MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DO DELTA DO PARNAÍBA
CAMPUS MINISTRO REIS VELOSO
CURSO DE BACHARELADO EM BIOMEDICINA

YASMIM ALVES AIRES MACHADO

AVALIAÇÃO DA ATIVIDADE ANTILEISHMANIA DO ÁCIDO LITOCÓLICO SOBRE *Leishmania (Leishmania) amazonensis*

PARNAÍBA
2023

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Trabalho de Conclusão de Curso apresentado ao Curso de Biomedicina da Universidade Federal do Delta do Parnaíba, *Campus* Ministro Reis Velloso, como requisito obrigatório para obtenção do título de Bacharel em Biomedicina.

Orientador: Prof. Dr. Klinger Antonio da Franca Rodrigues

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Aprovado em 30 / 08 / 2023

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Resumo

As leishmanioses são doenças ocasionadas por parasitos pertencentes ao gênero *Leishmania*. Estas doenças têm impacto na pele, mucosas e órgãos internos dos indivíduos afetados. Consideradas Doenças Tropicais Negligenciadas (DTNs), impactam áreas de grande carência socioeconômica e sem infraestruturas básicas de higiene e saneamento. O tratamento é realizado através de medicamentos de elevada toxicidade, culminando em inúmeros efeitos adversos e, em certas situações, há manifestação de resistência por parte do parasito. Nesse cenário, a exploração de novos compostos antileishmania tem fomentado a investigação no âmbito de substâncias naturais e seus derivados. O ácido litocólico, um ácido biliar secundário, tem evidenciado atividades biológicas antimicrobianas, antifúngicas e também como potencial inibidor da biossíntese de esteróis em espécies da família Tripanosomatidae, como *Trypanossoma cruzi* e *Trypanossoma brucei*, além de demonstrar atividade *in sIlico* contra *Leishmania (Leishmania) amazonensis*. Diante do exposto o trabalho teve como objetivo avaliar a atividade biológica do ácido litocólico sobre *L. amazonensis,* bem como sua citotoxicidade. O ácido litocólico foi eficaz em inibir o crescimento de formas promastigotas (CI_{50} 4,95 μM) em concentrações não tóxicas a macrófagos RAW 264.7. A CC_{50} obtida para macrófagos foi de 83,71 μM. No modelo de infecção em macrófagos com *L. amazonensis*, o ácido litocólico foi capaz de diminuir a porcentagem de infecção e o número de amastigotas por macrófago. O composto testado apresentou índice de seletividade superior aos fármacos disponíveis no mercado, sendo 22 vezes e 15 vezes mais seletivo do que antimoniato de meglumina e a anfotericina B, respectivamente. Os macrófagos tratados com ácido litocólico apresentaram aumento da síntese de NO, sendo uma possível via de ativação de macrófagos no combate à infecção. Os resultados sugerem que o ácido litocólico é um composto com atividade antileishmania *in vitro* promissora e um possível candidato a ensaios *in vivo*.

Palavras-chave: Ácido litocólico; *L. amazonensis*; citotoxicidade, óxido nítrico.

1. INTRODUÇÃO

As leishmanioses são um espectro de infecções parasitárias causada por diferentes espécies do gênero *Leishmania,* prevalentes principalmente em países localizados em áreas tropicais ou subtropicais, geralmente subdesenvolvidos. Considerada a segunda doença parasitária de maior incidência, atrás apenas da malária, atualmente, cerca de 12 milhões de pessoas estão infectadas e estima-se que a cada ano ocorra 700.000 a 1 milhão de novas infecções. A estimativa global é de aproximadamente 350 milhões de pessoas suscetíveis às leishmanioses (Mesquita et al., 2022; WHO, 2023).

A transmissão da doença é feita por fêmeas de insetos vetores dos gêneros *Phlebotomus,* novo mundo, e *Lutzomyia,* velho mundo (Kumari et al., 2022). A infecção manifesta-se de formas distintas como Leishmaniose Tegumentar (LT) e Leishmaniose Visceral (LV). A LT promove lesões dérmicas, ocorrendo desde nódulos ulcerados e não ulcerados até o tipo mucocutâneo, afetando as mucosas do nariz, palato bucal, garganta, faringe e laringe (Kumari et al., 2022). Em contrapartida, a LV, popularmente calazar, causa danos principalmente ao fígado, baço e medula óssea, sendo essa a forma considerada mais grave por acometer órgãos internos e pode ser fatal em 95 % dos casos que não seja feito o correto tratamento (Blaizot et al., 2021).

As leishmanioses não dispõem de vacinas preventivas para humanos e os fármacos comumente usados possuem muitos registros de cepas resistentes a eles (Tiwari et al., 2018). O tratamento clássico é dividido em medicamentos de primeira linha, como os antimoniais pentavalentes, presentes no mercado como antimoniato de meglumina e estibogluconato de sódio, os quais são usados desde a década de 1940. Em casos de resistência ou presença de comorbidades, o tratamento é feito

com os medicamentos de segunda linha, como anfotericina B, um anfifúngico, e mitelfosina, um anticancerígeno e a única opção para o tratamento via oral (Tiwari et al., 2018; Tabrez et al., 2021).

