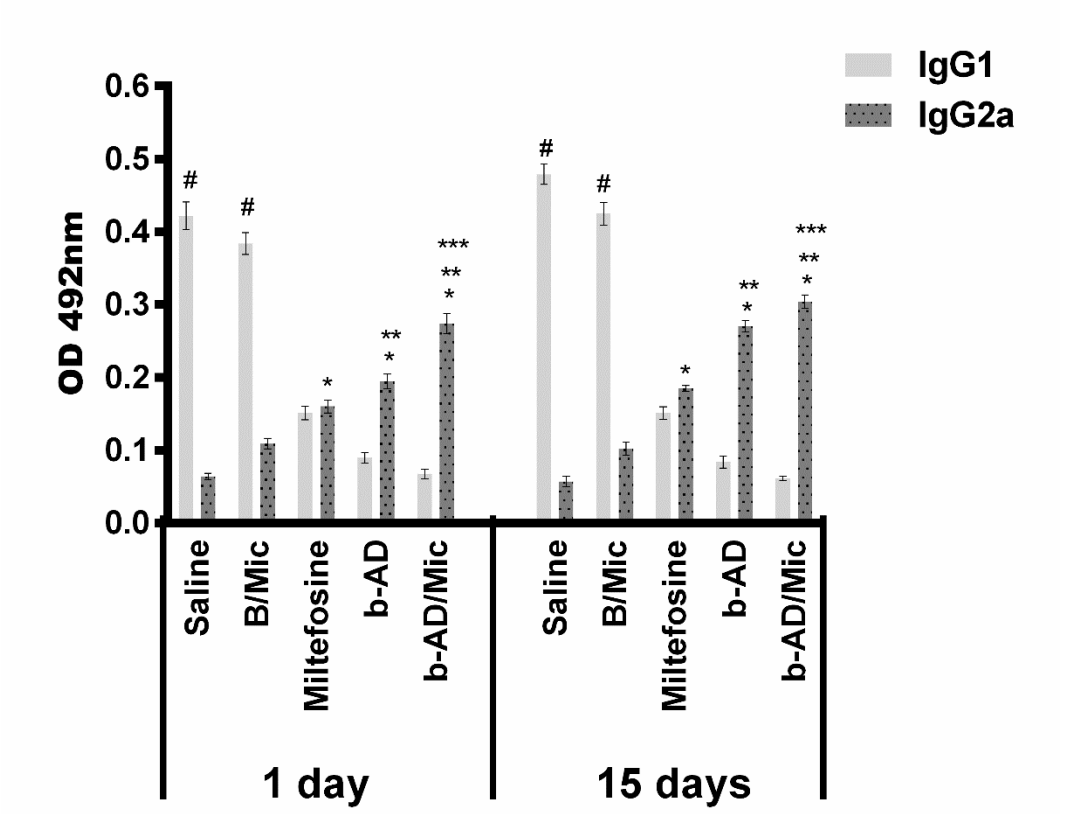

Figura 14. **Humoral response developed in the treated and infected mice**. Sera samples were collected of treated and *L. infantum*-infected animals, one and 15 days post-therapy, when levels of antileishmanial IgG1 and IgG2a isotype antibodies were estimated by an ELISA technique. Bars indicate the mean ± standard deviation of the groups. (*) indicate significant difference in relation to the saline and B/Mic groups ($P < 0.05$). (**) indicate statistically significant difference in relation to the miltefosine group ($P < 0.05$). (***) indicate statistically significant difference in relation to the b-AD group ($P < 0.05$). ($^{\#}$) indicate statistically significant difference in relation to the miltefosine, b-AD and b-AD/Mic groups ($P < 0.05$).

Evaluating the *in vivo* toxicity

The hepatic and cardiac toxicity was evaluated in the treated and infected animals. Results showed higher levels of ALT, AST and CK-MB enzymes in saline and B/Mic groups mice, suggesting that organic alterations could occur in these animals caused by infection and/or treatment. On the other hand, miltefosine, b-AD and b-AD/Mic groups mice produced lower levels of these enzymatic markers, with animals treated with b-AD/Mic being those presenting the lowest levels of AST, ALT and CK-MB *(Figura 15).*

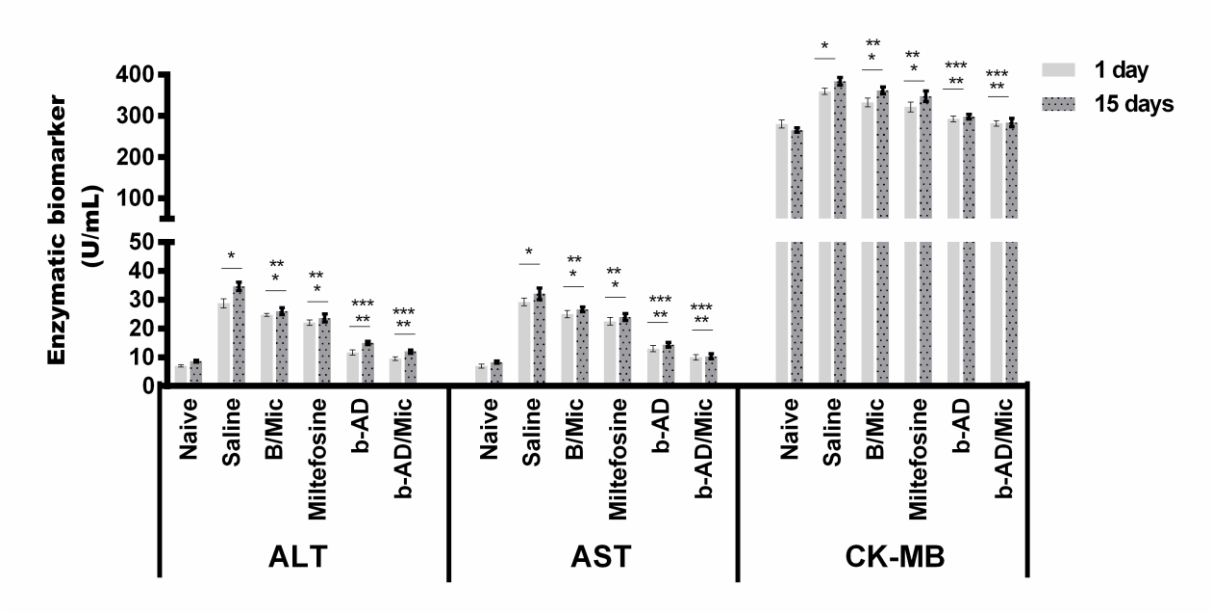

Figura 15. **Evaluation of *in vivo* toxicity.** Levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatine kinase muscle brain fraction (CK-MB) enzymes were estimated using sera samples collected of treated and infected mice, one and 15 days post-treatment. Sera of naive (non-infected and non-treated) mice were used as control. Bars represent the mean ± standard deviation of the groups. (*) indicate significant difference in relation to the saline and B/Mic groups ($P < 0.05$). (**) indicate statistically significant difference in relation to the miltefosine group ($P < 0.05$). (***) indicate statistically significant difference in relation to the b-AD group ($P < 0.05$). ($^{\#}$) indicate statistically significant difference in relation to the miltefosine, b-AD and b-AD/Mic groups ($P < 0.05$).

Discussion

Drugs currently used for VL treatment are toxic, costly, present long duration time and/or variable efficacy [61]. In this context, novel antileishmanial agents are needed and plant products may represent a valid strategy for drug development, especially those compounds or derivatives in clinical use, such as cardenolide derivatives. Cardiac glycosides such as digoxin are used to heart diseases, and several compounds of this class have been described to induce a plethora of biological responses, including anti-inflammatory and antiprotozoal activities [14, 29, 68]. Following the rationale to identify novel antileishmanial targets; in the present work, a cardenolide derivative called β-acetyl-digitoxin, which was obtained from *Digitalis lanata* leaves, was evaluated regarding its *in vitro* and *in vivo* activity against *L.*

infantum. The obtained results disclosed the effectiveness of b-AD against the parasites, especially when it was tested incorporated in Poloxamer 407-based polymeric micelles. This composition was more efficient in reducing the parasite load in the treated and infected animals, and also in inducing a more polarized and specific antileishmanial Th1-type response.

The occurrence of active VL depends on the activation of T cell subsets, being Th1-type cytokines, such as IFN-γ, GM-CSF and IL-12, among others related to the protective response, while IL-4, IL-5, IL-6, IL-10, among others related to the development of the disease [16, 18, 42]. Usually, the protective immunity depends on the induction of specific Th1-type response, which activates macrophages to kill intracellular parasites through nitric oxide-mediated mechanism [30]. In this study, considering the analysis of T-cell populations and cytokine production, both $CD4^+$ and $CD8^+$ T subtypes were showed to be origin of IFN-γ in mice that were treated with miltefosine, b-AD or b-AD/Mic. The Th1-type profile was related to the parasitism control in distinct organs of the animals, when the parasite load was evaluated through limiting dilution technique and qPCR. On the other hand, control groups mice showed higher parasitism in these systemic organs, as well as the development of Th2-type immune response. Parasitological and immunological evaluations performed one and 15 days post-treatment indicated the maintenance of the positive therapeutic response in the treated animals, suggesting then a possible long-term efficacy of b-AD against *L. infantum*.

In our study, b-AD showed higher therapeutic efficacy, when it was incorporated in Poloxamer 407 (Pluronic® F127)-based polymeric micelles. The employ of micelles as delivery for antileishmanial molecules has presented good therapeutic efficacy against *Leishmania* spp. [20, 34, 37]. In a study developed, AmpB-containing polymeric micelles were tested against *L. donovani*, and results showed that the composition was 100 times more active against parasites as compared to the use of free AmpB [23]. In other work, AmpB-containing and chitosan-coated Poloxamer 407-based micelles were prepared and the composition was tested against *Leishmania*, with results showing low toxicity of formulation in mammalian cells, higher uptake by host´s macrophages and higher effectivity against infection by the parasites [54].

Our group has also used polymeric micelles as delivery systems for antileishmanial candidates. In a study, AmpB-containing Poloxamer P407 micelles

were prepared and tested against *L. amazonensis*. Results showed that the composition induced significant reductions in the parasite load in infected BALB/c mice, as compared to the other groups. The Th1-type immunity was developed in these treated animals, which was also associated with the therapeutic response against infection [37]. In another study, clioquinol-containing Pluronic® F127 polymeric micelles (ICHQ/Mic) were developed and also tested against *L. amazonensis.* Results showed that treated mice developed a Th1-type immune response, which was associated with significant reductions in the parasitism in livers, spleens, BMs and dLNs of the animals, when compared to data found in the control groups [64].

