FERNANDA TOMIOTTO PELLISSIER

EFEITO ANTILEISHMANIA DE DIFERENTES EXTRATOS DO PEQUI (*Caryocar coriaceum*)

Londrina
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Dissertação apresentada ao Programa de Pós Graduação em Patologia experimental do Departamento de Ciências Patológicas da Universidade Estadual de Londrina, como requisito parcial à obtenção do título de mestre em Patologia Experimental.

Orientador: Prof. Dr. Wander Rogério Pavanelli.

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Ficha de identificação da obra elaborada pelo autor, através do Programa de Geração Automática do Sistema de Bibliotecas da UEL

Pellissier, Fernanda Tomiotto.
EFEITO ANTILEISHMANIA DE DIFERENTES EXTRATOS DO PEQUI (Caryocar coriaceum) / Fernanda Tomiotto Pellissier. - Londrina, 2017.
57f. : il.

Orientador: Wander Rogério Pavanelli.
Dissertação (Mestrado em Patologia Experimental) - Universidade Estadual de Londrina, Centro de Ciências Biológicas, Programa de Pós-Graduação em Patologia Experimental, 2017.
Inclui bibliografia.

1. Leishmaniose - Tese. 2. Extratos naturais - Tese. 3. Pequi - Tese. 4. Compostos alternativos - Tese. I. Pavanelli, Wander Rogério . II. Universidade Estadual de Londrina. Centro de Ciências Biológicas. Programa de Pós-Graduação em Patologia Experimental. III. Título.

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Londrina, 17 de fevereiro de 2017.

Dedico este trabalho à minha eternamente amada avó Ilma Rossi Tomiotto, que partiu no decorrer do último ano e que sempre se encheu de orgulho ao falar dos estudos da neta. Gostaria muito que ela estivesse aqui para ver mais esta etapa sendo cumprida.

AGRADECIMENTOS

Agradeço à Deus, quem me trouxe até aqui, me guiou, me confortou, me instruiu e colocou pessoas maravilhosas ao meu redor. Senhor, minha eterna gratidão à Ti.

Ao meu orientador, professor Dr. Wander Rogério Pavanelli, que me acolheu em seu laboratório há cerca de seis anos, quando eu mal sabia como um laboratório funcionava. Professor, você acompanhou meu crescimento e fez parte dele. Obrigada pelos conselhos, incentivo e até pelas brigas durante todos estes anos. Isso tudo contribuiu muito para o meu desenvolvimento profissional e pessoal.

À professora Dra. Ivete Conchon Costa, que abriu as portas de seu laboratório e me transmitiu um pouco da sua paixão por um parasito tão complexo e completo que *Leishmania* é. Obrigada por me ensinar também sobre a profissão da docência, com respeito e carinho pelo conteúdo e pelos alunos.

À professora Dra. Idessania Nazareth Costa por contribuir estreitamente na minha formação e também pela amizade e companheirismo.

À professora Ms. Francine Nesello Melanda por não poupar esforços para me transmitir conhecimento e por todas as demonstrações de amizade e cuidado.

À todos os colegas de laboratório que foram parte ativa na construção de minha vida acadêmica e deixaram a rotina mais leve e divertida. Em especial Milena, Bruna, Manoela, João Paulo e Allan sem os quais, este trabalho não existiria.

Ao Pedro S. R. Dionísio, que sempre esteve à maxima distância de um grito no corredor para me socorrer, que também me aconselhou e partilhou do amor de Jesus.

Ao Jesus Antônio Vargas (Zui) por toda a ajuda e paciência nas infinitas pesagens dos extratos.

À todos os professores e funcionários do Departamento de Ciências Patológicas.

Ao Laboratório de Microscopia Eletrônica e de Microanálise (LMEM), bem como aos responsáveis técnicos Osvaldo Capelo, Daiani Barbieri e Edson.

À professora Dr^{a}. Selene M. de Morais e à Ms. Daniela R. Alves pelo

fornecimento dos extratos utilizados neste trabalho.

Agradeço à CAPES e CNPq pela bolsa concedida e financiamento do meu projeto.

Aos integrantes da banca de qualificação pelo tempo e dedicação despendidos na correção deste trabalho.

Gostaria de agradecer também aos meus pais, que sempre incentivaram meus estudos e me deram totais condições de chegar até aqui e, sem eles, eu não chegaria. Que vibraram em minhas conquistas e sofreram comigo nos momentos difíceis durante este último e turbulento ano. Estendo este agradecimento ao meu irmão, Raul.

Ao meu esposo, Louis, que acompanhou de perto todos os passos dados e nem por um segundo deixou de me apoiar e me incentivar. Cuidou de mim nos momentos tristes e estressantes, viveu comigo cada vitória e até ajudou nos experimentos tarde da noite e aos fins de semana. Você é reflexo do amor de Deus na minha vida.

À todos os citados e aos possíveis esquecidos, obrigada!

Não andem ansiosos por coisa alguma, mas em tudo, pela oração e súplicas, e com ação de graças, apresentem seus pedidos a Deus.
E a paz de Deus, que excede todo o entendimento, guardará os seus corações e as suas mentes em Cristo Jesus.

Filipenses 4:6,7

TOMIOTTO-PELLISSIER, Fernanda. **Efeito antileishmania de diferentes extratos do pequi (*Caryocar coriaceum*).** 2017. 57 f. Dissertação (Mestrado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2017.

RESUMO

A Leishmaniose Tegumentar Americana (LTA) é uma zoonose causada por protozoários do gênero *Leishmania*. Os fármacos disponíveis para o tratamento da LTA apresentam: toxicidade, baixa eficiência e dificuldade de administração, tais fatores implicam na busca por terapias alternativas. Plantas do gênero *Caryocar* são encontradas no cerrado brasileiro e seus frutos são utilizados como alimento e na medicina popular. Estudos com diferentes extratos destas plantas demonstraram efeito anti-inflamatório, antifúngico, antibacteriano, analgésico, antineoplásico, imunomodulador, cicatrizante e leishmanicida. Entretanto, não existem estudos que tenham verificado o efeito de extratos provenientes da espécie *C. coriaceum* sobre protozoários *Leishmania.* Neste sentido, o objetivo do presente estudo foi investigar o efeito dos extratos de *C. coriaceum* sobre *Leishmania amazonensis*. Para isso foram utilizados diferentes extratos, de acordo com a origem e o solvente utilizado, respectivamente: (1) polpa do fruto, etanol; (2) casca do fruto, etanol; (3) folha, acetato de etilo; (4) folha, metanol. Na triagem fitoquímica, realizada com o intuito de verificar a presença de alcalóides, catequinas, esteróides, fenóis, flavonóides, saponinas e taninos, foi verificado que os extratos 1 e 3 apresentaram todos os metabólitos investigados, enquanto no extrato 2 não foram verificados catequinas, e no extrato 3, catequinas e esteróides estavam ausentes. Quando quantificados os fenóis e flavonóides, observou-se que o extrato 2 era o mais abundante em fenóis, enquanto o extrato 3 se destacou pela maior quantidade de flavonóides. Para os ensaios biológicos, formas promastigotas de *L. amazonensis* foram mantidas em meio 199 e utilizadas em fase estacionária. O ensaio antipromastigota foi realizado através da contagem em câmara de Neubauer onde verificou-se que todos os extratos apresentaram efeito antileishmania após 24, 48 e 72h de tratamento. Este dado foi confirmado através de microscopia eletrônica de varredura (MEV), na qual foram observadas alterações morfológicas nos parasitos tratados com os diferentes extratos na concentração de 50 µg/mL. Ao caracterizar os mecanismos pelos quais os extratos induziram a morte do parasito, verificamos, por sonda catiônica (TMRE) que os tratamentos induziram despolarização mitocondrial e, por sonda H_2DCFDA, que os extratos levaram a produção de espécies reativas de oxigênio (ERO) em promastigotas. De forma semelhante, foi verificado que os tratamentos provocaram danos na membrana plasmática, pela marcação com iodeto de propídio (IP) e a marcação com anexina V revelou que o tratamento induz a exposição à fosfatidilserina, eventos sugestivos de morte por processo apoptose-*like* tardio/ necrose. Em seguida, verificamos o efeito anti-amastigota dos extratos em macrófagos infectados com *L. amazonensis.* Primeiramente, observamos que o intervalo de concentrações não alterou a dos macrófagos peritoneais de camundongos BALB/c. Na sequência, os macrófagos foram infectados com os parasitos (1:5), e analisado o efeito nas formas amastigotas. Nossos resultados mostraram que o tratamento com os extratos foi capaz de reduzir o número de amastigotas por macrófago, macrófagos infectados e também a quantidade de parasitos viáveis recuperados. Adicionalmente, através do ensaio de CBA

(cytometric bead array), verificamos que os extratos não foram capazes de modular os níveis das citocinas investigadas, nem a síntese dos mediadores microbicidas, óxido nítrico (NO) e ERO, analisados por ensaio de Griess e marcação com sonda fluorescente H_2DCFDA, respectivamente. Com base nos dados obtidos, podemos inferir que extratos de diferentes partes da planta de *C. coriaceum* exercem efeito leishmanicida, atuando em formas promastigotas através de mecanismos apoptose-*like* tardios/necrose e em formas amastigotas de forma direta, ou seja, independente da ativação de macrófagos.

Palavras-chave: Leishmaniose. Apoptose. Leishmanicida.

TOMIOTTO-PELLISSIER, Fernanda. **Antileishmanial effect of different extracts from pequi (*Caryocar coriaceum*)**. 2017. 57 p. Dissertation (Master´s degree in Experimental Pathology) – Universidade Estadual de Londrina, Londrina, 2017.

ABSTRACT

American Cutaneous Leishmaniasis (ACL) is a zoonosis caused by protozoa of *Leishmania* genus. The drugs available for ACL treatment present toxicity, low efficiency and difficult administration; such factors imply the search for alternative therapies. Plants of the *Caryocar* genus are found in the Brazilian cerrado and its fruits are used as food and in folk medicine. Studies with different extracts from these plants demonstrated anti-inflammatory, antifungal, antibacterial, analgesic, antineoplastic, immunomodulatory, wound healing and leishmanicidal effects. However, there are no studies that have verified the effect of *C. coriaceum* extracts on *Leishmania* protozoa. In this sense, the objective of the present study was to investigate the effect of *C. coriaceum* extracts on *Leishmania amazonensis*. For this, different extracts were used, according to the origin and solvent used, respectively: (1) fruit pulp, ethanol; (2) fruit peel, ethanol; (3) leaf, ethyl acetate; (4) leaf, methanol. In the phytochemical screening carried out in order to verify the presence of alkaloids, catechins, steroids, phenols, flavonoids, saponins and tannins, it was verified that extracts 1 and 3 presented all the metabolites investigated, while in extract 2, catechins were not verified, and in extract 3, catechins and steroids were absent. When quantifying the phenols and flavonoids, it was observed that extract 2 was the most abundant in phenols, while extract 3 was highlighted by the greater amount of flavonoids. For the biological assays, promastigote forms of *L. amazonensis* were maintained in 199 medium and used in stationary phase. The antipromastigote assay was performed by counting in the Neubauer chamber where all extracts were found to have antileishmania effect after 24, 48 and 72 hours of treatment. This data was confirmed by scanning electron microscopy (SEM), in which morphological changes were observed in the parasites treated with the different extracts at the concentration of 50 µg/mL. For characterizing the mechanisms by which the extracts induced the death of the parasite, we verified by cationic probe (TMRE) that the treatments induced mitochondrial depolarization and, by H2DCFDA probe, that the extracts led to the production of reactive oxygen species (ROS) in promastigotes. Similarly, treatments were found to cause damage to the plasma membrane by propidium iodide (PI) labeling and annexin V staining revealed that the treatment induces exposure to phosphatidylserine, events suggestive of late apoptosis-like/ necrosis death. Then, we verified the anti-amastigote effect of the extracts in macrophages infected with *L. amazonensis*. First, we observed that the concentrations did not alter that of the peritoneal macrophages of BALB/c mice. Subsequently, macrophages were infected with the parasites (1:5), and the effect on the amastigote forms was analyzed. Our results showed that treatment with the extracts was able to reduce the number of infected macrophages, amastigotes by macrophage and also the amount of viable parasites recovered. Additionally, through the CBA (cytometric bead array) assay, we found that the extracts were not able to modulate investigated cytokine levels nor the synthesis of microbicidal mediators, nitric oxide (NO) and ROS, analyzed by Griess assay and labeling with H_2DCFDA fluorescent probe, respectively. Based on the data obtained, we can infer that

extracts from different parts of the *C. coriaceum* plant exert leishmanicidal effect, acting in promastigote forms through late apoptose-like/ necrosis mechanisms and in amastigote forms directly, that is, independent of the macrophages activation.

Keywords: Leishmaniasis. Apoptosis. Leishmanicidal.

LISTA DE ILUSTRAÇÕES

FIGURAS DA DISSERTAÇÃO

FIGURAS DO ARTIGO CIENTÍFICO

LISTA DE ABREVIATURAS E SIGLAS

ACL	American Cutaneous Leishmaniasis
AMB	Amphotericin B (anfotericina B)
B.O.D.	Bioquimic Oxygen Demand
BALB/c	Linhagem de Camundongos isogênicos
C57BL/6	Linhagem de Camundongos isogênicos
CaCl2	Cloreto de Cálcio
CBA	Cytometric Bead Array
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
CDC	Center Diseases Control
CO2	Dióxido de carbono
DMSO	Dimetilsulfóxido
DNDi	Drugs for Neglected Diseases iniciative
ERO	Espécies Reativas de Oxigênio
FBS	Fetal Bovine Serum
FITC	Isotiocianato de fluoresceína
H2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
H2O2	Peróxido de hidrogênio
H3PO4	Ácido fosfórico
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)
IF	Impact Factor
IL	Interleucina
IL-10	Interleucina-10
IL-13	Interleucina-13
IL-17	Interleucina-17
IL-1β	Interleucina-1 beta
IL-4	Interleucina-4
INF-γ	Interferon gama
IP	Iodeto de propídeo
LC	Leishmaniose Cutânea
LTA	Leishmaniose Tegumentar Americana
LV	Leishmaniose Visceral

MCP-1	Monocyte chemoattractant protein-1
MEV	Microscopia Eletrônica de Varredura
mL	Mililitro
mm	Milímetro
mM	Milimolar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	n-Acetilcisteína
NaCl	Cloreto de sódio
NaNO2	Nitrito de Sódio
nm	nanômetro
nM	nanomolar
NO	Oxido nítrico / Nitric oxide
ºC	Graus Celsius
PBS	Phosphate-Buffered Saline (tampão fosfato)
pg	Picograma
pH	Potencial hidrogeniônico
PI	Propidium iodide
PS	Phosphatidilseryne
ROS	Reactive oxygen species
SEM	Scaning Electron Microscopy
T CD4+	Linfócito T CD4+
TGF-β	Transforming Growth Fator beta
Th1	Linfócito T auxiliar tipo 1
Th17	Linfócito T auxiliar tipo 17
Th2	Linfócito T auxiliar tipo 2
TMRE	Tetramethylrhodamine ethyl ester
TNF-α	Tumor Necrosis Factor alfa
USA	United States of America
WHO	World Heatlh Organization
µg	Micrograma
µL	Microlitro
µM	Micromolar

SUMÁRIO

1. INTRODUÇÃO

1.1 Leishmaniose

Leishmaniose é um termo genérico que define um grupo de doenças infecciosas causadas por protozoários do gênero *Leishmania*, transmitidas aos humanos através da picada de fêmeas de insetos hematófagos (Diptera: Psychodidae: Phlebotomidae). As doenças são classificadas em dois grandes grupos de acordo com os órgãos acometidos: leishmaniose cutânea (LC), que afeta pele e/ou mucosas, e leishmaniose visceral (LV), caracterizada pelo acometimento de órgãos internos. A LC é a forma mais comum da doença enquanto a LV provoca os quadros mais severos e potencialmente fatais (WHO, 2016).

A leishmaniose apresenta distribuição mundial, com 12 milhões de casos registrados e números estimados de pessoas vivendo em regiões endêmicas chegando a 350 milhões. A situação da doença no Brasil é preocupante, uma vez que o país se encontra na lista dos 10 países que concentram 75% dos casos de LC, e dos seis países que acumulam 90% dos casos de LV (WHO, 2014) .