As alternativas terapêuticas utilizadas para as leishmanioses apresentam alto custo, difícil administração, via parenteral extremamente incômoda na maioria dos casos, necessidade de internação do paciente durante o tratamento, além da alta cardiotoxicidade, nefrotoxicidade e hepatotoxicidade, que acaba em limitar seu uso (Chakravarty, Sundar, 2019). Com isso, devido às limitações, há a urgente necessidade de buscar novas alternativas terapêuticas que apresentem resultados mais eficazes e que sejam opções mais seguras ao paciente.

Substâncias naturais provenientes de plantas medicinais surgem como uma perspectiva promissora na exploração de novas abordagens terapêuticas, dado que essas plantas abrigam uma abundância de moléculas bioativas. Isso pode representar uma opção menos prejudicial e potencialmente mais eficaz para o tratamento das leishmanioses. A família Annonaceae possui uma grande diversidade de compostos naturais, e na literatura as pesquisas evidenciam a atividade biológica de diversos de seus metabólitos secundários (Vila-Nova et al. 2011; Brígido et al. 2020).

O ácido litocólico (AcL), ou ácido 3α-hidroxi-5β-colan-24-óico, é um ácido biliar secundário hidrofóbico obtido a partir da 7-desidroxilação bacteriana do ácido quenodesoxicólico e do ácido ursodesoxicólico (He et al., 2017; Fiorucci et al., 2018). Também é considerado um metabólito secundário terpenoide isolado de espécies de Annonaceae (Menezes et al., 2022). Estudos tem demonstrado o AcL como um aliado na contenção da inflamação hepática (Sinha et al., 2020), com atividades biológicas antimicrobianas e antifúngicas (Do Nascimento et al., 2015;

Bellini et al., 1984), inibição tumoral e efeito indutor de apoptose em linhagens de células cancerígenas (El Kihel et al., 2008; Arlia-Ciommo et al., 2014). Mostrando-se também como potencial inibidor da biossíntese de esteróis, uma via metabólica importante na família *Trypanosomatidae*, demonstrados em *T. cruzi* e *T. brucei* (Vandeweerd; Black, 1990; García Liñares et al., 2015; Musikant et al., 2019), apresentando também comprovada atividade antiepimastigota de *T. cruzi* (Barros De Menezes et al., 2022)

As atividades *in vitro* do ácido litocólico frente as leishmanioses ainda não são exploradas na literatura, entretanto estudo *in silico* dispõem uma boa atividade contra formas promastigotas e amastigotas de *Leishmania (Leishmania) amazonensis* (De Menezes et al., 2022). Dessa forma, este estudo tem como objetivo avaliar o ácido litocólico quanto a inibição do crescimento de formas promastigotas de *L. amazonensis*, sua citotoxicidade contra células, avaliar o seu desempenho frente a infecção de macrófagos RAW 264.7 e os possíveis mecanismos de ação.

2. MATERIAL E MÉTODOS

2.1. Reagentes

Meio de cultura Schneider para insetos, meio Roswell Park Memorial Institute 1640 (RPMI 1640), reagente de Griess, diacetato de 2′,7′-diclorodihidrofluoresceína (H2DCFDA), solução antibiótica estabilizada (penicilina 10.000 U/mL; estreptomicina 10 µg/mL) e solução antibiótica antimicótica estabilizada (penicilina 10.000 U/mL; estreptomicina 10 µg/mL; anfotericina B 25 µg/mL) foram obtidos da Sigma Aldrich (St. Louis, MO, EUA). 3-(4,5-dimetiltiazol-2-il)-2,5-di-fenil brometo de tetrazólio (MTT – Amresco, Ohio, EUA). O fármaco anfotericina B foi adquirido da Cristália (São

Paulo, SP, Brasil). O antimoniato de meglumina (Glucantime®) foi obtido da Aventis Pharma (São Paulo, SP, Brasil). O soro fetal bovino (SFB) foi adquirido da Cultilab (São Paulo, SP, Brasil). O dodecil sulfato de sódio (SDS) foi obtido da Mallinckrodt Chemicals (St. Louis, EUA). O kit de coloração rápida de panótipos foi adquirido da Laborclin (Curitiba, PR, Brasil). O nitrito de sódio (NaNO2) foi adquirido da Vertec Fine Chemistry (Rio de Janeiro, RJ, Brasil).

2.2. Ácido litocólico

O ácido litocólico, $C_{24}H_{40}O_3$, 98% de pureza, foi cedido pelo Dr. Josean Fechine Tavares, professor adjunto da Universidade Federal da Paraíba. O composto foi solubilizado em DMSO. Para alcançar as concentrações desejadas, as soluções foram diluídas, garantindo que a quantidade de DMSO não excedesse 0,5%.

