MILENA MENEGAZZO MIRANDA SAPLA

ATIVIDADE ANTI-LEISHMANIA DO ÁCIDO CAURENÓICO E DA *TRANS* -CHALCONA:

EFEITO IMUNOMODULADOR E MECANISMO DE AÇÃO

Londrina
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Tese apresentada ao Programa de Pós-graduação em Patologia Experimental da Universidade Estadual de Londrina, como requisito à obtenção do título de Doutora em Patologia Experimental .

Orientador: Prof. Dr. Wander R. Pavanelli

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Londrina, 28 de setembro de 2017.

Dedico este trabalho aos meus amados Menegazzos, Mirandas e Saplas.

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Pablo Neruda

SAPLA, Milena Menegazzo Miranda. **Atividade Anti-Leishmania do Ácido Caurenóico e da *Trans*-Chalcona:** efeito imunomodulador e mecanismo de ação. 2017. 101 p. Tese de Doutorado (Pós-graduação em Patologia Experimental) – Universidade Estadual de Londrina, Londrina-PR, 2017.

RESUMO

Leishmanioses são doenças infecciosas causadas por protozoários do gênero *Leishmania* que exibem amplo espectro de apresentação clínica incluindo diferentes formas cutâneas e a forma visceral. Essa diversidade de manifestações é atribuída principalmente à diferença entre as espécies do parasito, o estado imunológico do hospedeiro. O tratamento atual da leishmaniose se baseia na eliminação das formas intracelulares, no entanto os fármacos disponíveis apresentam: eficácia variável, alto custo, dificuldade de administração e podem provocar graves efeitos colaterais. Sendo assim, é cada vez mais necessário a busca por novos compostos que possam ser uma alternativa terapêutica para o tratamento da leishmaniose. O ácido caurenóico (AC) e *trans*-chalcona (TC) são moléculas químicas isoladas de diferentes fontes naturais, e apresentam diversas propriedades biológicas, dentre elas: anti-inflamatória, antimicrobiana e antioxidante. Entretanto, não existem estudos que tenham verificado o efeito dessas moléculas sobre protozoários de *Leishmania* visando elucidar os principais mecanismos de ação e propriedades imunomoduladoras envolvidas. Neste sentido, o objetivo do presente estudo foi investigar o efeito *in vitro* do AC e TC sobre *Leishmania amazonensis,* bem como os mecanismos de morte envolvidos na eliminação do parasito. Previsões *in silico* mostraram que TC apresenta bom potencial para fármaco com alta biodisponibilidade oral e absorção intestinal. Para verificar a ação das moléculas sobre formas promastigotas, foram utilizados AC nas concentrações de 10-90 μM e TC em 2-12μM. A partir dos resultados obtidos, observou-se que ambos os tratamentos promoveram a eliminação das formas promastigotas. TC induziu morte por mecanismos apoptose-*like* tardio, devido à produção de espécies reativas de oxigênio, despolarização mitocondrial, exposição de fosfatidilserina e permeabilização da membrana do parasito. Em relação a ação antiamastigota intracelular, AC induziu perfil pró-inflamatório nos macrófagos infectados, com aumento nos níveis de óxido nítrico (NO), dependente de cNOS. Além disso, o AC induziu o aumento na produção de IL-1β e expressão do componente ativador de inflamassoma NLRP12. Esses resultados demonstram a capacidade leishmanicida de AC sobre amastigotas intracelulares por um mecanismo NLRP12 / IL-1β / cNOS / NO. Já o tratamento com TC promoveu a redução na produção de citocina inflamatória (TNF-α) e regulatórias (IL-10 e TGF-β) e das espécies reativas de oxigênio e NO. O tratamento com TC ainda induziu a expressão do fator nuclear eritróide 2 (Nrf2), responsável pela proteção celular em relação ao estresse oxidativo, modulando assim os níveis de ferro ligado a transferrina, colaborando para a eliminação do parasito. Com base nos dados obtidos, podemos inferir que ambos os compostos apresentam atividade anti-leishmania e imunomoduladora, sendo AC e TC moléculas promissoras para o desenvolvimento futuro de uma terapia eficaz e não tóxica para o tratamento da leishmaniose.

Palavras-Chave: Leishmaniose. *Trans*-chalcona. Ácido caurenóico. NLRP12. Ferro.

SAPLA, Milena Menegazzo Miranda. **Anti-Leishmania Activity of Caurenoic Acid and *Trans*-Chalcone:** immunomodulatory effect and mechanism of action. 2017. 101 p. Thesis (PhD degree in Experimental Pathology) – Universidade Estadual de Londrina, Londrina, 2017.

ABSTRACT

Leishmaniases are an infectious disease caused by protozoa of the genus *Leishmania* that exhibit a broad spectrum of clinical manifestations including different cutaneous and visceral form. This diversity of manifestations is mainly attributed to the difference between the species of the parasite and the immune response of the host.The current treatment of leishmaniasis is based on the elimination of intracellular forms, however the available drugs present: variable efficacy, high cost, the difficulty of administration and can cause serious side effects. Therefore, it is increasingly necessary to search for new compounds that may have a therapeutic alternative for the treatment of leishmaniasis. Kaurenoic acid (KA) and trans-chalcone (TC) are chemical molecules isolated from different natural sources, and have several biological properties, among them: anti-inflammatory, antimicrobial and antioxidant. However, there are no studies that have verified the effect of these molecules on *Leishmania* protozoa in order to elucidate the main mechanisms of action and immunomodulatory properties involved. In this sense, the objective of the present study was to investigate the in vitro effect of CA and TC on *Leishmania amazonensis*, as well as the mechanisms of death involved in parasite elimination. *In silico* predictions have shown that TC has good potential for a drug with high oral bioavailability and intestinal absorption. To verify the action of the molecules on promastigote forms, KA were used in concentrations of 10-90 μM and TC in 2-12 μM. From the obtained results, it was observed that both treatments promoted the elimination of the promastigote forms. TC induced death by late apoptosis-like mechanisms, due to the production of reactive oxygen species, mitochondrial depolarization, phosphatidylserine exposure and membrane permeabilization of the parasite. In relation to the intracellular antiamastigote action, KA-induced a proinflammatory profile in the infected macrophages, with an increase in cNOS-dependent nitric oxide (NO) levels. In addition, KA-induced increased production of IL-1β and expression of the inflammasome-activating component NLRP12. These results demonstrate the leishmanicidal ability of KA against intracellular amastigotes by triggering an NLRP12/IL-1β/cNOS/NO mechanism. On the other hand, TC treatment promoted a reduction in the production of inflammatory (TNF-α) and regulatory (IL-10 and TGF-β) cytokines and the reactive oxygen species and NO. The treatment with TC also induced the expression of erythroid nuclear factor 2 (Nrf2), responsible for cellular protection in relation to oxidative stress, modulating the levels of iron bound to transferrin, contributing to the elimination of the parasite. Based on the data obtained, we can infer that both compounds have anti-leishmania and immunomodulatory activity, being KA and TC promising molecules for the future development of an effective and non-toxic therapy for the treatment of leishmaniasis.

Key words: Leishmaniasis. *Trans*-chalcone. Kaurenoic acid. NLRP12. Iron.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

%	porcentagem
∆ψm	potencial de membrana mitocondrial
°C	graus celsius
µg	micrograma
µL	microlitros
µM	micromolar
ACL	do inglês *American Cutaneous Leishmaniasis*
ADMET	absorção distribuição excreção metabolização e toxicidade
Akt	do inglês *protein kinase b*
ANOVA	análise de variância
Amb	anfotericina B
APAF-1	do inglês *apoptotic protease activating fator 1*
ARE	do inglês *antioxidant-response elements*
B.O.D	do inglês *Biochemical Oxygen Demand*
BALB/c	camundongo de linhagem isogênica
BAX	do inglês *BCL2-associated X protein*
BCL2	do inglês *B-cell lymphoma 2*
BSA	do inglês *Bovine Serum Albumins*
C57BL/6	camundongo de linhagem isogênica
$CaCl_2$	cloreto de cálcio
$CaCO_2$	carbonato de cálcio
CC50	do inglês *cytotoxicity concentration 50%*
CIDEA	do inglês *Cell death–inducing DFFA-like effector A*
do inglês *Cell death-inducing DFFA-like effector A*	
cm^2	centímetros quadrados
cNOS	do inglês *constitutive Nitric Oxide Synthase*
CO_2	dióxido de carbono
COX_2	ciclooxigenase-2
CYP	do inglês *Cytochrome p*
DAB	do inglês *3,3'-diaminobenzidine*
DAMPS	do inglês *danger-associated molecular patterns*

DMSO	dimetilsulfóxido
DNA	do inglês *deoxyribonucleic acid*
DNDi	do inglês *Drugs for Neglected Diseases initiative*
DTH	do inglês *Delayed-tyte Hypersensitivity*
DTNs	Doenças Tropicais Negligenciadas
EIMS	do inglês *electron impact mass spectometry*
ELISA	do inglês *Enzyme-Linked Immunosorbent Assay*
EROs	espécies reativas de oxigênio
FBS	do inglês *Fetal Bovine Serum*
FDA	do inglês *Food and Drug Administration*
Fe^{2+}	íon ferro ferroso
Fe^{3+}	íon ferro férrico
FSC-H	do inglês *Forward SCatter Height*
g	grama
GP63	glicoproteína de 63 kDa
h	horas
H_2DCFDA	2’7’ diclorofluoresceína
H_2O_2	peróxido de hidrogênio
H-acc	do inglês *number of Hydrogen bond aceptor*
H-Don	do inglês *number of Hydrogen bond donor*
HEPES	ácido 4-(2-hidroxietil)piperazina-1-etanosulfónico
HepG2	linhagem celular humana de câncer fígado
HerG	do inglês *The human ether-a-go-go related gene*
HIV	do inglês *Human Immunodeficiency Virus*
HO-1	heme oxigenase -1
IC50	do inglês *inhibitory concentration* 50%
ICAM-1	do inglês *Intercellular Adhesion Molecule 1*
ICC	do inglês *Immunocytochemistry*
ICR	linhagem de camundongo não isogênico
IFN-γ	interferon gama
IL-10	interleucina 10
IL-12	interleucina 12
IL-13	interleucina 13
IL-1β	interleucina 1β
IL-4	interleucina 4
iNOS	do inglês *inducible nitric oxide synthase*

KA	do inglês Kaurenoic Acid
L.	*Leishmania*
LC	Leishmaniose cutânea
LCD	Leishmaniose cutânea difusa
LMC	Leishmaniose mucocutânea
L-NAME	do inglês *L-NG-Nitroarginine Methyl Ester*
Log-P	do inglês *Log of partition-coefficient*
Log-S	do inglês *Predicted aqueous solubility*
LPG	do inglês *Lipophosphoglycan*
LPS	lipopolissacarídeo
LTA	Leishmaniose Tegumentar Americana
$LTB_{4.}$	leucotrieno B_4
LV	Leishmaniose visceral
M	Molar
M1	macrófago tipo 1
M2	macrófago tipo 2
MCP-1	do inglês *Monocyte Chemoattractant Protein-1*
MDA	malondealdeído
MHz	mega-hertz
mL	mililitro
mm	milímetros
mM	milimolar
MM	massa molar
MTT	brometo de 3-(4,5-dimetiltiazol-2-il)-2,5- difeniltetrazólio
NAC	N-acetilcisteína
NADPH	nicotinamida adenina dinucleotídeo fosfato
$NaNO_2$	nitrito de sódio
NaOH	hidróxido de sódio
ND	do inglês *not determined*
NF-κB	do inglês *nuclear fator kappa B*
NLR	do inglês *Nod-like receptor*
Nlrp12	do inglês *NLR Family pyrin domain containing 12*
nm	nanômetro
nM	nanomolar
NMR	do inglês *Nuclear Magnetic Ressonance*
NO	do inglês *Nitric Oxyde*

Nrf2	do inglês *Nuclear factor erythoide 2*
OD	do inglês *Optical density*
PAMPS	do inglês *pathogen-associated molecular patterns*
PBS	do inglês *phosphate buffered saline*
PDA	do inglês Photodiode Array Detector
pg	picograma
PGE_2	prostaglandina E-2
PGLA	do inglês *Poly D, L-lactide-co-glycolide*
pH	potencial hidrogeniônico
PI	do inglês *Propidium iodide*
Pi3K	do inglês *Phosphoinositide 3-kinase*
PKDL	do inglês *Post-kala-azar dermal Leishmaniasis*
PLA	do inglês *Poly D, L-lactide acid*
PPAR α/γ	do inglês *Peroxisome proliferator activated receptor* α/γ
ppm	partes por milhão
PS	do inglês *Phosphatidylserine*
RB	do inglês *number of rotatable bonds*
RNS	do inglês *Reactive Nitrogen Species*
ROS	do inglês *Reactive Oxigen Species*
rpm	rotação por minuto
RPMI 1640	Meio de cultura (*Roswell Park Memorial Institute*)
SEM	do inglês *Scanning electron microscopy*
SI	do inglês *Selectivity Index*
SINAN	Sistema de Informação de Agravos de Notificação
SOD	superóxido dismutase
STAT3	do inglês *signal transducers and activators of transcription 3*
T.	*Trypanosoma*
TC	*trans*-chalcona
$TCD4^+$	linfócitos T auxiliares
$TCD8^+$	linfócitos T citotóxico
TEP	do inglês *Tetraethoxypropane*
TGF-β	do inglês *Transforming growth factor-beta*
TH1	do inglês *T-helper 1*
TH2	do ingês *T-helper 2*
TMRE	do inglês *Tetramethylrhodamine,ethyl ester*

TNF-α	do inglês *Tumor necroses fator alpha*
tPSA	do inglês *molecular polar surface area*
TRAP	do inglês *Total antioxidante capacity of plasma*
Treg	linfócitos T reguladores
V.	*Viannia*
Vegf	do inglês *Vascular endotelial growth factor*
VLC	do inglês *Vacuum liquid chromatograpy*
WHO	do inglês *World Health Organization*

SUMÁRIO

1 INTRODUÇÃO

1.1 Formas clínicas e epidemiologia das leishmanioses

Leishmanioses fazem parte de um grupo de doenças causadas por mais de vinte espécies de protozoários flagelados do gênero *Leishmania* os quais são transmitidos ao homem por meio da picada de insetos flebotomíneos. São reconhecidas pela WHO como doenças tropicais negligenciadas, uma vez que acometem principalmente populações economicamente desfavorecidas de regiões tropicais, não despertando, assim, o interesse das indústrias farmacêuticas em relação à pesquisa e desenvolvimento de novos medicamentos (FDA, 2014).