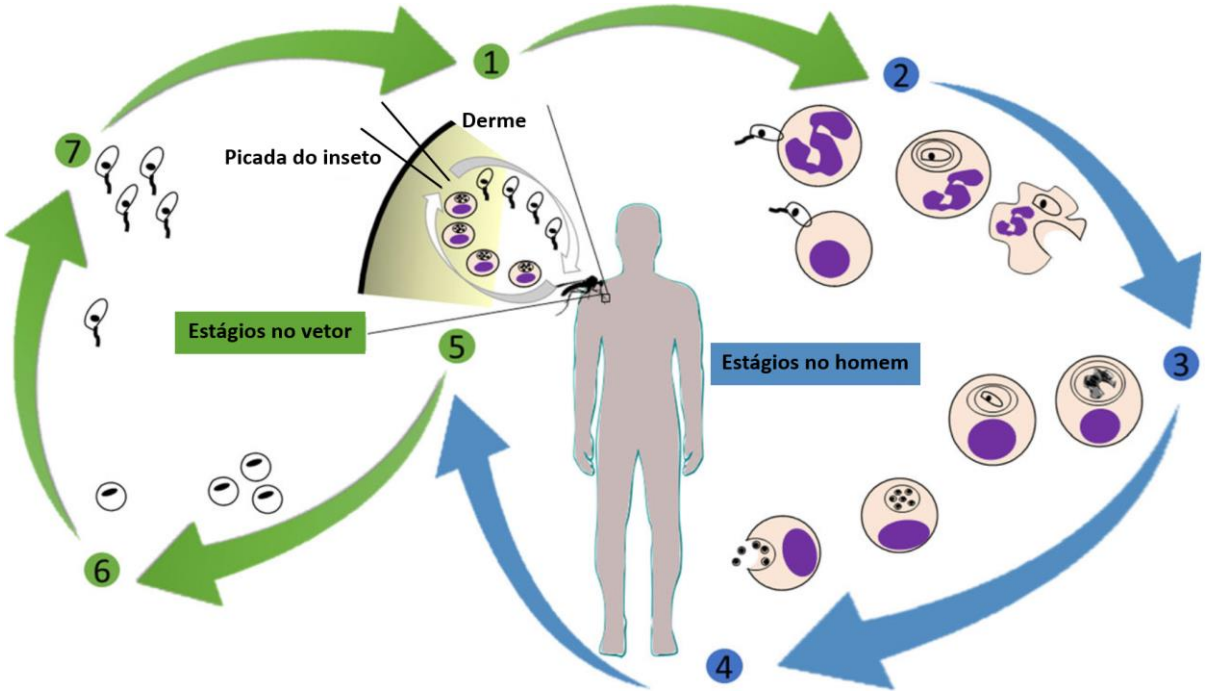
Nas Américas, a LC é denominada Leishmaniose Tegumentar Americana (LTA) agrupada em três principais formas clínicas: leishmaniose cutânea localizada, cutânea difusa e cutâneo-mucosa. A forma cutânea localizada caracteriza-se pela presença de lesões únicas ou múltiplas na derme com epiderme ulcerada. Já a doença cutânea difusa é crônica e progride durante toda a vida do paciente, com lesões não ulceradas e papulares por toda a pele. Por fim, a forma cutâneo-mucosa é caracterizada por lesões primárias localizadas com ulcerações na pele e lesões secundárias destrutivas envolvendo cartilagens e mucosas. Dentre as regiões acometidas estão nariz, faringe, boca e laringe, sendo comuns casos de perda da estrutura cartilginosa do nariz, podendo atingir lábios e disseminar-se pelo rosto (ALVAR; CROFT; OLLIARO, 2006; GONTIJO, 2003)

As formas da doença são determinadas por ambos: espécie do parasito e resposta imune desencadeada pelo hospedeiro. Atualmente são conhecidas várias espécies de *Leishmania* que causam a LC, sendo as que ocorrem no Brasil: *Leishmania (Viannia) braziliensis; Leishmania (Viannia) guyanensis; Leishmania (Viannia) lainsoni; Leishmania (Viannia) shawi; Leishmania (Viannia) naiffi,*

Leishmania (Viannia) lindenbergi e Leishmania (Leishmania) amazonensis (BRITO et al., 2012).

O gênero *Leishmania* foi descrito por Ross em 1903 e engloba parasitos pertencentes à ordem Kinetoplastidae e família Trypanosomatidae. São espécies de protozoários unicelulares que possuem duas principais formas evolutivas durante seu ciclo biológico: a forma promastigota é flagelada livre e encontrada colonizando o trato digestivo do inseto vetor, enquanto a forma amastigota, dotada de um flagelo rudmentar indistiguível por microscopia óptica, é encontrada no interior das células fagocíticas de hospedeiros mamíferos (GONTIJO et al., 1995)

Figura 1 - Ciclo biológico de *Leishmania* spp.



Fonte: Adaptado de BORGHI et al. (2017). (1) Durante o repasto sanguíneo fêmeas hematófagas regurgitam formas promastigotas infecciosas (metacíclicas) na pele de hospedeiros vertebrados; (2) os parasitos são fagocitados no tecido dérmico; (3) além dos parasitos fagocitados, macrófagos também fagocitam os neutrófilos apoptóticos infectados; (4) no interior dos macrófagos, as formas promastigotas se diferenciam em amastigotas aflageladas, onde se multiplicam, podendo ser liberadas após a lise celular; (5) o ciclo é completado quando outro inseto ingere fagócitos infectados durante o repasto sanguíneo; (6) no intestino do vetor, formas amastigotas se diferenciam em promastigotas; (7) os parasitos se proliferam e diferenciam-se em promastigotas metacíclicos, perpetuando seu ciclo de vida.

O ciclo biológico do parasito inicia-se com o repasto sanguíneo de fêmeas do inseto vetor, as quais inoculam formas promastigotas infectantes na derme de um

hospedeiro vertebrado. As formas flageladas são, então, fagocitadas por células do sistema mononuclear fagocitário, diferenciando-se em formas amastigotas, que por sua vez, se multiplicam por divisão binária. Após a multiplicação, as células hospedeiras, principalmente macrófagos, são rompidas liberando as formas amastigotas, as quais serão novamente fagocitadas por outras células ou poderão contaminar um novo vetor, no momento do repasto sanguíneo. No aparelho digestório do inseto, as formas amastigotas diferenciam-se em promastigotas, sofrem metaciclogênese, tornam-se formas promastigotas metaciclícas e migram para a porção anterior do intestino dos vetores, podendo então reiniciar o ciclo no próximo repasto sanguíneo (BORGHI et al., 2017; CUVILLIER et al., 2003; HANDLER et al., 2015) **(Figura 1)**.

1.2 Resposta imunológica na infecção por *Leishmania* spp.

Como supracitado, os principais fatores determinantes na evolução da LC são os fatores de virulência do parasito e a resposta imune do hospedeiro. Apesar de se tratar de um parasito unicelular, as diferentes espécies de *Leishmania* possuem sofisticados mecanismos de desvio da resposta imune do hospedeiro (GUPTA; OGHUMU; SATOSKAR, 2013; OLIVIER; GREGORY, 2005), a qual, para ser bem sucedida na eliminação do protozoário também necessita de estratégias refinadas que até hoje não são totalmente compreendidas para as diferentes espécies.

O modelo mais utilizado para validar a relação entre resposta imune e padrões de resistência ou susceptibilidade tem sido através de infecções experimentais com *Leishmania* spp. em diferentes linhagens de camundongos, através das quais verificou-se que algumas linhagens são geneticamente susceptíveis à infecção por *L. major*, com desenvolvimento de lesões cutâneas e ativação preferencial da subpopulação de linfócitos T $CD4^+$ do padrão Th2, com produção sustentada de interleucina (IL)-4 e IL-13. Já o padrão de resistência apresentada por outras linhagens de camundongos é estabelecido pela sobreposição da subpopulação de linfócitos Th1 ativados, que por sua vez secretam principalmente INF-γ e TNF-α que em conjunto levam à ativação dos macrófagos, síntese de IL-1β e com consequente aumento na produção de óxido nítrico (NO) e espécies reativas de oxigênio (ERO), seguido de sucesso na eliminação do parasito (SACKS; NOBEN-TRAUTH, 2002).

No entanto, a resposta desenvolvida pela infecção por *L. amazonensis* não segue um padrão tão rígido, diferindo em muitos aspectos da resposta contra *L. major*. Sabe-se que todas as linhagens de camundongos isogênicos (incluindo, portanto, BALB/c e C57BL/6) desenvolvem lesões progressivas com vacúolos parasitóforos repletos de parasitos em infecção por *L. amazonensis* (SOONG et al., 1997). Esta susceptibilidade generalizada não parece estar diretamente relacionada à resposta do tipo Th2 mas sim à ativação de células T CD4+ que produzem níveis relativamente baixos de diversas citocinas de ambos os padrões (SOONG et al., 1997; XIN; LI; SOONG, 2007).

Interessantemente, essa resposta imune não polarizada para os perfis Th1/Th2 observada em camundongos infectados com *L. (L.) amazonensis,* é semelhante à resposta observada em infecções humanas, validando a relevância biológica deste modelo de infecção experimental para o estudo de doenças humanas (OSORIO Y FORTÉA et al., 2007; PEREIRA; ALVES, 2008; SILVEIRA et al., 2009)

1.3 Morte celular em *Leishmania* spp.

A morte celular é uma parte essencial da biologia das células, exercendo funções cruciais tanto em situações fisiológicas quanto patológicas de organismos multicelulares. No entanto, também é conhecida a ocorrência de diferentes tipos de mortes em organismos unicelulares, como *Leishmania* spp. (LEE et al., 2002).

O termo apoptose descreve um processo de morte celular, cujas alterações incluem o arredondamento célula, redução do volume celular (picnose), condensação da cromatina, formação de bolhas na membrana plasmática (*blebbing*) e fagocitose por macrófagos residentes sem desencadeamento de resposta inflamatória. Pouca ou nenhuma modificação ultraestrutural das organelas citoplasmáticas é observada na apoptose (JIMÉNEZ-RUIZ et al., 2010).

Do ponto de vista molecular, exposição de fosfatidilserina, fragmentação oligonucleossomal de DNA, ativação de caspases e perda do potencial de membrana mitocondrial com manutenção da integridade da membrana plasmática são caraterísticas de células em processo de apoptose (JIMÉNEZ-RUIZ et al., 2010).

Em tripanossomatídeos foi decrito um processo que compartilha aspectos morfológicos e moleculares da apoptose de metazoários **(Figura 2)**. No entanto,

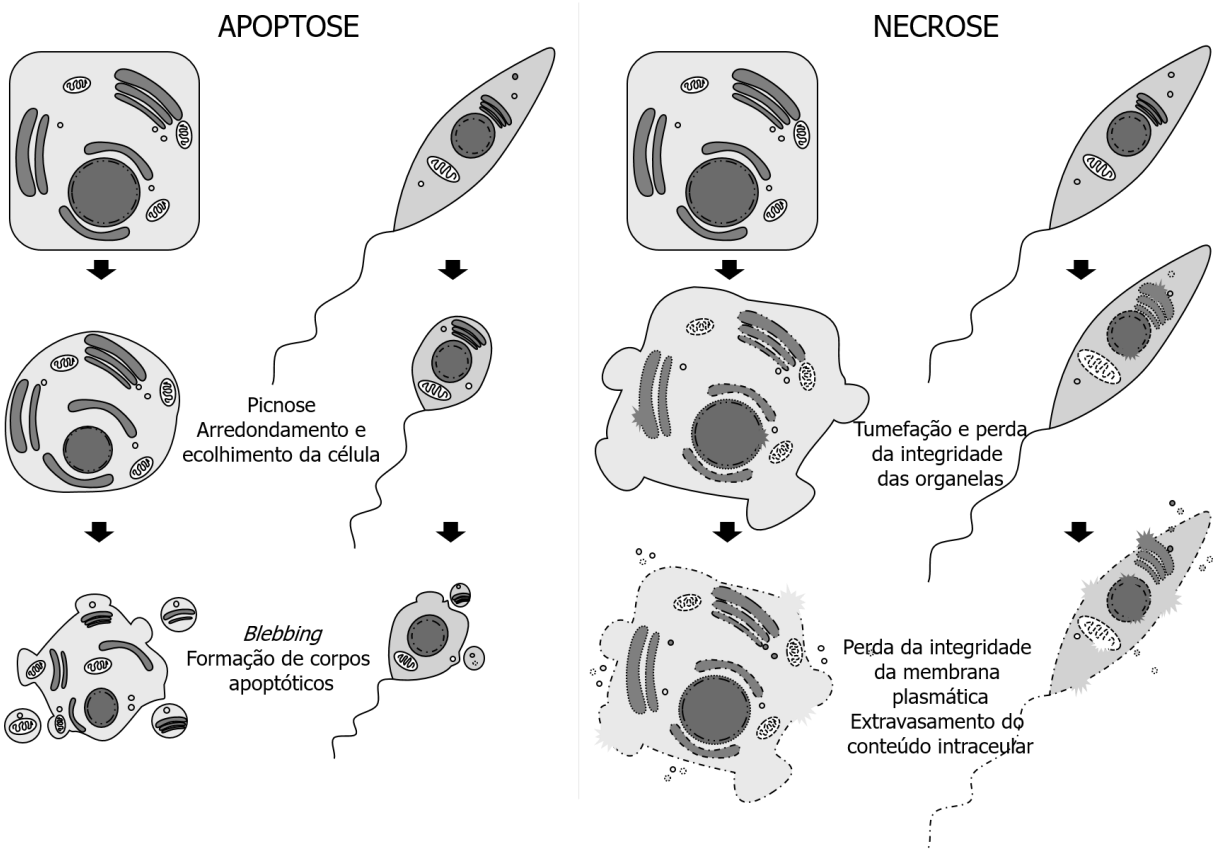
estes protozoários não possuem muitas das moléculas aceitas hoje como mediadores canônicos da apoptose (por exemplo membros da família Bcl-2, caspases, família de receptores relacionados ao TNF), por isso o processo de morte celular programada nestes organismos é chamado de apoptose-*like* (KACZANOWSKI; SAJID; REECE, 2011; PROTO; COOMBS; MOTTRAM, 2013; SMIRLIS et al., 2010; SMIRLIS; SOTERIADOU, 2011).

Apesar de ter sido descrita a função de mecanismos relacionados à apoptose em *Leishmania* na entrada "silenciosa" em células fagocíticas (fagocitose sem disparo do *burst* oxidativo), estabelecimento da infecção e progressão da doença (WANDERLEY et al., 2009; WANDERLEY; BARCINSKI, 2010), vários trabalhos demonstraram que eventos apoptose-*like* estão envolvidos com a ação letal de diferentes substâncias sobre *Leishmania* spp. (AWASTHI et al., 2016; CARDOSO et al., 2016; DOROODGAR et al., 2016; FOROUTAN-RAD et al., 2016; GARCIA et al., 2013, 2017; KHAN et al., 2017; MARINHO et al., 2014; SAHA et al., 2016; VERMA; SINGH; DEY, 2007)

A necrose, por outro lado, diz respeito a um processo de morte que envolve a liberação do conteúdo intracelular com consequente danos às células adjacentes e desencadeamento de resposta inflamatória (FESTJENS; VANDEN BERGHE; VANDENABEELE, 2006). Morfologicamente é caracterizada por aumento do volume celular e nuclear, desorganização do citoplasma, inchaço das organelas, perda da integridade da membrana plasmática e, por fim, perda do conteúdo intracelular (JIMÉNEZ-RUIZ et al., 2010).

Na ausência de consenso sobre marcadores bioquímicos, a permeabilização precoce da membrana plasmática é considerada a principal característica da necrose tanto em organismos multicelulares quanto em seres unicelulares **(Figura 2)**. Além disso, tanto em metazoários quanto em tripanossomatídeos, a morte celular necrótica é amplamente identificada pela ausência de marcadores apoptóticos. (CORRAL et al., 2016; JIMÉNEZ-RUIZ et al., 2010; ZANGGER; MOTTRAM; FASEL, 2002). Alguns estudos enfatizam a indução deste tipo de morte em parasitos tratados com substâncias como a alicina, encontrada no alho, e nanopartículas de prata biosintetizadas com extratos de *Euphorbia prostrata*, planta nativa do Caribe (CORRAL et al., 2016; ZAHIR et al., 2015).

Figura 2 – Alterações morfológicas presentes na apoptose e necrose em células de metazoários e tripanossomatídeos.



Fonte: o próprio autor.

1.4 Tratamento da LC

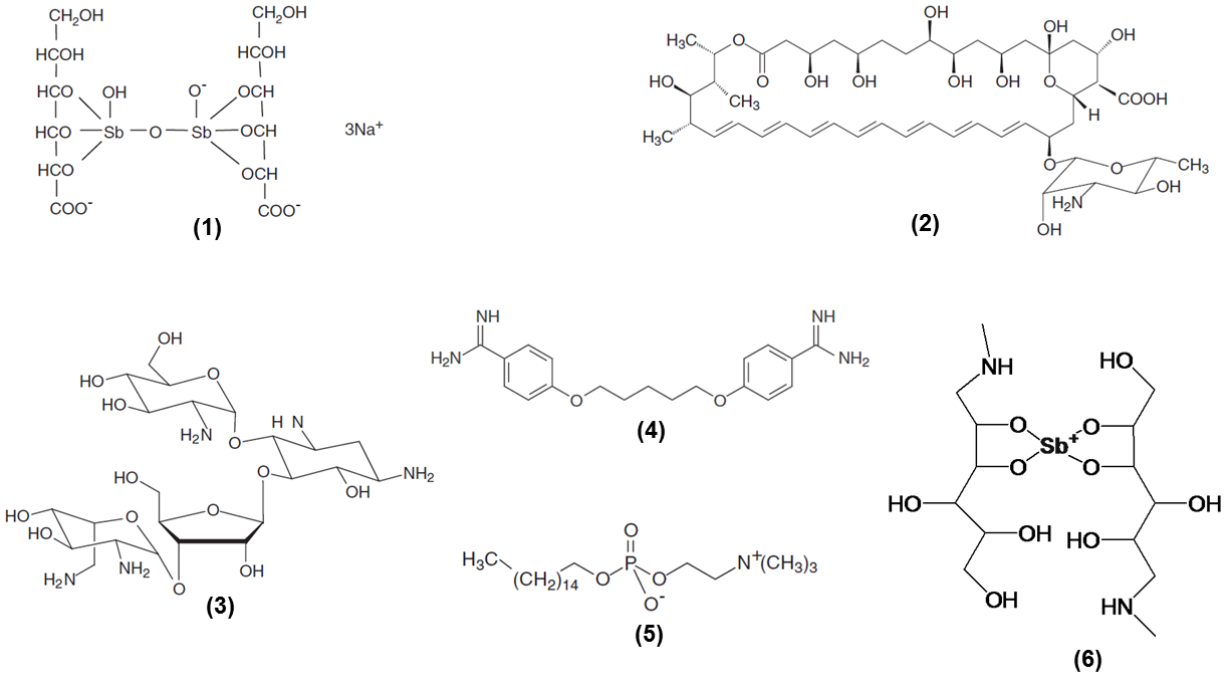
O tratamento da leishmaniose se baseia na eliminação das formas amastigotas do parasito. Contudo, a localização intramacrofágica destas formas dificulta uma atuação eficaz dos atuais medicamentos, sendo crucial para a susceptibilidade quimioterápica dos parasitos, que é também influenciada pela presença de transportadores mediadores do influxo e do efluxo de fármacos para as células (RODRIGUES et al., 2006).

Os fármacos de primeira escolha utilizados no tratamento da LC são os antimoniais pentavalentes, como o antimoniato de N-metil glucamina (Glucantime®) e o estibogluconato de sódio (Pentostam®). Nos casos de tratamento insatifatório com os antimoniais, o tramento de segunda escolha é efetuado com a administração de anfoterina B. Além destes, pentamidina, miltefosina e paramomicina também tem

sido empregadas como tratamento alternativo (ALVAR; CROFT; OLLIARO, 2006) **(Figura 3)**.