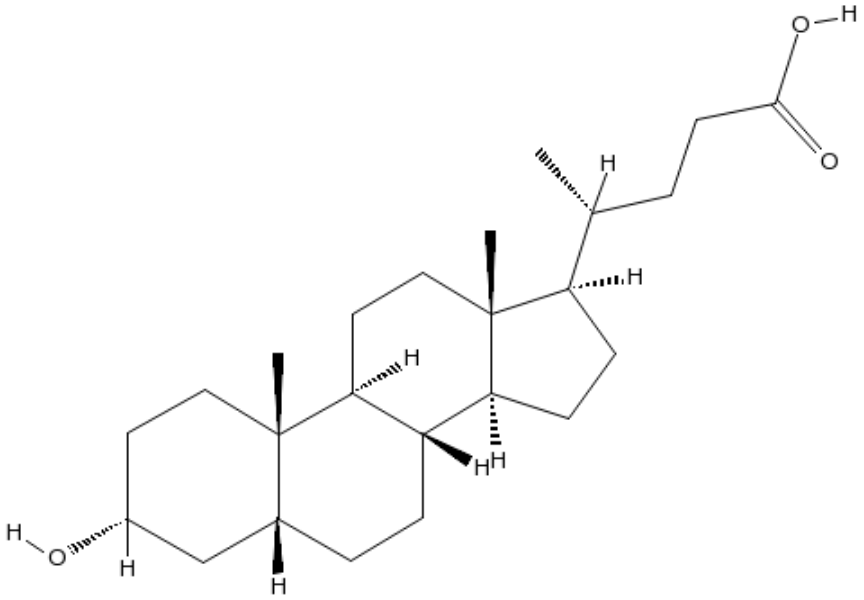

Fig. 1. Estrutura molecular do ácido litocólico.

2.3. Parasitos

Promastigotas da espécie *Leishmania (Leishmania) amazonensis* (IFLA/BR/67/PH8), foram cultivadas e mantidas em meio de Schneider para insetos, com suplementação feita com 20 % de soro fetal bovino (SFB) e 1 % de penicilina/estreptomicina (10.000 UI/10 mg/mL) com pH de 7,4 e mantidas a 26 °C em estufa de demanda biológica de oxigênio (BOD). Amastigotas axênicas foram obtidas a partir da transformação de formas promastigotas de *L. amazonensis*, cultivando-as em meio Schneider suplementado a temperatura de 26 ºC durante 5 dias, até que atingissem a fase estacionária. Após esse período, as culturas foram ressuspensas em meio Schneider, suplementado com 5 % de SFB e 1 % de penicilina/estreptomicina, com o pH 5,5. Após a ressuspensão, os mesmos foram incubados a 32 ºC em estufa BOD por três dias para diferenciação em amastigotas.

2.4. Macrófagos murinos e células da linhagem VERO

Os macrófagos murinos da linhagem RAW 264.7, foram cultivados em meio *Eagle Modificado por Dulbecco* (DMEM), com suplementação de 10 % de SFB, 1 % de solução antibiótica e antimicótica, penicilina/estreptomicina/anfotericina B (10.000 UI / 10 mg/mL / 25 µM) e mantidos a temperatura de 37 °C com 5 % de CO_2. Células da linhagem VERO foram cultivadas em meio *Roswell Park Memorial Institute 1640* (RPMI) suplementado a 10 % de SFB e 1 % de antibiótico e antimicótico, sendo incubados em estufa a 37°C e 5 % de CO_2. Os repiques celulares foram feitos sempre após as células atingirem a confluência de 100 % em todo o frasco de cultura, em torno de 72h.

2.5. Atividade antileishmania do AcL sobre formas promastigotas e amastigotas axênicas de L. amazonensis

A atividade biológica contra promastigotas e amastigotas axênicas foram avaliadas a partir do teste colorimétrico com sal brometo de 3-(4,5-dimetiltiazol-zil)-2,5-difeniltetrazolio (MTT). Promastigotas e amastigotas axênicas de *L. amazonensis* na proporção de $1x10^6$ por poço foram adicionadas em placas de fundo chato de 96 poços, acrescidos de ácido litocólico diluído em meio Schneider suplementado em concentrações seriadas (100 µM – 0,78 µM). As placas foram incubadas em estufa de demanda biológica no período de 72 h a temperatura de 26 ºC para promastigotas, e 32 ºC para amastigotas axênicas. Decorrido esse período, para observar a porcentagem de inibição no crescimento das formas parasitárias, foi adicionado 10 µL de MTT a cada poço. As placas foram encubadas novamente por 4 h e posteriormente, para a dissolução dos cristais de formazan, 50 µL da solução de dodecil sulfato de sódio (DSS) a 10 % em água destilada foram adicionados. As leituras foram realizadas em leitor de placa ELISA a 540 nm.

2.6. Citotoxicidade do ácido litocólico sobre macrófagos RAW 264.7 e células VERO

O ensaio para avaliação da citotoxicidade foi realizado em placas de cultura celular de 96 poços usando o MTT para observação da viabilidade celular. Placas contendo 100 µL de meio DMEM suplementado, receberam o semeio de $1x10^5$ macrófagos RAW 264.7 ou células VERO por poço e foram incubadas por 4 h a 37°C com 5 % de CO_2 para que ocorresse a adesão celular. Após esse tempo, foram adicionados o meio DMEM suplementado juntamente com concentrações do ácido litocólico (200 µM - 12,5 µM). Após a aplicação do ácido litocólico, as placas foram incubadas por 72 h de a 37°C e 5 % de CO_2. Decorrido esse tempo, 10 µL de MTT

foram adicionados e incubados por mais 4h. Por fim, a placa foi levada para leitura em um espectrofotômetro a 540nm. Os resultados são expressos em termos de concentração citotóxica média (CC_{50}). O índice de seletividade (IS) foi determinado dividindo-se os valores de CC_{50} pelos de CI_{50} (Rodrigues et al., 2015b).