Cardenolides have been used to treat congestive heart failure and arrhythmias [1, 11, 32, 52]. In the present study, preliminary data suggest that the mechanism of action of our cardenolide derivative in *L. infantum* occurred in the parasite mitochondria causing cell death, since b-AD altered the mitochondrial membrane potential and stimulated the ROS production by the parasites. In fact, the presence of high levels of ROS can cause increase in the levels of lipid peroxidation and reduction in the membrane fluidity, leading to the loss of cell viability [55, 58]. Mitochondria has been considered as a target when distinct antileishmanial agents are tested [7, 28, 35, 57, 63]; however, contrarily to observed in mammalian cells, where this organelle is abundant in their content, *Leishmania* spp. and other Trypanosomatides present only one mitochondria, which exhibits functional and morphological differences, such as the presence of own genetic material, peculiarities in the functionality of the electron transport chain and existence of a non-canonical antioxidant machinery [2, 24, 40]. In this context, and based on the data presented here, b-AD induced was able to induce the establishment of a stress environment in the parasites, which contributed to the *Leishmania* death. Additionally, and due to the low toxicity found in murine and human cells; one could speculate that this molecule will not be toxic to treat against human VL, mainly when administered in low doses and/or incorporated in delivery system such as polymeric micelles.

Miltefosine is used as an oral drug for the treatment against VL; however, its effectiveness has been variable between the populations in the world [51, 60]. In addition, due to its long half-life and long therapy duration, parasite resistance against this drug has been also documented [45, 47]. Here, miltefosine induced significant reductions in the parasitism in the treated animals, as compared to values found in the

controls; however, data obtained in b-AD/Mic-treated mice showing more significant reductions in the parasite load suggest that the micellar composition was more effective in reduce the organ parasitism in treated and infected animals. Taken together, these results suggest then that b-AD/Mic could be then considered effective against *L. infantum* infection in our experimental mammalian model.

Infection by *L. infantum* usually causes primary parasitism in the liver of the animals that tends to decrease, while the splenic parasitism tends to increase when the infection becomes chronic [12, 43]. In this context, animals´ spleens could be considered as a marker of chronic infection [22, 36]. In our study, parasitism in this organ was evaluated by two distinct techniques and results showed that, when both assays were performed, lower splenic parasitism was found in b-AD/Mic-treated mice, as compared to the other groups, including the treatment using miltefosine. This fact could be considered relevant and therapeutic schemes testing the association of this cardenolide derivative with other antileishmanial drugs could be considered, with the purpose to reduce the side effects, decrease parasite resistance and allow to the prescription of lower drug doses to achieve satisfactory therapeutic results [30, 48, 59, 65].

Limitations of the study include the absence of other therapeutic schedules employing lower number of doses and other antileishmanial agents, such as amphotericin B, as well as the absence of parasitological and immunological evaluations performed in longer periods of time post-treatment; aiming to verify a possible long-term therapeutic efficacy induced by b-AD/Mic. In addition, the absence of evaluation of the levels of urea and creatinine in the sera samples of treated and infected animals could be considered as a limitation of the work, since antileishmanial agents, such as amphotericin B [38, 50], cardenolide derivatives [9, 17], among others, can cause renal toxicity, mainly if administered in high and/or inappropriately doses [9]. In this context, additional studies are certainly necessaries to be performed, aiming to solve such questions. However, preliminary data presented here, describing the parasitological and immunological therapeutic response found in mice treated with b-AD/Mic, as well as the low cardiac and hepatic toxicity, suggest that this molecule could be considered in future studies as a therapeutic agent against VL.

Conflict of interest

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

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CONSIDERAÇÕES FINAIS DO ARTIGO 1

Os resultados obtidos sugerem a eficácia seletiva da molécula β-acetil-digitoxina (b-AD) contra a espécie *L. infantum*, com valores melhores que fármacos de referência atualmente utilizados no tratamento da LV. A composição micelar contendo b-AD foi mais eficiente na redução da carga parasitária nos animais infectados e tratados, além de induzir ao desenvolvimento de uma resposta imune celular e humoral antileishmanial do tipo Th1 mais polarizada.

5.2. Artigo 2: estudo da Digitoxigenina contra *L. infantum*

Este artigo foi submetido ao periódico *Parasitology Research,* na data de 21 de agosto de 2020 e aceito para publicação em 04 de novembro de 2020 (Anexo 2).

Title

Digitoxigenin presents an effective and selective antileishmanial action against *Leishmania infantum* and is a potential therapeutic agent for visceral leishmaniasis

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ABSTRACT

Treatment for visceral leishmaniasis (VL) is hampered mainly by drug toxicity, their high cost and parasite resistance. Drug development is a long and pricey process and therefore drug repositioning may be an alternative worth pursuing. Cardenolides are used to treat cardiac diseases, especially those obtained from *Digitalis* species. In the present study, cardenolide Digitoxigenin (DIGI) obtained from a methanolic extract of *Digitalis lanata* leaves was tested for its antileishmanial activity against *Leishmania infantum* species. Results showed that 50% *Leishmania* and murine macrophage inhibitory concentrations (IC_{50} and CC_{50}, respectively) were of 6.9±1.5 and 295.3±14.5 µg/mL, respectively. With Amphotericin B (AmpB) deoxycholate, used as a control drug, values of 0.13±0.02 and 0.79±0.12 µg/mL, respectively, were observed. Selectivity index (SI) values were of 42.8 and 6.1 for DIGI and AmpB, respectively. Preliminary studies suggested that the mechanism of action for DIGI is to cause alterations in the mitochondrial membrane potential, to increase the levels of reactive oxygen species and induce accumulation of lipid bodies in the parasites. DIGI was incorporated into Pluronic® F127-based polymeric micelles and the formula (DIGI/Mic) was used to treat *L. infantum*-infected mice. Miltefosine was used as a control drug. Results showed that animals treated with either Miltefosine, DIGI or DIGI/Mic presented significant reductions in the parasite load in their spleens, livers, bone marrows and draining lymph nodes, as well as the development of a specific Th1-type response, when compared to the controls. Results obtained one day after treatment were corroborated with data corresponding to 15 days after therapy. Importantly, treatment with DIGI/Mic induced better parasitological and immunological responses when compared to Miltefosine- and DIGI-treated mice. In conclusion, DIGI/Mic has the potential to be used as a therapeutic agent to protect against *L. infantum* infection and it is therefore worth of consideration in future studies addressing VL treatment.

Keywords: Treatment; Digitoxigenin; drug repositioning; visceral leishmaniasis; Amphotericin B deoxycholate; Miltefosine.

Introduction

Leishmaniases are protozoal vector-borne diseases that affect both humans and animals. There are approximately 380 million people at risk of contracting infection and 2.0 million cases registered annually (WHO, 2018). This disease complex is caused by some species of the *Leishmania* genus, being the tegumentary and visceral leishmaniases the main clinical forms of the disease (Grimaldi and Tesh, 1993). Visceral leishmaniasis (VL) is a worldwide distributed infectious disease that, when symptomatic, causes fever, hepatosplenomegaly and pancytopenia, among others (Burza et al., 2018). Its diagnosis is accomplished by molecular and/or conventional parasitological techniques, as well as by immunological assays. However, accurate diagnosis depends on the current clinical status and immune response of the infected hosts, with false-negative and/or false-positive results being frequently encountered (Sakkas et al., 2016; Jamal et al., 2017; Van Griensven and Diro, 2019).

VL treatment has long relied on the use of pentavalent antimonials. However, long periods of parenteral administration cause toxicity to the patients, who present with symptoms such as cardiac arrhythmias, prolonged QT interval, ventricular premature beats, ventricular tachycardia and fibrillation (Sundar and Chakravarty, 2015). In addition, parasite resistance has been reported, limiting their effectiveness as clinical antileishmanial drugs (Sundar and Singh, 2018). Other therapeutic agents are also available and are currently being employed to treat VL. Despite presenting high efficacy, toxicity and/or high costs are also limitations for their widespread use within a clinical setting (Chakravarty and Sundar, 2019). Amphotericin B (AmpB) deoxycholate is one these alternative drugs, which toxic effects include nephrotoxicity, hypokalemia and myocarditis (Mohamed-Ahmed et al., 2012). AmpB-containing lipid formulations were developed with the aim to reduce side effects. Besides their low toxicity, these formulations have shown to induce high therapeutic responses but their high cost is still a limiting factor (Sundar et al., 2019). Miltefosine was originally used as an anti-tumor agent and its leishmanicidal potential was reported in the 1980s (Dorlo et al., 2012). It was the first drug used to treat VL via oral administration. Miltefosine inhibits the biosynthesis of the glycosyl phosphatidyl inositol receptor, a key molecule for *Leishmania* intracellular survival. Unfortunately, Miltefosine is teratogenic and parasite resistance has been also registered (Rijal et al., 2013; Srivastava et al., 2017).

In this context, there is currently and urgent need to identify novel effective, non-toxic, low-cost antileishmanial agents. Drug discovery is a long and expensive process, and leishmaniasis is a neglected disease (Hughes et al., 2011). Thus, drug repositioning may well represent an attractive alternative, since compounds with known biological functions have been proved to be effective against parasites (Andrade-Neto et al., 2018). Cardenolides are glycosides able to inhibit Na^+/K^+-ATPases by obstructing the Na^+/K^+ pump mechanism (Mijatovic and Kiss, 2013; Patel et al., 2019). These compounds are clinically used to treat cardiac diseases, such as congestive heart failure (Gheorghiade et al., 2009), and they have been reported also to possess antitumor (Slingerland et al., 2013), antimalarial (Chan et al., 2016) and anti-oxidant (Xu et al., 2017) activities, among others (Eid et al., 2012; Gurel et al., 2017). In the present study, a cardenolide derivate called Digitoxigenin (DIGI) was obtained from a methanolic extract of *Digitalis lanata* leaves and its antileishmanial activity was evaluated against *L. infantum* species.

Experimental results showed that DIGI was effective against parasites, presenting a higher selectivity index (SI) than AmpB, with corresponding high efficacy in the treatment of infected murine macrophages. Preliminary studies showed that DIGI induced alterations in the parasite mitochondrial membrane potential ($\Delta\Psi m$), stimulated the production of reactive oxygen species (ROS) and the accumulation of lipid bodies in the parasites. Furthermore, studies *in vivo* showed that DIGI administered either in a free format or incorporated into Poloxamer 407-based polymeric micelle system (DIGI/Mic) significantly reduced the parasite load in the spleens, livers, bone marrows (BM) and draining lymph nodes (dLNs) of *L. infantum*-infected mice, one and 15 days post-treatment. Immunological data demonstrated that both DIGI and DIGI/Mic stimulated also the development of an antileishmanial Th1-type cellular and humoral immune response one and 15 days after treatment, suggesting the potential of this cardenolide derivate as an effective alternative therapeutic agent against VL.

Materials and Methods

Ethics statement, experimental animals and chemicals

The study was approved by the Committee for the Ethical Handling of Research Animals of Federal University of Minas Gerais (UFMG; Belo Horizonte, Minas Gerais, Brazil), with protocol number 085/2017. BALB/c mice (female, 8 weeks old) were purchased from the Institute of Biological Sciences of UFMG and they were kept under pathogen-free conditions. Poloxamer 407 (Pluronic® F127), Miltefosine and AmpB were acquired from Sigma-Aldrich (catalog numbers 16758, 58066-85-6 and 1397-89-3, respectively; St. Louis, USA).