No entanto, um tratamento eficaz continua a ser o desafio mais importante na leishmaniose, pois os poucos medicamentos disponíveis apresentam elevada toxicidade e alto custo, além disso, a terapia requer ambiente ambulatorial, portanto pacientes que vivem em áreas rurais remotas e têm acesso limitado aos centros de tratamento; os tratamentos são de longa duração e por isso os pacientes muitas vezes não conseguem completar um ciclo completo, favorecendo o desenvolvimento de parasitos resistentes aos medicamentos (ALVAR; CROFT; OLLIARO, 2006).

Figura 3 – Estrutura química de fármacos utilizados no tratamento da leishmaniose: (1) pentostam, (2) anfotericina B, (3) paramomicina, (4) pentamidina, (5) miltefosina e (6) glucantime.



Fonte: adaptado de Alvar et al. (2006).

Além disso, outras variáveis que influenciam o insucesso do tratamento da leishmaniose é a diversidade de espécies de *Leishmania*, bem como as variações de fatores de virulência e patogenicidade, o que configura quadros clínicos variados, com respostas terapêuticas diversas. Além disso, a desnutrição e as co-infecções aumentam as taxas de mortalidade em consequência da doença, em especial em

indivíduos imunocomprometidos, os quais tornam-se incapazes de eliminar os parasitos por mecanismos naturais de defesa (ALVAR; CROFT; OLLIARO, 2006; LAULETTA LINDOSO et al., 2016; VAN GRIENSVEN et al., 2014).

1.5 Compostos naturais como alternativa no tratamento da Leishmaniose Cutânea

O desenvolvimento de fármacos para tratamentos rápidos, seguros, eficazes e acessíveis para o tratamento da leishmaniose tem sido um desafio das entidades de pesquisa com impacto mundial (DNDi, 2015). O reino vegetal fornece compostos que vem sendo utilizados direta ou indiretamente na terapia de doenças desde a origem da humanidade. Porém, logo após a segunda guerra mundial, houve disseminação de fármacos sintéticos, avanço dos antimicrobianos e da vacinação em massa, ocasionando o desuso da terapêutica natural. Contudo, devido à ineficácia, alto custo e toxicidade de alguns fármacos, atualmente há um interesse crescente na busca por produtos naturais que possuam atividade sobre patógenos humanos de forma geral, em especial, *Leishmania* spp. (SEN; CHATTERJEE, 2011).

Existem aproximadamente 250.000 espécies de plantas em todo o mundo, das quais apenas uma fração foi estudada até agora e mostraram potentes propriedades quimioterapêuticas (SEN; CHATTERJEE, 2011). Neste contexto, pode-se afirmar que o Brasil é um país privilegiado, pois abriga uma flora amplamente diversificada abrangida por diferentes biomas, tais quais Floresta Amazônica, Mata Atlântica, Pantanal, Caatinga e Cerrado.

Na tentativa desenvolver um tratamento para leishmaniose, alguns extratos naturais já foram testados, como por exemplo aqueles provenitentes de *Serjania lethalis, Myrciaria dubia, Satureja hortensis, Artemisia dracundus, Coccina grandis, Scrophularia striata*, entre outros (ALVES PASSOS et al., 2017; CORREIA et al., 2016; DOS SANTOS THOMAZELLI et al., 2016; MIRZAEI et al., 2016; PRAMANIK et al., 2016; SILVA et al., 2013; ZAHIRI et al., 2016), além disso, compostos ativos das plantas foram isolados, identificados e testatos sobre *Leishmania* spp., como Hispidulina, Condrocurina, Amentoflavona, Annonacina, Isoanonacina, Rolliniastatina, Ácido caurenóico e Curcumina (FOULADVAND; BARAZESH; TAHMASEBI, 2013; MIRANDA et al., 2015; ULLAH et al., 2016). No entanto, ainda

há muito a ser explorado e o aprimoramento dos estudos é necessário para que um fármaco efetivo e com baixa toxicidade seja desenvolvido.

1.6 Plantas do gênero *Caryocar*

O pequizeiro é uma espécie arbórea nativa do Cerrado brasileiro, pertencente à família Caryocaraceae. Diversas espécies do gênero *Caryocar* são popularmente conhecidas por pequi, piqui, piqui-do-cerrado, amêndoa-de-espinho, grão-de-cavalo e pequiá. A espécie de maior incidência no Brasil é *Caryocar brasiliense* Camb, porém *C. villosum, C. coriaceum, C. barbinerve, C. crenatum e C. edulis* também são encontrados neste bioma brasileiro. As plantas tem ocorrência em todo o Cerrado, com distribuição nos estados da Bahia, Ceará, Maranhão, Mato Grosso, Mato Grosso do Sul, Minas Gerais, Pará, Piauí, São Paulo e Tocantins, dada ampla distribuição tem sido considerada, como a árvore símbolo do Cerrado (ALMEIDA, S.P. DE; SILVA, 1994; MACEDO J.F., 2005; RATTER; RIBEIRO; BRIDGEWATER, 1997).

O gênero *Caryocar* engloba espécies nativas do cerrado de enorme interesse econômico, principalmente devido ao uso do seu fruto na culinária, onde os frutos são consumidos tanto *in natura*, quanto no preparo de sucos, sorvetes, licores, geléias e em pratos típicos desta região brasileira. Além disso, os óleos extraídos da planta são utilizados na fabricação de cosméticos e a casca e as folhas são usadas como tinturas (ROESLER et al., 2007).

Popularmente, folhas e frutos do pequi são utilizados para tratamento de afecções respiratórias, oftalmológicas e hepáticas, e as sementes são usadas como afrodisíacas. O óleo da polpa de pequi é amplamente usado como agente tônico contra asma, gripe, resfriado e doenças broncopulmonares (ALMEIDA, S.P. DE; SILVA, 1994; ROESLER et al., 2007).

Estas espécies tem ganhado a atenção de pesquisadores devido às propriedades anti-inflamatória (TORRES et al., 2016), antifúngica (BREDA et al., 2016), antibacteriana (PAULA-JU et al., 2006), antinociceptiva (de OLIVEIRA et al., 2015), antineoplásica (COLOMBO et al., 2015; SUFFREDINI et al., 2007), imunomoduladora (GUSMAN et al., 2015), cicatrizante (de OLIVEIRA et al., 2010) e nematicida (NOGUEIRA et al., 2012). Além disso, Paula-Ju e colaboradores (2006) verificaram potente ação leishmanicida de extratos hidroetanólicos provenientes de

folhas de *C. brasiliense* sobre formas promastigotas de *L. amazonensis*. Entretanto, não há estudos que verifiquem o potencial de extratos da espécie *C. coriaceum* sobre *Leishmania* spp.

As plantas utilizadas no presente trabalho foram obtidas no Campus da Universidade Estadual do Ceará (UECE) lat: -3.792222 long: -38.556111, na cidade de Fortaleza (Brasil) **(Figura 4)**. Folhas de *C. coriaceum* foram apresentados e identificados no Herbário Prisco Bezerra sob o código EAC57060.

Figura 4 – Folhas de *C. coriaceum* idenficados no Herbário Prisco Bezerra.

Fonte: Herbário Prisco Bezerra.

2. OBJETIVOS

2.1 Objetivos gerais

Avaliar *in vitro* o efeito do tratamento com extratos obtidos de diferentes partes da planta *Caryocar coriaceum* sobre formas promastigotas e amastigotas de *L. amazonensis*.

2.2 Objetivos específicos

- Verificar as principais características fitoquímicas dos diferentes extratos, bem como quantificar fenóis e flavonóides nos mesmos;
- Avaliar a ação direta dos extratos sobre as formas promastigotas;
- Compreender o mecanismo de morte induzido pelos extratos sobre formas promastigotas;
- Avaliar a citotoxidade dos extratos sobre macrófagos murinos;
- Avaliar a ação antiamastigota dos extratos em macrófagos infectados;*;*
- Avaliar a atividade imunomoduladora dos diferentes extratos em macrófagos infectados ou não por *L. amazonensis*;
- Quantificar óxido nítrico (NO) e espécies reativas de oxigênio (ERO) sintetizadas por macrófagos*.*

3. REFERÊNCIAS

ALMEIDA, S.P. DE; SILVA, J. A. DA. Piqui e buriti: importancia alimentar para a populacao dos cerrados. **EMBRAPA Cerrados**, p. 38, 1994.

ALVAR, J.; CROFT, S.; OLLIARO, P. Chemotherapy in the Treatment and Control of Leishmaniasis. **Advances in parasitology**, v. 61, p. 223–274, 2006.

ALVES PASSOS, C. L. et al. Anti-Leishmania amazonensis activity of Serjania lethalis A. St.-Hil. **Parasitology International**, v. 66, n. 1, p. 940–947, fev. 2017.

AWASTHI, B. P. et al. Plumbagin, a plant-derived naphthoquinone metabolite induces mitochondria mediated apoptosis-like cell death in Leishmania donovani: an ultrastructural and physiological study. **Apoptosis**, v. 21, n. 8, p. 941–953, 17 ago. 2016.

BORGHI, S. M. et al. Leishmania infection: painful or painless? **Parasitology Research**, v. 116, n. 2, p. 465–475, 9 fev. 2017.

BREDA, C. A. et al. Phytochemical Analysis and Antifungal Activity of Extracts from Leaves and Fruit Residues of Brazilian Savanna Plants Aiming Its Use as Safe Fungicides. **Natural Products and Bioprospecting**, v. 6, n. 4, p. 195–204, 11 ago. 2016.

BRITO, M. E. F. DE et al. Cutaneous leishmaniasis in northeastern Brazil: a critical appraisal of studies conducted in State of Pernambuco. **Revista da Sociedade Brasileira de Medicina Tropical**, v. 45, n. 4, p. 425–429, 2012.

CARDOSO, B. et al. Antileishmanial Activity of a Calophyllum brasiliense Leaf Extract. **Planta Medica**, v. 83, n. 01/02, p. 57–62, 25 maio 2016.

COLOMBO, N. B. R. et al. Caryocar brasiliense camb protects against genomic and oxidative damage in urethane-induced lung carcinogenesis. **Brazilian Journal of Medical and Biological Research**, v. 48, n. 9, p. 852–862, set. 2015.

CORRAL, M. J. et al. Allicin Induces Calcium and Mitochondrial Dysregulation Causing Necrotic Death in Leishmania. **PLoS Neglected Tropical Diseases**, v. 10, n. 3, p. 1–22, 2016.

CORREIA, V. C. DE S. et al. Evaluation of the antiplasmodial and leishmanicidal potential of Myrciaria dubia (Myrtaceae) extract. **Revista da Sociedade Brasileira de Medicina Tropical**, v. 49, n. 5, p. 586–592, out. 2016.

CUVILLIER, A. et al. Abortive infection of Lutzomyia longipalpis insect vectors by aflagellated LdARL-3A-Q70L overexpressing Leishmania amazonensis parasites. **Cellular Microbiology**, v. 5, n. 10, p. 717–728, out. 2003.

DE OLIVEIRA, F. F. B. et al. Antinociceptive and anti-inflammatory effects of Caryocar coriaceum Wittm fruit pulp fixed ethyl acetate extract on zymosan-induced arthritis in rats. **Journal of Ethnopharmacology**, v. 174, p. 452–463, nov. 2015.

DE OLIVEIRA, M. L. M. et al. In vivo topical anti-inflammatory and wound healing activities of the fixed oil of Caryocar coriaceum Wittm. seeds. **Journal of Ethnopharmacology**, v. 129, n. 2, p. 214–219, 2010.

DNDI. DRUGS FOR NEGLECTED DISEASES INICIATIVE. **Leishmaniasis Fact Sheet**. Disponível em: <http://www.dndi.org/wp-content/uploads/2016/10/Factsheet_2015_Leish.pdf>. Acesso em: 28 dez. 2016.

DOROODGAR, M. et al. Tamoxifen Induces Apoptosis of Leishmania major Promastigotes in Vitro. **The Korean journal of parasitology**, v. 54, n. 1, p. 9–14, fev. 2016.

DOS SANTOS THOMAZELLI, A. P. F. et al. Brazilian propolis promotes immunomodulation on human cells from American Tegumentar Leishmaniasis patients and healthy donors infected with L. braziliensis. **Cellular Immunology**, v. 311, p. 22–27, 2016.

FESTJENS, N.; VANDEN BERGHE, T.; VANDENABEELE, P. Necrosis, a well-orchestrated form of cell demise: Signalling cascades, important mediators and concomitant immune response. **Biochimica et Biophysica Acta (BBA) - Bioenergetics**, v. 1757, n. 9–10, p. 1371–1387, set. 2006.

FOROUTAN-RAD, M. et al. Holothuria leucospilota Extract Induces Apoptosis in Leishmania major Promastigotes. **Iranian journal of parasitology**, v. 11, n. 3, p. 339–349, 2016.

FOULADVAND, M.; BARAZESH, A.; TAHMASEBI, R. Evaluation of in vitro antileishmanial activity of curcumin and its derivatives “ Gallium curcumin , Indium curcumin and Diacethyle curcumin ”. p. 3306–3308, 2013.

GARCIA, F. P. et al. Eupomatenoid-5 isolated from leaves of piper regnellii induces apoptosis in leishmania amazonensis. **Evidence-based Complementary and Alternative Medicine**, v. 2013, 2013.

GARCIA, F. P. et al. A3K2A3-induced apoptotic cell death of Leishmania amazonensis occurs through caspase- and ATP-dependent mitochondrial dysfunction. **Apoptosis**, v. 22, n. 1, p. 57–71, 19 jan. 2017.

GONTIJO, B. Leishmaniose tegumentar americana American cutaneous leishmaniasis. **Medicina Tropical**, v. 36, n. 13, p. 71–80, 2003.

GONTIJO, C. M. F. et al. The development of species of Leishmania Ross, 1903 in Lutzomyia longipalpis (Lutz & Neiva, 1912). **Memórias do Instituto Oswaldo Cruz**, v. 90, n. 3, p. 367–373, jun. 1995.

GUPTA, G.; OGHUMU, S.; SATOSKAR, A. R. Mechanisms of immune evasion in leishmaniasis. **Advances in applied microbiology**, v. 82, p. 155–84, jan. 2013.

GUSMAN, G. S. et al. Evaluation of the Effects of Some Brazilian Medicinal Plants on the Production of TNF- α and CCL2 by THP-1 Cells. **Evidence-based complementary and alternative medicine : eCAM**, v. 2015, p. 497123, 2015.

HANDLER, M. Z. et al. Cutaneous and mucocutaneous leishmaniasis. **Journal of the American Academy of Dermatology**, v. 73, n. 6, p. 897–908, dez. 2015.

JIMÉNEZ-RUIZ, A. et al. Apoptotic markers in protozoan parasites. **Parasites & Vectors**, v. 3, n. 1, p. 104, 9 nov. 2010.

KACZANOWSKI, S.; SAJID, M.; REECE, S. E. Evolution of apoptosis-like programmed cell death in unicellular protozoan parasites. **Parasites & vectors**, v. 4, p. 44, 25 mar. 2011.

KHAN, H. et al. *In-vitro* antileishmanial potential of peptide drug hirudin. **Chemical Biology & Drug Design**, v. 89, n. 1, p. 67–73, jan. 2017.

LAULETTA LINDOSO, J. A. et al. Leishmaniasis–HIV coinfection: current challenges. **HIV/AIDS - Research and Palliative Care**, v. Volume 8, p. 147–156, out. 2016.

LEE, N. et al. Programmed cell death in the unicellular protozoan parasite Leishmania. **Cell Death and Differentiation**, v. 9, n. 1, p. 53–64, 21 jan. 2002.

MACEDO J.F. **Pequi: do plantio à mesa**. Belo Horizonte: EPAMIG. Boletim Técnico,76, 2005.

MARINHO, F. A. et al. The Calpain Inhibitor MDL28170 Induces the Expression of Apoptotic Markers in Leishmania amazonensis Promastigotes. **PLoS ONE**, v. 9, n. 1, p. e87659, 31 jan. 2014.

MIRANDA, M. M. et al. Kaurenoic Acid Possesses Leishmanicidal Activity by Triggering a NLRP12/IL-1β/cNOS/NO Pathway. **Mediators of inflammation**, v. 2015, p. 392918, 2015.

MIRZAEI, F. et al. In vitro anti-leishmanial activity of Satureja hortensis and Artemisia dracunculus extracts on Leishmania major promastigotes. **Journal of Parasitic Diseases**, v. 40, n. 4, p. 1571–1574, 14 dez. 2016.

NOGUEIRA, F. A. et al. In vitro and in vivo efficacy of aqueous extract of Caryocar brasiliense Camb. to control gastrointestinal nematodes in sheep. **Parasitology Research**, v. 111, n. 1, p. 325–330, 11 jul. 2012.

OLIVIER, M.; GREGORY, D. J. Subversion Mechanisms by Which Leishmania Parasites Can Escape the Host Immune Response : a Signaling Point of View. **Clinical Microbiology Reviews**, v. 18, n. 2, p. 293–305, 2005.

OSORIO Y FORTÉA, J. et al. Unveiling pathways used by Leishmania amazonensis amastigotes to subvert macrophage function. **Immunological Reviews**, v. 219, n. 1, p. 66–74, out. 2007.

PAULA-JU, W. DE et al. Leishmanicidal, antibacterial, and antioxidant activities of Caryocar brasiliense Cambess leaves hydroethanolic extract. **Revista Brasileira de Farmacognosia**, v. 16, p. 625–630, dez. 2006.

PEREIRA, B. A. S.; ALVES, C. R. Immunological characteristics of experimental murine infection with Leishmania (Leishmania) amazonensis. **Veterinary**

Parasitology, v. 158, n. 4, p. 239–255, 20 dez. 2008.

PRAMANIK, A. et al. Coccinia grandis (L.) Voigt Leaf Extract Exhibits Antileishmanial Effect Through Pro-inflammatory Response: An In Vitro Study. **Current Microbiology**, p. 1–9, 31 out. 2016.

PROTO, W. R.; COOMBS, G. H.; MOTTRAM, J. C. Cell death in parasitic protozoa: regulated or incidental? **Nature Reviews Microbiology**, v. 11, n. 1, p. 58–66, 3 dez. 2013.