2.7. Avaliação da atividade antileishmania do ácido litocólico sobre amastigotas intramacrofágicas de L. amazonensis

Em placas de cultura de células de 24 poços contendo lamínulas estéreis redondas de 13 mm, macrófagos RAW 264.7, na concentração de $1x10^5$ células/mL em 1 mL de meio RPMI, foram adicionados e incubadas a 37°C e 5 % de CO_2 por 4 h para a adesão celular. Após este período, o meio foi substituído por um novo meio contendo formas promastigotas em fase estacionária de crescimento de *L. amazonensis*, na proporção de 10 promastigotas por macrófago em cada poço. Após 4 h de incubação a 37 °C e 5 % de CO_2, foram realizadas três lavagens com PBS pré-aquecido a 37 °C, para remover macrófagos não aderidos e promastigotas livres. Em seguida, 1 mL de meio RPMI completo foi adicionado em cada poço contendo concentrações seriadas de 12,5 µM a 1,56 µM de ácido litocólico. As culturas foram então incubadas a 37 °C e 5 % de CO_2 por um período de 72h, ao final, as lamínulas foram removidas, fixadas e coradas com Panótico rápido e montadas em lâminas. Em microscopia óptica com aumento de 1000 x, em cada lamínula foram contados 300 macrófagos e o número de amastigotas por cada macrófago. Os resultados foram expressos como porcentagem de células infectadas e número de amastigotas por macrófago. Os sobrenadantes do ensaio de infecção foram armazenados crio-preservados a -20°C para posterior avaliação da produção de óxido nítrico (NO) (Noleto Dias et al., 2020).

2.8. Produção de espécies reativas de oxigênio (EROs)

A fim de avaliar os efeitos do ácido litocólico na geração de espécies reativas de oxigênio (EROs) por macrófagos infectados com *L. amazonensis*, utilizou-se o ensaio com H_2DCFDA (2,7-dicloro dihidro fluoresceína diacetato). Os macrófagos foram incubados em placas de 96 poços contendo meio RPMI completo por 3 horas a 37 °C e 5 % de CO_2 para permitir a adesão. Em seguida, os macrófagos foram infectados com promastigotas de *L. amazonensis* na proporção de 10 promastigotas para 1 macrófago e incubados por 4 horas. Após esse período, o composto em teste foi adicionado nas concentrações de 1,56 µM a 12,5 µM por 72 horas. Em seguida, 10µL de H_2DCFDA foram adicionados para alcançar uma concentração final de 20 µM, e os macrófagos foram incubados no escuro a 37 °C por 30 minutos. A intensidade de fluorescência foi medida utilizando um espectrofluorômetro com excitação a 485 nm e emissão a 528 nm (Nunes et al., 2021).

2.9. Produção de óxido nítrico (NO)

A quantificação da produção de NO a partir dos sobrenadantes dos ensaios de infecção foi feita com base nos níveis de nitrito por meio do reagente de Griess. Foram adicionados 100 µL do sobrenadante da infecção em uma placa de 96 poços. Simultaneamente, foram adicionadas concentrações seriadas de nitrito de sódio ($NaNO_2$) em meio RPMI à placa, para a interpolação da curva padrão. O reagente de Griess foi adicionado e incubado por 10 min em temperatura ambiente. Ao final do experimento, a análise foi realizada utilizando um leitor de placas a 540 nm para NO (Garcia et al, 2021). Foi utilizado lipopolissacarídeo de *Escherichia coli* (LPS) a 2 µg/mL como controle positivo.

2.10. Análise estatística

Os ensaios foram realizados em triplicata e todos em 3 experimentos independentes. As variações entre resultados foram analisadas por ANOVA unidirecional e a significância estatística foi calculada usando testes post-hoc de Tukey, considerando o valor de $p<0,05$ como nível máximo de significância. Os valores da concentração inibitória média (CI_{50}), concentração efetiva média (CE_{50}) e os valores da concentração de citotoxicidade média (CC_{50}), com intervalos de confiança de 95 %, foram calculados usando regressão não linear. Para o cálculo do índice de seletividade (IS) foi utilizada a fórmula: IS (índice de seletividade) = CC_{50}/CI_{50} ou CE_{50}.

3. RESULTADOS

3.1. Atividade antileishmania do ácido litocólico sobre formas promastigotas de L. amazonensis

O ácido litocólico apresentou citotoxicidade contra promastigotas de *L. amazonensis* em todas as concentrações testadas, apresentando inibição de 91,54 %, 89,95 %, 80,18 %, 74,77 %, 55,42 %, 30,69 %, 2,34 % e 0,93 % nas concentrações de 100 µM, 50 µM, 25 µM, 12,5 µM, 6,25 µM, 3,12 µM, 1,56 µM e 0,78 µM, respectivamente (Fig. 2), com uma concentração inibitória média (CI_{50}) de 4,95 µM (Tabela 1). As concentrações inibitórias médias demonstram que anfotericina B apresentou atividade antileishmania, com CI_{50} de 0,35 µM e CE_{50} de 0,51 µM, entretanto o antimoniato de meglumina apresenta a baixa atividade contra o parasita com CI_{50} e CE_{50} de 21564 µM e 1805 µM, respectivamente (Tabela 1).

3.2. Citotoxicidade do ácido litocólico sobre macrófagos RAW 264.7 e células VERO

Os resultados da avaliação da citotoxicidade do ácido litocólico em macrófagos RAW *264.7* e células VERO estão apresentados nas Fig. 3A e 3B, respectivamente. Observa-se que na concentração de 12,5 µM, houve viabilidade de 100 % dos macrófagos e das células de linhagem VERO. Nas concentrações de 25 µM e 50 µM, 100 µM e 200 µM, o AcL causou citoxicidade nos macrófagos, exibindo porcentagem de viabilidade de 95,56 %, 81,69 %, 41,10 % e 22,04 %, respectivamente (Fig. 3A), resultando na concentração citotóxica média (CC_{50}) de 83,71 µM (Tabela 1). Em VERO, o ácido litocólico também se demonstrou citotóxico nas concentrações de 25 µM e 50 µM, 100 µM e 200 µM, com porcentagens de 84,03 %, 69,35 %, 42,66 % e 11,66 %, originando CC_{50} de 76,58 µM (Tabela 1).