As doenças possuem quatro formas clínicas principais: cutânea (LC), cutânea difusa (LCD), mucocutânea (LMC) e visceral (LV), as quais tendem a variar entre e dentro das regiões, refletindo a diferença entre as espécies do parasito, o ciclo epidemiológico, o estado imunológico, bem como a resposta determinada geneticamente pelo hospedeiro (Murray et al., 2005; Reithingher et al., 2007).

Leishmaniose cutânea é a forma mais comum da doença, sendo determinada pelo surgimento de úlceras únicas ou múltiplas na derme, localizada geralmente na região exposta do tegumento, tipicamente caracterizadas por bordas elevadas, contornos regulares e leito da lesão granuloso e com presença ou não de exsudato purulento. A infecção cutânea pode permanecer subclínica ou tornar-se clinicamente aparente após período variável de incubação, que dura em média várias semanas (Machado et al., 2002; Magill, 2005).

A Leishmaniose cutânea difusa é uma variação da LC, caracterizada pela cronicidade e refratariedade ao tratamento devido à anergia do paciente. Caracteriza-se pelo maciço comprometimento dérmico, com formação de lesões difusas não ulceradas, distribuídas amplamente na pele, assumindo caráter crônico e progressivo que persiste por toda a vida do paciente (Ashford, 2000).

A Leishmaniose mucocutânea é considerada mais agressiva e caracteriza-se pela habilidade do parasito de se disseminar (metástase) para tecidos da mucosa oral e/ou nasal por vias linfáticas ou hematogênicas, resultando em lesões destrutivas secundárias de curso crônico, envolvendo nariz, faringe, boca e laringe (Barral et al., 1991; Modabber, 1993). Estima-se que 3 a 5% dos casos de LC

desenvolvam lesão mucosa, visto que a forma clássica de LMC é secundária à lesão cutânea, devido à má resolução da doença ou por terapia inadequada. Ao contrário da LC, a LM nunca evolui para cura espontaneamente, apresenta dificuldades de responder ao tratamento e é potencialmente fatal (Marsden, 1986; Goto;Lindoso, 2010)

A Leishmaniose visceral, também conhecida por calazar, é fatal se não for tratada. É caracterizada por febre alta irregular, perda de peso, aumento do baço e fígado, e anemia embora 5% dos infectados não apresentem qualquer sintomatologia (Hashim et al.,1994).

Segundo a Organização Mundial de Saúde (2016), 102 países, áreas e territórios são endêmicos para as leishmanioses. Estima-se que 0,7 à 1,3 milhão de novos casos de LC ocorram anualmente no mundo e 200 a 400 mil de casos de LV. Cerca de 95% dos casos de LC ocorrem nas Américas, na bacia do Mediterrâneo, no Oriente Médio e Ásia Central. Em relação a LV, mais de 90% dos novos casos ocorrem em Bangladesh, Brasil, Etiópia, Índia e Sudão (Alvar et al.,2012, WHO, 2015).

Outro fator alarmante na epidemiologia das leishmanioses refere-se ao número de pessoas co-infectadas com *Leishmania*-HIV, principalmente pacientes acometidos pela LV, as quais apresentam elevadas taxas de recaída e mortalidade. A infecção concomitante por HIV aumenta 100 e 2320 vezes o risco de desenvolver LV ativa. No sul da Europa, até 70% dos casos de leishmaniose visceral em adultos estão associados com a infecção pelo HIV. No Brasil, a incidência de co-infecção por LV/HIV aumentou de 0,32 casos/100.000 habitantes em 2007 para 1,08 casos/100.000 habitantes em 2010 em um estudo no Norte do Brasil (Albuquerque et al., 2014). Outro estudo recente, mostrou que 51% dos 90 pacientes com LV diagnosticados em um centro de referência urbana no Estado de Minas Gerais - Brasil confirmaram co-infecção com HIV (Lindoso et al., 2014).

LC é causada por várias espécies do parasito e de acordo com a distribuição geográfica pode ser dividida em LC do Velho Mundo, muitas vezes benigna e auto-limitada, presente no Sudeste Europeu, Sul da Europa, do Oriente Médio, partes do sudoeste da Ásia, Ásia Central e África, sendo causada principalmente pelas espécies *L. aethiopica, L. donovani, L. infantum, L. major* e *L. tropica*. A LC do Novo Mundo ocorre no México e América Latina, sendo conhecida como Leishmaniose Tegumentar Americana (LTA) podendo causar um amplo

espectro de manifestações, desde benignas a doença grave, incluindo o envolvimento de mucosas. É causada por múltiplas espécies do subgênero *Leishmania*: *L. amazonensis, L. infantum, L. mexicana, L. venezuelensis* e subgênero *Viannia*: *L. braziliensis, L. shawi, L. guyanensis, L. panamensis, L. peruviana* (WHO,2015).

LMC é causada no Novo Mundo está mais comumente associada à espécie *L. braziliensis*, portanto, salvo exceções, a LMC é principalmente encontrada na América do Sul (Reithinger et al., 2007). Esta forma clinica pode também ser causada por *L. panamensis, L. guyanensis, L. amazonensis* e *L. infantum* (Aliaga et al., 2003). A LCD é a forma mais rara, causada principalmente pelas espécies *L. mexicana*, *L. amazonensis* e *L. tropica* (Gontijo; Carvalho, 2003).

O Brasil é considerado a área endêmica de maior extensão territorial no continente para LTA e um dos países com as mais elevadas taxas de notificação da doença. No período entre 2000 e 2013, foram registrados 345.722 casos de LTA no país (SINAN, 2015). Em 2013, a região Norte representou 46,4% dos casos, seguida pelas regiões Nordeste (29,5%), Sudeste (6,3%), Centro-Oeste (16,1%) e Sul (1,6%) (SINAN, 2015). Atualmente está confirmada em todos os estados brasileiros tendo como as principais espécies circulantes: *L. braziliensis, L. amazonensis* e *L. shawi*, esta última encontrada apenas na região amazônica (Ministério da Saúde, 2007).

No Paraná, a LTA é endêmica com notificação em 276 dos 399 municípios, principalmente das regiões norte e oeste. No estado, a doença vem sendo notificada desde 1980 com número crescente de casos. Em 2013, dados do Sistema de Informação de Agravos de Notificação (SINAN, 2015), confirmam 528 casos notificados (Ministério da Saúde/SVS - Sistema de Informação de Agravos de Notificação - Sinan Net, 2017).

Apesar dos avanços nos estudos acerca do parasito, bem como dos conhecimentos sobre a doença, a LTA constitui um problema de Saúde Pública, não apenas por sua alta incidência, ampla distribuição e grande complexidade, mas também devido à possibilidade do aparecimento de lesões destrutivas, desfigurantes e até mesmo incapacitantes (Gontijo; Carvalho, 2003; Lonardoni et al., 2006; Monteiro et al., 2008). Além disso, vale ressaltar que as medidas de controle, incluindo a atual terapia contra a LTA ainda são insatisfatórias, devido principalmente à limitada

eficácia, tratamento prolongado, alto custo e toxicidade (Chen et al., 2001; Croft; Coombs, 2003; Singh; Sivakumar, 2004; Richard; Werbovetz, 2010).

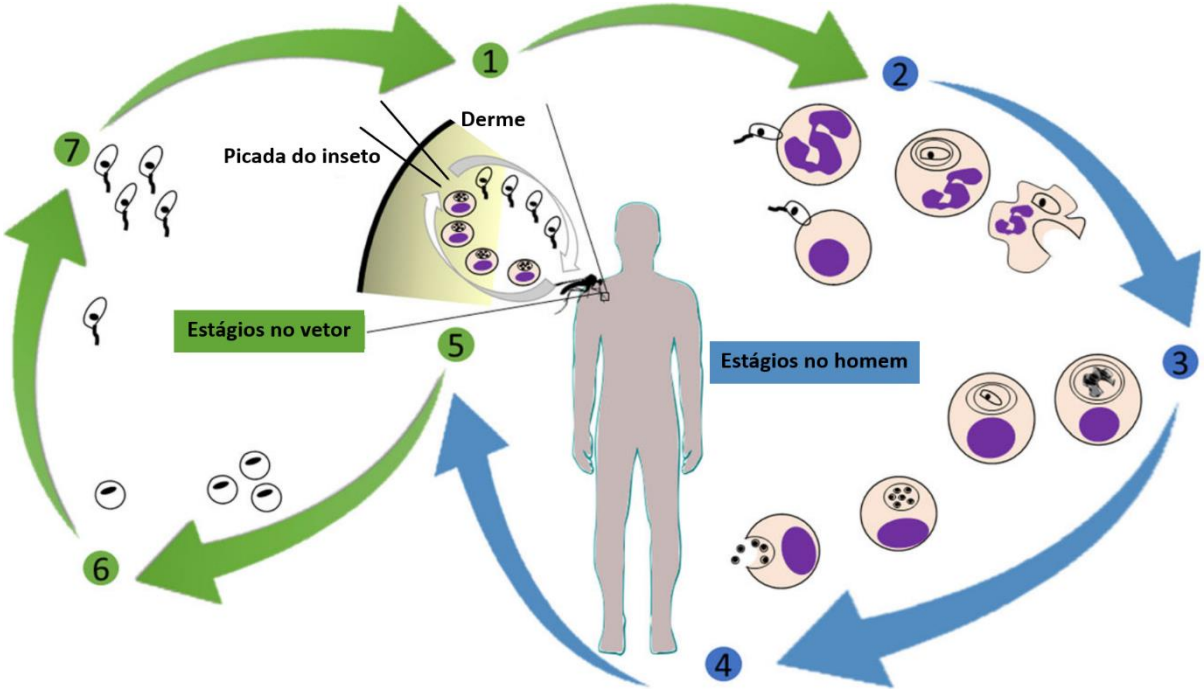
1.2 Agente etiológico e ciclo biológico

A Leishmaniose é causada por diferentes espécies de protozoários que se incluem na ordem Kinetoplastida, na família Trypanosomatidae e no gênero *Leishmania* Ross 1903. São parasitos unicelulares, com ciclo de vida heteroxeno, em que os hospedeiros invertebrados são fêmeas hematófagas do gênero *Phlebotomus* - no Velho Mundo e *Lutzomyia* - no Novo Mundo. O hospedeiro invertebrado é determinante para a epidemiologia da doença, uma vez que a presença do inseto vetor é decisiva na disseminação do parasito e está estritamente relacionada com as condições climáticas típicas das regiões tropicais. Por outro lado, os hospedeiros vertebrados são os humanos e outras espécies de mamíferos silvestres e domésticos (WHO, 2010).

No Brasil já foram identificadas sete espécies do parasito causadoras de LTA, sendo seis do subgênero *Viannia*: *L. braziliensis*, *L. guyanensis, L. lainsoni*, *L. naiffi, L. lindenberg, L. shawi* e uma espécie do subgênero *Leishmania: L. amazonensis* (Ministério da Saúde, 2007). No estado do Paraná, somente *L. amazonensis* e *L. braziliensis* foram relatadas (Silveira et al., 1990; Luz et al., 2000).