RATTER, J. A.; RIBEIRO, J. F.; BRIDGEWATER, S. The Brazilian Cerrado Vegetation and Threats to its Biodiversity. **Annals of Botany**, v. 80, p. 223–230, 1997.

RODRIGUES, A. M. et al. Factors associated with treatmet failure of cutaneous leishmaniasis with meglumine antimoniate. **Revista da Sociedade Brasileira de Medicina Tropical**, v. 39, n. 2, p. 139–145, abr. 2006.

ROESLER, R. et al. Atividade antioxidante de frutas do cerrado. **Ciência e Tecnologia de Alimentos**, v. 27, n. 1, p. 53–60, mar. 2007.

SACKS, D.; NOBEN-TRAUTH, N. The immunology of susceptibility and resistance to Leishmania major in mice. **Nature reviews. Immunology**, v. 2, n. 11, p. 845–58, nov. 2002.

SAHA, S. et al. A novel spirooxindole derivative inhibits the growth of leishmania donovani parasites both in vitro and in vivo by targeting type IB topoisomerase. **Antimicrobial Agents and Chemotherapy**, v. 60, n. 10, p. 6281–6293, 2016.

SEN, R.; CHATTERJEE, M. Plant derived therapeutics for the treatment of Leishmaniasis. **Phytomedicine**, v. 18, n. 12, p. 1056–1069, set. 2011.

SILVA, S. S. et al. Brazilian Propolis Antileishmanial and Immunomodulatory Effects. v. 2013, 2013.

SILVEIRA, F. T. et al. Immunopathogenic competences of *Leishmania* (*V.*) *braziliensis* and *L.* (*L.*) *amazonensis* in American cutaneous leishmaniasis. **Parasite Immunology**, v. 31, n. 8, p. 423–431, ago. 2009.

SMIRLIS, D. et al. Targeting essential pathways in trypanosomatids gives insights into protozoan mechanisms of cell death. **Parasites & vectors**, v. 3, n. 1, p. 107, 2010.

SMIRLIS, D.; SOTERIADOU, K. Trypanosomatid apoptosis: “Apoptosis” without the canonical regulators. **Virulence**, v. 2, n. 3, p. 253–256, 2011.

SOONG, L. et al. Role of CD4+ T cells in pathogenesis associated with Leishmania amazonensis infection. **Journal of immunology (Baltimore, Md. : 1950)**, v. 158, n. 11, p. 5374–83, 1 jun. 1997.

SUFFREDINI, I. B. et al. In vitro cytotoxic activity of Brazilian plant extracts against human lung, colon and CNS solid cancers and leukemia. **Fitoterapia**, v. 78, n. 3, p. 223–226, abr. 2007.

TORRES, L. R. DE O. et al. Pequi (Caryocar brasiliense Camb.) almond oil attenuates carbon tetrachloride-induced acute hepatic injury in rats: Antioxidant and anti-inflammatory effects. **Food and Chemical Toxicology**, v. 97, p. 205–216, 2016.

ULLAH, N. et al. Plants as Antileishmanial Agents: Current Scenario. **Phytotherapy Research**, v. 30, n. 12, p. 1905–1925, 2016.

VAN GRIENSVEN, J. et al. Leishmaniasis in immunosuppressed individuals. **Clinical Microbiology and Infection**, v. 20, n. 4, p. 286–299, abr. 2014.

VERMA, N. K.; SINGH, G.; DEY, C. S. Miltefosine induces apoptosis in arsenite-resistant Leishmania donovani promastigotes through mitochondrial dysfunction. **Experimental Parasitology**, v. 116, n. 1, p. 1–13, 2007.

WANDERLEY, J. L. M. et al. Cooperation between Apoptotic and Viable Metacyclics Enhances the Pathogenesis of Leishmaniasis. **PLoS ONE**, v. 4, n. 5, p. 1–11, 29 maio 2009.

WANDERLEY, J. L. M.; BARCINSKI, M. A. Apoptosis and apoptotic mimicry: the Leishmania connection. **Cellular and Molecular Life Sciences**, v. 67, n. 10, p. 1653–1659, 7 maio 2010.

WHO. **Small bites big threats**. 2014. Disponível em: <http://www.paho.org/world-health-day-2014/wp-content/uploads/2014/02/Leishmaniasis.pdf?ua=1>. Acesso em:

28 dez. 2016.

WHO. **Weekly epidemiological record Relevé épidémiologique hebdomadaire**. 2016. Disponível em: <http://www.who.int/wer/2016/wer9122.pdf>. Acesso em: 27 dez. 2016.

XIN, L.; LI, Y.; SOONG, L. Role of interleukin-1beta in activating the CD11c(high) CD45RB- dendritic cell subset and priming Leishmania amazonensis-specific CD4+ T cells in vitro and in vivo. **Infection and immunity**, v. 75, n. 10, p. 5018–26, out. 2007.

ZAHIR, A. A. et al. Green Synthesis of Silver and Titanium Dioxide Nanoparticles Using Euphorbia prostrata Extract Shows Shift from Apoptosis to G0/G1 Arrest followed by Necrotic Cell Death in Leishmania donovani. **Antimicrobial agents and chemotherapy**, v. 59, n. 8, p. 4782–99, ago. 2015.

ZAHIRI, M. et al. Therapeutic Effect of Scrophularia striata Ethanolic Extract against Localized Cutaneous Leishmaniasis Caused by Leishmania major (MRHO/IR/75/ER). **Iranian journal of public health**, v. 45, n. 10, p. 1340–1347, out. 2016.

ZANGGER, H.; MOTTRAM, J. C.; FASEL, N. Cell death in Leishmania induced by stress and differentiation: programmed cell death or necrosis? **Cell Death and Differentiation**, v. 9, n. 10, p. 1126–1139, 16 set. 2002.

ARTIGO CIENTÍFICO

Caryocar coriaceum leaf extracts present leishmanicidal activity by inducing death of promastigote forms by apoptosis-like process and amastigotes by iron depletion

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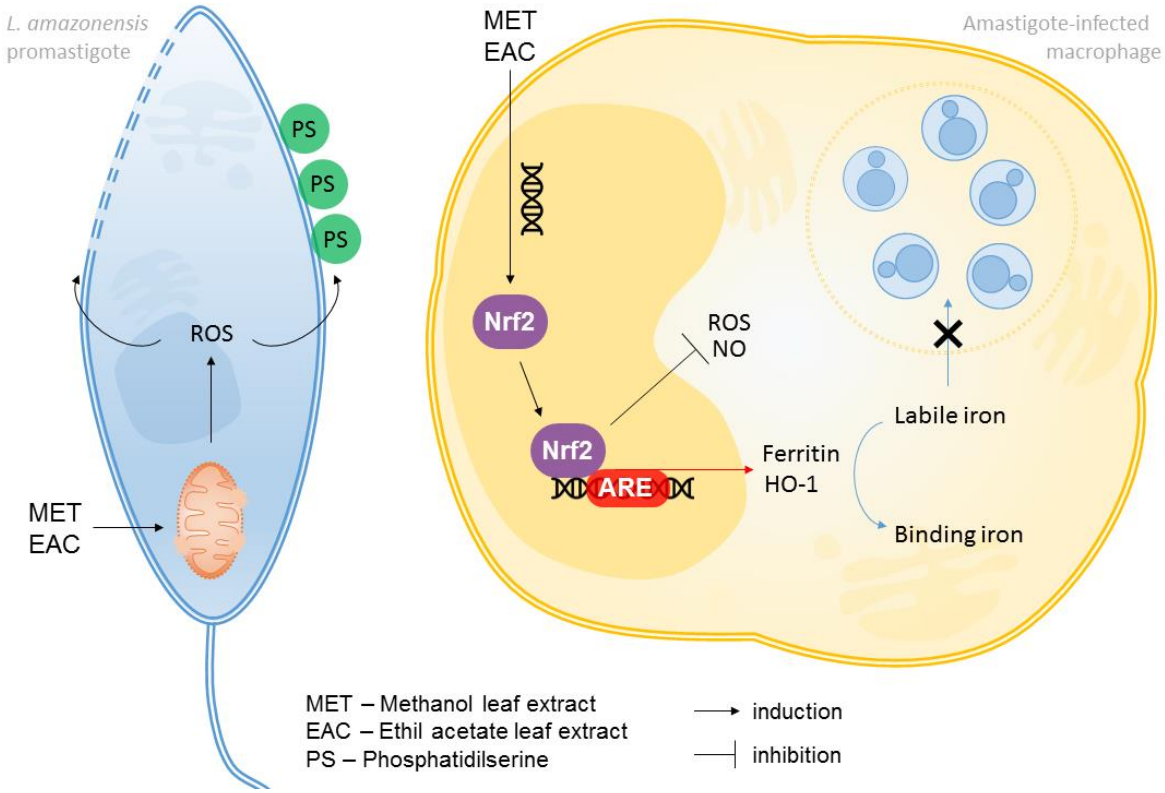
ABSTRACT

Ethnopharmacological relevance: Plants belonging to the *Caryocar* genus are found in Brazilian Cerrado, where fruits are used as food and in folk medicine to treat respiratory, ophthalmological, and hepatic diseases.

Aim of the study: To assess the *in vitro* effect of two different solvents extracts obtained from leaves of *Caryocar coriaceum* plant on *L. amazonensis* promastigote and amastigote forms. **Materials and methods:** Use of ethyl acetate (EAC) and methanol (MET) leaf extracts tested at 25; 50 and 100 µg/mL. Performance of antipromastigote assay using a Neubauer chamber's counting and scanning electron microscopy. Fluorescent techniques were applied to carry out the death mechanism by using a TMRE and H_2DCFDA probe, propidium iodide, and annexin V labeling. Subsequently, cytotoxicity was assessed through MTT assay. The anti-amastigote assays, in turn, were performed by infecting BALB/c mice macrophages with *L. amazonensis* promastigotes (1:5) followed by the verification of different parameters: infection index, cytokine production, lipoperoxidation, reactive oxygen species (ROS) and nitric oxide (NO) measurement, total iron and total iron bound capacity levels, Nrf2, heme oxygenase (HO-1), and ferritin expression. **Results:** *C. coriaceum* extracts showed antipromastigote effect after 24, 48 and 72 hours of treatment. The extracts also induced loss of mitochondrial membrane potential as well as reactive oxygen species production on promastigotes, damage on plasma membrane, and phosphatidylserine exposure on extracellular parasites. In addition, we verified that most parasites were going through a late apoptosis-like process. The range of concentrations used did not alter the viability of peritoneal macrophages of BALB/c mice; therefore, we observed that the treatment with extracts was able to reduce the infection on host cells. Thereafter, the CBA revealed that the extracts were able to significantly improve the levels of TNFα, IL-6, MCP-1, and IL-10, but they also reduced the levels of MDA and ROS without interfering on NO levels. In addition, both EAC and MET upregulated NRF-2/HO-1/Ferritin expression and modulated the labile iron pool in infected macrophages. **Conclusion:** Based on the data obtained, it is possible to infer that different solvent extracts of the *C. coriaceum* leaves exert leishmanicidal effect, acting on promastigote forms through apoptosis-like mechanisms and intracellular amastigote forms involving an antioxidant response, which culminates in a depletion of available iron for *L. amazonensis* replication.

Key words: *Leishmania amazonensis*, Nrf2, heme oxygenase-1, ferritin, reactive oxygen species, cytokines.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Leishmania (Leishmania) amazonensis is among the American Cutaneous Leishmaniasis-causing agents (ACL), which is a zoonosis whose pathogenesis depends both on the virulence factors of the parasite strain and the host immune response. The clinical manifestations may range from a single granulomatous skin lesion to diffuse lesions, including a possibility to affect the mucous membranes or even progress to visceral disease (Barral et al., 1991).

In order to control this disease many challenges appear such as the factor that *Leishmania* parasites have a sophisticated survival mechanism including the downregulation of microbicidal mechanisms of macrophages (Cecílio et al., 2014; Liu and Uzonna, 2012a; Olivier and Gregory, 2005). In addition, studies have demonstrated that *Leishmania* parasites are able to sequester hosts nutrients, as scavenge labile iron pool, and uses them for survival and replication inside macrophages (Das et al., 2009a; Zaidi et al., 2017).

The chemotherapy currently available to treat ACL is based on pentavalent antimonials such as sodium stibogluconate (Pentostam®) and antimoniate N-methyl-glucamine (Glucantime®). In the case of lack of response, second-line drugs such as amphotericin B or pentamidines are applied; however, the available drugs present toxicity, low efficiency and difficulty of administration, which leads researches to seek for alternative treatments (Alvar et al., 2006).

Therefore, the search for therapeutic alternatives, including the use of natural products, considering the discovery of new action mechanisms with less toxicity than conventional treatments has been the objective of many researchers.

Brazilian cerrado is a biome occupying vast areas of the country; its large diversity of plants has been of greatinterest regarding the varied biological properties with potential use in medicine involved (Bailão et al., 2015; Correia et al., 2016; Justino et al., 2016; KELLNER and KELLNER, 2016).

This type of vegetation shelters plants belonging to the *Caryocar* genus whose fruits are used as food and in folk medicine. Scientific studies have demonstrated their antioxidant and anti-inflammatory effects in the reduction of interleukin (IL)-6, leukotriene-4 and 5 as well as tumor necrosis factor (TNF) receptor in a rats-acute liver injury model (Torres et al., 2016) and inhibition of TNF-α production on *in vitro* cell line (Gusman et al., 2015). Wound healing and anti-inflammatory effect on mice skin lesions were also reported (de Oliveira et al., 2010). Its microbicidal action has been demonstrated as antifungal on *in vitro* activity against *Alternaria solani* and *Venturia pirina* (Breda et al., 2016), antibacterial on *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Paula-Ju et al., 2006). In addition, Paula-Ju et al., 2006b described antioxidant and antipromastigote of hydroethanolic extracts of leaves of *Caryocar brasiliense* effect on *L. amazonensis*. However, no studies have verified the potential of *Caryocar coriaceum* (pequi) extracts on *Leishmania* spp neither their mechanisms of action.

In this context, this study aimed at investigating the effect of extracts from ethyl acetate and methanol *C. coriaceum* leaves on promastigote forms as well as on intracellular amastigote forms of *L. amazonensis*. We

further investigated a putative mechanism of action of this compound as a modulator of molecules such as cytokines, nitric oxide, and labile iron pool in experimental models.

2. MATERIALS AND METHODS

2.1 *Leishmania (Leishmania) amazonensis* maintenance

Promastigotes forms of *L. (L.) amazonensis* (MHOM/BR/1989/166MJO) were maintained in culture medium 199 (GIBCO, Invitrogen, New York, USA) pH 7.18-7.22 supplemented with 10%-fetal bovine serum (FBS) (GIBCO, Invitrogen, New York, USA), 10mM-HEPES buffer, 0.1%-human urine, 0.1%-L-glutamine, 10U/mL-penicillin and 10µg/mL-streptomycin (Invitrogen-GIBCO) and 10%-sodium bicarbonate. The cell culture was maintained in a B.O.D at 25°C in a 25 cm^2 culture flask. All experiments used promastigote forms at the stationary growth phase (5-day culture).

2.2 *Caryocar coriaceum* extracts

The leaf extracts of *C. coriaceum* were kindly supplied by Dr. Selene Maia de Morais of the State University of Ceará, and obtained at the Campus of the State University of Ceará (UECE) lat: -3.792222 long: -38.556111, in the city of Fortaleza (Brazil). Leafs of *C. coriaceum* were presented and identified in the Herbarium Prisco Bezerra (UFC) under code EAC57060. The leaves were washed with tap water, dried in an oven and cut into pieces with a maximum of $1cm^3$.

Subsequently, the material obtained was subjected to soxhlet- extraction firstly with ethyl acetate and next with methanol during four days for each until reaching the exhaustion of metabolites extraction of the total mass. Both treatments were performed in the 12h light cycle and without shaking. After the exhaustion, all crude extracts were filtered and concentrated under vacuum until the complete elimination of the organic solvent. Thereafter, the crude extracts were kept at room temperature for further use and fractionation.

The extracts were named according to the solvent, respectively: (EAC) ethyl acetate and (MET) methanol. The extracts were diluted in dimethyl sulfoxide (DMSO) 0.01% for the biological assays.

2.3 Antipromastigote assay

L. amazonensis promastigote forms (10^6 cells/mL) were treated with *C. coriaceum* extracts 25, 50 and 100 µg/mL. Parasites were counted on a Neubauer chamber after 24, 48 and 72h of treatment. *L. amazonensis* promastigote was used as control and maintained in the culture medium, DMSO 0.01% or amphotericin B (AMB) 1 µM.

2.4 Scanning electron microscopy of promastigotes

Scanning electron microscopy of promastigotes forms was performed according to (Silva et al., 2013), with the parasites (10^6) treated with 50 µg/mL of EAC and MET extracts and incubated for 24 hours.

Subsequently, the promastigotes were collected and subjected to -washing and 2.5% glutaraldehyde-fixation in 0.1 M of Sodium cacodylate buffer containing 1 mM $CaCl_2$. After fixation, the samples were placed in poly-L-lysine treated coverslips and dehydrated with graded ethanol baths, CO_2 dry point, gold coated, and observed through scanning electron microscopy (FEI QUANTA 200 scanning electron microscope).

2.5 Determination of parasites cell volume

Promastigotes (10^6 cells/mL) were treated or untreated with with 50 µg/mL of EAC or MET as well as incubated for 24 hours at 24ºC, harvested, and washed with PBS. Subsequently, the parasites were analyzed using a BD Accuri™ C6 Plus personal flow cytometer. Histograms were generated, and FSC-H represented the cell volume. A total of 10,000 events were acquired in the region which corresponded to the parasites.

2.6 Determination of mitochondrial membrane potential

In order to assess the inner mitochondrial membrane potential, we conducted a tetramethylrhodamine ethyl ester (TMRE) staining (Sigma, St. Louis, MO, USA). For this purpose, promastigote forms (10^6 cells/mL) which had been treated for 24h with 50µg/mL of EAC or MET, washed and incubated with 25 nM of TMRE for 30 min at 25°C, were rewashed in PBS and immediately analyzed on a fluorescence microplate reader (Victor X3, PerkinElmer, Finland). An excitation wavelength of 480 nm and an emission wavelength of 580 nm were applied.