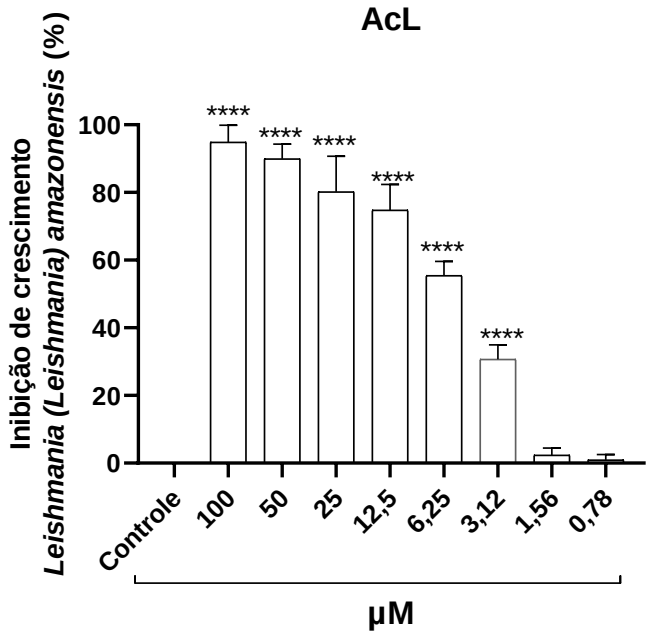

Fig. 2. Efeito do ácido litocólico em formas promastigotas de *Leishmania (Leishmania) amazonensis.* Formas promastigotas de *L. amazonensis* em fase logarítimica de crescimento foram cultivadas utilizando meio de Schneider completo, ao qual foram adicionadas concentrações em série de ácido litocólico. A avaliação da inibição do crescimento dos parasitas foi realizada por meio do ensaio do MTT após um período de 72 horas de tratamento. Os resultados expressam a média ± erro padrão de três experimentos independentes realizados em triplicata, considerando o grupo (DMSO 0,5 % em meio Schneider completo) como 0 % de inibição. A comparação entre os grupos foi realizada por One-way ANOVA seguido pelo pós-teste de Tukey, considerando (*) $p < 0,05$ vs. controle; (**) $p < 0,01$ vs. controle; (***) $p < 0,001$ vs. controle; (****) $p < 0,0001$ vs. controle.

Tabela 1 - Atividade antileishmania, efeito citotóxico sobre células mamíferas e valores de índice de seletividade (IS) calculados para ácido litocólico (AcL), anfotericina B e antimoniato de meglumina.

Compostos	RAW 264.7	VERO	Promastigotas		Amastigotas Intramacrofágicas	
	CC_{50} µM	CC_{50} µM	CI_{50} µM	SI	CE_{50} µM	SI
AcL	83,71	76,58	4,95	16,91	2,34	35,77
Anfotericina B	0,39	2,46	0,35	1,11	0,13	3
Antimoniato de meglumina	16.433	22.831	21.564	0,76	763,6	21,52

Índice de seletividade (IS) = CC_{50}/CI_{50} ou CE_{50}.

3.3. Avaliação da atividade antileishmania do ácido litocólico sobre amastigotas intramacrofágicas de Leishmania (Leishmania) amazonensis

Os resultados advindos do ensaio de tratamento de macrófagos infectados com *L. amazonensis* e tratados com ácido litocólico durante 72h, estão apresentados na Fig 3. Foram utilizados dois parâmetros: porcentagem de macrófagos infectados (Fig. 3A) e o número de amastigotas por macrófagos (Fig. 3B). Quanto a porcentagem de macrófagos infectados, observa-se que o ácido litocólico gerou a diminuição na taxa de infecção em todas as concentrações testadas, chegando a 99 % na concentração de 12,5 µM.

No que se refere ao número de amastigotas por macrófago, o tratamento com AcL surtiu em uma redução significativa no número de amastigotas internalizadas, onde na maior concentração, 12,5 µM, observa-se a redução de 100 %. O ácido litocólico gerou uma concentração efetiva contra 50 % das amastigotas internalizadas (CE_{50}) de 2,34 µM (Tabela 1). Quanto aos fármacos de referência, a anfotericina B demonstrou CE_{50} de 0,13 µM e o antimoniato de meglumina obteve CE_{50} de 763,6 µM (Tabela1).