Este parasito apresenta ciclo biológico heteroxêno com morfologia variável durante o ciclo. No inseto, apresenta-se como promastigota, com flagelo livre na porção anterior do corpo celular alongado. Já no hospedeiro vertebrado, após a internalização de promastigotas por células do sistema monocítico fagocitário, o pH ácido do vacúolo parasitóforo aliado à temperatura do hospedeiro vertebrado auxiliam a diferenciação das promastigotas em amastigotas intracelulares. Essas formas apresentam um flagelo atrofiado na região anterior do corpo arredondado do parasito (Cunningham, 2002).

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, 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.

Ao picar o indivíduo ou animal parasitado, os flebotomíneos ingerem juntamente com o sangue ou a linfa, macrófagos periféricos infectados com formas amastigotas de *Leishmania* sp. Uma vez no tubo digestório do inseto, essas formas são liberadas e se diferenciam em promastigotas procíclicas, formas delgadas com flagelo típico que emerge da extremidade anterior. Essas formas se multiplicam por divisão binária e colonizam o tubo digestório do vetor. Quando atingem grande número, diferenciam-se em formas promastigotas metacíclicas infectivas, em um processo chamado de metaciclogênese migrando para as porções anteriores do estômago e proventrículo do vetor. No próximo repasto sanguíneo, ocorre a

regurgitação destas formas infectivas em um novo hospedeiro vertebrado, as quais são fagocitadas principalmente por macrófagos, formando vacúolos parasitóforos no qual se diferenciam na forma amastigota e as que evadem dos mecanismos microbicidas, multiplicam-se recomeçando o ciclo (Ponte-Sucre, 2003; CDC, 2015) (**Figura 1**).

1.3 Interação parasito-hospedeiro e resposta imune na LTA

O processo de instalação da LTA é determinado por uma complexa associação, principalmente entre os fatores relacionados à virulência do parasito e a resposta imunológica do hospedeiro. Tudo tem início quando formas promastigotas metacíclicas, inoculadas na derme do hospedeiro, interagem com proteínas do soro, sistema complemento, saliva e fluidos digestivos do inseto, assim como com os receptores celulares. Nesta fase inicial da infecção, um dos grandes desafios do parasito é estabelecer residência no interior dos fagócitos, que compõem a primeira linha de defesa na leishmaniose, sem que estes disparem seus mecanismos de defesas (Genaro, 2005).

É sabido que no local da picada do inseto, ocorre uma rápida e intensa infiltração de neutrófilos, os quais são as primeiras células “hospedeiras” do parasito (Laskay et al., 2008; Peters et al., 2008). Um recrutamento substancial de monócitos/macrófagos, células hospedeiras definitivas para este protozoário, compõem uma segunda onda de células infiltrantes, e após dois dias de infecção, os parasitos são encontrados principalmente dentro de células monocíticas (van Zandbergen, 2004; Ribeiro-Gomes et al., 2004).

Neutrófilos apresentam papel complexo e multifacetado durante as fases da infecção. Ribeiro-Gomes e Sacks (2012) sugeriram que a influência da resposta dos neutrófilos no desfecho infecção depende criticamente sobre o momento do seu recrutamento e ao tecido infectado. Estas células têm como principal função fagocitar a grande maioria (80 a 90%) dos parasitos presentes, e produzir quimiocinas e citocinas que recrutam e ativam diferentes tipos celulares e influenciam o desenvolvimento da resposta imune adaptativa durante a infecção por *Leishmania* sp. (Scapini et al., 2000; Peters et al., 2008).

A atividade leishmanicida de neutrófilos *in vitro* sugere que estas células podem representar um componente importante da resposta imune inicial contra as formas promastigotas de *L. amazonensis* (Guimaraes-Costa et al., 2009; Carlsen et al., 2013). No entanto, tal como evidenciado pela persistência do parasito no local da infecção, esta ação leishmanicida de neutrófilos é claramente insuficiente para controlar o estabelecimento da infecção e o desenvolvimento da doença clínica (Carlsen et al.,2015).

Alguns estudos já demonstraram que formas promastigotas de *Leishmania* spp. podem sobreviver por períodos prolongados em neutrófilos sem sofrer danos letais (Laufs et al.,2002; Mollinedo et al.,2010). Os neutrófilos que não são capazes de eliminar o parasito podem atuar como "cavalos de Tróia" sendo prontamente internalizados pelos fagócitos vizinhos (como macrófagos ou células dendríticas) por vias que não desencadeiam mecanismos de defesa destas células, aumentando a infectividade e persistência do parasito (van Zandbergen, 2004; Ribeiro-Gomes et al., 2012).

Em relação aos macrófagos, uma vez recrutados, fagocitam parasitos livres e neutrófilos infectados, tornando-se as células hospedeiras definitivas para a replicação do parasito, bem como efetores responsáveis pela destruição deste protozoário.

No entanto, estas células podem ser ativadas por diferentes sinais que levam ao desenvolvimento de respostas funcionalmente distintas com diferentes resultados da doença. Assim, a ativação efetiva de macrófagos através IFN-γ é crucial para a eliminação deste patógeno intracelular e desencadeamento do "burst" oxidativo durante o processo de fagocitose do parasito. Neste processo, a célula hospedeira aumenta intensamente a sua atividade respiratória liberando espécies reativas de oxigênio (superóxidos, peróxido de hidrogênio e radicais hidroxila) bem como o óxido nítrico (NO), moléculas com elevada capacidade microbicida (Qadoumi *et al*., 2002; Cunningham, 2002).

Em contraste, a ativação de macrófagos pelas citocinas IL-4 e IL-13 induz a biossíntese de poliaminas através da ativação da enzima arginase, favorecendo a sobrevivência do parasito nos macrófagos infectados e a progressão da doença (Launois et al*.,* 2002; Awasthi; Mathur; Saha, 2004, Kropf et al., 2005).

A fim de sobreviver dentro dos macrófagos, esses tripanossomatídeos desenvolveram mecanismos sofisticados para subverter funções microbicidas destas células. As formas metacíclicas são altamente resistentes à lise mediada pelo sistema complemento, devido principalmente à presença das moléculas de lipofosfoglicanos (LPG) em sua superfície (Sacks et al*.,* 1995). Além disso, esta molécula protege o protozoário contra a ação dos radicais livres gerados no "burst" oxidativo e retarda a fusão do vacúolo parasitóforo com o vacúolo lisossomal, permitindo que a forma promastigota diferencie-se em amastigota (Awasthi; Mathur; Saha, 2004; Rodríguez et al.,2011)

A presença da glicoproteína de peso molecular 63 kDa (gp63) no glicocálice do parasito promove a degradação das enzimas lisossomais presentes nos vacúolos parasitóforos, permitindo a sobrevida dos parasitos, seu desenvolvimento e multiplicação no interior dos macrófagos (Cunningham, 2002).

Em relação à sintese de NO, já foram descritos mecanismos de como estes parasitos são capazes de modular negativamente a produção do principal elemento microbicida. O protozoário *Leishmania* dispõe da enzima arginase, que atua no seu próprio metabolismo, e compete juntamente com a enzima óxido nítrico sintase induzível (iNOS) pelo aminoácido arginina (Wanasen; Soong 2008; da Silva et al.,2012a). A proteína de membrana cinetoplastida-11, tem sido descrita como um análogo estrutural de NG-monometil-L-arginina, um inibidor bem conhecido da síntese de NO que também atua por competição com L-arginina (Jardim et al.,1995). Calegari-Silva et al. (2015), demonstraram que *L. amazonensis* é capaz modula a síntese de NO através da ativação da via fosfatidilinositol-3-cinase/proteína AKT (PI3K/Akt) que induz a formação do homodímero repressor transcricional do fator nuclear-κ*B* (NF-κB) regulando negativamente a enzima iNOS.

O estabelecimento do parasito no hospedeiro, associado ao desenvolvimento da resposta imune adaptativa, são fatores que estão intimamente relacionados com o quadro clínico da leishmaniose. Desta forma, para se entender melhor os fatores que conferem os fenótipos de resistência ou susceptibilidade à infecção por *Leishmania* sp*.,* estudos mais detalhados sobre a imunidade tem sido realizados em modelos murinos.

Camundongos C57BL/6 infectados por *L. major* são resistentes à infecção, enquanto camundongos da linhagem BALB/c são susceptíveis (Sacks;

Noben-Trauth, 2002). É sabido que a resistência apresentada pelos camundongos C57BL/6 é estabelecida pela ativação preferencial da subpopulação de linfócitos Th1, que por sua vez produz várias citocinas, principalmente INF-γ e TNF-α, que em conjunto levam a ativação dos macrófagos, aumento na atividade da enzima iNOS, com consequente aumento na produção de NO, seguido de eliminação do parasito (Cunningham, 2002; Qadoumi *et al*., 2002). Já em BALB/c, a ativação preferencial da subpopulação Th2, com produção de IL-4 e IL-13 leva à suscetibilidade à infecção (Launois et al*.,* 2002; Awasthi; Mathur; Saha, 2004).

Contudo, embora a resposta Th1 esteja relacionada ao fenótipo de resistência, o aparecimento de lesões leishmanióticas típicas estão diretamente relacionadas a uma exacerbação desta resposta, com participação importante no dano tecidual (Pirmez et al*.,* 1993; Lessa et al*.,* 2001; Laskay et al*.,* 2008; Peters et al., 2008). O espectro imunológico observado em pacientes com leishmaniose varia de indivíduos com resposta de células tipo TH1 exacerbada, caracterizada por reação de hipersensibilidade tardia (DTH), altos níveis de IFN-γ e aumento no número de células T citotóxicas $CD8^+$ como na forma localizada LCL à indivíduos com células T pouco responsivas, altos níveis de anticorpos e IL-10, encontrados na forma clínica LCD (Revisão de Scott; Novais, 2016). Em LMC, a capacidade de resposta das células T ao antígeno de *Leishmania* aumenta ainda mais para o pólo de DTH, resultando em danos nos tecidos, mas baixa carga parasitária (Scott; Novais, 2016) (**Figura 2**).

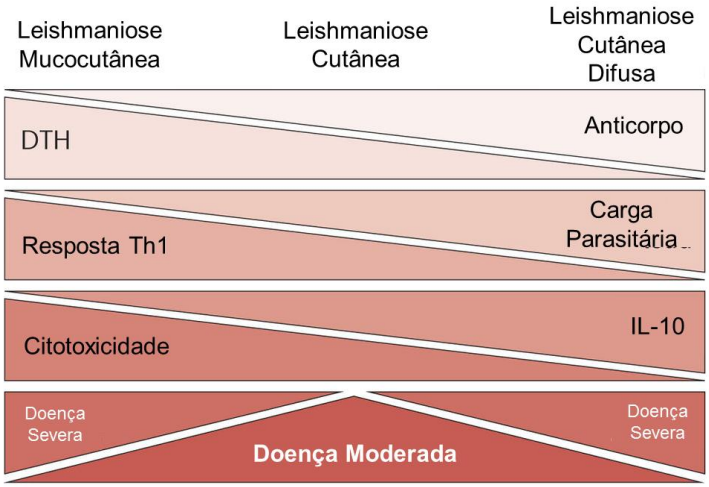
Vale ressaltar que além do perfil Th1/Th2, tem sido demonstrado nos últimos anos o papel importante das células Th17 e T reguladoras (Tregs) nas infecções por *Leishmania* spp*.* Células Th17 possuem propriedades pró-inflamatórias, e secretam principalmente IL-17. Esta citocina atua principalmente no recrutamento e ativação de neutrófilos, o que contribui para o agravamento da doença (Lopez-Kostka *et al*., 2009). Camundongos BALB/c, considerados susceptíveis à infecção por *Leishmania* spp., produzem níveis mais elevados de IL-17 após a infecção com *L. major*. De fato, foi observado que em camundongos BALB/c com mutação pontual para IL-17 houve uma redução no número de neutrófilos, no tamanho das lesões, e na quantidade de parasitos (Lopez Kostka et al*.,* 2009).

Já com relação às células T reguladoras (Tregs), tem sido verificado que em modelos murinos de leishmaniose cutânea causada por *L. major* e *L. amazonensis,* essas células favorecem a eliminação do parasito e o controle do

desenvolvimento da lesão, mesmo em camundongos susceptíveis (Belkaid et al., 2002; Mendez et al., 2004; Aseffa et al., 2002; Xu et al., 2003; Sousa et al., 2014). Este papel protetor das Tregs reforçado, uma vez que a redução da população destas células no início da infecção esta correlacionada com o aumento da resposta inflamatória, e consequente agravamento da lesão na leishmaniose cutânea (Barth et al., 2012).