2.7 Determination of reactive oxygen species (ROS) generation on *L. amazonensis*

In order to assess the ROS generation in promastigote forms of *L. amazonensis*, 10^6 parasites were incubated with 50µg/mL for each treatment along 24h. Subsequently, they were washed in PBS (pH 7.4) and loaded with 10µM of a permeant probe diacetate 2',7'-dichlorofluorescein (H_2DCFDA) (Sigma, St. Louis, MO, USA) diluted in DMSO, and incubated in the dark for 45 min, 24°C. Reactive oxygen species (ROS) were measured as an increase in fluorescence caused by the conversion of non-fluorescent dye to highly fluorescent 20,70-dichlorofluorescein, with an excitation wavelength of 488 nm and emission wavelength of 530 nm on a fluorescence microplate reader (Victor X3, PerkinElmer, Finland).

2.8 Determination of phosphatidylserine exposure in parasite membrane

Phosphatidylserine (PS) exposure was detected by applying Annexin-V FITC (Invitrogen, Eugene, USA), a calcium-dependent phospholipid binding protein used as an apoptosis marker. Promastigotes (10^6 cells/mL) were treated with 50 µg/mL of the EAC or MET for 24 h at 25ºC. Afterwards, the parasites were washed and resuspended in 100 µL of binding buffer (140 mM NaCl, 5 mM $CaCl_2$, and 10 mM HEPES-Na, pH 7.4), followed by the addition of 5 µL of the calcium-dependent phospholipid binding protein Annexin-V FITC for 15 min at room temperature. After the incubation, the binding buffer (400 µL) was also added.

Data acquisition was performed using a fluorescence microplate reader (Victor X3, PerkinElmer, Finland) at an excitation wavelength of 488 nm and an emission wavelength of 520 nm. In order to compare the different treatments, the fluorescent values obtained were normalized to the respective number of cells.

2.9 Determination of promastigotes membrane integrity

Promastigotes (10^6 cells/mL) treated with 50 µg/mL of the different extracts for 24h at 25°C were harvested, washed with PBS and directly incubated with propidium iodide (PI) (Sigma, St. Louis, MO, USA) (0.50 µg/mL) for 5 min according to the manufacturer's instructions. Immediately thereafter, the promastigotes were analyzed using a fluorescence microplate reader (Victor X3, PerkinElmer, Finland) with an excitation wavelength of 480 nm and an emission wavelength of 580 nm. The fluorescent values obtained were normalized to the total number of cells for each treatment.

2.10 Co-determination of annexin V and propidium iodide label

Promastigotes (10^6 cells/mL) under the same abovementioned conditions were washed and resuspended in 100 μL of assay buffer 1x (Santa cruz Biotechnology), followed by the addition of a mix containing 1 μL of annexin-V FITC and 5 μL of PI (Santa cruz Biotechnology). Data acquisition and analysis were performed using a BD Accuri™ C6 Plus personal flow cytometer. A total of 10,000 events were acquired in the region which had been established as corresponding to the parasites. Annexin-V-stained cells (PI-positive or -negative) were considered apoptotic, and PI-positive-cells were classified as necrotic (Doroodgar et al., 2016; Ghaffarifar et al., 2015).

2.11 Animals and Ethics Committee

BALB/c mice were kindly provided by the Carlos Chagas Institute /Fiocruz-PR, Curitiba, Brazil. The animals weighing approximately 25-30 g and aged 6-8 weeks were kept under sterile conditions and used according to protocols approved by the Institutional Animal Care and Committee. This study was approved by the Ethics Committee for Animal Experimentation of the State University of Londrina (13134.2016.62).

2.12 Viability of peritoneal macrophages

The cytotoxic effects of *C. coriaceum* extracts in peritoneal macrophages were tested based on mitochondrial oxidation through MTT (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) (Sigma, St. Louis, MO, USA) assay as described by (Mosmann, 1983). Macrophages ($5x10^5$ cells/mL) were recovered from the peritoneal cavity of BALB/c mice with cold PBS supplemented with 3% of FBS and then cultured in 24-well plates with 200 µL of RPMI 1640 medium (10% FBS) for 2 h (37°C, 5% CO_2). Adherent cells were incubated with 25, 50 and 100 µg/mL of EAC or MET extract and cultured for 24 h under the same conditions. After this period, the culture was washed with PBS and added with MTT (5

mg/mL), followed by further incubation for 3 h. The culture was used as control under the same conditions without treatment; DMSO 0.01% was the vehicle, and the positive control was H_2O_2 0.4%. The MTT product (formazan crystals) was diluted with 300 µL of DMSO (Sigma, St. Louis, MO, USA), transferred to 96 wells plates and read using a spectrophotometer (Thermo Scientific, Multiskan GO) at 550 nm. The results were expressed as a percentage of viability compared to the control group calculated with the following formula:

$$\%\ Viable\ macrophages = \frac{OD\ samples\ of\ extracts\ treated}{OD\ sample\ untreated} \times 100$$

2.13 Anti-amastigote assay

Peritoneal macrophages ($5x10^5$ cells/mL) were cultured in 24-well plates containing 13 mm glass coverslips and incubated with 200µL of RPMI 1640 medium for 2h at 37°C and 5% CO_2. The adherent macrophages were infected with *L. amazonensis* promastigotes of ($1x10^6$ cells/mL) for 2h. After infection, the non-internalized promastigotes were removed through PBS-washing PBS and the cells treated with EAC or MET (25, 50 or 100 µg/mL), RPMI 1640 medium (control) or DMSO 0.01% (vehicle) and AMB 1 µM (positive control) for 24h (37°C, 5% CO_2). Subsequently, the cells were stained with Giemsa (Laborclin, Pines-PR Brazil) and 20 fields analyzed through optical microscope (Olympus BX41, Olympus Optical Co., Ltd., Tokyo, Japan) (1000x magnification) in order to determine the % of infected macrophages and number of amastigote per macrophage. The supernatant was stored for the measurement of cytokines and nitric oxide.

2.14 Promastigote recovery test

Promastigotes recovery assay was performed as previously described by (Silva et al., 2013). In brief, peritoneal macrophages were infected with *L. amazonensis* and treated with concentrations of *C. coriaceum* extracts in the same conditions described for the anti-amastigote assay. Viable amastigotes are known to have the ability to differentiate into promastigotes when exposed to ideal conditions; therefore, after 24 h of treatment the cell culture was washed with PBS and incubated with 199 culture media at 24°C in order to induce the differentiation of intracellular viable amastigotes in promastigote free forms. Free promastigotes recovered (FPR) were counted on a Neubauer chamber for three consecutive days and the number of recovery promastigotes normalized using the following equation:

$$\%\ FPR = \frac{number\ of\ FPR\ of\ extracts\ treated}{number\ of\ FPR\ of\ untreated} \times 100$$

2.15 Cytokines measurement

The Cytometric Bead Array Assay (CBA, BD Biosciences®) was applied to measure the levels of TNF-α, MCP-1, IL-6 and IL-10 in supernatants from non-infected and *L. amazonensis*-infected macrophages

treated with EAC or MET following the manufacturer's instructions. Data acquisition and analysis were performed using a BD Accuri™ C6 Plus personal flow cytometer.

2.16 Determination of nitrite as estimative of NO levels

Nitric oxide (NO) was determined through the Griess method. Briefly, supernatant aliquots (60 µL) of anti-amastigote assay were centrifuged at 5000 rpm for 2 min and a volume of 50 µL of the supernatant was recovered and added with 50 µL of Griess reagent (1% sulfanilamide and 0.1% of N-(1-Naphthyl) ethylenediamine in orthophosphoric acid (H_3PO_4) 5 %). After a 10-minute incubation at room temperature, the samples were placed in 96-well microplates. A calibration curve was made using dilutions of NaNO2, and the absorbance was determined at 550 nm on a microplate reader (Thermo Scientific, Multiskan GO).

2.17 Measurement of Malondialdehyde Levels (MDA)

MDA is a lipid peroxidation metabolite derived from oxidative stress occurrence. Accordingly, the MDA levels were determined through a High-Performance Chromatography (HPLC) according to description by Victorino et al., 2013 with a few modifications. The analyses were conducted using an Alliance e2695 HPLC (Waters, Milford, USA) equipped with a SecurityGuard ODS-C18 (4 × 3.0 mm, Phenomenex), C18 reverse phase column (Eclipse XDBC18; 4.6 × 250 mm, 5 µm, Agilent) as well as a photodiode array detector (Photodiode ArrayDetector (PDA), 2998) using Empower 2 software (Waters, Milford, USA). The preparation of MDA standards used 1,1,3,3- tetraethoxypropane (TEP). After the treatment with EAC or MET, as described in anti-amastigote assay, aliquots containing 500 µL of cells + supernatants were deproteinized by adding 20% trichloroacetic acid, subsequently reacted with 1mL of thiobarbituric acid. The mobile phase was 70% 10 mM KH_2PO_4 buffer, pH 7.0, and 40% HPLC-grade methanol. Readings were obtained at 532 nm, following an eight-minute isocratic flow at the rate of 1 mL/min. The results were expressed in nM MDA.

2.18 Peritoneal macrophage Reactive Oxygen Species generation

In order to assess the ROS generation, *L. amazonensis*-infected macrophages ($5x10^5$ cells/mL - infected and treated under the same conditions described in the anti-amastigote assay) were washed in PBS (pH 7.4) and loaded with 2µM of a permeant probe diacetate 2',7'-dichlorofluorescein (H_2DCFDA) (Sigma, St. Louis, MO, USA) diluted in DMSO, incubated in the dark for 30 min, 37°C, 5% CO_2. ROS were measured as an increase in fluorescence caused by the conversion of non-fluorescent dye to highly fluorescent 20,70-dichlorofluorescein, with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a fluorescence microplate reader (Victor X3, PerkinElmer, Finland).

2.19 Determination of iron concentration and total bound iron

The determination of iron concentration in supernatants of the anti-amastigote assay was performed utilizing the Dimension® automated system. The method is an adaptation of the direct assay for iron, developed by Smith et al., 1984, and requires the Flex® reagent cartridge for its implementation. The dispensation, shaking and processing of the samples are performed automatically through the Dimension® system. Briefly, in acidic conditions (pH 4.5) and in the presence of a reducing agent (ascorbic acid), it occurs the release of iron bound to transferrin. The resulting product (Fe^{2+}) forms a blue complex with 3-(2-piridil)-5,6-bis-2- (5-furyl sulfonic acid)-1,2,4-triazine (Ferene®). The absorbance of the complex is measured using a biochromatic endpoint technique (600, 700nm). The test principle for the total bound iron is similar: the samples are automatically mixed with a serum iron solution to saturate all available sites of iron binding in transferrin. In non-acidic conditions (pH 8.6), only saturated iron in excess, unbound, is available to be reduced to ferric iron by ascorbic acid forming the blue complex with Ferene®. The subsequent addition of acid (pH 4.5) releases the iron bound to transferrin, this supplemental iron is reduced to ferric iron by ascorbic acid, forming an increased amount of blue complex with Ferene®. The increase in absorbance during the change of pH 8.6 to pH 4.5 is proportional to the concentration of iron bound to transferrin.

2.20 Relative Quantification of Nrf2, ferritin and HO-1 mRNA by Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The cell culture was performed as aforementioned in the anti-amastigote assay. RNA extraction was performed with 10^6 cells using SV Total RNA Isolation System (Promega, USA) following the manufacturer's procedure. RNA concentration was determined through absorbance (260 nm) measurements using a spectrophotometer (SynergyHT, Biotek, USA). Complementary DNA was synthesized using 500 ng of total RNA in a reverse transcription reaction by MMLV reverse transcriptase (Invitrogen, USA) following the manufacturer's procedure. Real-time RT-PCR quantitative mRNA analyses were performed in Rotor-Gene Q equipment (Qiagen, Germany) using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, USA), with a final volume of 20 µl. The reaction mixture also contained 2 µM primers and 100 ng of cDNA template. The sequences of primers used for Nrf2 were Nrf2-F 5'-TCACACGAGATGAGCTTAGGGCAA-3' and Nfr2-R 5'-TACAGTTCTGGG CGGCGACTTTAT-3', for heme-oxygenase-1 were HO-1-F 5′-CCCAAAACTGGCCTGTAAAA-3′ and HO-1-R 5′-CGTGGTCAGTCAACATGGAT-3′, for ferritin-L were ferritin-F 5'- TTCCAGGATGTGCAGAAGCC-3' and ferritin-R 5'-AAGAGGGCCTGATTCAGGTTC-3' and for β-actin were b-actin-F 5′-AGCTGCGTTTTACACCCTTT-3′ and b-actin-R 5′-AAGCCATGCCAATGTTGTCT-3′. Cycling conditions were 10 minutes at 95 ºC, and 40 cycles of 30 seconds at 95 ºC, 30 seconds at 62 ºC, and 30 seconds at 72 ºC, followed by melting curve analysis (70 to 95 ºC at 0.5 ºC/second). The calculation to

determine the qualified levels of gene expression by reference to β-actin was achieved through the cycle threshold method.

2.21 Statistical analysis

Data were expressed as a mean ± standard error of the mean (SEM). At least three independent experiments were performed, each with duplicate datasets. Data were analyzed using the GraphPad Prism statistical software (GraphPad Software, Inc., USA, 500.288). Significant differences between the groups were determined through one-way ANOVA, followed by Tukey's test for multiple comparisons. Differences were considered statistically significant upon $p \leq 0.05$.

3. RESULTS

3.1 *C. coriaceum* extracts inhibit the proliferation of *L. amazonensis* promastigote forms

The antileishmanial effect of EAC and MET was assessed by determining the proliferation of parasites. All tested concentrations of the extracts proved significant proliferation of *L. amazonensis* from 24h in relation to the control or vehicle groups, with no difference between both, except for the 25 μg/mL of MET, whose effect occurred only after 48h and 72h of treatment. At 48 and 72h of treatment, parasites treated with 50 and 100 μg/mL of EAC were similar to amphotericine-B treatment, whereas the treatment with MET 50, 100 μg/mL and EAC 25 μg/mL remained similar to their 24h effect, having persisted the reduction in the number of parasites (Figure 1).

As we obtained similar results according to the different extracts concentration in this assay, the next experiments investigating the effect of treatments on promastigote forms were performed with the intermediate concentration of EAC and MET (50 μg/mL). As the extracts in this concentration showed an effect within 24 hours, all the experiments were performed along such period.

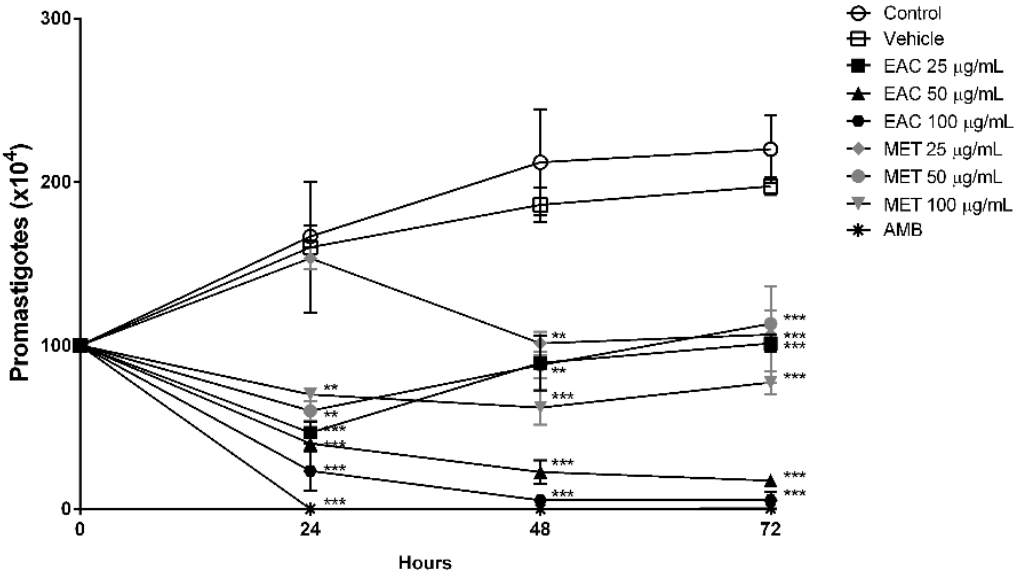


Figure 1 – Antipromastigote effect of *C. coriaceum* leaves extracts. *L. amazonensis* promastigotes forms were subjected to 25, 50 and 100 μg/mL of ethyl acetate (EAC) or methanol (MET) leaf extract. The parasite viability was assessed at 0, 24, 48 and 72 h by neubauer chamber. Non-treated parasites were used as control; DMSO 0.01% was employed as vehicle control, and amphotericin B (AMB) 1 μM was the positive control . The values represent the mean ± SEM of three independent experiments performed in duplicate. ** Significant difference against the control group ($p \leq 0.01$), *** ($p \leq 0.001$).

3.2 The *C. coriaceum* extracts induce loss of typical morphology and reduce the cell volume of promastigote forms

Morphological alterations in *L. amazonensis* promastigotes treated with EAC and MET (50 μg/mL, 24h) were revealed through scanning electron microscopy. Both treatments caused rounding and reduction of parasite body size. MET treatment also showed rupture of the plasma membrane and cell lysing. These data were confirmed through a flow cytometer analysis, which indicated a reduction of cell volume of EAC- and MET-treated parasites.