Extraction and purification of DIGI

DIGI was obtained according to the method reported by Pádua et al. (2005), with few modifications. Briefly, a methanolic extract from *Digitalis lanata* leaves (kindly donated by Prof. Wolfgang Kreis, University of Erlangen-Nuremberg, Germany), which is an industrial refuse resulting from the extraction of digoxin, was resuspended (17 g) in 200 mL acetone and sonicated for 30 min at room temperature. The acetonic extract obtained was filtrated through a sintered Büchner funnel (nº 1-100 to 160 µm pore sizes) and the process was repeated with the insoluble material using 100 mL acetone. The acetone extract was filtered over 30 g of activated charcoal powder under reduced pressure to remove chlorophyll and other pigments. The active charcoal was washed with acetone (100 mL) and the filtrate was evaporated to dryness to allow the dry extract to enrich in cardenolides (10 g). The dry extract was dissolved in 400 mL methanol and stirred on an oil bath until the temperature reached 55ºC. Then, 350 mL 1 M hydrochloric acid was added and the solution was stirred for 35 min at room temperature. Next, it was partitioned with dichloromethane (3×200 mL) and the organic layer was neutralized by partition with 50 mL 3% (w/v) $NaHCO_3$ aqueous solution. The organic layer was then washed with water (3×100 mL), dried over anhydrous sodium sulfate, and dichloromethane was evaporated to dryness to get the dried residue (7.0 g), which was dissolved in dichloromethane (100 mL) and filtered under reduced pressure over silica gel (50.0 g; 0.04-0.063 mm) deposited in a sintered Buchner funnel

(nº 4-10 to 16 µm pore sizes). The silica gel was sequentially washed using mixtures of dichloromethane (A) and ethyl acetate (B): 100% A (100 mL), 95% A/5% B (950 mL), 90% A/10% B (750 mL), and 70% A/30% B (250 mL). Forty-one fractions were collected (50 mL each); the solvent was removed in a fume hood for 18 h at room temperature, and fractions were submitted to thin-layer chromatography analysis over silica gel (eluent: ethyl acetate; spray reagent: Kedde). Fractions containing DIGI (fractions 25 to 34) were pooled and used to purify the molecule (1.4 g; purity > 98%; *Figura 16*).

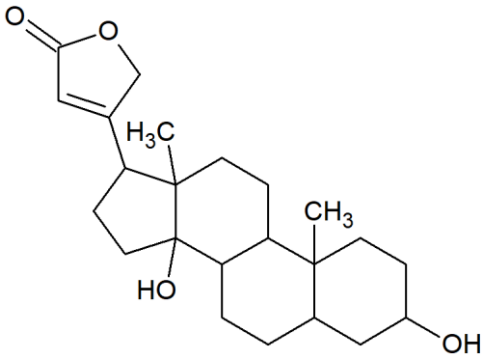

Figura 16. **Chemical structure of the digitoxigenin.**

Parasites

L. infantum (MHOM/BR/1970/BH46) parasites were grown at a 24°C in complete Schneider's medium (Sigma-Aldrich, USA), supplemented with 20% (v/v) heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich, USA) and 20 mM L-glutamine pH 7.4 (Coelho et al., 2003).

Antileishmanial activity

50% *L. infantum* inhibitory concentration (IC_{50}) was evaluated by incubating parasite stationary promastigotes (10^6 cells) with DIGI (0 to 50.0 µg/mL) or AmpB (0 to 10.0 µg/mL; Sigma-Aldrich, USA) in 96-well culture plates (Nunc, Nunclon, Roskilde, Denmark) for 48 h at 24°C. Cell viability was assessed using the 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (Sigma-Aldrich, USA) method. Optical density (OD) values were measured in 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 in Microsoft Excel software (version 10.0) (Tavares et al., 2018).

Cytotoxicitiy assay

Cytotoxicity was evaluated *ex vivo* in murine macrophages and human red blood cells, for which concentrations inhibiting 50% of macrophages (CC_{50}) and red blood cells (RBC_{50}) were determined. Briefly, murine cells (5×10^5) or a 5% human red blood cells suspension were incubated in the presence of DIGI (0 to 100.0 µg/mL) or AmpB (0 to 10.0 µg/mL) in RPMI 1640 medium for 48 h (murine macrophages) or 1 h (red blood cells) at 37°C in 5% CO_2. Macrophage viability was assessed by MTT method. The red blood cells suspension was centrifuged at 1,000 × *g* for 10 min at 4°C, after which percentage of cell lysis was evaluated spectrophotometrically at 570 nm. The absence of (blank) or 100% hemolysis were determined by replacing DIGI with an equal volume of phosphate buffered saline pH 7.4 (PBS) or distilled water, respectively. CC_{50} and RBC_{50} values were calculated by sigmoidal regression of dose-response curves in Microsoft Excel software (version 10.0) (Sousa et al., 2019). SI was calculated as the ratio between CC_{50} and IC_{50} values.

Treatment of infected macrophages

To evaluate the efficacy of DIGI for treating infected macrophages, murine cells (5×10^5) were plated on round glass coverslips in 24-well plates in RPMI 1640 medium supplemented with 20% (v/v) FBS and 20 mM L-glutamine pH 7.4, and incubated for 24 h at 37°C in 5% CO_2. Stationary promastigotes were then added to the wells at a ratio of 10 parasites per macrophage and cultures were incubated further for 48 h at 37°C in 5% CO_2. Free parasites were removed by extensive washing with RPMI 1640 medium and infected macrophages were treated with DIGI (0, 2.5, 5.0 and 10.0 µg/mL) or AmpB (0, 0.25, 0.5 and 1.0 µg/mL) for 48 h at 24°C in 5% CO_2. After fixation with 4% paraformaldehyde, cells were washed, stained with Giemsa and the infection percentage, the number of amastigotes per infected macrophage and the reduction in the infection percentage were determined by counting 200 cells, in triplicate, using an optical microscope (Tavares et al., 2018).

Evaluation of mitochondrial membrane potential and integrity

Stationary promastigotes (10^7 cells) were cultured in the absence or presence of DIGI (13.8 µg/mL, corresponding to 2× the IC_{50} value) during 24 h at 25°C. Parasites were washed with PBS and incubated with MitoTracker Red CM-H2XROS (500 nM; Invitrogen, USA) or propidium iodide (1.0 µg/mL; Sigma-Aldrich) for 30 and 15 min, 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. Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazon (FCCP, 5.0 µM; Sigma-Aldrich, USA) and pre-heated parasites at 65ºC for 10 min were used as positive controls (Sousa et al., 2019).

Production of reactive oxygen species (ROS)

Stationary promastigotes (10^7 cells) were cultured in the absence or presence of DIGI (13.8 µg/mL, corresponding to 2× the IC_{50} value) for 24 h at 25°C. Parasites were washed with PBS and incubated with 20 µM cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Sigma-Aldrich, USA) for 30 min 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 485 and 528 nm, respectively. H_2O_2-treated parasites (4.0 mM; Sigma-Aldrich, USA) were used as positive control (Sousa et al., 2019).

Infection, treatment and toxicity assay

Mice (n=12 per group) were infected subcutaneously with 10^7 *L. infantum* stationary-phase promastigotes and 60 days post-infection, they were grouped and treated with one of the following therapeutic regimens: 50 µL of PBS (saline group); 50 µL of empty micelles (10 mg/kg body weight) (B/Mic group); 2 mg Miltefosine/kg body weight (Miltefosine group); 50 µL of free DIGI (5 mg/kg body weight) (DIGI group) or

50 μL of DIGI-containing micelles (5 mg/kg body weight) (DIGI/Mic group). Except for Miltefosine which was administered orally, all other regimens were administered subcutaneously, every two days for a total period of 10 days. Half of the animals were euthanized one and 15 days post-therapy, when biochemical, immunological and parasitological analyses were performed. To evaluate toxicity *in vivo*, sera samples from the infected and then treated mice were collected one and 15 days post-treatment, with which the levels of Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and Creatine kinase - muscle brain fraction (CK-MB) markers were measured using commercial kits (Labtest Diagnostica®, Belo Horizonte), according to the manufacturer's instructions.

Cytokine and nitrite production

Spleens were collected from the infected and treated mice one and 15 days post-treatment and spleen cells (5×10^6 cells/mL) were incubated in DMEM (medium) supplemented with 20% (v/v) FBS and 20 mM L-glutamine at pH 7.4, or stimulated with *L. infantum* Soluble *Leishmania* Antigen (SLA, 50 μg/mL) for 48 h at 37 °C in 5% CO_2. IFN-γ, IL-4, IL-10, IL-12p70 and GM-CSF levels were measured in the culture supernatant by capture ELISA technique, using commercial kits (BD Pharmingen®, San Diego, CA, USA) according to the manufacturer's instructions. Nitrite production was evaluated in these same supernatants by Griess method. The IFN-γ source in Miltefosine-, DIGI- and DIGI/Mic-treated mice was evaluated by addition of anti-CD4 (GK 1.5) or anti-CD8 (53-6.7) monoclonal antibodies (5 μg each; Pharmingen®, USA) to the stimulated cultures. Appropriate isotype-matched controls [rat IgG2a (R35-95) and rat IgG2b (95-1)] were used. In addition, IFN-γ, TNF-α and IL-10-producing $CD4^+$ and $CD8^+$ T cell frequency was evaluated in the saline-, B/Mic-, Miltefosine-, DIGI- and DIGI/Mic-treated mice by flow cytometry using cells collected 15 days post-treatment. Briefly, splenocytes (5×10^6 cells/mL) were cultured in RPMI 1640 (medium) or stimulated with SLA (50 μg/mL) for 48 h at 37 °C in 5% CO_2. IFN-γ, TNF-α and IL-10-producing $CD4^+$ and $CD8^+$ T cell frequency were determined by their relative flow cytometry size (forward laser scatter – FSC) and granularity (side laser scatter – SSC). After selection of the R1 region of containing FSCLow and SSCLow phenotype cells, IFN-γ^+, TNF-α and IL-10^+ T cell frequency was calculated based on the analysis of

density plot distribution of CD4/FL1 or CD8/FL1 versus IFN-γ/FL2$^+$, TNF-α/FL2$^+$ and IL-10/FL2$^+$ cells. Results were expressed as indexes, which were calculated as the ratio between CD4$^+$ and CD8$^+$ T-cell percentages in the stimulated versus unstimulated (control) cultures (Mendonça et al., 2018).

Humoral response

The levels of anti-parasite IgG1 and IgG2a isotype antibodies in sera samples collected from infected and treated mice were evaluated by ELISA assay, one and 15 days post-treatment. *L. infantum* SLA was used as antigen (1.0 µg/well) and sera samples were diluted 1:100 in PBS-T (PBS plus 0.05% (v/v) Tween 20). Anti-mouse IgG1 and IgG2a horseradish-peroxidase conjugated antibodies (Sigma-Aldrich, USA) were used at a 1:10,000 dilution in PBS-T. Reactions were developed using H_2O_2, ortho-phenylenediamine and citrate-phosphate buffer at pH 5.0 for 30 min and in the dark, and stopped by addition of 2 N H_2SO_4. OD values were measured in an ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Canada) at 492 nm.