De fato, a complexa interação parasito-hospedeiro nas infecções experimentais de linhagens de camundongos com *L. amazonensis* diferem em muitos aspectos das causadas por *L. major*. A maioria das cepas de camundongos infectados por *L. amazonensis* desenvolve lesão, caracterizando um padrão de resposta bastante distinto do observado em infecção por *L. major* e ausência de polarização para o perfil Th1 (Osorio y Fortea et al., 2007; Pereira; Alves, 2008; Silveira et al., 2009). Além disso, uma resposta dominante do tipo Th2 raramente se desenvolve nos camundongos susceptíveis à *L. amazonensis* (Afonso; Scott, 1993; Lemos de Souza *et al*., 2000).

Figura 2 - Espectro da Leishmaniose varia de níveis elevados de imunidade mediada por células a níveis elevados de anticorpos.



Fonte: Adaptado de SCOTT; NOVAIS, 2016. Espectro das formas clinicas da leishmaniose cutânea humana. O espectro imunológico observado em pacientes com leishmaniose varia de indivíduos com forte resposta de células T, caracterizados por reação de hipersensibilidade tardia (DTH), níveis elevados de IFNγ e um aumento do número de células T citotóxicas $CD8^+$ à indivíduos que não possuem resposta DTH, mas apresentam

altos títulos de anticorpos e níveis elevados de IL-10. Entre esses extremos estão os pacientes que desenvolvem lesões que podem se auto curar ou tornar-se crônica, apresentando níveis intermediários de respostas de células T e anticorpos.

1.4 Morte Celular

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).

Os processos de morte celular podem ser classificados de acordo com os aspectos funcionais (patológica, fisiológica, programada ou acidental), critérios enzimáticos (com envolvimento ou não de nucleases ou proteases), alterações morfológicas ou características imunológicas (imunogênica ou não-imunogênica). Dentre os tipos de morte celular destacam-se a apoptose, necrose e autofagia (Menna-Barreto; de Castro, 2015).

A apoptose representa um tipo de morte celular programada, desencadeada por fatores imunológicos, externos, tóxicos, infecciosos ou, ainda, pela própria necessidade de controle de crescimento e substituição celular (Andrade, 2003). Esse processo de morte celular é caracterizado por alterações como: arredondamento célula, redução do volume celular (picnose), condensação da cromatina, formação de bolhas na membrana plasmática (*blebbing*), externalização de fosfatidilserina, perda do potencial de membrana mitocondrial ($\Delta\Psi m$), e fagocitose por macrófagos residentes sem desencadeamento de resposta inflamatória. Pouca ou nenhuma modificação ultraestrutural das organelas citoplasmáticas é observada na apoptose (Jimenez-Ruiz et al.,2010). Estas alterações, independente do estímulo, são decorrentes da ativação de caspases que apresentam importância fundamental no processo de apoptose (Pereira, 2006).

Em tripanossomatídeos foi decrito um processo que compartilha aspectos morfológicos e moleculares da apoptose de metazoários. 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), sugerindo que a apoptose em tripanossomatídeos

é um caminho independente de caspase e Bcl-2, por isso o processo de morte celular programada nestes organismos é chamado de apoptose-like (Revisão de Menna-Barreto; de Castro, 2015).

A necrose, outro tipo de morte celular, tem como os principais contribuintes da sua propagação e execução o cálcio e as espécies reativas de oxigênio. Estes, direta ou indiretamente causam diversos danos aos constituintes celulares, resultando em ruptura de organelas e afetando a integridade celular. A morte celular por necrose apresenta algumas peculiaridades como aumento do volume celular, agregação da cromatina, desorganização do citoplasma, perda da integridade da membrana plasmática e ruptura da célula (Rodrigues; Seabra; De Souza, 2006, Kroemer et al., 2009, Jiménez-Ruiz et al., 2010). Estas alterações, independente do estímulo, são decorrentes das alterações lisossomais, que perdem a capacidade de conter as hidrolases (proteases, lipases, glicosidases, nucleases) no seu interior. Estas enzimas livres no citosol são ativadas e iniciam o processo de autólise, degradando praticamente todos os substratos celulares (Pereira, 2006). Durante o processo necrótico ocorre liberação do conteúdo celular com consequentes danos às células vizinhas e reação inflamatória local (Ziegler; Groscurth, 2004). Além disso, tanto em metazoários quanto em tripanossomatídeos, a morte celular necrótica é amplamente identificada pela ausência de marcadores apoptóticos (Menna-Barreto; de Castro, 2015).

A autofagia constitui um tipo de morte celular regulada, entretanto, na maioria dos casos, este processo é uma resposta citoprotetora ativada em células que estão sofrendo algum estresse (Galluzzi et al., 2012; Proto et al., 2013). Durante este processo, a célula recicla os seus componentes intracelulares. Quando a autofagia é induzida, ocorre a formação de uma dupla membrana ao redor do conteúdo celular, resultando na formação de uma estrutura denominada autofagossomo. Em seguida, ocorre a fusão do autofagossomo ao lisossomo, originando o autofagolisossomo, e no interior desta estrutura, o conteúdo celular é reciclado em componentes reutilizáveis, através da ação de hidrolases ácidas lisossomais (Kiel, 2010; Jain et al., 2013).

Os metabólitos gerados por este processo de degradação são liberados no citosol para que entrem novamente no processo metabólico celular (Jain et al., 2013). Uma característica deste tipo de morte celular é a ocorrência de maciça

vacuolização autofágica no citoplasma da célula (Smirlis et al., 2010; Galluzzi et al., 2012).

1.5 Tratamento

O tratamento atual da Leishmaniose se baseia na eliminação das formas intracelulares (amastigotas) de *Leishmania* sp. Contudo, a localização intramacrofágica dificulta a atuação dos fármacos, bem como a presença de mediadores do efluxo de fármacos do interior do parasito, provocando resistência dos mesmos (Croft; Sundar; Fairlamb, 2006; Rodrigues et al*.,* 2006).

As formas de tratamento baseiam-se, desde a década de 1940, no uso de antimoniais pentavalentes, como o antimonato de *N*-metil glucamina (Glucantime®) e o estibogluconato de sódio (Pentostam®) como fármacos de primeira escolha tanto para o tratamento da LC quanto da LV. Esses medicamentos são de administração parenteral obrigatória por pelo menos 20 dias, apresentam eficácia variável, alto custo e podem provocar graves efeitos colaterais como: mialgia, artralgia, aumento sérico das enzimas hepáticas, pancreatite, disfunção gastrintestinal, dores musculares difusas, enrijecimento das articulações, arritmias, pancitopenia, insuficiência renal reversível e cardiotoxicidade (Singh; Sivakumar, 2004; Sundar; Chakravarty, 2014). Aliado a isso, existem relatos de a cura clínica não ser acompanhada de cura parasitológica, pois tem sido observados parasitos em cicatrizes de pacientes após tratamento (Lucumi et al*.,* 1998; Rath *et al*., 2003; Croft; Barrett; Urbina, 2005).

Embora o uso de antimonias pentavalentes esteja em vigor a mais de 70 anos, o mecanismo de ação desses compostos permanece um enigma (Sundar; Chakravarty, 2014). Acredita-se que a ação ocorra por inibição de adenosina (ATP) e guanosina trifosfatos (GTP ou AGP) (Berman, 1988), através do bloqueio da atividade glicolítica e oxidativa de ácidos graxos de amastigotas, diminuindo a capacidade de fosforilação de ADP a ATP culminando na depleção do ATP intracelular (Berman, 1988; Koff; Rosen, 1994). Outra hipótese compreende a possível conversão do antimonial pentavalente, uma espécie de pró-fármaco, à forma trivalente (SbIII) ativa (Sereno et al., 1998; Kothari et al., 2007; Lima et al., 2007).

Os fármacos de segunda escolha incluem Anfotericina B e Pentamidina. Além destes, Miltefosina e a Paramomicina também tem sido usados como alternativas nos casos de resistência aos antimoniais, mas não possuem um

índice terapêutico tão favorável e também apresentam várias reações adversas (Bray et al., 2003; Berman, 2006).

Outra variável que influencia na descoberta de novos fármacos para o tratamento da LTA é a diversidade de espécies de *Leishmania* no Brasil, o que configura quadros clínicos variados, com respostas terapêuticas diversas. Além disso, a desnutrição e as co-infecções (*Plasmodium* sp, HIV, etc.) aumentam as taxas de mortalidade desta patologia (Paredes et al., 2003).

Desta forma, ao avaliarmos o atual cenário do tratamento da LTA, é possível identificar a necessidade da pesquisa de novos fármacos, com estrutura e mecanismos de ação distintos dos compostos disponíveis, que apresentem além da ação leishmanicida mais eficaz e menos tóxica ao paciente, propriedades anti-inflamatórias e antioxidantes importantes para a resolução desta doença.

1.6 Produtos Naturais como Alternativa Terapêutica

O uso de produtos naturais com propriedades terapêuticas é tão antigo quanto a própria humanidade e por um longo tempo o uso de plantas em forma de chás, tinturas, pós e cataplasmas foram utilizados como compostos terapêuticos (Rates, 2001; Maciel et al., 2002; Balunas; Kinghorn, 2005).

É crescente o interesse em terapias de produtos naturais derivados de plantas, pois muitas vezes a medicina convencional pode ser ineficiente com muitos efeitos colaterais assim como no uso abusivo e incorreto de fármacos sintéticos, além do alto custo ao consumidor, sendo as plantas de fácil acesso a população (Rates, 2001).

O estudo dos componentes vegetais constitui um campo imenso de novos conhecimentos científicos, podendo contribuir para o aprimoramento da medicina tradicional. As vantagens para desenvolvimento de pesquisas farmacológicas com plantas são de grande alcance social, permitindo que as informações sejam repassadas à população (Maciel et al., 2002).

1.6.1 Ácido Caurenóico

O ácido caurenóico (AC) ou ácido ent-kaur-16-em-19-oic é um diterpeno do tipo caurano com diversas atividades biológicas já comprovadas, dentre elas ação antiparasitária (Batista, Chiari; de Oliveira, 1999; Izumi et al., 2012; Santos

et al., 2013), antimicrobiana (Yatsuda et al., 2005; Ambrosio et al., 2008), antifúngica (Santos, 2005; Baccarin et al., 2009), antinociceptiva (Baccarin et al., 2009), vasorrelaxante e hipotensiva (Tirapelli et al., 2004; Tirapelli et al., 2010) e hipoglicemiante (Bresciani et al., 2004) (**Figura 3**).

Santos et al. (2013), verificaram que *in vitro* o efeito direto do AC extraído do óleo de copaíba nas formas promastigotas e amastigotas de *L. amazonensis*. Através de microscopia eletrônica de varredura e citometria de fluxo confirmou alterações morfológicas e estruturais no parasito, principalmente aumento da permeabilidade da membrana plasmática e despolarização da membrama mitocondrial. Em estudo semelhante, porém utilizando *Trypanosoma cruzi* em seu modelo, Izumi et al. (2012), verificaram que o AC teve moderada atividade contra esses parasitos, não sendo capaz de causar lipoperoxidação na membrana de *T. cruzi.*

Ao utilizar óleo de copaíba (*Copaifera martii*) para o tratamento de leishmaniose experimental, Santos et al. (2011) obtiveram resultados positivos no tratamento oral com melhora significativa das lesões na pata. Os principais compostos químicos do óleo de copaíba utilizados neste trabalho eram representados por diterpenos (62,3%) e sesquiterpenos (37,7%), sendo o AC um dos principais diterpenos presentes. Fernandes e Freitas (2007), atribuíram todas as propriedades biológicas da *Copaifera* spp. ao grupo de sesquiterpenos e diterpenos.

No modelo de edema de pata induzido por carregenina, Lim et al., (2009) e Choi et al., (2011) verificaram que o AC extraído de *Aralia continentalis* foi capaz de inibir o processo inflamatório, inibindo a ação da cicloxigenase-1 (COX-1), cicloxigenase-2 (COX-2) - precursores da síntese de prostaglandinas-2 (PGE_2), importantes mediadores da inflamação - e diminuição dos níveis de IL-8, a qual é um potente quimioatraente para neutrófilos - células que apresentam papel importante no agravamento do processo inflamatório (Akdis, et al, 2011). Choi et al. (2011), demonstrou ainda que a atividade anti-inflamatória do ácido caurenóico a nível celular se dá através da inibição do fator nuclear κB (NFκB), da produção de NO e PGE_2.