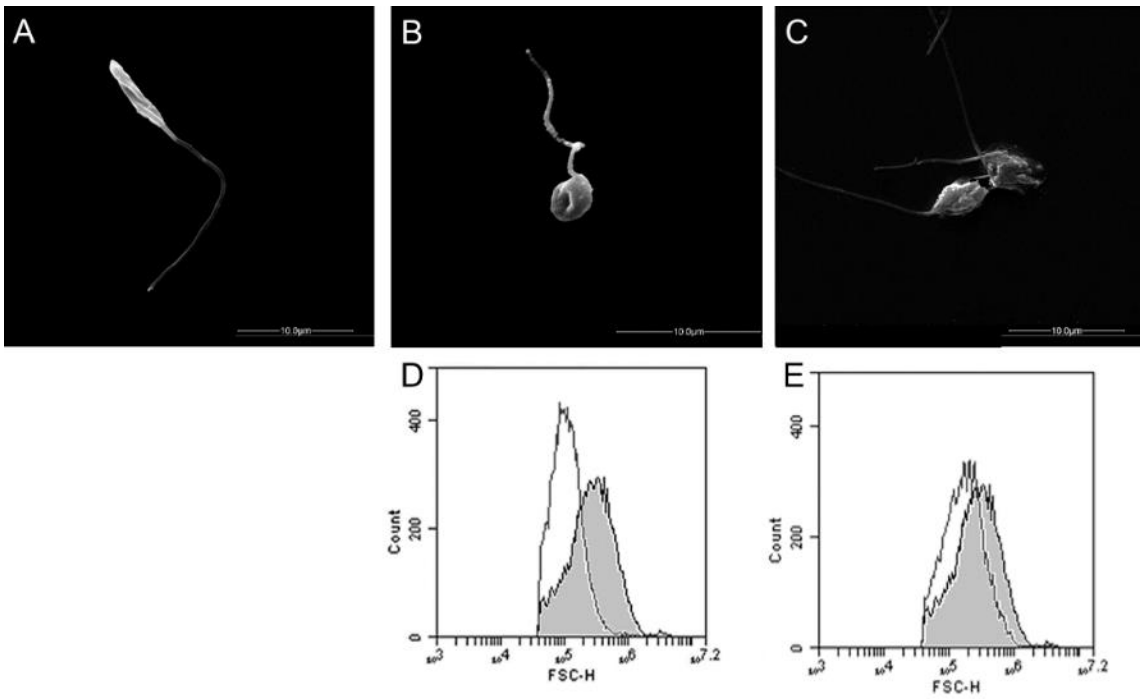


Figure 2 – Morphological changes of treated promastigote forms. Scanning electron microscopy images of *L. amazonensis* promastigote forms incubated either in the absence (A) or presence of 50 μg/mL of ethyl acetate (EAC) (B) or methanol (MET) (C) leaf extract for 24 h. Cell volume in promastigote forms of *L. amazonensis* treated with EAC (D) or MET (E) (50 μg/mL). FSC-H were considered a function of cell size. The gray area corresponds to the control group (i.e., untreated parasites) and the white area corresponds to the treated group. Typical histograms of at least three independent experiments are shown.

3.3 The antipromastigote effect of *C. coriaceum* extracts is due mitochondrial depolarization, increase in ROS production, phosphatidylserine exposure and damage in plasma membrane of the protozoan

Having verified the antipromastigote effect of *C. coriaceum* extracts, we decided to elucidate the mechanism through which the parasites were being eliminated. Based on this, we assessed the mitochondrial membrane integrity in both EAC- and MET- (50 μg/mL, 24h) treated parasites using TMRE, a fluorescent marker which complexes with active mitochondria. The treatment with extracts were found to having similarly decreased the total TMRE fluorescence intensity in relation to the control group, indicating loss of integrity of this organelle (Figure 3A).

We also assessed the effects of total ROS production on treated promastigotes using H_2DCFDA, a fluorescent probe which primarily detects H_2O_2 and hydroxyl radicals and fluoresces after forming dichlorofluorescein (Myhre et al., 2003). The results showed that both tested extracts increased total ROS production on promastigotes forms at concentration 50 μg/mL against the control group (Figure 3B).

In addition, we investigated whether the cell death mechanism triggered by the treatments involved apoptosis by marking the externalization of PS. Our data showed that treated parasites have increased annexin V labeling compared with the control group, indicating an increase in PS externalization (Figure 3C). The EAC treatment showed a more expressive PS labeling in comparison with MET extract (Figure 3C).

After these results, we studied whether the mechanism of cell death triggered by the extracts treatment also involves the plasma membrane integrity. For this purpose, treated parasites were stained with PI, which diffuses across permeable membranes and binds to nucleic acids. As shown in Figure 3D, EAC and MET extracts increased PI stained in promastigotes forms, being the highest increase in the forms treated with EAC.

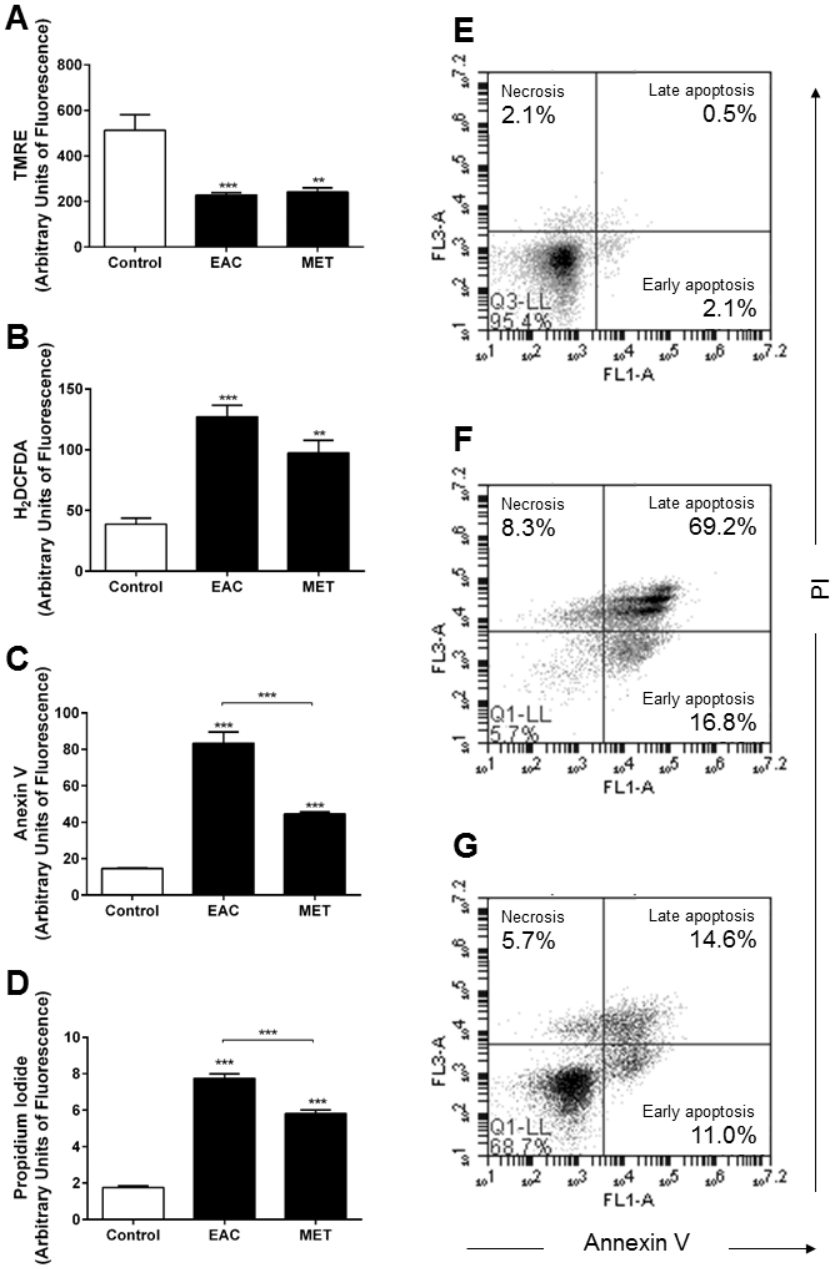


Figure 3 – *C. coriaceum*-induced death mechanism in *L. amazonensis* promastigote forms submitted to a 24 h treatment with 50 µg/mL of the extracts. The following methods were used for the respective assessments: (A) TMRE assay for fluorometric analysis of the mitochondrial membrane potential, (B) H_2DCFDA probe for reactive species of oxygen measurement, (C) Annexin V labeling for phosphatidylserine exposition, and (D) propidium iodide staining for the analyses of plasma membrane integrity. Data represent the mean ± SEM of three independent experiments performed in duplicate. ** Significant difference in relation to control ($p \leq 0.01$). Co-staining of EAC (F) or MET (G) treated promastigotes with PI and annexin V–FitC analyzed by flow cytometry. As control was used untreated parasites (E). Typical dot plots of at least three independent experiments are shown. EAC – ethyl acetate leaf extract; MET – methanol leaf extract.

In order to differentiate the cell death mechanism in the necrotic, apoptotic or late-apoptotic process, we performed an annexin V/ PI co-staining in treated promastigotes. Promastigotes with a single label for annexin (annexin V+) was considered apoptotic, PI + as necrotic and the double mark for annexin V+/ PI+ as a late-apoptotic process. Promastigotes which were Annexin V+ had a percentage of 16.8 and 11% after

treatment with EAC and MET, respectively. The PI+ cells were 8.3% for EAC and 5.7% for MET. Lastly, the annexin V+/ PI+ promastigotes were 69.2 and 14.6% for EAC and MET, respectively, indicating that most of the parasites were undergoing late apoptosis-like death in both conditions (Figure 3F, G).

3.4 Low concentrations of *C. coriaceum* extracts do not alter the viability of peritoneal macrophages

As an attempt to verify whether the *C. coriaceum* extracts has a toxic effect on murine macrophage, we performed the MTT assay and verified that the concentrations of 25, 50 and 100 μg/mL were not toxic during the period assessed (24 h) for both EAC and MET (Figure 4). For this reason, we decided on these concentrations to proceed with the following experiments.

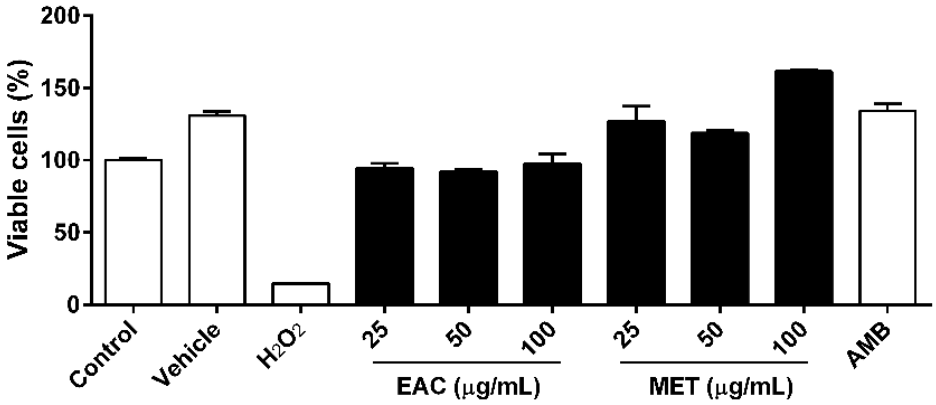


Figure 4 – Cytotoxicity of *C. coriaceum* extracts. Peritoneal BALB/c macrophages were submitted to a 24 hour-treatment using the 25, 50 and 100 μg/mL of ethyl acetate (EAC) or methanol (MET) leaf extracts and viability analyzed through MTT assay. Untreated parasites were used as control; 0.01% DMSO was used as the vehicle, and 0.4% H_2O_2 was a positive control. Amphotericin B (AMB) was used at 1μM. The values represent the mean ± SEM of three independent experiments performed in duplicate.

3.5 *C. coriaceum* extracts act on intracellular amastigotes forms and decreases the recovery of promastigotes forms from infected macrophages

In order to verify the effect of *C. coriaceum* extracts on amastigotes forms of *L. amazonensis*, we performed the anti-amastigote assay which assess the percentage of infected macrophages and the amount of amastigotes after a 24-hour treatment. The treatment with all tested concentration (25-100 μg/mL) of EAC and MET proved able to induce a significant reduction in the percentage of infected macrophages and amount of amastigotes per macrophage, showing similar effect to the standard drug (Figure 5A and B).

In order to confirm the reduction of infection, we performed a recovery assay of promastigotes forms in which the culture of infected macrophages was submitted to ideal conditions for the differentiation of viable amastigotes in free promastigote forms. All tested concentrations showed to have promoted at least 50% of reduction in recovery promastigotes forms, after 48 and 72h of culture (Figure 5C).

No statistical difference was indicated according to the different treatments (EAC, MET or AMB) or according to the tested extracts concentrations (25, 50 and 100 μg/mL). Therefore, aiming at minor side effects involving further therapeutic applications, the lowest concentration (25 μg/mL) was chosen for the following experiments.

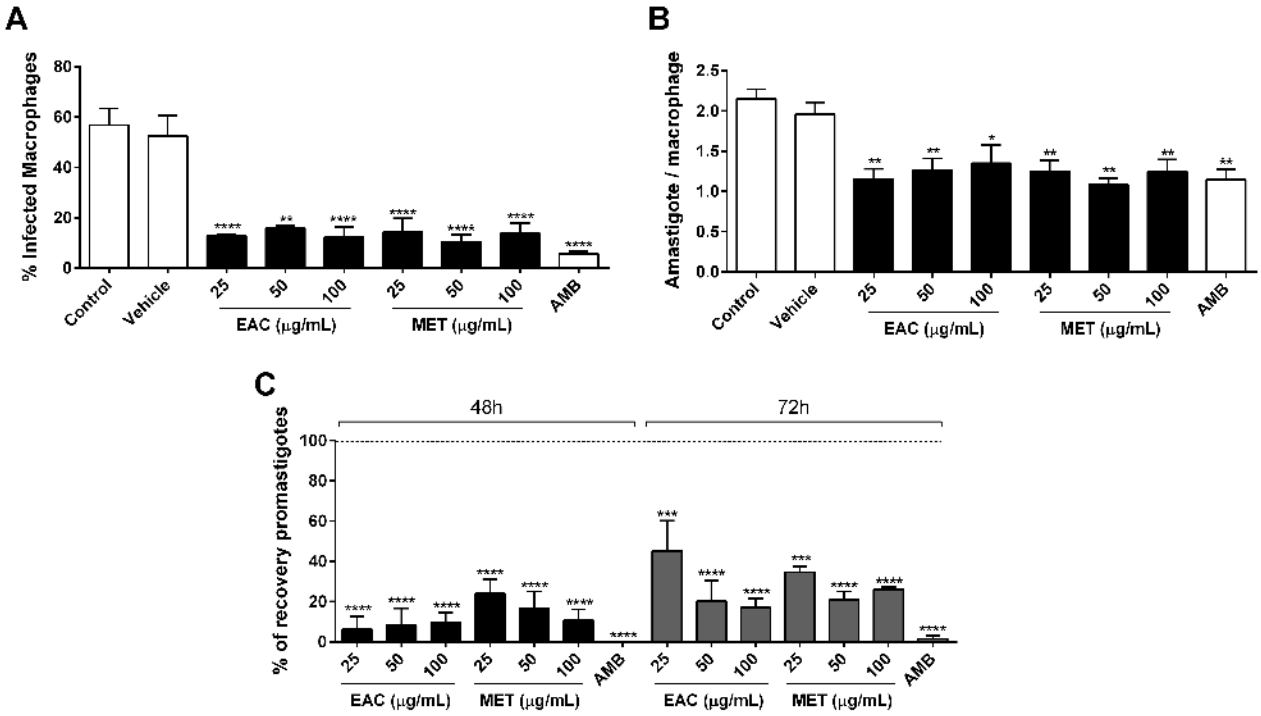


Figure 5 – *C. coriaceum* extracts effect on *L. amazonensis*-infected macrophages. Infected cells were submitted to a 24-hour treatment using 25, 50 and 100 μg/mL of the leaf extracts. (A) Assessment of the amount of amastigotes per macrophage and (B) percentage of infected macrophages. (C) 48 and 72 h of *L. amazonensis*-infected macrophages incubation 199 media at 24ºC. The number of recovered parasites was measured through a Neubauer chamber and the values converted to %. Dashed line indicates the control group (100%). Data represent the mean ± SEM of three independent experiments performed in duplicate. Infected non-treated cells were used as control; 0.01% DMSO was used as the vehicle. EAC – ethyl acetate extract, MET – methanol extract, AMB – amphotericin B. * Significant difference in relation to control ($p \leq 0.05$) ** ($p \leq 0.01$), *** ($p \leq 0.001$), **** ($p \leq 0.0001$).

3.6 *C. coriaceum* leaf extracts induce cytokine secretion by macrophages

In addition to the antileishmanial effect, the immunomodulatory properties are essential for the development of new alternative to leishmaniasis therapy. Once EAC and MET treatment reduced the amount of infected cell and recovered the promastigotes, we examined the immunomodulatory activity of the extracts triggered through this experimental model.

The treatment with EAC and MET was able to increase the levels of TNF-α, MCP-1, IL-6 and IL-10 significantly in comparison with *L. amazonensis*-infected non-treated cells (Control) (Figure 6A, B, C and D, respectively).