Parasite load

Parasite load in the spleens, livers, BMs and dLNs collected from the infected and treated mice one and 15 days post-treatment was evaluated by limiting dilution technique (Tavares et al., 2018). Briefly, organs were macerated in a glass tissue grinder using sterile PBS and tissue debris were removed by centrifugation at 150 × *g*. Cells were concentrated by centrifugation at 2,000 × *g*, 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.

Splenic parasitism

The splenic parasite load was evaluated also by qPCR technique, as described previously (Duarte et al., 2016; Antonia et al., 2018). Briefly, spleen DNA was extracted, resuspended and the parasite load was estimated by qPCR using the primers *Forward* (CCTATTTTACACCAACCCCCAGT) and *Reverse* (GGGTAGGGGCGTTCTGCGAAA). Mouse β-actin gene (*Forward*: CAGAGCAAGAGAGGTATCC and *Reverse*: TCATTGTAGAAGGTGTGGTGC) was used as an endogenous control. Reactions were processed in an ABI Prism 7500 Sequence Detection System (96 wells-plate; Applied Biosystems) using 2x SYBR™ Select Master Mix (5 µL; Applied Biosystems, USA) supplemented with each primer at a final concentration of 2 mM (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 15 s at 95°C followed by 1 min at 60°C. After each cycle, fluorescence data were collected. A standard curve was run in parallel, which was performed in duplicate and expressed as the number of *L. infantum* organisms per total DNA.

Statistical analysis

Statistical analysis was performed using GraphPad Prism™ (version 6.0 for Windows). Results were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni´s post-test for comparison between the groups. Results were expressed as mean ± standard deviation. Two independent experiments, presenting similar results, were performed. Differences were considered significant when $P < 0.05$.

Results

Analysis of antileishmanial activity, cytotoxicity and treatment of infected macrophages

Antileishmanial activity (IC_{50}) and cytotoxicity (CC_{50}) were evaluated against *L. infantum* stationary promastigotes and murine macrophages, respectively. Results showed IC_{50} and CC_{50} values of 6.9±1.5 and 295.3±14.5 µg/mL for DIGI, respectively, with a corresponding SI value of 42.8, and of 0.13±0.02 and 0.79±0.12 µg/mL for AmpB, respectively, with a SI value of 6.1 *(Tabela 4).* The hemolytic activity in human red blood cells showed RBC_{50} values of 384.6±20.4 and 11.7±2.3 µg/mL for DIGI and AmpB, respectively. Infection percentages of treated macrophages were of 21.1%±2.5% for DIGI (10.0 µg/mL) and of 23.4%±3.3% for AmpB (1.0 µg/mL), with 0.5±0.1 and 1.0±0.2 amastigotes per infected macrophage, respectively *(Tabela 5).* If left untreated, the infection percentage and number of amastigote per infected macrophage were of 68.7%±4.0% and 3.9±0.5, respectively. The inhibition of infection using pre-treated parasites showed also that DIGI caused significant reduction in the parasite infection degree after incubation with it *(Tabela 6)*.

***Tabela 4. In vitro* biological assays.** *L. infantum* stationary promastigotes were incubated with DIGI (0 to 50 µg/mL) or AmpB (0 to 1.0 µg/mL) for 48 h at 24°C. Cell viability was analyzed by MTT method, and the 50% *Leishmania* inhibitory concentration (IC_{50}) was calculated by sigmoidal regression of the corresponding dose-response curve. Murine macrophages were also incubated with DIGI (0 to 100 µg/mL) or AmpB (0 to 10.0 µg/mL), and the 50% macrophage inhibitory concentration (CC_{50}) was determined by applying a dose-response curve. The selectivity index (SI) was calculated by the ratio between the CC_{50} and IC_{50} values. The inhibition 50% of human red cells (RBC_{50}) viability was calculated by incubating a 5% red cells suspension with DIGI (0 to 100.0 µg/mL) or AmpB (0 to 10.0 µg/mL) for 1 h at 37°C in 5% CO_2. The lyses percentage was evaluated spectrophotometrically, and the absence (blank) or 100% of hemolysis were determined by replacing DIGI for an equal volume of PBS or distilled water, respectively. Results were expressed as mean ± standard deviation.

Compound	IC_{50} (µg/mL)	CC_{50} (µg/mL)	SI	RBC_{50} (µg/mL)
Digitoxigenin	6.9±1.5	295.3±14.5	42.8	384.6±20.4
Amphotericin B	0.13±0.02	0.79±0.12	6.1	11.7±2.3

Tabela 5. **Treatment of infected macrophages**. Murine macrophages were incubated (5 x 10^5 cells) in RPMI 1640 medium added with 20% FBS and 20 mM L-glutamine at pH 7.4, for 24 h at 37°C in 5% CO_2. *L. infantum* stationary promastigotes were used to infect macrophages (at a ratio of 10 parasites per one macrophage) for 48 h at 37°C in 5% CO_2. Free parasites were removed by extensive washing with medium and infected macrophages were treated with DIGI (0, 2.5, 5.0 and 10.0 µg/mL) or AmpB (0, 0.25, 0.5 and 1.0 µg/mL) for 48 h at 24°C in 5% CO_2. The percentage of infected macrophages, the infectiveness reduction and the number of recovered amastigotes per cell were determined by counting 200 macrophages, in triplicate. Results are expressed as mean ± standard deviation.

Compound	Concentration (µg/mL)	Percentage of infected macrophages after treatment	Infectiveness reduction (%)	Number of amastigotes per macrophage
Digitoxigenin	10.0	21.1±2.5	69.3	0.5±0.1
	5.0	30.5±4.0	55.6	1.0±0.4
	2.5	44.4±3.8	35.4	2.3±0.6
	0	68.7±4.0	(-)	3.9±0.5
Amphotericin B	1.0	23.4±3.3	65.9	1.0±0.2
	0.50	32.1±3.8	53.3	1.4±0.3
	0.25	44.5±5.2	35.2	2.5±0.9
	0	68.7±4.0	(-)	3.9±0.5

Tabela 6. **Inhibition of infection using pre-treated parasites.** The inhibition of infection using pre-treated parasites was performed by incubating *L. infantum* promastigotes (5 x 10^6 cells) with DIGI (0, 2.5, 5.0 and 10.0 µg/mL) or AmpB (0, 0.25, 0.5 and 1.0 µg/mL), for 4 h at 24°C. Parasites were washed in RPMI 1640 and used to infect murine macrophages (at a ratio of 10 parasites per one macrophage) for 24 h at 37°C in 5% CO_2. The percentage of infected macrophages, the infectiveness reduction and the number of recovered amastigotes per cell were determined by counting 200 macrophages, in triplicate. Results are expressed as mean ± standard deviation.

Compound	Concentration (µg/mL)	Infection percentage using pre-treated parasites	Infectiveness reduction (%)	Number of amastigotes per macrophage
Digitoxigenin	10.0	9.5±1.4	86.5	0.2±0.1
	5.0	16.7±1.6	76.2	0.8±0.3
	2.5	31.2±3.5	55.6	1.8±0.4
	0	70.3±4.3	(-)	3.9±0.4
Amphotericin B	1.0	25.4±3.0	63.9	1.2±0.3
	0.50	32.4±5.1	53.9	1.8±0.5
	0.25	49.3±3.2	29.9	2.6±0.3
	0	70.3±4.3	(-)	3.9±0.4

Evaluation of the mechanism of action in *L. infantum* promastigotes

A proof of concept for the mechanism of action of DIGI was evaluated in *L. infantum* stationary promastigotes. Results showed that the molecule caused an alteration in the ΔΨm in the order of 24.8%, when compared to the untreated controls, while FCCP-treated promastigotes, used as positive control, showed a value of 30.9% *(Figura 17).* Treatment with DIGI generated also structural alterations on the integrity of *Leishmania* membrane in the order of 78.6%, in comparison to an alteration of 165.3% caused by heat-treatment of the parasites (control) *(Figura 18).* In addition, treatment with DIGI resulted in parasite oxidative stress with an increase in ROS levels of 87.5%, compared to an increase of 321.0% after treatment with H_2O_2 used as a control *(Figura 19).*

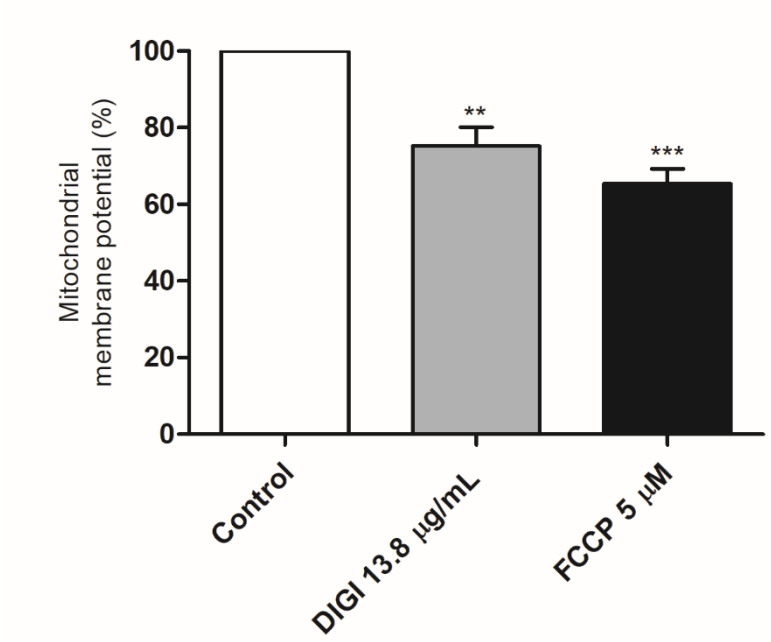

Figura 17. **Evaluation of the mitochondrial membrane potential.** *L. infantum* stationary promastigotes (10^7 cells) were cultured in the absence (control) or presence of DIGI (33.80 µg/mL, corresponding to two times the IC_{50} value) for 24 h at 25°C. Cells were incubated for 30 min in the dark with 500 nM MitoTracker Red CM-H2XROS. After washing twice with PBS, treated promastigotes were transferred to a black 96-well plate and fluorescence intensity was measured using a fluorometer, with excitation and emission wavelengths of 540 and 600 nm, respectively. Promastigotes pre-heated with carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazon (FCCP, 5.0 µM) were used as positive control. Bars indicate the mean plus standard deviation of the groups. (**) and (***) indicate statistically significant difference in relation to the non-treated control (P<0.01 and P<0.001, respectively).