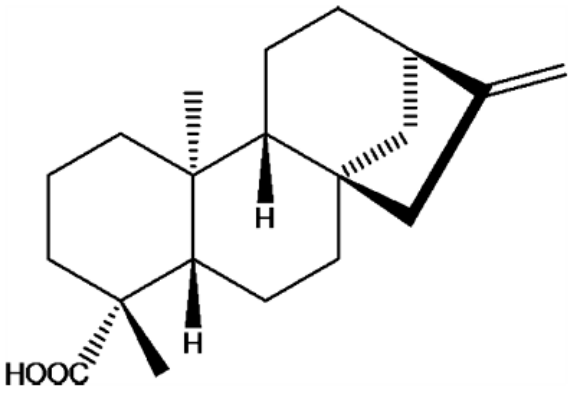
Em estudo controverso, Liu et al. (2011) verificou *in vitro* que na concentração de 1nM o AC não foi capaz de suprimir a atividade de NFκB, inalterando a expressão gênica de COX-2, óxido nítrico, IL-1β, TNF-α e IL-12 nesta concentração estudada.

Mizokami et al. (2012), no modelo *in vivo* de dor inflamatória induzida por ácido acético, verificou que o AC possui um efeito analgésico ao inibir citocinas como TNF-α, IL-1β, COX-2 e PGE_2

Em modelo de contração de artérias isoladas de ratos, Tirapelli et al. (2004) verificou que o pré-tratamento com AC reduziu as contrações induzidas por fenilefrina e cloreto de potássio (KCl) tanto no endotélio da aorta íntegra quanto nos anéis aórticos e causou o relaxamento do vaso. Isto esta relacionado ao fato do AC bloquear os canais de cálcio pela ativação da via óxido nítrico/Gaunosina monofosfato cíclico (NO/GMPc) e abertura dos canais de potássio. Também concluiu que este efeito vasorelaxante está relacionado a ativação da isoforma NO sintase neuronal e endotelial (Tirapelli, 2004).

Em modelo de colite experimental induzida por ácido acético, Paiva et al. (2002) concluiu que o ácido caurenóico é um eficaz anti-inflamatório que leva à uma redução da atividade da mieloperoxidase uma enzima presente nos leucócitos e utilizada de forma indireta a análise de migração de leucócitos, especialmente neutrófilos, para o local da inflamação, e diminuiu também a concentração de malondialdeido (MDA), um indicador da lipoperoxidação lipídica. Diante disso houve uma redução do infiltrado inflamatório, edema e lesão tecidual (Paiva et al, 2002).

Figura 3 - Estrutura química do ácido caurenóico.



1.6.2 *trans*-chalcona

As chalconas compreendem um importante grupo de compostos naturais especialmente abundantes em frutas e vegetais (Di Carlo *et al*., 1999).

Estruturalmente, estes compostos são flavonóides de cadeia aberta com dois anéis aromáticos ligados por uma porção enona de três-carbonos, sendo a forma *trans* da molécula considerada a mais estável termodinamicamente, desta forma, o isômero mais abundante nas plantas (Hijova, 2006) (**Figura 4**).

Esses compostos apresentam um amplo espectro de atividades biológicas, incluindo ação anti-bacteriana (Friis-Moller et al., 2002, Tsukiyama et al., 2002), anti-helmíntica, antimalárica (Chen et al., 1997; Go et al., 2004) e tripanocida (Lunardi *et al*., 2003)

Recentemente, Tajuddeen e colaboradores (2017) analisaram a atividade leishmanicida de 278 chalconas sintético e 34 chalconas de origem vegetal. Esta grande variedade de moléculas deve-se a estrutura química simples que permite uma multiplicidade de substituições resultando em um grande número de derivados (Gomes et al., 2017). No entanto, essas alterações na composição química da molécula resultam em mudanças importantes nas atividades biológicas promissoras e mecanismo de ação muitas vezes não estudados.

Chalconas naturais apresentam intensa atividade leishmanicida (Chen et al., 1994; Liu et al., 2003; Hermoso et al., 2003, Piñero et al., 2006, Lunardi *et al*., 2003).

Torres-Santos et al., (1999 a, b) verificaram que a metoxichalcona, isolada a partir do extrato de *Piper aduncum,* exerce atividade microbicida sobre *L. amazonensis* em ensaios *in vitro* e *in vivo.* Estes estudos demonstraram que a ação inibidora sobre as formas amastigotas é, aparentemente, um efeito direto sobre os parasitos por desorganização da mitocôndria.

Também já foi demonstrado que a licochalcona-A é capaz de alterar a estrutura da mitocôndria do parasito e inibir a sua função (Zhai et al*.,* 1995). O mecanismo de ação foi elucidado mostrando que esta chalcona inibe a respiração do parasito e a atividade das desidrogenases mitocondriais (Zhai et al*.,* 1999).

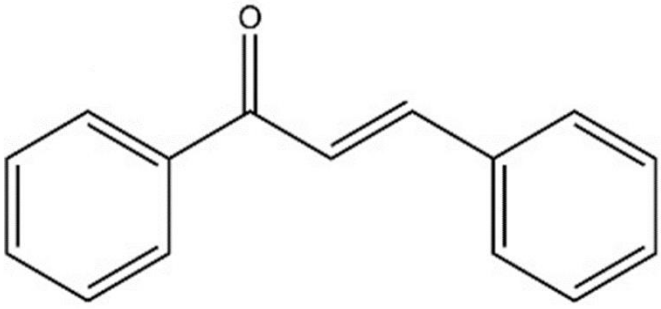
Em relação à *trans*-chalcona, Piñero et al. (2006), demonstraram o efeito leishmanicida sobre formas promastigotas de *L. braziliensis, L. tropica, L. infantum* e *L. amazonensis* e ainda relataram que administração de *trans*-chalcona via implante de polímeros, promove a redução do desenvolvimento da lesão em camundongos BALB/c infectados por *Leishmania (L.) amazonensis.*

Sabe-se que flavonóides são compostos conhecidos por sua atividade antioxidante e moduladora de células sinalizadoras que conduzem a processos inflamatórios (Haraguchi et al., 1998, Kalt et al., 2010). De fato, um estudo mostra que a *trans*-chalcona foi capaz de aumentar a resistência de células HepG2 ao desafio oxidativo, elevando a sobrevivência destas diminuindo a formação de produtos de peroxidação lipídica após a exposição destas células ao H_2O_2 (Sikander et al*.,* 2011) e reduziu fibrose em modelo *in vivo* (Karkhaneh et al., 2016; Srivastava et al., 2016).

Trans-chalcona bloqueou a indução de isquemia induzida por VEGF e ICAM-1 devido à inibição de ativação de STAT3 e NF-κB (Lamoke et al., 2011) e induziu apoptose via mitocondrial no em linhagens de célula de câncer de mama humano (MCF-7 e MDA-MB-231) e células de osteossarcoma humano através da regulação da expressão do gene p53 (Bortolotto et al., 2017; Silva et al., 2016; Hsu et al., 2006; Shen et al., 2007).

Martinez et al. (2017a, b) mostraram que a administração sistêmica e a formulação tópica de *trans*-chalcona apresentaram mecanismos anti-inflamatórios e antioxidantes na inflamação da pele induzida por irradiação ultravioleta (UV) em camundongos *hairless*, reduzindo o estresse oxidativo. A formulação tópica de *trans*-chalcona foi capaz de aumentar a expressão de mRNA do fator nuclear eritroide 2 (Nfr2) e heme-oxigenase-1 (OH-1), melhorando o potencial antioxidante neste modelo experimental (Martinez et al., 2017b).

Figura 4 - Estrutura química da *trans*-chalcona.



2 JUSTIFICATIVA

Conforme exposto, a quimioterapia para LTA não é satisfatória, devido à toxicidade dos fármacos atualmente utilizadas, dificuldade de administração, longo tempo de tratamento, e o surgimento de cepas resistentes. Portanto, sabendo da importância desta doença, de suas complicações anatomofisiológicas, associada ao surgimento de cepas com diferentes sensibilidades e resistência aos fármacos disponíveis, faz-se necessário e urgente, a busca de novos fármacos para o tratamento da leishmaniose.

Uma série de estudos tem demonstrado o efeito leishmanicida de produtos naturais e seus múltiplos mecanismos de ação sobre as formas de *Leishmania* spp em experimentos *in vitro* e *in vivo*. Esses compostos são capazes de agir diretamente sobre alvos cruciais para sobrevivência do parasito, moderando a resposta imune exacerbada e consequente o dano tecidual, característicos desta doença.

Baseados nas evidências experimentais encontradas acreditamos que o ácido caurenóico e a *trans*- chalcona possam exercer atividade leishmanicida e/ou imunomoduladora sobre macrófagos peritoneais de camundongos BALB/c infectados ou não com formas promastigotas de *Leishmania amazonensis* por diferentes mecanismos de ação.

3. OBJETIVO GERAL

Avaliar *in vitro* a atividade biológica dos compostos naturais – ácido caurenóico (AC) e *trans*-chalcona (TC) em formas promastigotas e amastigota de *Leishmania amazonensis*, bem como elucidar os principais mecanismos de ação e propriedades imunomoduladoras envolvidas.

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3.1. Objetivos específicos

- Efetuar o estudo *in silico* da estrutura de TC quanto às regras de Lipinski e Veber e das propriedades de ADMET;
- Determinar as concentrações de AC e TC capazes de inibir diretamente o crescimento das formas promastigotas de *L. amazonensis*;
- Elucidar os mecanismos de morte de TC sobre formas promastigotas de *L. amazonensis;*
- Determinar *a* concentração citotóxica (CC50) de TC sobre macrófagos peritoneais e índice de seletividade;
- Analisar a ação de AC e TC sobre macrófagos murinos infectados por amastigotas de *L. amazonensis*;
- Quantificar a produção das citocinas (IFN-γ, TNF-α, IL-1β, IL-10, TGF- β e IL-12)
- Determinar a quantidade de NO e EROS no sobrenadante de macrófagos infectados por *L. amazonensis* e tratados com AC e TC;
- Delinear os mecanismos de ação leishmanicida desencadeados por AC e TC, em cultura de macrófagos infectados por *L. amazonensis*.

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4 PRODUÇÃO CIENTÍFICA

4.1 ARTIGO 1

Kaurenoic Acid Possesses Leishmanicidal Activity by Triggering a NLRP12/IL-1β/cNOS/NO Pathway

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Research Article

Kaurenoic Acid Possesses Leishmanicidal Activity by Triggering a NLRP12/IL-1β/cNOS/NO Pathway

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Leishmania amazonensis (*L. amazonensis*) infection can cause severe local and diffuse injuries in humans, a condition clinically known as American cutaneous leishmaniasis (ACL). Currently, the therapeutic approach for ACL is based on Glucantime, which shows high toxicity and poor effectiveness. Therefore, ACL remains a neglected disease with limited options for treatment. Herein, the *in vitro* antiprotozoal effect and mechanisms of the diterpene kaurenoic acid [*ent*-kaur-16-en-19-oic acid] (KA) against *L. amazonensis* were investigated. KA exhibited a direct antileishmanial effect on *L. amazonensis* promastigotes. Importantly, KA also reduced the intracellular number of amastigote forms and percentage of infected peritoneal macrophages of BALB/c mice. Mechanistically, KA treatment reestablished the production of nitric oxide (NO) in a constitutive NO synthase- (cNOS-) dependent manner, subverting the NO-depleting escape mechanism of *L. amazonensis*. Furthermore, KA induced increased production of IL-1β and expression of the inflammasome-activating component NLRP12. These findings demonstrate the leishmanicidal capability of KA against *L. amazonensis* in macrophage culture by triggering a NLRP12/IL-1β/cNOS/NO mechanism.

1. Introduction

American cutaneous leishmaniasis (ACL) is a devastating illness caused by the protozoa *Leishmania* spp. ACL displays distinct clinical manifestations depending on both the parasite strain and the capability of the host to mount an effective immune response. Therefore, this disease may clinically appear in the host as cutaneous, mucocutaneous, or diffuse forms [1].

The treatment of ACL is based on a highly toxic chemotherapy with the antimonials sodium stibogluconate (Pentostam) and antimonate N-methyl-glucamine (Glucantime). In case of lack of response, second-line drugs such as amphotericin B or pentamidines are used [2]. However, these drugs frequently exhibit high toxicity, which has been related to its restricted use and resistance resulting in more restrictions in chemotherapy [2–5]. These considerations reveal the urgency to develop new therapeutic agents for the treatment of this disease.

Therefore, the search for more effective and less toxic chemotherapeutic agents for the treatment of ACL is increasing. There are various reported studies of synthetic compounds and natural products as potential sources of leishmanicidal activity [6, 7]. An interesting molecule towards this aim is kaurenoic acid [*ent*-kaur-16-en-19-oic acid] (KA), a diterpene obtained from various Brazilian plants [8, 9]. This molecule has been reported as showing a wide variety of biological activities such as antiprotozoal [10, 11], antimicrobial [12], antinociceptive [13], vasorelaxant, hypotensive [14, 15], and anti-inflammatory [13, 16, 17] activities. Moreover, some immunomodulatory properties of KA have been reported in these models.