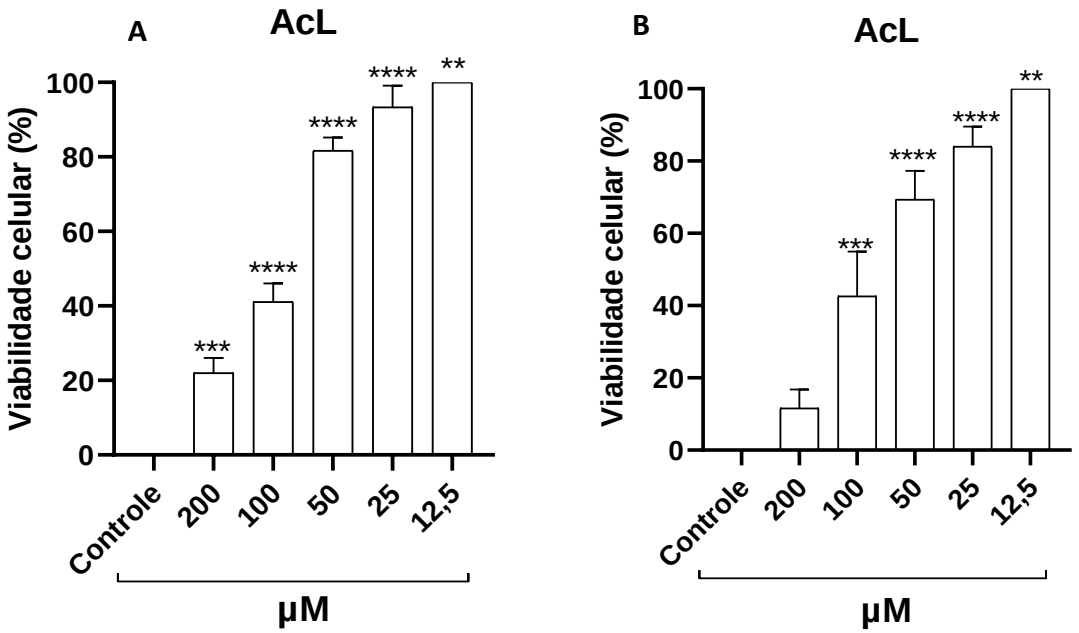

Fig. 3. Efeitos citotóxicos do ácido litocólico sobre macrófagos RAW 264.7 e células VERO Citotoxicidade avaliada pelo teste colorimétrico do MTT. Macrófagos RAW 264.7 (A) e células VERO (B) foram cultivados em placa de 96 poços em meio DMEM na concentração de $1x10^5$ por poço, levados para aderência no período de 4 horas, concentrações seriadas de ácido litocólico foram adicionadas e encubadas por 72h. A viabilidade celular foi avaliada pelo ensaio do MTT após 72 h de tratamento. Os resultados expressam a média ± erro padrão de três experimentos independentes realizados em triplicata, considerando o controle (DMSO 0,5 % em DMEM completo) como 100 % de viabilidade. A comparação entre os grupos foi realizada por One-way ANOVA seguido pelo pós-teste de Tukey, considerando (*) $p < 0,05$ vs. controle; (**) $p < 0,01$ vs. controle; (***) $p < 0,001$ vs. controle; (****) $p < 0,0001$ vs. controle.

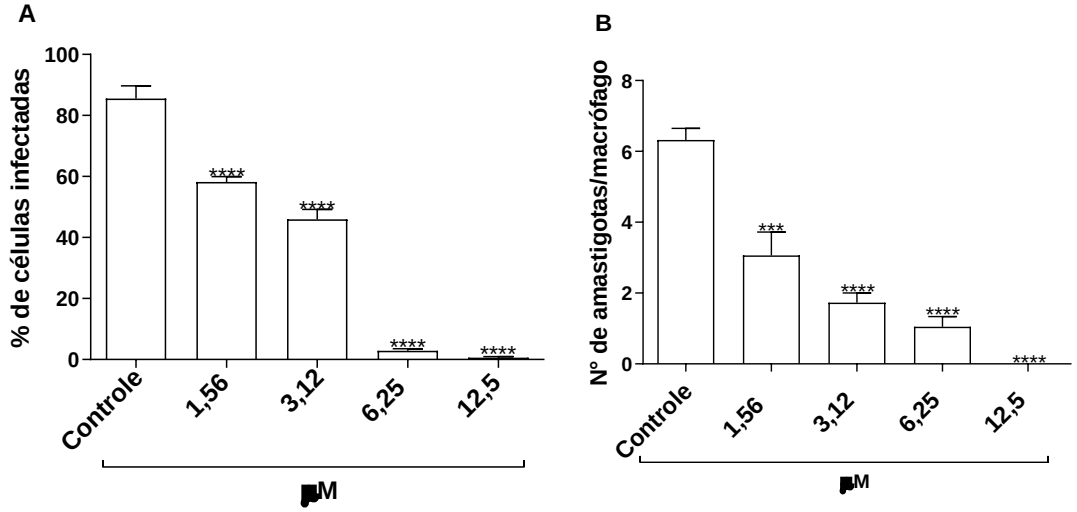

Fig. 4. Atividade antileishmania do ácido litocólico contra formas amastigotas internalizadas em macrófagos após 72 h de exposição. Percentual de infecção (A) e número de amastigotas por macrófago (B). Macrófagos RAW 264.7 em placas de 24 poços contendo lamínulas redondas, foram infectados com formas promastigotas de *L. amazonensis* na proporção de 10 leishmanias por célula e tratados por 72h com ácido litocólico em concentrações decrescentes de 12,5 µM – 1,56 µM. Após as 72h, a avaliação da

porcentagem de células infectadas e o número de amastigotas por macrófago foi feita por contagem em microscopia óptica das lamínulas circulares. Os resultados expressam a média ± erro padrão de três experimentos independentes realizados em triplicata, considerando o controle (DMSO 0,5 % em DMEM completo) como 0 % de inibição. A comparação entre os grupos foi realizada por One-way ANOVA seguido pelo pós-teste de Tukey, considerando (*) $p < 0,05$ vs. controle; (**) $p < 0,01$ vs. controle; (***) $p < 0,001$ vs. controle; (****) $p < 0,0001$ vs. controle.