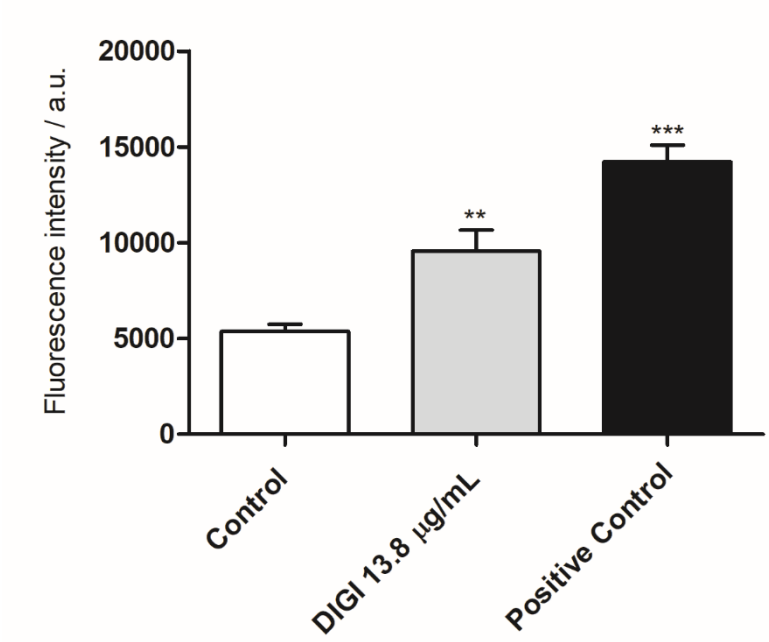

Figura 18. **Evaluation of mitochondrial membrane integrity.** Stationary promastigotes (10^7 cells) were cultured in the absence or presence of DIGI (13.8 µg/mL, corresponding to two times the IC_{50} value) for 24 h at 25°C. Parasites were washed in PBS and incubated with propidium iodide (1.0 µg/mL) for 15 min 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. Pre-heated parasites for 10 min at 65°C were used as positive control. Bars indicate the mean plus standard deviation of the groups. (**) and (***) indicate statistically significant difference in relation to the non-treated control (*P*<0.01 and *P*<0.001, respectively).

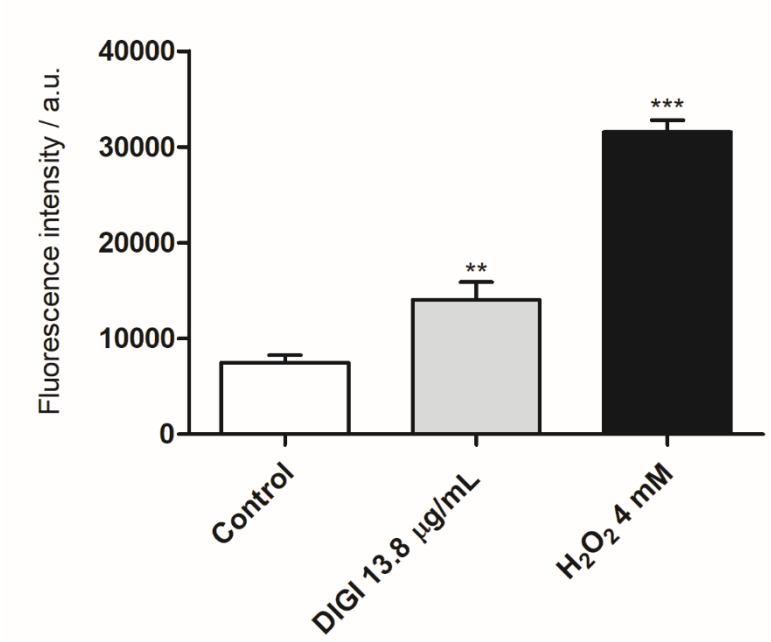

***Figura 19*. Production of the reactive oxygen species.** *L. infantum* promastigotes (10^7 cells) were cultured in the absence (control) or presence of DIGI (13.8 µg/mL, corresponding to two times the IC_{50} value) for 24 h at 25°C. Cells were incubated for 30 min in the dark with 20 µM cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). Fluorescence intensity was measured using a fluorometer, with excitation and emission wavelengths of 485 and 528 nm, respectively. H_2O_2-treated parasites (4.0 mM) were used as positive control. Bars indicate the mean plus standard deviation of the groups. (**) and (***) indicate statistically significant difference in relation to the non-treated control (P<0.01 and P<0.001, respectively).

Analysis of treatment toxicity

Organic toxicity of DIGI was evaluated by dosage of hepatic and cardiac markers in sera samples collected from the infected and treated mice, one and 15 days post-treatment. Results showed that Miltefosine-, DIGI- and DIGI/Mic-treated mice presented lower levels of Alanine aminotransferase (ALT) *(Figura 20A),* Aspartate transaminase (AST) *(Figura 20B)* and Creatine kinase-muscle brain fraction (CK-MB) *(Figura 20C)* enzymes, when compared to the controls. Notably, DIGI/Mic-treated mice presented the lowest levels of both hepatic and cardiac markers in comparison to the other treated groups.

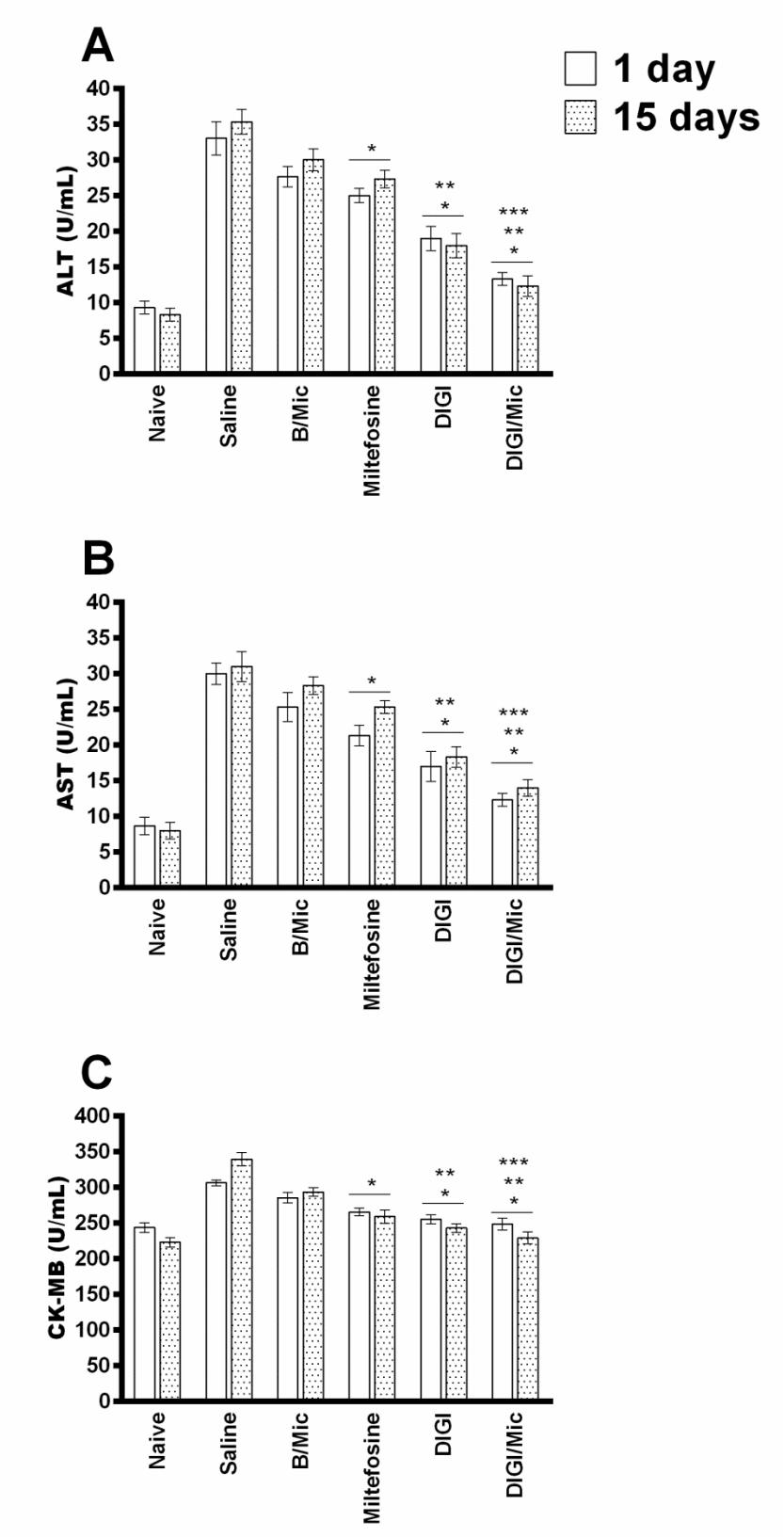

Figura 20. **Evaluation of *in vivo* toxicity.** Alanine aminotransferase (ALT) (A), aspartate aminotransferase (AST) (B) and creatine kinase muscle brain fraction (CK-MB) (C) enzymes were measured in sera samples of treated and infected animals, one and 15 days post-treatment.

Samples of non-treated and non-infected (naive) mice were used as control. Bars represent the mean ± standard deviation of the groups. (*) indicate significant difference in relation to the saline and B/Mic groups ($P < 0.05$). (**) indicate statistically significant difference in relation to the miltefosine group ($P < 0.05$). (***) indicate statistically significant difference in relation to the DIGI group ($P < 0.05$).

Evaluation of the immune response generated after treatment

The cellular response to treatment after infection was evaluated one and 15 days post-treatment on *in vitro* cultures of murine spleen cells, with were stimulated with SLA *L. infantum*. One day after therapy, Miltefosine-, DIGI- and DIGI/Mic-treated mice produced significantly higher levels of IFN-γ, IL-12 and GM-CSF, associated with low IL-4 and IL-10 production. On the other hand, spleen cells of the control groups (saline- and B/Mic-receiving mice) produced significantly higher levels of antileishmanial IL-4 and IL-10 *(Figura 21A).* Analysis of the source of IFN-γ production in the treated animals using anti-CD4 or anti-CD8 monoclonal antibodies showed that the production of this cytokine declined significantly when both monoclonal antibodies were added to the cultures *(Figura 21B).* Evaluation of the humoral response indicated also that Miltefosine-, DIGI- and DIGI/Mic-treated mice produced significantly higher levels of anti-parasite IgG2a antibody in comparison to IgG1 *(Figura 21C).* Furthermore, evaluation of nitrite secretion in the cell supernatant showed significantly higher levels of this molecule in Miltefosine, DIGI- or DIGI/Mic-treated mice, when compared to the controls *(Figura 21D).* All data mentioned above corresponding to one day after therapy were consistent with findings fifteen days post-treatment, since Miltefosine-, DIGI- and DIGI/Mic-treated mice continued to produce significantly higher levels of IFN-γ, IL-12 and GM-CSF, as well as low IL-4 and IL-10 levels *(Figura 22A).* As observed one day post-treatment, both T cell subtypes continued to produce IFN-γ after 15 days of therapy *(Figura 22B),* with a maintained Th1-type humoral response corresponding to significantly higher levels of anti-parasite IgG2a isotype antibodies in comparison to IgG1 levels *(Figura 22C).* Nitrite secretion was likewise increased in the Miltefosine-, DIGI- and DIGI/Mic-treated groups *(Figura 22D).* Moreover, a flow cytometry analysis revealed that Miltefosine-, DIGI- and DIGI/Mic-treated mice presented higher IFN-γ and TNF-α-producing $CD4^+$ and $CD8^+$ T cell frequencies when

compared to the control groups, which instead showed a higher IL-10-producing T-cell frequency *(Figura 23).*