The antiprotozoal activity of this diterpene involves its direct action in altering cell membrane integrity and mitochondrial membrane depolarization in promastigote and amastigote forms of *L. amazonensis* [18] and epimastigote forms of *Trypanosoma cruzi* [19], respectively. This direct activity of KA against the protozoa is not enough to reflect its overall potential as a therapeutic leishmanicidal drug, since *Leishmania* spp. parasites replicate intracellularly in macrophages and have several escape mechanisms against microbicidal molecules that are not dependent on the direct action of antileishmanial drugs [20–23].

Members of the Nod-like receptor (NLR) family of proteins have emerged as important innate immune sensors of pathogen-associated molecular patterns (PAMPS) and damage-associated molecular patterns (DAMPS) [24]. NLRs are the key components of the inflammasome that regulate the maturation of the potent inflammatory cytokine interleukin- (IL-) 1β [25].

Accordingly, Lima-Junior et al. found that the NLRP-3 inflammasome is engaged in the response against *L. amazonensis* restricting parasite replication. Additionally, IL-1β seems to be important for host resistance to infection by inducible (i) NOS-mediated production of NO, a major host defense mechanism against *Leishmania* spp. [26].

Taking into account the above-mentioned evidence, KA was evaluated for its *in vitro* effect on susceptible macrophages from BALB/c mice infected with *L. amazonensis* promastigote forms. Accordingly, we performed *in vitro* assays to investigate the direct effect of KA on parasites as well as its modulatory action on *Leishmania*infected macrophages. We further investigated a putative mechanism of action of this compound as a modulator of proinflammatory molecules such as oxygen reactive species, NO, cytokines, and inflammasome.

2. MATERIALS AND METHODS

2.1. Parasite. L. amazonensis (MHOM/BR/1989/166MJO) was used in promastigote forms, kept in culture medium 199 (Invitrogen-GIBCO) supplemented with 10% fetal

bovine serum (Invitrogen-GIBCO), 1 M Hepes, 0.1% human urine, 0.1% L-glutamine, 10 U/mL penicillin and 10 μg/mL streptomycin (Invitrogen-GIBCO), and 10% sodium bicarbonate (complete medium for promastigotes—CMP). Cell cultures were incubated at 25°C in 25 cm^2 flasks.

2.2. Animals. Female BALB/c mice weighing approximately 25–30 g and aged 6–8 weeks were obtained from Fundac¸ao˜ Osvaldo Cruz, FIOCRUZ, Curitiba, Brazil. Mice were kept under pathogen-free conditions and used according to protocols approved by the Ethics Committee of the State University of Londrina (protocol number 33064/2012.42). Every effort was made to minimize the number of animals used and their suffering.

2.3. Plant Material. KA used in this paper was obtained from *Sphagneticola trilobata*. The crude extract was obtained from dried roots, which were pulverized and extracted with dichloromethane and partitioned with *n*-hexane and ethyl acetate; all solvents were dried under reduced pressure. The hexane fraction was subjected to vacuum liquid chromatography (VLC) by increasing gradient polarity. The second fraction produced an amorphous compound (200 mg), which was washed with cold methanol and analyzed by high performance liquid chromatography (HPLC) methods, yielding 96% purity. The identification was performed by 1H and 13 C nuclear magnetic resonance (NMR), electron impact mass spectrometry (EIMS), and comparison with literature data [27]. The stock solution of KA was dissolved in 2% dimethyl sulfoxide (DMSO) (Invitrogen-Gibco). However, DMSO concentration did not exceed 0.2% in all experiments.

2.4. Viability of Promastigotes. The viability of *L. amazonensis* promastigote forms treated with KA was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [28]. Promastigote forms (10^6/100 μL) were incubated with different concentrations of KA (10, 30, 50, 70, and 90 μM) or with KA solvent (0.2% DMSO) and maintained in culture for 24, 48, and 72 h at 25°C. Thereafter, 10 μL of MTT (5 mg/mL) were added, followed by incubation for an additional 4 h at 24°C. The MTT formazan product was diluted with 300 μL of DMSO, transferring to 96-well plates and measured in a spectrophotometer with absorbance determined at 550 nm. The results were expressed as percentage MTT reduction relative to the control group calculated as the following formula: (viable promastigotes)% = (OD of drug-treated sample/OD of untreated sample) × 100.

2.5. Cell Proliferation Kinetics. Promastigote forms (10^6/mL) incubated in CMP were treated with different concentrations of KA (10, 30, 50, 70, and 90 μM) or with KA solvent (0.2% DMSO) and cultured for 5 days at 25°C. Promastigotes were counted in a Neubauer chamber after 24, 48, 72, and 120 h.

2.6. Phagocytic Assay. Macrophages (5×10^5/mL) were obtained from the peritoneal cavity by the injection of 2 mL of RPMI 1640 culture medium (Invitrogen-GIBCO) supplemented with 10% fetal bovine serum (Invitrogen-GIBCO) and cultured on 24-well plates containing 13 mm diameter glass coverslips. Cells were preincubated with 200 μL of RPMI medium for 2 h for adherence and incubated with promastigote forms (5 : 1) for 2 h. KA (50, 70 or 90 μM) or medium was added, followed by 24 h incubation at 37°C and 5% CO2. The cells were stained with Giemsa to establish the phagocytic index of infection (by percentage) and the parasites/macrophage (mean). The supernatant was utilized to measure the levels of malondialdehyde (MDA), total antioxidant capacity of plasma (TRAP), nitric oxide (NO), and cytokines

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2.7. Measurement of the Total Antioxidant Capacity of Samples (Trapping Antioxidant Parameter (TRAP)). Samples (50 μL of supernatant with cells) obtained from the phagocytic assay were analyzed as previously described by Repetto et al. [29], by using the chemiluminescence-based method. Soluble vitamin E (Trolox) was employed as a standard antioxidant. The chemiluminescence curves were obtained using the Glomax luminometer (Promega), and the results were expressed in nM Trolox.

2.8. Measurement of Malondialdehyde Levels (MDA) by High Performance Chromatography. MDA levels were determined as resultant of oxidative stress occurrence, which causes lipid peroxidation and the production of this metabolite. Accordingly, we used HPLC as previously described by Victorino et al. [30], with slight modifications. The analyses were conducted with an Alliance e2695 HPLC (Waters, Milford, MA, 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), and a photodiode array detector (Photodiode Array Detector (PDA), 2998). Analyses were conducted using Empower 2 software (Waters, Milford, MA, USA). MDA standards were prepared using 1,1,3,3tetraethoxypropane (TEP). Aliquots containing 250 μL of cells + supernatants were deproteinized by adding 20% trichloroacetic acid and reacted with 1 mL of thiobarbituric acid. The mobile phase was 70% 10 mM KH2PO4 buffer, pH 7.0, and 40% HPLC-grade methanol. Readings were obtained at 532 nm, following an 8 min isocratic flow at the rate of 1 mL/min. The results were expressed in nM MDA.

2.9. Determination of Nitrite Levels as Estimate of NO Production. The determination of nitrite in supernatants collected from the phagocytic tests was used to measure the concentration of nitric oxide (NO) according to Panis et al. [31], with some modifications. Briefly, the supernatant aliquotsweredeproteinizedbyadding50 μLof75 mMZnSO4 and 70 μL of NaOH and shaking and centrifuging for 5 min at 10,000 rpm and 25°C. The supernatant was recovered and diluted in glycine buffer (45 g/L, pH 9.7). Cadmium granules were rinsed with distilled sterile water and added to a 5 mM CuSO4 in glycine-NaOH buffer (15 g/L, pH 9.7), followed by 5 min incubation, and the copper-coated cadmium granules were used within 10 min. Activated granules were added to glycine buffer-diluted supernatant and the suspension stirred for 10 min. Aliquots of 200 μL were recovered in appropriate tubes for nitrite determination, and the same volume of Griess reagent was added. After 10 min incubation at room temperature, tubes were centrifuged at 10,000 rpm for 2 min at 25°C and added to 96-well microplates in triplicate. A calibrationcurvewaspreparedusingdilutionsofNaNO2,and the absorbance was determined at 550 nm in a microplate reader.

2.10. Cytokine Determination. The supernatants obtained from the phagocytic assay were used to determine the levels of IL-1β, IL-12, TNF-α, IFN-γ, TGF-β, and IL-10 using eBioscience commercial kits capture enzyme-linked immune sorbent assay (ELISA) (San Diego, CA, USA). According to the manufacturer's instructions, absorbance was read at 450 nm using a spectrophotometer and the results are expressed in pg/mL based on a standard curve. The sensitivity of the test was 8 pg/mL for IL-1β, TNF-α, and TGF-β, 15 pg/mL for IL-12 and IFN-γ, and 32 pg/mL for IL-10.

2.11. Immunocytochemical Labeling for NLRP12 and iNOS. Immunocytochemistry of NLRP12 and inducible nitric oxide synthase (iNOS) was performed on coverslip-adherent cells (cells prepared according to the protocol described in the phagocytic assay) using the labeled streptavidin

biotin method with the LSAB kit (DAKO Japan, Kyoto, Japan) without microwave treatment. The coverslips were incubated with 10% Triton X-100 for 1 h, washed 3 times with PBS, and treated for 40 min at room temperature with 10% BSA. In addition, coverslips were incubated overnight at 4°C with the primary antibody (anti-NLRP12 rabbit polyclonal antibody diluted 1 : 300 (Abcam, catalog number ab93113) and anti-iNOS rabbit monoclonal antibody diluted 1 : 200 (BD Biosciences, catalog number 610599)). After secondary antibody treatment (2 h, room temperature), horseradish peroxidase activity was visualized by treatment with H2O2 and 3,3□-diaminobenzidine (DAB) for 5 min. In the last step, the sections were weakly counterstained with Harry's hematoxylin (Merck). For each case, negative controls were performed by omitting the primary antibody. Intensity and localization of immunoreactivity against primary antibody used were examined in all coverslips using a photomicroscope (Olympus BX41, Olympus Optical Co., Ltd., Tokyo, Japan). Color photomicrographs of representative areas (×40 objective lens) were digitally acquired for image analysis. For determining a semiquantitative scoring, images were evaluated by using the color deconvolution tool from Image J software (NIH, USA). Pixels were categorized as previously described by Chatterjee et al. [32] as strong positive (3+), positive (2+), weak positive (1+), and negative (0).

2.12. cNOS Inhibition Assay. Peritoneal macrophages were challenged with *L. amazonensis* and treated with KA as described in the phagocytic assay method. Before the treatment with KA, the cells were incubated with 20 μM NGnitro-L-arginine methyl ester (L-NAME) for 2 h at 36°C and 5% CO2 [33], aiming to cause pharmacological blockage of constitutive NOS. The supernatants were utilized to measure NO levels (as previously described).

2.13. Statistical Analysis. Three independent experiments were performed, each with triplicate datasets. Data were expressed as mean ± standard error of the mean. Data were analyzed using the GraphPad Prism statistical software (GraphPad Software, Inc., USA, 500.288). Significant differences between the treatments were determined by one-way ANOVA, followed by Tukey's test for multiple comparisons. $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Kaurenoic Acid Exerts Leishmanicidal Effect against Promastigote and Amastigote Forms of L. amazonensis. In the first set of experiments, the antileishmanial effect of KA was investigated against the promastigote forms of *L. amazonensis.* We observed that KA at concentrations of 50, 70, and 90 μM reduced promastigote viability according to an MTT assay of 24 h by 30, 31, and 34%, respectively (Figure 1(a)), the reduction was maintained for 72 h. We also observed 28.2, 45.8, and 51.5% decrease in the proliferation of the promastigote forms at concentrations of 50, 70, and 90 μM, respectively, after 120 h with 24 h of pretreatment (Figure 1(b)). Therefore, we chose testing all concentrations of KA for 24 h treatment of macrophages.

In attempt to verify if KA could enhance the leishmanicidal capacity of macrophages, we initially challenged these cells with promastigote forms of *L. amazonensis* for 2 h, for phagocytosis. Afterwards, the cells were treated with KA (at concentrations ranging from 50 to 90 μM) for 24 h. Macrophages and amastigotes were counted to establish the phagocytic index, indicating the extent of infection as the number of parasites per macrophage.

Regarding the percentage of infected macrophages, 50, 70, and 90 μM KA, respectively, caused a decrease of 26.6, 25.6, and 28.4% of infected macrophages when compared to the

untreated infected macrophages (Figure 1(c)). Moreover, the mean number of amastigotes per macrophage was significantly decreased at the concentrations of 70 and 90 μM, by 21.5 and 20.3%, respectively (Figure 1(d)).

3.2. Effect of Kaurenoic Acid on Macrophages Is Not Associated with ROS Production. In order to assess the involvement of KA in modulating the respiratory burst of macrophages during the *Leishmania* challenge, we measured the oxidative stress status of these cells by quantifying its total antioxidant capacity (TRAP) and MDA formation.