3.4. Produção de espécies reativas de oxigênio (EROs) e óxido nítrico (NO) induzidas pelo ácido litocólico

No sobrenadante da infecção, foi examinada a geração de óxido nítrico (NO) por meio da medição indireta de nitritos. Foi observado um aumento significativo de NO nos grupos tratados com as maiores concentrações de AcL (6,25 µM e 12,5 µM), em comparação com o grupo controle positivo (Fig. 4A).

Entretanto, no experimento de produção de espécies reativas de oxigênio (EROs) por macrófagos infectados com *L. amazonensis* e tratados com o AcL no período de 72 horas, não se observou aumento em nenhuma concentração testada (Fig. 4B).

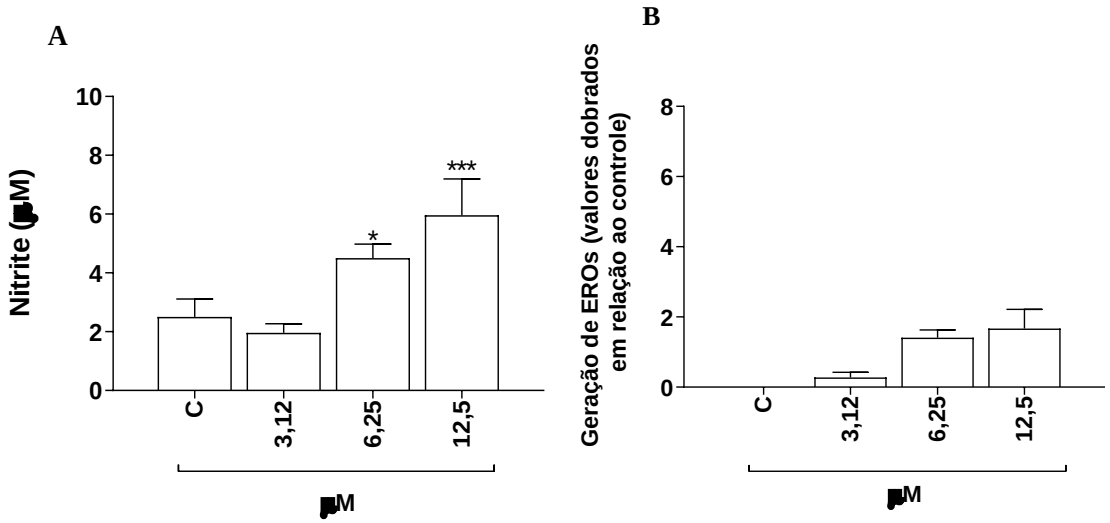

Fig. 5. Níveis de óxido nítrico (NO) e espécies reativas a oxigênio (EROs) produzidos por macrófagos infectados com *L. amazonensis* tratados com AcL. Produção de NO (A) e EROs (B) em macrófagos infectados com *L. amazonensis* e tratados com o AcL. A produção de NO foi avaliada utilizando reagente de Griess e a produção de EROs foi avaliada através do diacetato de 2',7'-diclorofluoresceína (H_2DCFDA). Os resultados expressam a média ± erro padrão de três experimentos independentes realizados em triplicata. A comparação entre os grupos foi realizada por One-way ANOVA seguido pelo pós-teste de Tukey, considerando (*) $p < 0,05$ vs. controle; (**) $p < 0,01$ vs. controle; (***) $p < 0,001$ vs. controle.

4. DISCUSSÃO

Na presente situação em que o tratamento das leishmanioses é realizado através de medicamentos altamente tóxicos e dispendiosos, resultando em uma série de efeitos adversos, é imperativo buscar alternativas terapêuticas urgentes para abordar esse grupo de doenças (Brígido et al. 2020). Produtos naturais derivados de plantas medicinais são uma base promissora na busca de novos tratamentos, pois são fontes ricas em moléculas bioativas, podendo ser uma opção menos tóxica e mais eficaz ao tratamento das leishmanioses.

A atividade antipromastigota do AcL foi realizada utilizando o ensaio colorimétrico do MTT, o qual avalia a viabilidade das células testadas, onde células viáveis irão reduzir o sal tetrazólio a formazan, uma molécula insolúvel em água e de coloração púrpura (Ghasemi et al., 2021). O AcL foi efetivo em inibir o crescimento de formas promastigotas de *L. amazonensis* em baixas concentrações, com valor de CI_{50} abaixo de outros compostos extraídos de Annonaceae considerados agentes antileishmania potenciais, como o triterpeno ácido ursólico, com CI_{50} de 14,1 µg/mL, e o lupeol com CI_{50} de 39,06 µg/mL (Souza et al., 2017; Bilbao-Ramos et al., 2020).

Visando a investigação da toxicidade do composto em estudo, um dos parâmetros utilizados foi o IS, que representa a atividade da droga testada frente as células e aos parasitas. A análise *in vitro* do AcL em macrófagos RAW 264.7 e em células VERO demonstrou a segurança do composto apresentando bons valores de IS. O intuito desse teste foi verificar se esse composto poderia afetar a viabilidade celular dos macrófagos que não foram expostos a *L. amazonensis* e das células de linhagem VERO.