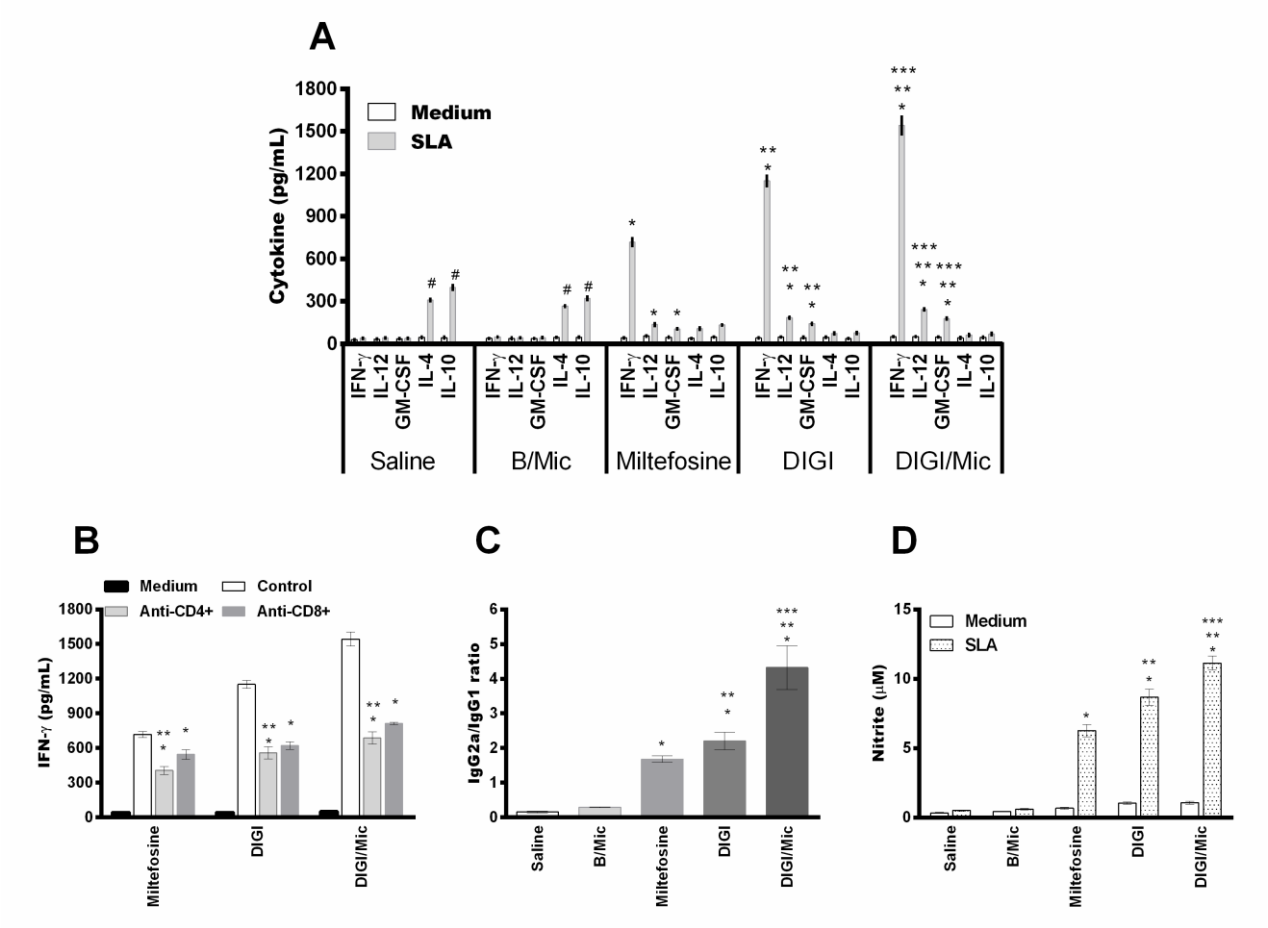

Figura 21. **Immune response developed one day after treatment.** Spleens of the treated and *L. infantum*-infected mice (n=6 per group) were collected one day after treatment, when spleen cells were *in vitro* cultured in DMEM (medium) or stimulated with SLA (50.0 μg/mL) for 48 h at 37°C in 5% CO_2. IFN-γ, IL-4, IL-10, IL-12p70 and GM-CSF levels were measured in the cell supernatant by a capture ELISA (A). The *in vitro* cultures were also incubated in the absence or presence of anti-CD4 or anti-CD8 monoclonal antibodies (5 μg/mL each) for 48 h at 37°C in 5% CO_2. The IFN-γ production was then evaluated in the cell supernatant (B). Sera samples of the animals were also collected and anti-parasite IgG1 and IgG2a antibody levels were measured by an indirect ELISA, when ratios between IgG2a and IgG1 levels were calculated (C). The cell supernatant used to quantify cytokines was also employed to evaluate the nitrite production and results are shown (D). Bars indicate the mean ± standard deviation of the groups. (*) indicate significant difference in relation to the saline and B/Mic groups ($P < 0.05$). (**) indicate statistically significant difference in relation to the miltefosine group ($P < 0.05$). (***) indicate statistically significant difference in relation to the DIGI group ($P < 0.05$). ($^{\#}$) indicate statistically significant difference in relation to the miltefosine, DIGI and DIGI/Mic groups ($P < 0.05$).

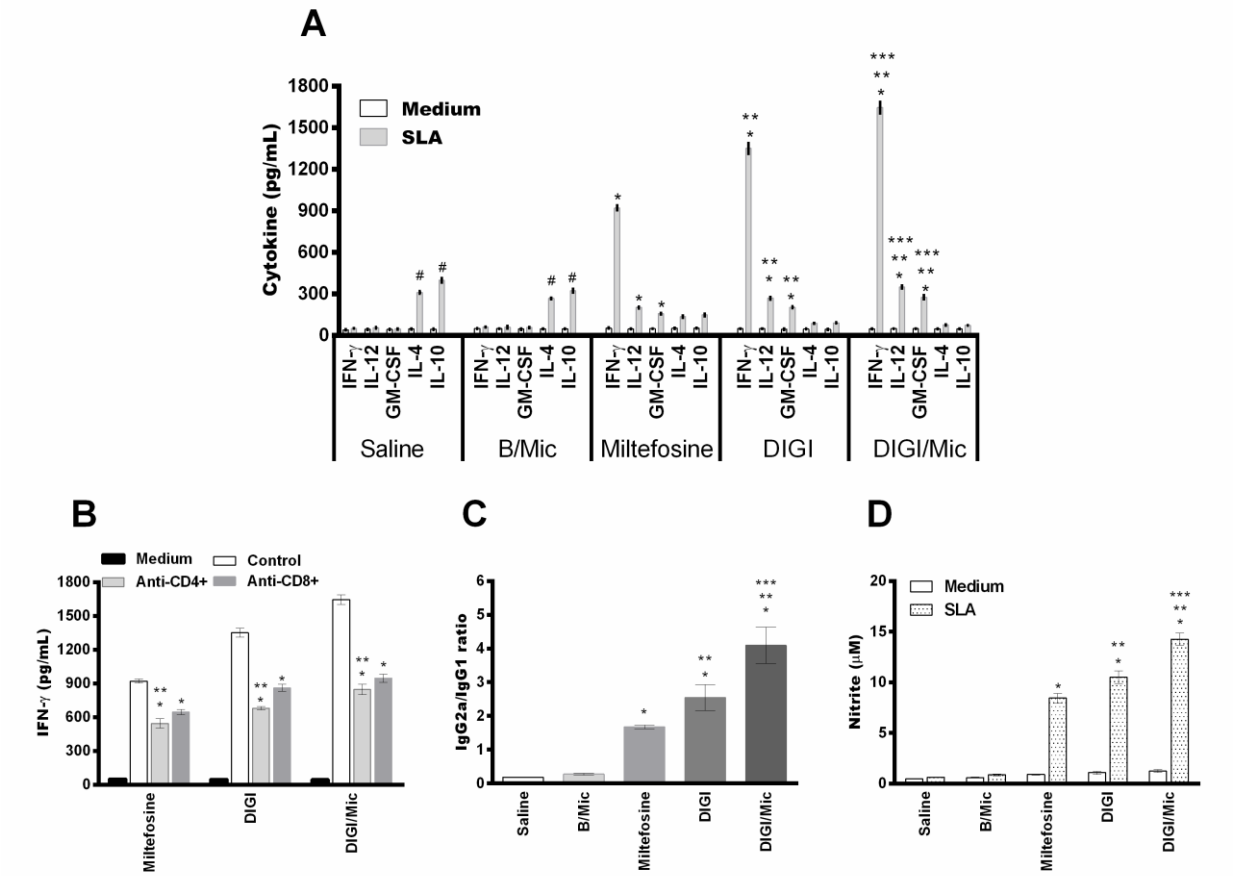

Figura 22. **Immunological profile developed 15 days after treatment.** Spleens of the treated and *L. infantum*-infected mice (n=6 per group) were collected 15 days after treatment, when spleen cells were *in vitro* cultured in DMEM (medium) or stimulated with SLA (50.0 µg/mL) for 48 h at 37°C in 5% CO_2. IFN-γ, IL-4, IL-10, IL-12p70 and GM-CSF levels were measured in the cell supernatant by a capture ELISA (A). The *in vitro* cultures were incubated in the absence or presence of anti-CD4 or anti-CD8 monoclonal antibodies (5 µg/mL each) for 48 h at 37°C in 5% CO_2. The IFN-γ production was then evaluated in the cell supernatant (B). Sera samples of the animals were also collected and anti-parasite IgG1 and IgG2a antibody levels were measured by an indirect ELISA, when ratios between IgG2a and IgG1 levels were calculated (C). The cell supernatant used to quantify cytokines was also employed to evaluate the nitrite production (D). Bars indicate the mean ± standard deviation of the groups. (*) indicate significant difference in relation to the saline and B/Mic groups ($P < 0.05$). (**) indicate statistically significant difference in relation to the miltefosine group ($P < 0.05$). (***) indicate statistically significant difference in relation to the DIGI group ($P < 0.05$). (#) indicate statistically significant difference in relation to the miltefosine, DIGI and DIGI/Mic groups ($P < 0.05$).