There was an increase in TRAP of macrophages infected with *L. amazonensis* treated with 50 and 70 μM KA, while TRAP was reduced by 90 μM KA compared to the infected control (Figure 2(a)). The MDA level was reduced at all KA concentrations with significant difference at 50 μM KA (Figure 2(b)).

3.3. Kaurenoic Acid Upregulates NO Levels in a cNOSDependent Mechanism. Concerning NO levels, our results showed that untreated macrophages infected with *L. amazonensis* displayed decreased levels of NO. Interestingly, the treatment with KA reestablished baseline NO levels at all concentrations tested (Figure 3(a)). Thus, we next investigated the enzymatic pathway involved in KA-induced NO production.

The expression of iNOS was assessed by immunocytochemistry. Our data showed that KA did not alter iNOS expression (Figure 3(b)).

Aiming to investigate the involvement of cNOS, we pretreated macrophages with the preferential cNOS inhibitor L-NAME. After the *Leishmania* challenge, macrophages were pretreated with L-NAME and then incubated with KA, resulting in a substantial reduction in NO production only at 90 μM, indicating that the augmented NO previously observed at 90 μM KA was probably dependent on cNOS activity (Figure 3(c)).

3.4. Kaurenoic Acid Promotes the Production of Active IL1β in Macrophages Infected with L. amazonensis. In order to determine the immunomodulatory action of KA on cytokines in infected macrophages, we measured the levels of IL-12, TNF-α, IFN-γ, TGF-β, IL-10, and IL-1β. We observed that the production of IL-12, TNF-α, IFN-γ, TGF-β, and IL-10 was not significantly different between the KA-treated groups and the control group (Figure 4). On the other hand, 70 and 90 μM KA augmented the levels of IL-1β (Figure 4(f)).

3.5. Kaurenoic Acid Upregulates NLRP12 Expression in Macrophages Infected with L. amazonensis. In this present study, the activation and participation of the inflammasome during the immune response to infection by intracellular pathogen were investigated. The augmented levels of IL1β induced during KA treatment of infected macrophages, combined with the lack of information about this complex in ACL, led us to investigate the role of NLRP12, one member of the subfamily of NLRP innate receptors. As shown in Figure 5, KA at 90 μM was able to upregulate NLRP12 expression in macrophages infected with *L. amazonensis*. These results indicate that the stimulation of macrophages with KA triggered the overexpression of NLRP12, with consequent activation of IL-1β.

4. DISCUSSION

The success of chemotherapy in ACL is mainly dependent on two factors: the microbicidal activity of the drug and the protective immune response triggered in the host during the treatment.

In the present study, we evaluated the therapeutic potential of KA treatment, which directly inhibited the viability and proliferation of *L. amazonensis* promastigote forms (Figure 1(a)). Previous *in vitro* studies demonstrated that KA has direct antileishmanial activity against promastigote and amastigote forms of *L. amazonensis* [19, 34,

35], and the main proposed mechanism was related to mitochondrial membrane depolarization in the protozoan. However, the immunomodulatory activity of KA in the macrophage experimental leishmaniasis model still remained to be determined.

Our results indicated that infected macrophages treated with KA were more effective during the leishmanicidal response against the intracellular forms of *L. amazonensis* (Figures 1(b) and 1(c)). The solvent (0.2% DMSO) did not affect the viability and proliferation of *L. amazonensis* promastigotes (Figures 1(a) and 1(b)). These findings suggested that this diterpene was able to reverse the downregulation of the killer machinery of macrophages caused by *Leishmania* infection [20, 36].

In order to elucidate the microbicidal effects of KA, we investigated the main leishmanicidal molecules produced by macrophages. The results showed that KA treatment was not able to enhance the oxidative burst of infected macrophages. On the other hand, even though *L. amazonensis* is capable of depleting NO levels [20], the results showed that treatment with KA was able to restore the levels of this microbicidal molecule (Figure 3(a)), but no alteration in the expression of iNOS was found.

In fact, NO is the main antileishmanial molecule produced in the early macrophage response against intracellular parasites. Besides iNOS, cNOS is also an important route for NO production [37, 38]. Some studies have demonstrated that KA induces cNOS-dependent activity in the disease context [13, 14], but the involvement of a cNOS mechanism for KA in parasitic infections has not been well elucidated.

Studies have demonstrated the importance of innate immune response-triggered cytokine production during the early stages of experimental leishmaniasis and have shown that some cytokines may drive the clinical manifestation of ACL by modulating resistance or susceptibility to infection [39]. IL-1β, IL-12, TNF-α, and IFN-γ are essential cytokines for the development of an effective immune response against *Leishmania* spp., leading to the activation of macrophages and promoting the microbicidal effects against this parasite [23, 26, 40, 41].

In the present experimental conditions, KA was able to increase the production of IL-1β despite of showing no effect on other cytokines evaluated, suggesting a selective effect of KA on IL-1β production/maturation system. IL-1β is a proinflammatory cytokine that becomes active after its cleavage by the inflammasome complex [42], and when active, this cytokine helps the activation of macrophages by enhancing the response against pathogens. In fact, a recent study showed that IL-1β was associated with resistance to *L. amazonensis, L. braziliensis,* and *L. major* infections. IL-1β maturation is dependent on the inflammasome NLRP3/ASC/caspase1 complex [26]. In addition to NLRP3, which is endogenously expressed during *Leishmania* infection, NLRP12 is an important NLR involved in the inflammatory response against parasites such as *Trypanosoma cruzi* [43]. In this sense, the present study also addressed whether the expression of NLRP12 increased during *Leishmania* infection of macrophages to determine if KA induces IL-1β production/maturation by a previously unrecognized mechanism.

Our findings showed that there was no induction of NLRP12 in macrophages during *Leishmania* infection. On the other hand, KA at 90 μM upregulated the expression of NLRP12 in infected macrophages, thus explaining the increased production of IL-1β induced by KA. These data are also in line with the increased cNOS-dependent production of NO, since it has been shown that during *Leishmania* infection a NLRP3/ASC/caspase-1/IL-1β/cNOS/NO pathway is triggered to kill this parasite. In the present study, the data suggest that KA triggers a NLRP12/IL-1β/cNOS/NO killing mechanism during *Leishmania* infection of macrophages from BALB/c mice. Importantly, KA-induced the

expression of a NLR (NLRP12) that is not endogenously activated to kill *Leishmania*, thereby upregulating unused endogenous mechanisms valuable to protect the host against *Leishmania* infection with additive effects for other NLRs such as NLRP3, as observed in *T. cruzi* infection [43]. Therefore, KA seems to unequivocally provide additional protective mechanisms against *Leishmania* infection. It is also possible that KA triggers similar mechanisms in other parasitic diseases, which remains to be determined.

In conclusion, the present study demonstrated that kaurenoic acid has therapeutic potential as a pharmacological approach against *Leishmania* infection. The mechanism of action of kaurenoic acid depends, at least in part, on triggering the NLRP12/IL-1β/cNOS/NO leishmanicidal pathway. Therefore, KA merits further preclinical and clinical studies as a possible therapy for *Leishmania* infection.

Conflict of Interests

The authors have no conflict of interests to declare.

Authors' Contribution

Milena Menegazzo Miranda, Carolina Panis, Suelen Santos da Silva, and Juliana Aparecida Macri equally contributed to this study.

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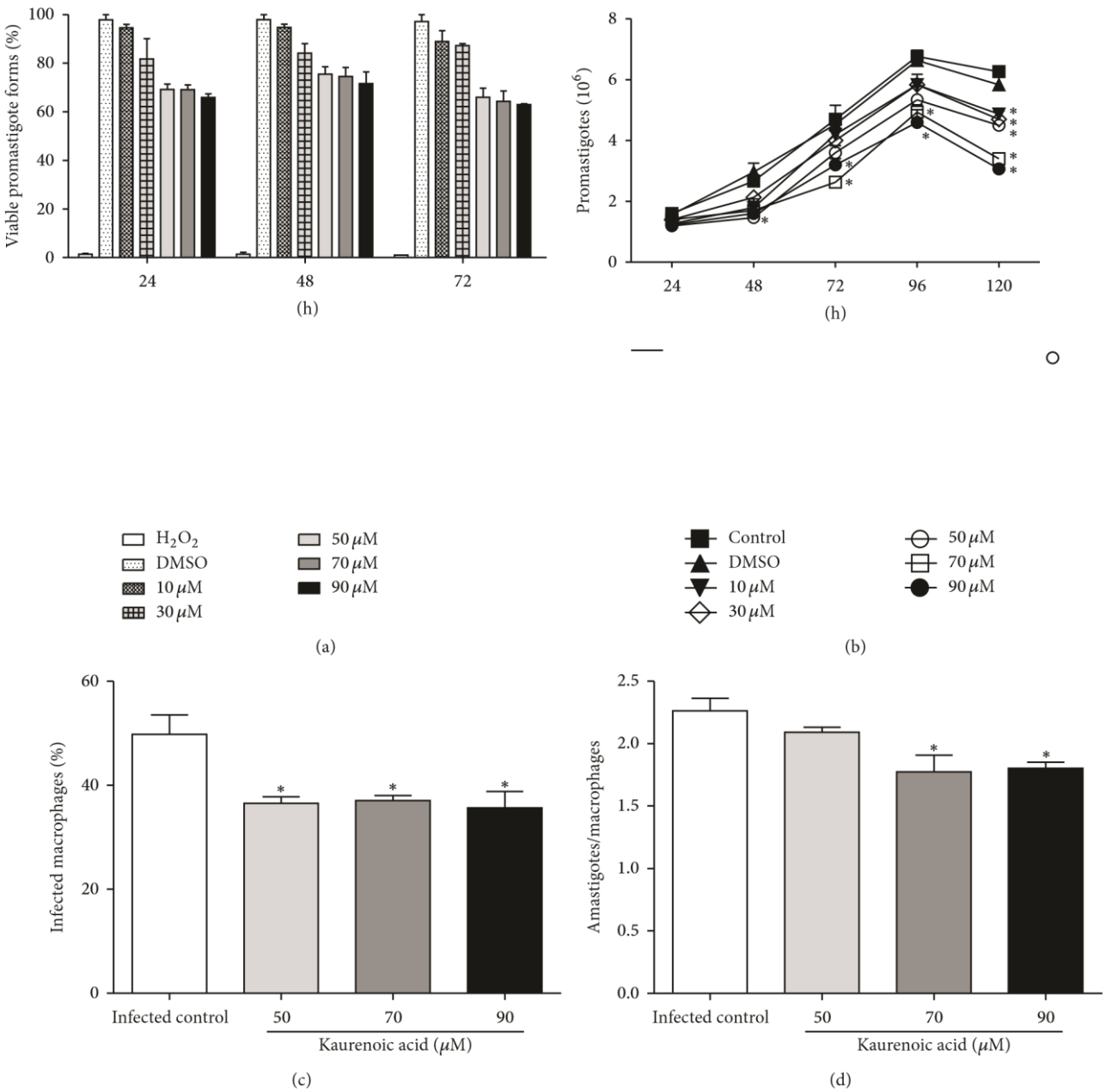

Figure 1: Kaurenoic acid has a leishmanicidal effect against promastigote and amastigote forms of *L. amazonensis*. MTT assay in promastigote forms of *L. amazonensis* treated with kaurenoic acid (10, 30, 50, 70, and 90 μM) or 0.2% DMSO for 24, 48, and 72 h (Panel (a)). Proliferation kinetics of *L. amazonensis* promastigote forms after pretreatment for 24 h with kaurenoic acid (10, 30, 50, 70, and 90 μM) or 0.2% DMSO for 24, 48, 72, and 120 h (Panel (b)). Percentage of infected macrophages (Panel (c)) and number of amastigotes per macrophage after 24 h of incubation with kaurenoic acid (50, 70, and 90 μM) (Panel (d)). Data represent mean ± SEM of three independent experiments (promastigotes) and six independent experiments (amastigotes). (*Significantly different from control ($P < 0.05$ compared with control group promastigotes in culture medium) (one-way ANOVA followed by Tukey's test).)