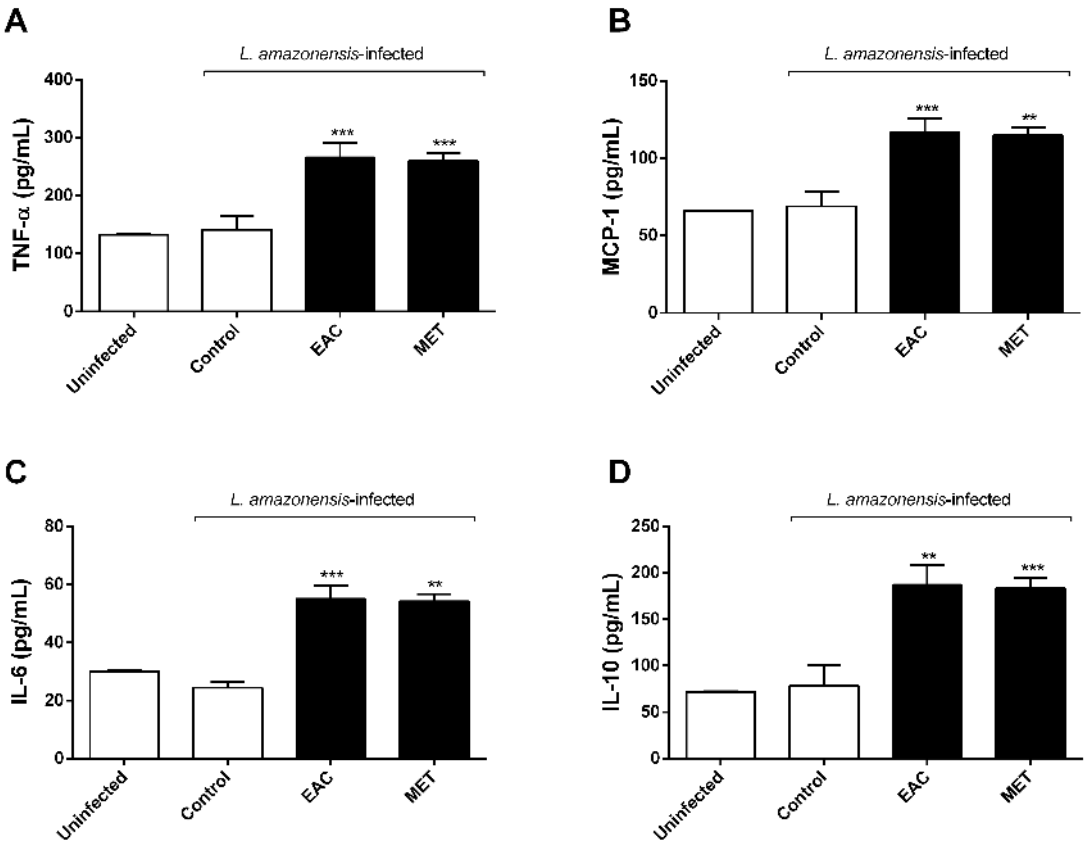


Figure 6 – Effect of *C. coriaceum* extracts on cytokine production of *L. amazonensis*-infected macrophages. The infected cells were submitted to a 24-hour treatment with the EAC and MET extracts at 25 μg/mL and the cytokine levels were verified by CBA. (A) TNF-α, (B) MCP-1, (C) IL-6 and (D) IL-10. Uninfected represents the non-infected and non-treated cells; control represents infected non-treated cells. EAC – ethyl acetate leaf extract; MET – methanol leaf extract. The values represent the mean ± SEM of three independent experiments performed in duplicate. ** Significant difference compared to control ($p \leq 0.01$), *** ($p \leq 0.001$).

3.7 The EAC and MET treatments reduce the amount of malondialdehyde (MDA) and ROS, but not affect the NO levels

Knowing that the treatment induced immunomodulation, we investigated the two major microbicidal molecules synthesized by macrophages to contain intracellular infection: ROS and NO. Our results demonstrated that the treatment with EAC and MET similarly reduced the cleavage of H_2DCFDA probe (Figure 7A), indicating a reduction of ROS generation; however, the extracts were not able to affect the production of NO, having maintained the low levels of NO resulting from *Leishmania* infection (Figure 7B). In order to corroborate these results, we analyzed the MDA levels, a lipid peroxidation metabolite derived from oxidative stress occurrence and verified that the treatments with EAC and MET were also able to reduce these levels (Figure 7C).

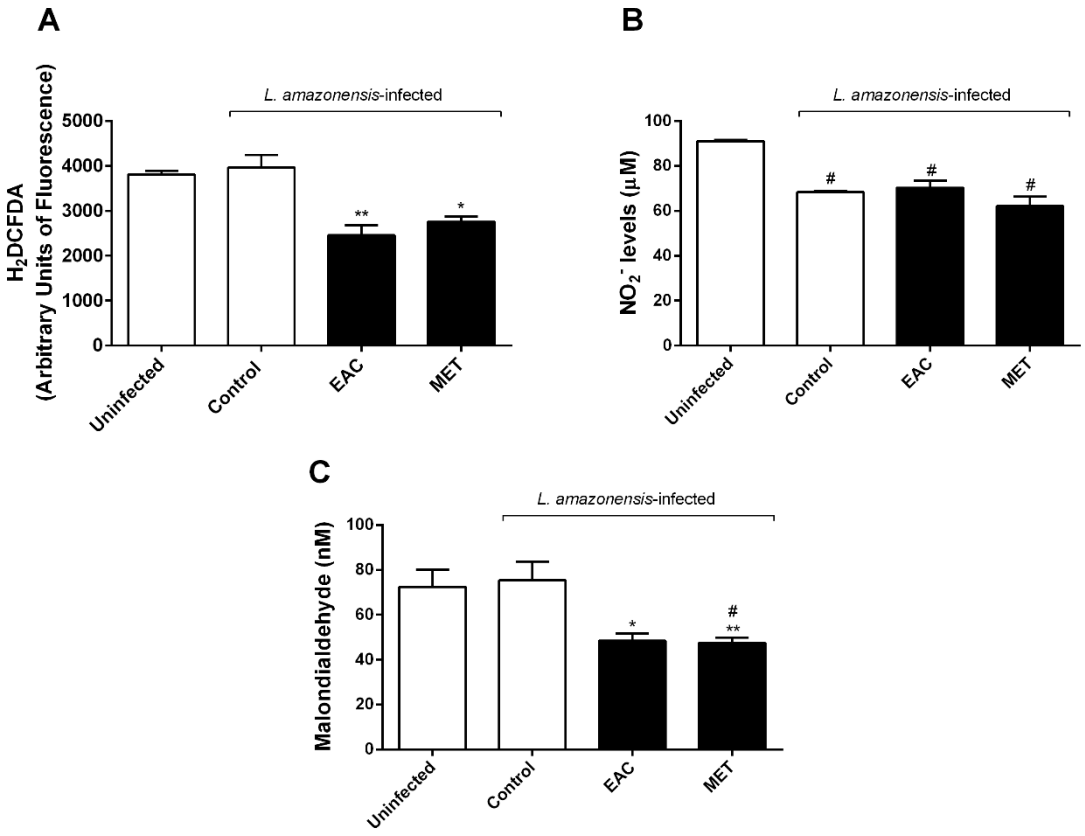


Figure 7 – Measurement of ROS, NO_2^- and MDA of EAC and MET treated cells. The following methods were used to assess the *L. amazonensis*-infected macrophages submitted to a 24-hour treatment using the ethyl acetate (EAC) and methanol (MET) extracts at 25 µg/mL: (A) MDA measurement through HPLC, (B) fluorescent probe H_2DCFDA for reactive oxygen species measurement, and (C) Griess method for nitrite levels. The values represent the mean ± SEM of three independent experiments performed in duplicate. Uninfected represents the non-infected and non-treated cells; control represents infected non-treated cells. * Significant difference compared to control, ** ($p \leq 0.01$). # Significant difference compared to uninfected group ($p \leq 0.05$).

3.8 *C. coriaceum* leaf extracts modulate iron pool of infected macrophages

Still with the objective to understand the parasites death pathway, we investigated the levels of iron and total binding iron capacity, since this metal is required as a cofactor for all *Leishmania* SOD enzymes, being essential for parasite survival and closely related to the differentiation of promastigote into virulent amastigotes (Flannery et al., 2011; Mittra et al., 2017, 2013).

The results showed that treatment with EAC and MET of *L. amazonensis*–infected macrophages did not alter the total iron concentration (Fig. 8A) but increased iron bound to transferrin (Fig. 8B) when compared to control, decreasing the labile bioavailability of this metal, being the highest increase in the total iron binding capacity found in EAC treatment.

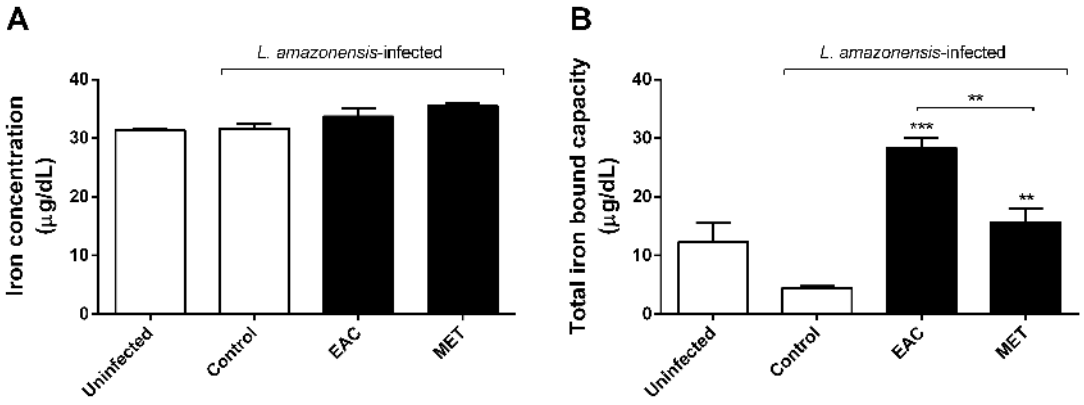


Figure 8 – Total iron concentration and total bound iron in *L. amazonensis*-infected macrophages treated with EAC and MET. The determination of labile iron concentration in supernatants was performed utilizing the Dimension® automated system. *L. amazonensis*-infected macrophages treated or not with 25 µg/mL of EAC (ethyl acetate extract) or MET (methanol extract) for 24h do not alter the iron concentration (A) but increased total iron bound capacity (B). Data represent mean ± SEM of three independent experiments. ** Significant difference compared to control ($p \leq 0.01$), *** ($p \leq 0.001$).

3.9 *Caryocar coriaceum* treatment upregulated the Nrf2, ferritin and heme oxygenase-1 (HO-1) expression

Previous studies have demonstrated the effect of Nrf2 as a key regulator of the antioxidant response which plays a central role in up-regulation of multiple genes, including HO-1 and ferritin involved in iron metabolism (Qaisiya et al., 2013). Therefore, we decided to investigate the Nrf2, ferritin and HO-1 expression on *L. amazonensis*-infected macrophages treated with the extracts, and we observed that the MET treatment significantly upregulated the expression of Nrf2 (Figure 9A) and both treatments also significantly upregulated the expression of ferritin and HO-1 (Figure 9B and C).

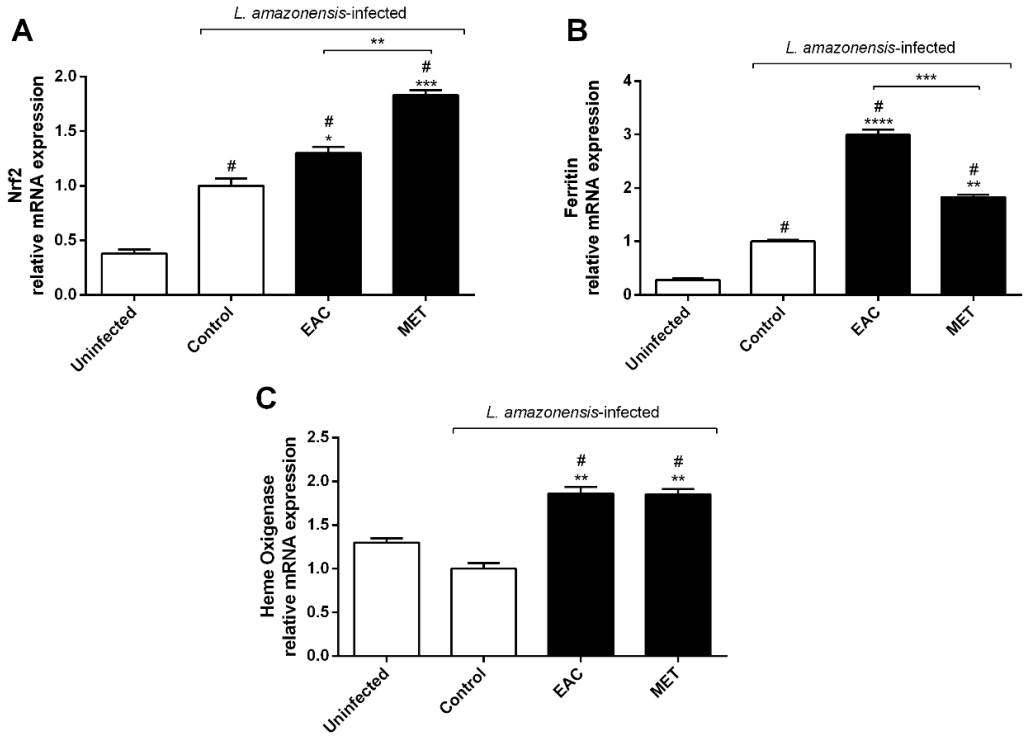


Figure 9 - Nrf2, ferritin and HO-1 expression in *L. amazonensis*-infected macrophages treated with EAC and MET. Real-time RT-PCR quantitative mRNA analyses were performed to quantify the expression of Nrf2, ferritin and HO-1. Uninfected (macrophages incubated with culture medium) and control (infected macrophages with *L. amazonensis*) were used. EAC – ethyl acetate leaf extract; MET – methanol leaf extract. Data represent mean ±SEM of three independent experiments. * Significant difference compared to control or between treatments when indicated ($p \leq 0.05$), **($p \leq 0.01$), ***($p \leq 0.001$), **** ($p < 0.0001$). # Significant difference compared to uninfected control ($p \leq 0.05$).

4. DISCUSSION

The current treatment against ACL is based on a highly toxic chemotherapy (Alvar et al., 2006) which drives the search for new alternative strategies, incliding the great attention directed to natural compounds. Thus, this study aimed at verifying the leishmanicidal action of ethyl acetate (EAC) and methanol (MET) leaf extracts from a Brazilian Cerrado plant, *C. coriaceum*.

A previous study demonstrated the anti-*L. amazonensis* promastigote action of the hydroethanolic extract from leaves of *C. brasiliense* (Paula-Ju et al., 2006b). Later on, we verified the action of EAC and MET extracts of *C. coriaceum* on *L. amazonensis* promastigotes with results showing that the EAC and MET extracts were able to reduce the number of viable parasites as well as induce both morphological and physiological alterations on promastigote forms using concentrations a hundred times smaller than those used by Paula-Ju et al. (2006).

Regarding the physiological changes, we observed that the extracts induced the depolarization of the mitochondrial membrane of the promastigote parasite. It is known that maintenance of mitochondrial integrity is essential for parasite survival, once *Leishmania* spp. have a unique mitochondrion which is the main site to generate cellular ATP through oxidative phosphorylation, thus becoming a promising antiparasitic target (Fidalgo and Gille, 2011; Monzote and Gille, 2010).

The dysfunction of the mitochondrial respiratory chain may produce a huge amount of ROS inside the organelle (Roy et al., 2008). As expected, a treatment with both extracts was able to promote ROS increase in promastigote forms. Furthermore, the cascade triggered by the loss of mitochondrial integrity followed by increased ROS production may lead to the death of the parasite through an apoptosis-like mechanism (Fidalgo and Gille, 2011; Mehta and Shaha, 2004).

Our data showed that the treatment induces a late apoptosis-like death in the majority of the population, characterized by double labeling with Annexin V and PI (Doroodgar et al., 2016; Ghaffarifar et al., 2015). Doroodgar et al. (2016) and Ghaffarifar et al. (2015) verified similar effects of Tamoxifen and Artemisina treatment on *Leishmania major* promastigotes, respectively.

Alltogether, these results showed that even though both extracts present similar results, the EAC treatment was more efficient at direct activity against the promastigote forms than MET. The analysis of both EAC and MET extracts revealed similar amount of phenols in both; however, EAC presented 2.6 times more flavonoids than MET (data not showed). The higher presence of flavonoids can explain the more pronounced results found in EAC treatment.

Once the effect of the extracts on promastigote forms had been proven, we sought to understand the action of these treatments on intracellular amastigote forms. We found that the treatments did not affect the viability of macrophages, the main host cells of *Leishmania* spp. (Liu and Uzonna, 2012b), but acted on the intracellular amastigotes. Since EAC and MET showed a killing effect on parasite, we investigated the potential mechanisms involved in amastigote elimination.

Macrophages are phagocytizing specialized cells which can subsequent destroy intracellular pathogens by activating a full arsenal of antimicrobial characteristics in phagosomes (Flannagan et al., 2009). The *Leishmania* elimination involved the secretion of proinflammatory molecules, as IFN-γ, TNF-α, IL-6 and MCP-1, which activate macrophages to produce ROS and NO as well as eliminate the parasites (Bhattacharyya et al., 2002; de Santana et al., 2017; Liew et al., 1990; Murray and Nathan, 1999). Our data showed an increase in proinflammatory cytokines, but not in ROS and NO. A possible explanation is the concomitant increase in IL-10 levels, which is responsible for controlling the inflammation and synthesis of proinflammatory mediators (Mosser and Zhang, 2008).

Our findings reinforce this role of IL-10 since even with the TNF-α, MCP-1 and IL-6 synthesis, the decrease in lipid peroxidation and ROS levels parameters demonstrates the prevalence of IL-10 on pro-inflammatory cytokines. Haddad and Fahlman (2002) demonstrated that IL-10 was described as the anti-inflammatory cytokine with an antioxidant properties. As *Caryocar* sp. extracts were also reported for having antioxidant capacity (Paula-Ju et al., 2006), they can act directly by scavenging ROS or indirectly by activating pathways which promote ROS degradation (Poljsak et al., 2013).

Having the mechanism of action of the extracts on the intracellular amastigotes forms not involve the production of ROS or NO, we investigated pathways incorporated with the iron metabolism, since this metal is fundamental for the replication and survival of the intracellular parasite. The success or failure of iron uptake by the pathogen influences the outcome of pathogenesis and is an important process in infection control (Zaidi et al., 2017).

Our data showed that EAC and MET did not alter the total iron pool, but increased the total iron bound to transferrin. Previous works showed that *L. donovani* uses only the labile iron pool for its survival and replication (Das et al., 2009b); in this sense, the increase in transferrin iron binding made the labile unavailable metal to the parasite.

Kedzierski et al., 2007 showed that antioxidant defense could help macrophages to eliminate *L. major*. As demonstrated previously, EAC and MET extracts acted in the reduce ROS production, MDA formation and iron availability, we assessed the genes responsible for the antioxidant response and iron metabolism.

A pivotal factor that regulates the antioxidant responses in a variety of disease models is the transcription factor Nrf2 (Paiva and Bozza, 2014). Nrf2 is responsible for regulating a battery of antioxidant and cellular protective genes, primarily in response to oxidative stress and genes containing ARE motifs, triggering H-ferritin and heme oxygenase 1 (HO-1) (Alam et al., 1999; Hintze and Theil, 2005). HO-1 is associated with

regulation of labile iron pool by increasing ferritin, a protective protein which acts by sequestering iron ions (Lin et al., 2008). In addition, this protein can be translocated to the nucleus, activating antioxidant mechanisms (Gozzelino et al., 2010). We showed for the first time that EAC and MET extracts increased the expression of Nrf2, HO-1 and Ferritin in *L. amazonensis*-infected macrophages.