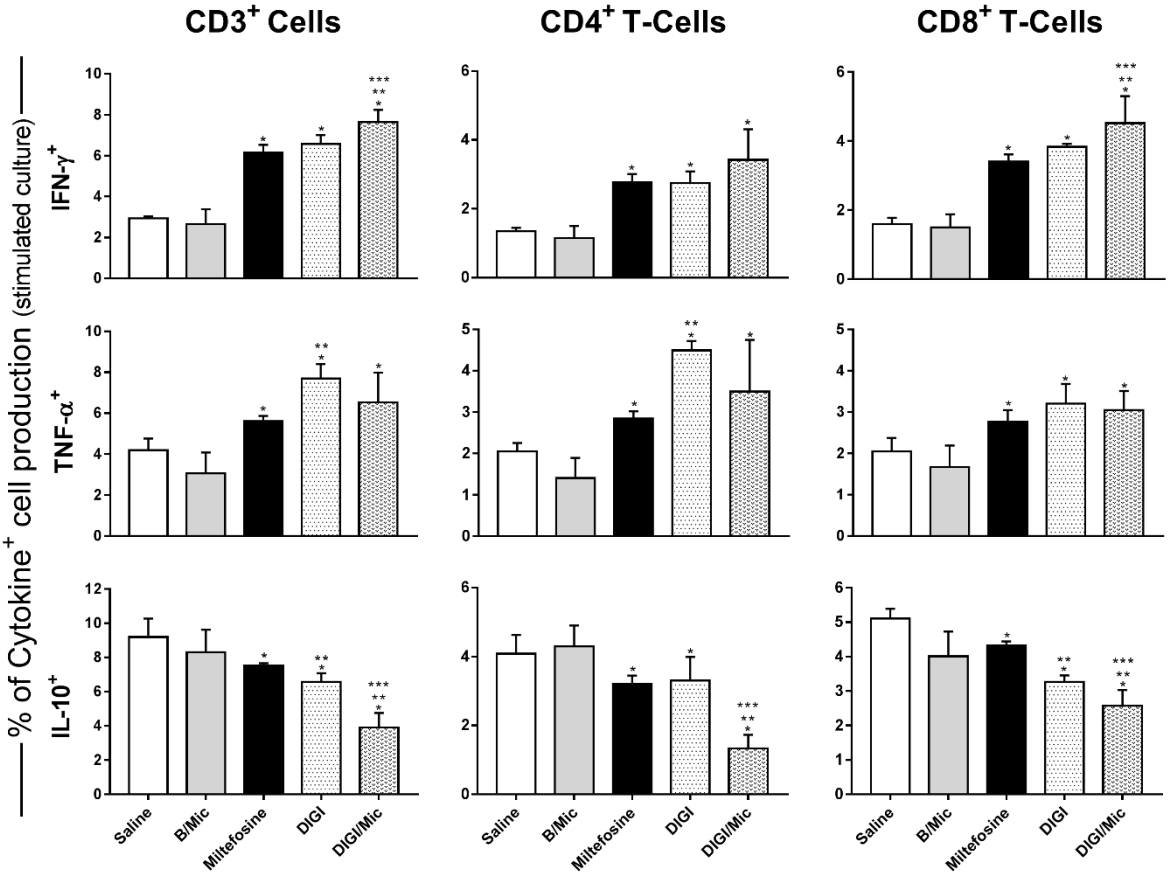

Figura 23. **Evaluation of the intracytoplasmic cytokine-producing T-cell frequency.** *L. infantum*-infected mice were treated and euthanized, 15 days post-therapy, when their spleen cells were collected and unstimulated (medium) or stimulated with *L. infantum* SLA. The IFN-γ, TNF-α and IL-10-producing T cell frequency was evaluated in the $CD4^+$ and $CD8^+$ T cell subpopulations. Results were expressed as indexes, which were calculated by the ratios between the $CD4^+$ and $CD8^+$ T-cell percentage in the SLA-stimulated versus unstimulated (control) cultures. Bars indicate the mean plus standard deviation of the groups. (*) indicate significant difference in relation to the saline and B/Mic groups ($P < 0.05$). (**) indicate statistically significant difference in relation to the miltefosine group ($P < 0.05$). (***) indicate statistically significant difference in relation to the DIGI group ($P < 0.05$).

Analysis of parasite load after treatment

The parasite load in the spleen, liver, BM and dLNs of the infected and treated mice was evaluated one and 15 days post-therapy. Results showed that mice treated with Miltefosine, DIGI and DIGI/Mic presented significant reductions in the parasitism in these organs, when compared to the controls *(Figura 24).* One day post-therapy, Miltefosine-, DIGI-, and DIGI/Mic-treated mice showed parasite load reductions in the

order of 2.3, 2.7 and 3.7-log in their livers *(Figura 24A),* of 3.0, 3.7 and 4.7-log in their spleens *(Figura 24B)*, of 3.6, 5.0 and 6.0-log in their dLNs *(Figura 24C)* and of 1.6, 2.0 and 2.6-log in their BMs (Figura 24A), respectively, when compared to the saline group. Fifteen days post-treatment, reductions in these groups were of 2.6, 3.6 and 4.6-log in their livers, of 3.3, 4.0 and 5.3-log in their spleens, of 3.6, 5.0 and 6.0-log in their dLNs, and of 1.7, 2.3 and 3.0-log in their BMs, respectively, when compared to the saline group. Notably, treatment with DIGI/Mic induced the most significant reductions in the organic parasitism, when compared to the other treated groups. A qPCR technique showed also that the splenic parasitism was significantly reduced after treatment with Miltefosine, DIGI or DIGI/Mic, and mice receiving DIGI/Mic presented the lowest parasite load, when compared to the other groups *(Figura 25).*

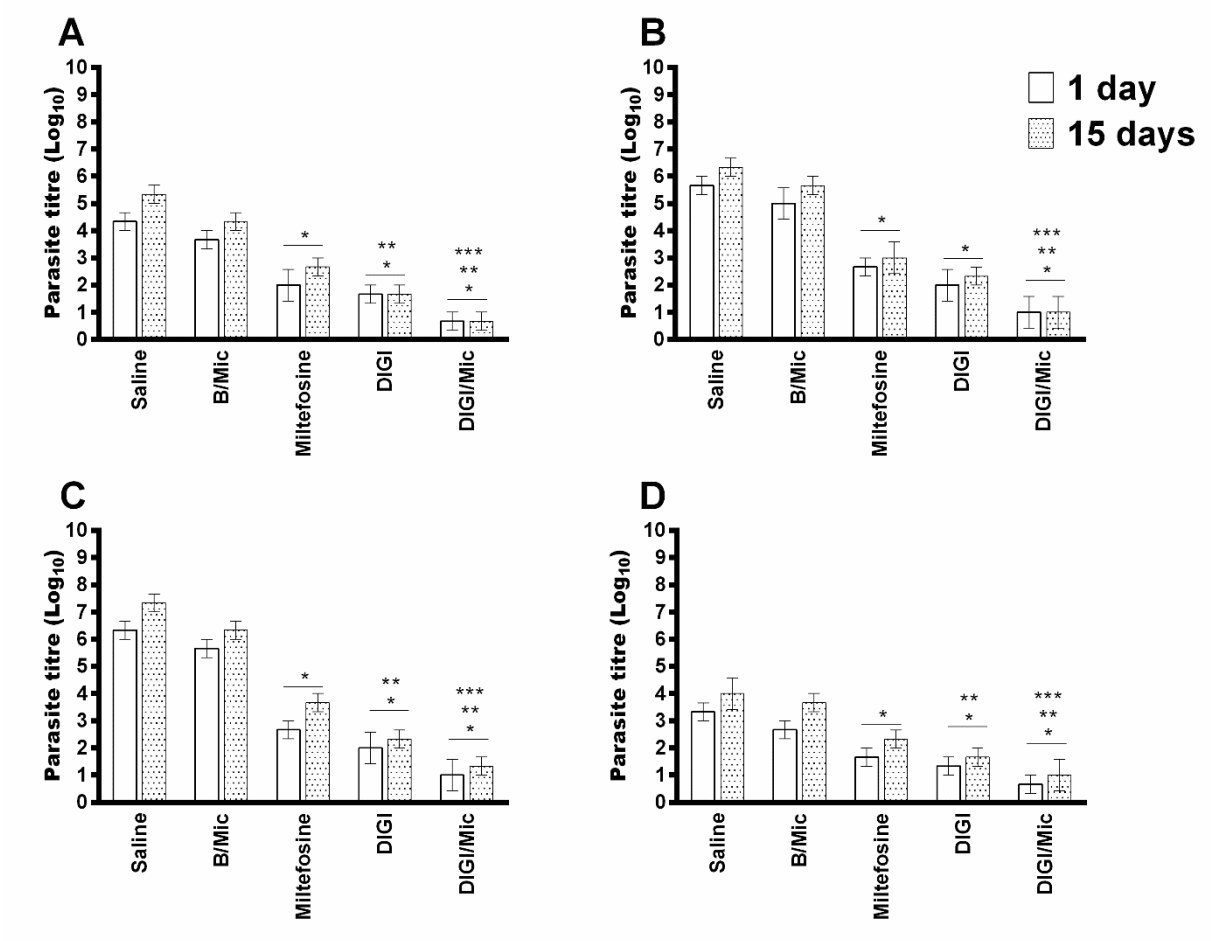

Figura 24. **Parasite burden evaluated by limiting dilution technique.** Mice were infected with *L. infantum* promastigotes and later received saline or were treated with empty micelles, miltefosine, DIGI or DIGI/Mic. One and 15 days after treatment, their livers (A), spleens (B), draining lymph nodes (C) and bone marrows (D) were collected, and the parasite load was investigated by a limiting dilution technique. Bars indicate the mean ± standard deviation of the groups. (*) indicate significant difference in relation to the saline and B/Mic groups ($P < 0.05$). (**)

indicate statistically significant difference in relation to the miltefosine group ($P < 0.05$). (***) indicate statistically significant difference in relation to the IVE group ($P < 0.05$).

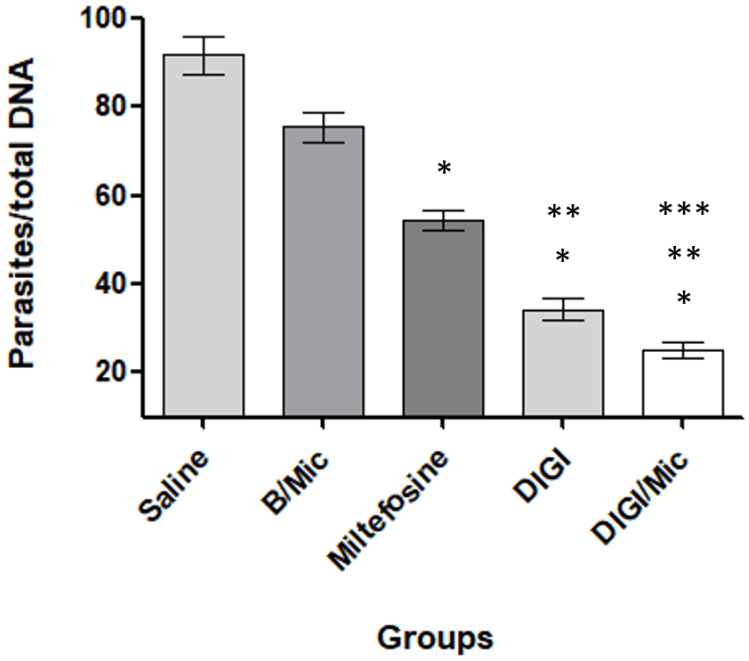

Figura 25. **Splenic parasite load evaluated by quantitative PCR (qPCR).** *L. infantum*-infected BALB/c mice (n=12 per group) were treated and, 15 days post-treatment, animals (n=6 per group) were euthanized, their spleen collected and the parasite load was estimated by qPCR technique. Results were expressed as the number of parasites per total DNA. Bars indicate the mean ± standard deviation of the groups. (*) indicate significant difference in relation to the saline and B/Mic groups ($P < 0.05$). (**) indicate statistically significant difference in relation to the miltefosine group ($P < 0.05$). (***) indicate statistically significant difference in relation to the DIGI group ($P < 0.05$).