A segurança dessas substâncias é considerada estabelecida quando o valor do IS ultrapassa 10 (RAMÍREZ-MACÍAS et al., 2012; PIRES et al., 2013). Os

fármacos de referência, antimoniato de meglumina e anfotericina B, contudo, não exibiram níveis aceitáveis de segurança, corroborando com a literatura, os quais são cardiotóxicos, hepatotóxicos e nefrotóxicos (Carvalho et al., 2017; Chakravarty, Sundar, 2019). Com base no IS calculado para o AcL nas formas promastigotas, é possível constatar a efetividade e a seletividade do composto, superando os fármacos de referência. O AcL demonstrou ser 22 vezes mais seletivo para promastigotas de *L. amazonensis* que o antimoniato de meglumina e 15 vezes mais seletivo para promastigotas de *L. amazonensis* do que a anfotericina B.

Uma vez determinadas as concentrações que conseguem inibir o desenvolvimento do parasita sem causar danos às células, procedeu-se à realização de ensaios com amastigotas intramacrofágicas. Essa análise foi realizada com o intuito de simular a infecção que ocorre *in vivo,* onde as células de defesa internalizam o parasita para o desenvolvimento da infecção, diferenciando em amastigota e replicando no seu interior (Lestinova et al., 2017; Dubie; Mohammed, 2020). Foram utilizados dois parâmetros para a avaliação: porcentagem de células infectadas e número de amastigotas por macrófago.

O AcL demonstrou efetividade frente à infecção, gerando uma diminuição na taxa de células infectadas, demonstrando uma efetividade superior ao antimoniato de meglumina, fármaco de referência que apresenta maior atividade contra amastigotas internalizadas (Wyllie et al., 2004; Carvalho et al., 2019). Ao compararmos seu desempenho com a anfotericina B, vemos uma efetividade superior do fármaco de referência, entretanto sua baixa seletividade em relação aos macrófagos, limitam sua utilidade na clínica com a observação de inúmeros efeitos adversos.

A maior responsividade contra as amastigotas intramacrofágicas, em relação a formas promastigotas, observada com a administração do AcL sugere uma possível ativação dos macrófagos mediante o tratamento (Rodrigues et al., 2015). Os macrófagos desempenham papel fundamental no processo infeccioso por *Leishmania* spp. atuando como células hospedeiras, mas a sua função microbicida pode ser influenciada por substâncias imunomoduladores que podem conferir resistência à infecção (GABRIEL et al., 2019).

No processo infeccioso por *Leishmania* spp*.,* a resistência à infecção é associada à promoção de uma resposta imunológica do tipo Th1, sendo caracterizada por níveis significativos de IFN-γ e TNF-α, citocinas predominantemente produzidas por células Natural Killer (NK) e células T (Dubie; Mohammed, 2020). Essas citocinas pró-inflamatórias induzem a expressão de óxido nítrico (NO) e espécies reativas de oxigênio (EROs), tendo a capacidade de afetar ou inativar enzimas que o parasita utiliza como defesa contra o sistema imunológico do hospedeiro, como a superóxido dismutase, além de agir nos componentes celulares podendo levar a alterações morfológicas, assim, controlando a carga parasitária (GABRIEL et al., 2019; Costa-Da-Silva et al., 2022).

Observa-se que o AcL induziu o aumento da produção de NO por macrófagos infectados com *L. amazonensis*, o que sugere a participação desse mecanismo de ação na atividade antileishmania do composto estudado. Por outro lado, não houve aumento de EROs nas condições testadas, sugerindo que o mecanimo envolvido na atividade antileishmania do AcL é independente da síntese de EROs.

5. CONCLUSÃO

Com base nos resultados obtidos, é possível inferir que o AcL revelou ser seletivo e eficaz para controlar as formas promastigotas de *L. amazonensis*. Além disso, o estudo também evidenciou que a atividade antileishmania pode estar associada a um mecanismo de ação indireto que amplia os níveis de estresse oxidativo nos macrófagos encarregados de combater a infecção. Esses achados respaldam a conclusão de que o AcL desponta como um possível candidato antileishmania, estimulando investigações futuras *in vivo* para o desenvolvimento de novos agentes para o tratamento das leishmanioses.

Declaração de contribuição de autoria

Yasmim Alves Aires Machado: Investigação, Conceitualização, Metodologia, Curadoria de dados e Redação. **Julyanne Maria Saraiva de Sousa:** Investigação. **Raiza Raianne Luz Rodrigues:** Investigação. **Vanessa Maria Rodrigues de Souza:** Investigação. **Airton Lucas dos Santos Sousa:** Investigação. **Josean Fechine Tavares:** Investigação. **Klinger Antonio da Franca Rodrigues:** Investigação, Conceitualização, Metodologia, Curadoria de dados, Redação, Aquisição de financiamento e Administração do projeto.

Conflito de interesse

Os autores confirmam que não têm conflitos de interesse.

Agradecimentos

Agradeço ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (número de concessão 403260/2021-3) e a Fundação de Amparo à Pesquisa do Piauí (FAPEPI) (número de concessão 004/2020-PPSUS) pelo financiamento desse trabalho.

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EXPERIMENTAL PARASITOLOGY

AUTHOR INFORMATION PACK

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DESCRIPTION

Experimental Parasitology emphasizes modern approaches to **parasitology**, including **molecular biology** and **immunology**. The journal features original research papers on the physiological, metabolic, immunologic, biochemical, nutritional, and chemotherapeutic aspects of **parasites** and **host-parasite relationships**.

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