Taking these results alltogether, it was demonstrated that EAC and MET were able to modulate the iron by increasing the iron bound to transferrin through the upregulation of Nrf2 expression, which massively stimulates the transcription of proteins HO-1 and ferritin responsible for controlling the bioavailability of labile iron pool, impairing the uptake of this metal by the parasite.

In conclusion, *C. coriaceum* leaf extracts EAC and MET have substantial *in vitro* antileishmanial activity, acting directly on promastigote forms leading to death by late apoptosis-like process as well as in *L. amazonensis*-infected macrophages by triggering an antioxidant response. The mechanism triggered by the extracts involves its capacity to activate responses mediated by Nrf2/HO-1/Ferritin, followed by a modulation of the labile iron pool, which culminates in a depletion of available iron for parasite replication and survival within macrophages.

GLOSSARY

ACL – american cutaneous leishmaniasis, AMB – amphotericin B, CBA – cytometric bead array, DMSO – dimethyl sulfoxide, EAC – ethyl acetate leaf extract, FBS – fetal bovine serum, FPR – free promastigote recovery, FSC-H – forward scatter-height, H_2DCFDA – diacetate 2',7'-dichlorofluorescein probe, HO-1 – heme oxygenase-1, HPLC – high performance liquid chromatography, IL – interleukin, MCP-1 – monocyte chemoattractant protein-1, MDA – malondialdehyde, MET – methanol leaf extract, mRNA – messenger RNA, MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide, NO – nitric oxide, Nrf2 – nuclar factor erythroid-2, OD – optical density, PBS – phosphate buffered saline, PI – propidium iodide, PS – phosphatidylserine, ROS – reactive oxygen species, RT-PCR – reverse transcription polymerase chain reaction, SEM – standard error of mean, TEP – 1,1,3,3- tetraethoxypropane, TMRE – tetramethylrhodamine ethyl ester probe, TNF – tumor necrosis factor.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

FUNDING

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

REFERENCES

Alam, J., Stewart, D., Touchard, C., Boinapally, S., Choi, A.M., Cook, J.L., 1999. Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. J. Biol. Chem. 274, 26071–8.

Alvar, J., Croft, S., Olliaro, P., 2006. Chemotherapy in the Treatment and Control of Leishmaniasis. Adv. Parasitol. 61, 223–274. doi:10.1016/S0065-308X(05)61006-8

Bailão, E., Devilla, I., da Conceição, E., Borges, L., 2015. Bioactive Compounds Found in Brazilian Cerrado Fruits. Int. J. Mol. Sci. 16, 23760–23783. doi:10.3390/ijms161023760

Barral, A., Pedral-Sampaio, D., Grimaldi Júnior, G., Momen, H., McMahon-Pratt, D., Ribeiro de Jesus, A., Almeida, R., Badaro, R., Barral-Netto, M., Carvalho, E.M., 1991. Leishmaniasis in Bahia, Brazil: evidence that Leishmania amazonensis produces a wide spectrum of clinical disease. Am. J. Trop. Med. Hyg. 44, 536–46.

Bhattacharyya, S., Ghosh, S., Dasgupta, B., Mazumder, D., Roy, S., Majumdar, S., 2002. Chemokine-Induced Leishmanicidal Activity in Murine Macrophages via the Generation of Nitric Oxide. J. Infect. Dis. 185, 1704–1708. doi:10.1086/340820

Breda, C.A., Gasperini, A.M., Garcia, V.L., Monteiro, K.M., Bataglion, G.A., Eberlin, M.N., Duarte, M.C.T., 2016. Phytochemical Analysis and Antifungal Activity of Extracts from Leaves and Fruit Residues of Brazilian Savanna Plants Aiming Its Use as Safe Fungicides. Nat. Products Bioprospect. 6, 195–204. doi:10.1007/s13659-016-0101-y

Cecílio, P., Pérez-Cabezas, B., Santarém, N., Maciel, J., Rodrigues, V., Cordeiro da Silva, A., 2014. Deception and manipulation: the arms of leishmania, a successful parasite. Front. Immunol. 5, 480. doi:10.3389/fimmu.2014.00480

Colombo, N.B.R., Rangel, M.P., Martins, V., Hage, M., Gelain, D.P., Barbeiro, D.F., Grisolia, C.K., Parra, E.R., Capelozzi, V.L., 2015. Caryocar brasiliense camb protects against genomic and oxidative damage in urethane-induced lung carcinogenesis. Brazilian J. Med. Biol. Res. 48, 852–862. doi:10.1590/1414-431X20154467

Correia, A.F., Silveira, D., Fonseca-Bazzo, Y.M., Magalhães, P.O., Fagg, C.W., da Silva, E.C., Gomes, S.M., Gandolfi, L., Pratesi, R., de Medeiros Nóbrega, Y.K., 2016. Activity of crude extracts from Brazilian cerrado plants against clinically relevant Candida species. BMC Complement. Altern. Med. 16, 203. doi:10.1186/s12906-016-1164-3

Das, N.K., Biswas, S., Solanki, S., Mukhopadhyay, C.K., 2009a. *Leishmania donovani* depletes labile iron pool to exploit iron uptake capacity of macrophage for its intracellular growth. Cell. Microbiol. 11, 83–94. doi:10.1111/j.1462-5822.2008.01241.x

Das, N.K., Biswas, S., Solanki, S., Mukhopadhyay, C.K., 2009b. *Leishmania donovani* depletes labile iron pool to exploit iron uptake capacity of macrophage for its intracellular growth. Cell. Microbiol. 11, 83–94. doi:10.1111/j.1462-5822.2008.01241.x

de Oliveira, F.F.B., de Araújo, J.C.B., Pereira, A.F., Brito, G.A.C., Gondim, D.V., Ribeiro, R. de A., de Menezes, I.R.A., Vale, M.L., 2015. Antinociceptive and anti-inflammatory effects of Caryocar coriaceum Wittm fruit pulp fixed ethyl acetate extract on zymosan-induced arthritis in rats. J. Ethnopharmacol. 174, 452–463. doi:10.1016/j.jep.2015.08.017

de Oliveira, M.L.M., Nunes-Pinheiro, D.C.S., Tomé, A.R., Mota, É.F., Lima-Verde, I.A., Pinheiro, F.G. de M., Campello, C.C., de Morais, S.M., 2010. In vivo topical anti-inflammatory and wound healing activities of the fixed oil of Caryocar coriaceum Wittm. seeds. J. Ethnopharmacol. 129, 214–219. doi:10.1016/j.jep.2010.03.014

de Santana, F.R., Dalboni, L.C., Nascimento, K.F., Konno, F.T., Alvares-Saraiva, A.M., Correia, M.S.F., Bomfim, M.D.C., Casarin, R.C.V., Perez, E.C., Lallo, M.A., Peres, G.B., Laurenti, M.D., Benites, N.R., Buchi, D.F., Bonamin, L.V., 2017. High dilutions of antimony modulate cytokines production and macrophage – Leishmania (L.) amazonensis interaction in vitro. Cytokine 92, 33–47. doi:10.1016/j.cyto.2017.01.004

Doroodgar, M., Delavari, M., Doroodgar, M., Abbasi, A., Taherian, A.A., Doroodgar, A., 2016. Tamoxifen Induces Apoptosis of Leishmania major Promastigotes in Vitro. Korean J. Parasitol. 54, 9–14. doi:10.3347/kjp.2016.54.1.9

Fidalgo, L.M., Gille, L., 2011. Mitochondria and trypanosomatids: Targets and drugs. Pharm. Res. 28,

2758–2770. doi:10.1007/s11095-011-0586-3
Flannagan, R.S., Cosío, G., Grinstein, S., 2009. Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. Nat. Rev. Microbiol. 7, 355–366. doi:10.1038/nrmicro2128
Flannery, A.R., Huynh, C., Mittra, B., Mortara, R.A., Andrews, N.W., 2011. LFR1 Ferric Iron Reductase of Leishmania amazonensis Is Essential for the Generation of Infective Parasite Forms. J. Biol. Chem. 286, 23266–23279. doi:10.1074/jbc.M111.229674
Ghaffarifar, F., Esavand Heydari, F., Dalimi, A., Hassan, Z.M., Delavari, M., Mikaeiloo, H., 2015. Evaluation of Apoptotic and Antileishmanial Activities of Artemisinin on Promastigotes and BALB/C Mice Infected with Leishmania major. Iran. J. Parasitol. 10, 258–67.
Gozzelino, R., Jeney, V., Soares, M.P., 2010. Mechanisms of Cell Protection by Heme Oxygenase-1. Annu. Rev. Pharmacol. Toxicol. 50, 323–354. doi:10.1146/annurev.pharmtox.010909.105600
Gusman, G.S., Campana, P.R. V, Castro, L.C., Castilho, R.O., Teixeira, M.M., Braga, F.C., 2015. Evaluation of the Effects of Some Brazilian Medicinal Plants on the Production of TNF- α and CCL2 by THP-1 Cells. Evid. Based. Complement. Alternat. Med. 2015, 497123. doi:10.1155/2015/497123
Haddad, J.J., Fahlman, C.S., 2002. Redox- and oxidant-mediated regulation of interleukin-10: an anti-inflammatory, antioxidant cytokine? Biochem. Biophys. Res. Commun. 297, 163–76.
Hintze, K.J., Theil, E.C., 2005. DNA and mRNA elements with complementary responses to hemin, antioxidant inducers, and iron control ferritin-L expression. Proc. Natl. Acad. Sci. 102, 15048–15052. doi:10.1073/pnas.0505148102
Justino, A.B., Pereira, M.N., Vilela, D.D., Peixoto, L.G., Martins, M.M., Teixeira, R.R., Miranda, N.C., da Silva, N.M., de Sousa, R.M.F., de Oliveira, A., Espindola, F.S., 2016. Peel of araticum fruit (Annona crassiflora Mart.) as a source of antioxidant compounds with α-amylase, α-glucosidase and glycation inhibitory activities. Bioorg. Chem. 69, 167–182. doi:10.1016/j.bioorg.2016.11.001
Kedzierski, L., Curtis, J.M., Kaminska, M., Jodynis-Liebert, J., Murias, M., 2007. In vitro antileishmanial activity of resveratrol and its hydroxylated analogues against Leishmania major promastigotes and amastigotes. Parasitol. Res. 102, 91–97. doi:10.1007/s00436-007-0729-y
Kellner, A.W.A. 2016. Understanding the Cerrado biome, medicinal properties of a Piperaceae, and consequences of seasonal variation in Amazonian upland lakes. An. Acad. Bras. Cienc. 88, 2029–2030. doi:10.1590/0001-37652016884
Liew, F.Y., Li, Y., Millott, S., 1990. Tumor necrosis factor-alpha synergizes with IFN-gamma in mediating killing of Leishmania major through the induction of nitric oxide. J. Immunol. 145, 4306–10.
Lin, Q.S., Weis, S., Yang, G., Zhuang, T., Abate, A., Dennery, P.A., 2008. Catalytic inactive heme oxygenase-1 protein regulates its own expression in oxidative stress. Free Radic. Biol. Med. 44, 847–855. doi:10.1016/j.freeradbiomed.2007.11.012
Liu, D., Uzonna, J.E., 2012a. The early interaction of Leishmania with macrophages and dendritic cells and its influence on the host immune response. Front. Cell. Infect. Microbiol. 2, 83. doi:10.3389/fcimb.2012.00083
Mehta, A., Shaha, C., 2004. Apoptotic death in Leishmania donovani promastigotes in response to respiratory chain inhibition: Complex II inhibition results in increased pentamidine cytotoxicity. J. Biol. Chem. 279, 11798–11813. doi:10.1074/jbc.M309341200
Mittra, B., Cortez, M., Haydock, A., Ramasamy, G., Myler, P.J., Andrews, N.W., 2013. Iron uptake controls the generation of *Leishmania* infective forms through regulation of ROS levels. J. Exp. Med. 210, 401–416. doi:10.1084/jem.20121368
Mittra, B., Laranjeira-Silva, M.F., Miguel, D.C., Perrone Bezerra de Menezes, J., Andrews, N.W., 2017. The iron-dependent mitochondrial superoxide dismutase SODA promotes *Leishmania* virulence. J. Biol. Chem. 292, 12324–12338. doi:10.1074/jbc.M116.772624
Monzote, L., Gille, L., 2010. Mitochondria as a Promising Antiparasitic Target. Curr. Clin. Pharmacol. 5, 55–66. doi:10.2174/157488410790410605
Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.
Mosser, D.M., Zhang, X., 2008. Interleukin-10: new perspectives on an old cytokine. Immunol. Rev. 226, 205–18. doi:10.1111/j.1600-065X.2008.00706.x
Murray, H.W., Nathan, C.F., 1999. Macrophage microbicidal mechanisms in vivo: reactive nitrogen versus

oxygen intermediates in the killing of intracellular visceral Leishmania donovani. J. Exp. Med. 189, 741–6. doi:10.1084/jem.189.4.741

Myhre, O., Andersen, J.M., Aarnes, H., Fonnum, F., 2003. Evaluation of the probes 2',7'-dichlorofluorescin diacetate, luminol, and lucigenin as indicators of reactive species formation. Biochem. Pharmacol. 65, 1575–82.

Nogueira, F.A., Fonseca, L.D., da Silva, R.B., de Paiva Ferreira, A. V., Nery, P.S., Geraseev, L.C., Duarte, E.R., 2012. In vitro and in vivo efficacy of aqueous extract of Caryocar brasiliense Camb. to control gastrointestinal nematodes in sheep. Parasitol. Res. 111, 325–330. doi:10.1007/s00436-012-2843-8

Olivier, M., Gregory, D.J., 2005. Subversion mechanisms by which Leishmania parasites can escape the host immune response : a Signaling Point of View 18, 293–305. doi:10.1128/CMR.18.2.293

Paiva, C.N., Bozza, M.T., 2014. Are reactive oxygen species always detrimental to pathogens? Antioxid. Redox Signal. 20, 1000–37. doi:10.1089/ars.2013.5447

Paula-Ju, W. de, Rocha, F.H., Donatti, L., Fadel-Picheth, C.M.T., Weffort-Santos, A.M., 2006. Leishmanicidal, antibacterial, and antioxidant activities of Caryocar brasiliense Cambess leaves hydroethanolic extract. Rev. Bras. Farmacogn. 16, 625–630. doi:10.1590/S0102-695X2006000500007

Poljsak, B., Šuput, D., Milisav, I., an, Milisav, I., 2013. Achieving the balance between ROS and antioxidants: when to use the synthetic antioxidants. Oxid. Med. Cell. Longev. 2013, 956792. doi:10.1155/2013/956792

Qaisiya, M., Zabetta, C.C., Bellarosa, C., Tiribelli, C., 2013. Bilirubin mediated oxidative stress involves antioxidant response activation via Nrf2 pathway. Cell. Signal. doi:10.1016/j.cellsig.2013.11.029

Roy, A., Ganguly, A., BoseDasgupta, S., Das, B.B., Pal, C., Jaisankar, P., Majumder, H.K., 2008. Mitochondria-Dependent Reactive Oxygen Species-Mediated Programmed Cell Death Induced by 3,3′-Diindolylmethane through Inhibition of F0F1-ATP Synthase in Unicellular Protozoan Parasite Leishmania donovani. Mol. Pharmacol. 74.

Silva, S.S., Thomé, G. da S., Cataneo, A.H.D., Miranda, M.M., Felipe, I., Andrade, C.G.T. de J., Watanabe, M.A.E., Piana, G.M., Sforcin, J.M., Pavanelli, W.R., Conchon-Costa, I., 2013. Brazilian Propolis Antileishmanial and Immunomodulatory Effects 2013.

Smith, F.E., Herbert, J., Gaudin, J., Hennessy, D.J., Reid, G.R., 1984. Serum iron determination using ferene triazine. Clin. Biochem. 17, 306–310. doi:10.1016/S0009-9120(84)90613-1

Suffredini, I.B., Paciencia, M.L.B., Varella, A.D., Younes, R.N., 2007. In vitro cytotoxic activity of Brazilian plant extracts against human lung, colon and CNS solid cancers and leukemia. Fitoterapia 78, 223–226. doi:10.1016/j.fitote.2006.11.011

Torres, L.R. de O., Santana, F.C. de, Torres-Leal, F.L., Melo, I.L.P. de, Yoshime, L.T., Matos-Neto, E.M., Seelaender, M.C.L., Araújo, C.M.M., Cogliati, B., Mancini-Filho, J., 2016. Pequi (Caryocar brasiliense Camb.) almond oil attenuates carbon tetrachloride-induced acute hepatic injury in rats: Antioxidant and anti-inflammatory effects. Food Chem. Toxicol. 97, 205–216. doi:10.1016/j.fct.2016.09.009

Victorino, V.J., Panis, C., Campos, F.C., Cayres, R.C., Colado-Simão, A.N., Oliveira, S.R., Herrera, A.C.S.A., Cecchini, A.L., Cecchini, R., 2013. Decreased oxidant profile and increased antioxidant capacity in naturally postmenopausal women. Age (Omaha). 35, 1411–1421. doi:10.1007/s11357-012-9431-9

Zaidi, A., Singh, K.P., Ali, V., 2017. Leishmania and its quest for iron: An update and overview. Mol. Biochem. Parasitol. 211, 15–25. doi:10.1016/j.molbiopara.2016.12.004

CONCLUSÃO

No presente trabalho foi demonstrada a ação antileishmania de extratos provenientes de folhas *Caryocar coriaceum*. Sobre formas promastigotas, os extratos agem induzindo uma morte por mecanismos apoptose-like, já sobre macrófagos infectados com formas amastigotas, os extratos induzem a morte do parasito e disparam uma resposta antioxidante. Em conjunto, nossos dados demonstram extratos de *C. coriaceum* como potencial agente terapêutico contra LTA, no entanto, mais estudos são necessários.