Discussion

The aim of the present study was to evaluate potential antileishmanial activity of Digitoxigenin, a cardenolide derivate, against stationary promastigotes, intracellular amastigotes and on a murine model which was previously infected with *L. infantum* promastigotes. Initially, *in vitro* results showed that DIGI reduced by 69.3% the intracellular parasite burden in treated macrophages and by 86.5% the infection of these cells using pre-treated parasites. In comparison, AmpB showed values of 65.9% and 63.9%, respectively. *In vivo*, treatment with DIGI or DIGI/Mic significantly reduced

the parasite load in the spleen, liver, BM and dLNs of infected mice, one and 15 days after treatment, both outperforming Miltefosine. These observations suggest a therapeutic action of DIGI against *L. infantum* infection.

Cardenolides have been used for the treatment of congestive heart failure and atrial arrhythmias (Scalese and Salvatore, 2017; Whayne, 2018). They act as inhibitors of the Na^+/K^+-ATPase by suppressing the sodium pump (Patel et al., 2019). In our study, preliminary data suggest that DIGI targets the *L. infantum* mitochondria, causing alterations in both the parasite mitochondrial membrane potential and integrity, as well as an increase in ROS production. Loss of mitochondrial membrane potential gives rise to a release of protons from the mitochondria into the cell cytosol, which has been reported to contribute to the cytosol acidification and subsequent parasite death (Su et al., 2019). Thus, experimental data on this study suggest a similar mechanism of action for DIGI in *L. infantum*. There are several studies reporting the effect of cardenolides in heart diseases (Campbell and Mc Donald, 2003; Biteker et al., 2017; Gurel et al., 2017), but evaluation of these compounds to treat VL is lacking. Although cardenolides have been reported as toxic compounds (Hauptman et al., 2016; Arbabian et al., 2018), the low doses of either DIGI on its free form or incorporated into a micellar composition (DIGI/Mic) tested in our study did not induce any significant toxicity when used to treat the infected hosts. Conversely, infected animals receiving Miltefosine did present with toxicity.

Antileishmanial drugs inductors of immunological response are key for effective parasite control, since they have a direct action on the *Leishmania* organisms at the same time they stimulate the development of a specific cellular and humoral response, thus allowing infection control (Kedzierski and Evans 2014; Dayakar et al. 2019). VL control usually depends on the magnitude of the Th1-type cellular response, which leads to the production of cytokines, such as IFN-γ, and subsequent activation of infected macrophages (Kaye et al. 2004). In this context, our results showed that treatment with DIGI or DIGI/Mic induced a significant increase in the levels of Th1-type cytokines, such as IFN-γ, IL-12, TNF-α and GM-CSF, one and 15 days post-treatment. On the other hand, cytokines like IL-4 and IL-10 are associated with progression of VL (Singh et al. 2012; Adem et al. 2016). In our study, treatment with DIGI or DIGI/Mic significantly reduced the levels of IL-4 and IL-10, both one and 15 days after therapy,

suggesting induction of a DIGI-specific Th1-type immune response profile following treatment.

Antileishmanial candidates have been incorporated into delivery systems to improve their therapeutic efficacy and reduce the toxicity of the corresponding pure compounds (Bruni et al., 2017; Wagner et al., 2019). In previous studies, we showed that micellar compositions using Poloxamer 407 (Pluronic® F127)-based polymeric micelles proved effective in mammalian models against *Leishmania* infection (Duarte et al., 2016; Oliveira-de-Siqueira et al., 2017; Mendonça et al., 2019). In a similar fashion, we also developed a clioquinol (ICHQ)-containing Pluronic® F127 polymeric micelle system (ICHQ/Mic) and tested it against *L. infantum* infection in BALB/c mice (Tavares et al., 2020). In agreement with the data presented in this report, findings on our previous study demonstrated that treatment with Miltefosine, ICHQ or ICHQ/Mic all induced a polarized Th1-type immune response in mice, with significant reductions in the parasite load in distinct organs. Importantly, data proved ICHQ/Mic to be the most effective in inducing immunological and parasitological protection in mice. In a similar fashion, Singh et al. (2017) developed an AmpB-containing micellar formulation based on Pluronic F127 micelles coated with chitosan. The authors showed higher antileishmanial activity *in vitro* of this composition in comparison to free AmpB, as well as lower cytotoxicity and hemolytic activity in mammalian cells. In addition, this micellar composition reduced the parasite load in *L. donovani*-infected Syrian hamsters, apart from stimulating the development of a Th1-type immune response in the treated animals. In this context, our current report is in complete agreement with our previous studies and with others described in the literature, with DIGI showing higher therapeutic efficacy when associated to polymeric micelles.

Polymeric micelles as delivery systems present characteristics that make them suitable for administration via subcutaneous route. These polymers can create a semi-rigid gel when in contact with the local tissue where they were administered, turning them into a reservoir system which maintains the active agent within the extracellular space (Tavares et al., 2020). In the course of hours, the gel matrix dilutes and the drug is gradually released into the bloodstream, enabling its systemic action to be exerted in a controlled manner (Mendonça et al., 2019). These properties could allow reduced doses of the formulations to be administered at longer time intervals, thus reducing toxicity to the mammalian hosts, even with those drugs considered as cardiotoxic, such

as cardenolide derivates. Therefore, future studies will need to be performed for a more comprehensive evaluation of DIGI as a VL-treating drug before its clinical application in other mammalian hosts, such as humans.

BALB/c mice usually develop a severe infection when a high number of stationary promastigotes, such as the infective inoculum employed here, is used and can result in the development of Th2-type immune response, susceptibility and visceralization of the infection. In the present study, control group mice developed severe infection, with high parasite load in distinct organs, loss of body weight and weakness. All animals remaining after this study were therefore sacrificed 15 days post-treatment, adhering to Ethical experimental procedures. Studies evaluating therapeutic agents against murine VL normally perform their analysis at a single time point after treatment (Craft et al., 2014; Raja et al., 2017; Want et al., 2017; Valle et al. 2019). In our study, however, we performed immunological and parasitological evaluations one and 15 days after therapy. Nevertheless, a dose-response analysis with an increasing dosage or prolonged exposure to DIGI could determine in a more precise manner the therapeutic efficacy of this cardenolide derivate for treating VL. We are aware this is a limitation of the work and thus additional experiments would be certainly required to fully characterize DIGI as an antileishmanial agent. Nevertheless, data presented here describe for the first time the antileishmanial activity of DIGI both *in vitro* and *in vivo*, with promising results suggesting that DIGI/Mic is worth considering in further evaluations for VL treatment.

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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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CONSIDERAÇÕES FINAIS DO ARTIGO 2

Os resultados obtidos utilizando Digitoxigenina (DIGI) também sugerem uma ação antileishmanial seletiva do composto contra *L. infantum* e que a composição micelar incorporando a molécula apresentou também melhor atividade *in vivo* para o tratamento contra a LV.

6. CONCLUSÃO

Os resultados descritos nesta dissertação apontam que as moléculas DIGI e b-AD apresentam ação seletiva contra *L. infantum* por meio de ensaios *in vitro* e *in vivo*, e que quando administradas na forma livre ou, principalmente, incorporadas em sistemas de *delivery* constituído por micelas poliméricas formadas por Poloxâmero P407, são eficazes para o tratamento contra a LV murina.

7. PERSPECTIVAS

- Avaliar a atividade antileishmanial das composições micelares contra a leishmaniose tegumentar murina.

- Realizar estudos para avaliar a eficácia terapêutica das composições micelares em outros modelos de mamíferos, tais como hamster.

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ANEXO 1 – Certificado do Comitê de Ética em Pesquisa Animal (CEUA) da UFMG.

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)
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***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

Belo Horizonte, 23/09/2018.

Atenciosamente,

Sistema Solicite CEUA UFMG
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ANEXO 2. Comprovação de publicação do artigo envolvendo DIGI.

Parasitology Research
https://doi.org/10.1007/s00436-020-06971-2

TREATMENT AND PROPHYLAXIS - ORIGINAL PAPER

Digitoxigenin presents an effective and selective antileishmanial action against *Leishmania infantum* and is a potential therapeutic agent for visceral leishmaniasis

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Abstract
Treatment for visceral leishmaniasis (VL) is hampered mainly by drug toxicity, their high cost, and parasite resistance. Drug development is a long and pricey process, and therefore, drug repositioning may be an alternative worth pursuing. Cardenolides are used to treat cardiac diseases, especially those obtained from *Digitalis* species. In the present study, cardenolide digitoxigenin (DIGI) obtained from a methanolic extract of *Digitalis lanata* leaves was tested for its antileishmanial activity against *Leishmania infantum* species. Results showed that 50% *Leishmania* and murine macrophage inhibitory concentrations (IC_{50} and CC_{50}, respectively) were of 6.9 ± 1.5 and 295.3 ± 14.5 μg/mL, respectively. With amphotericin B (AmpB) deoxycholate, used as a control drug, values of 0.13 ± 0.02 and 0.79 ± 0.12 μg/mL, respectively, were observed. Selectivity index (SI) values were of 42.8 and 6.1 for DIGI and AmpB, respectively. Preliminary studies suggested that the mechanism of action for DIGI is to cause alterations in the mitochondrial membrane potential, to increase the levels of reactive oxygen species and induce accumulation of lipid bodies in the parasites. DIGI was incorporated into Pluronic® F127-based polymeric micelles, and the formula (DIGI/Mic) was used to treat *L. infantum*–infected mice. Miltefosine was used as a control drug. Results showed that animals treated with either miltefosine, DIGI, or DIGI/Mic presented significant reductions in the parasite load in their spleens, livers, bone marrows, and draining lymph nodes, as well as the development of a specific Th1-type response, when compared with the controls. Results obtained 1 day after treatment were corroborated with data corresponding to 15 days after therapy. Importantly, treatment with DIGI/Mic induced better parasitological and immunological responses when compared with miltefosine- and DIGI-treated mice. In conclusion, DIGI/Mic has the potential to be used as a therapeutic agent to protect against *L. infantum* infection, and it is therefore worth of consideration in future studies addressing VL treatment.

Keywords Treatment · Digitoxigenin · Drug repositioning · Visceral leishmaniasis · Amphotericin B deoxycholate · Miltefosine

Introduction

Leishmaniases are protozoal vector-borne diseases that affect both humans and animals. There are approximately 380 million people at risk of contracting infection and 2.0 million cases registered annually (WHO 2018). This disease complex is caused by some species of the *Leishmania* genus, being the tegumentary and visceral leishmaniases, the main clinical forms of the disease (Grimaldi and Tesh 1993). Visceral leishmaniasis (VL) is a worldwide-distributed infectious disease that, when symptomatic, causes fever, hepatosplenomegaly, and pancytopenia, among others (Burza et al. 2018). Its diagnosis is accomplished by molecular and/or conventional parasitological techniques, as well as by immunological assays. However, accurate diagnosis depends on the current clinical status and immune response of the infected hosts, with false-negative and/or false-positive results being frequently

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