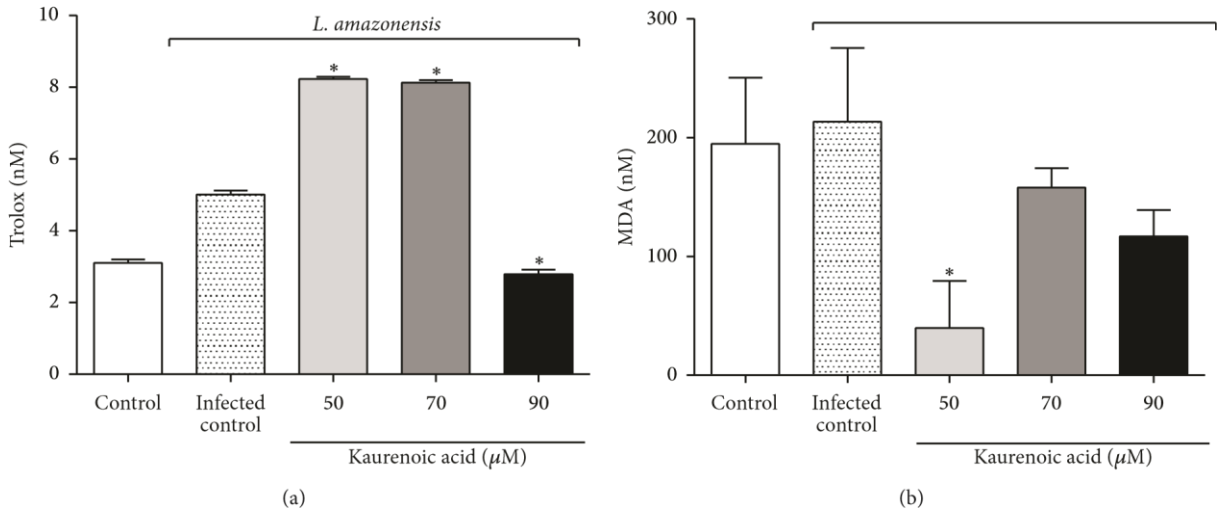

Figure 2: The effect of kaurenoic acid on macrophages is not associated with oxidative stress generation. Total antioxidant capacity (TRAP) (Panel (a)) and MDA levels (Panel (b)) were evaluated as markers of the oxidative status in supernatant or macrophages infected with *L. amazonensis* and treated with kaurenoic acid (50, 70, and 90 μM) for 24 h. Data represent the mean ± SEM of three independent experiments. (*Significantly different from infected cells ($P < 0.05$)) (one-way ANOVA followed by Tukey's test).

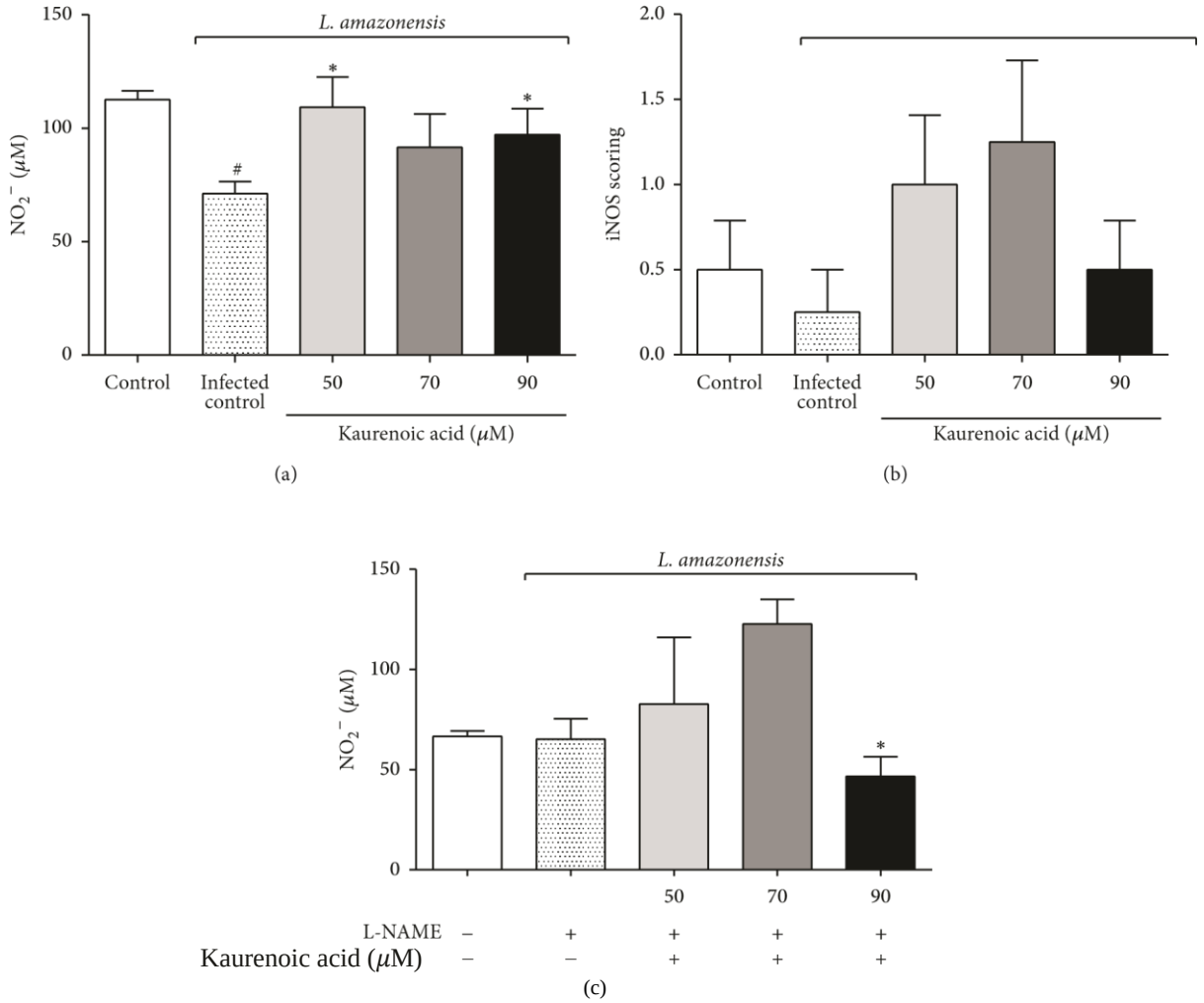

Figure 3: Kaurenoic acid exerted its effect by upregulating NO levels in a cNOS-dependent mechanism. NO levels (Panel (a)); immunocytochemistry scoring for inducible nitric oxide synthase (iNOS). Peritoneal macrophages infected with *L. amazonensis* and treated with kaurenoic acid (50, 70, and 90 μM) for 24 h (Panel (b)). Determination of NO in peritoneal macrophages infected with *L. amazonensis* and blocked with 20 μM L-NAME and treated with kaurenoic acid (50, 70, and 90 μM) for 24 h (Panel (c)). Data represent the mean ± SEM of three independent experiments. (*Significantly different from infected cells ($P < 0.05$). $^\#$Significantly different from control cells ($P < 0.05$)
(one-way ANOVA followed by Tukey's test).)

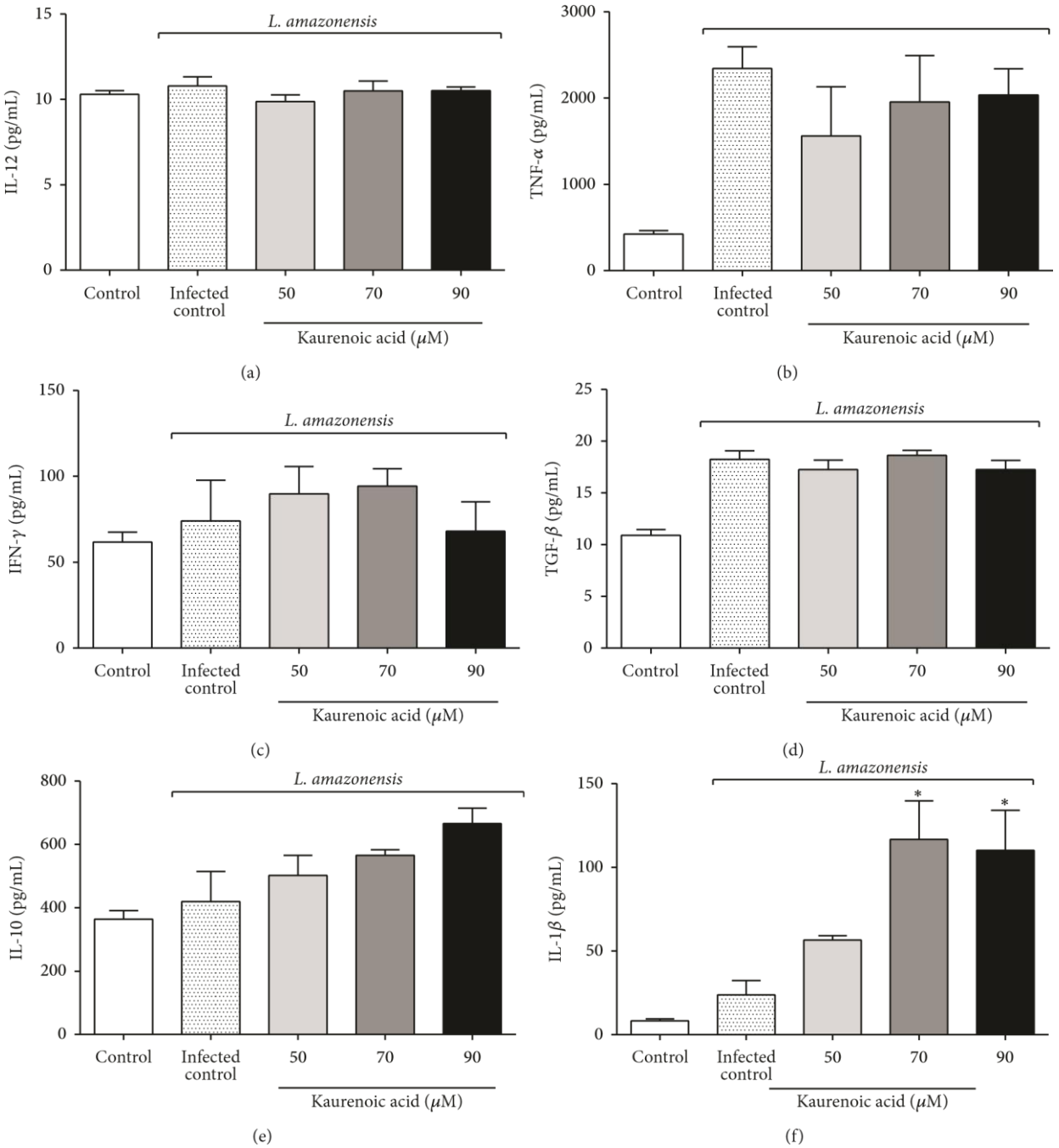

Figure 4: Kaurenoic acid promotes the production of active IL-1β in macrophages infected with *L. amazonensis*. Mapping the cytokine profiling produced *in vitro* by macrophages infected with *L. amazonensis* and treated with kaurenoic acid (50, 70, and 90 μM) for 24 h determined by ELISA. IL-12 production (Panel (a)), TNF-α production (Panel (b)), IFN-γ production (Panel (c)), TGF-β production (Panel (d)), IL-10 production (Panel (e)), and IL-1β production (Panel (f)). Data represent the mean ± SEM of three independent experiments. (*Significantly different from infected cells ($P < 0.05$) (one-way ANOVA followed by Tukey's test).)

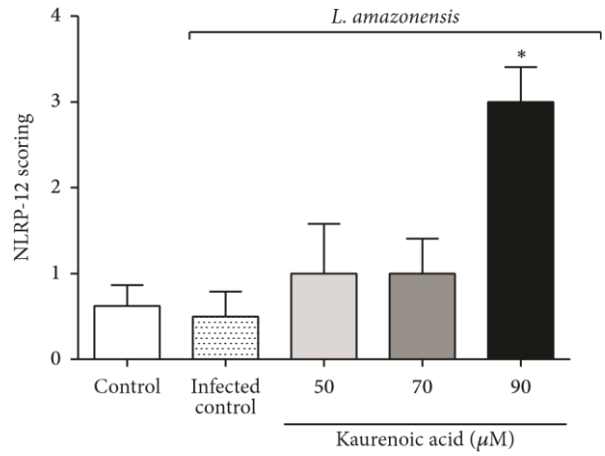

Figure 5: Kaurenoic acid upregulated the NLRP12 expression in macrophages infected with *L. amazonensis*. Immunocytochemistry scoring for NLRP12 in macrophages infected with *L. amazonensis* and treated with kaurenoic acid (50, 70, and 90 μM) for 24 h. Data represent the mean ± SEM of three independent experiments. (*Significantly different from infected cells ($P < 0.05$). (One-way ANOVA followed by Tukey's test).)

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4.2 ARTIGO 2

trans-Chalcone modulates *Leishmania amazonensis* infection *in vitro* by affecting iron availability and Nrf2 overexpression

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trans-Chalcone modulates *Leishmania amazonensis* infection *in vitro* by Nrf2 overexpression affecting iron availability

Milena Menegazzo Miranda-Sapla[a], Fernanda Tomiotto-Pellissier[a], João Paulo Assolini[a], Amanda Cristina Machado Carloto[a], Bruna Taciane da Silva Bortoleti[a], Manoela Daiele Gonçalves[a], Jean Henrique da Silva Rodrigues[b], Tiago Bervelieri Madeira[c], Celso Vataru Nakamura[b], Suzana Lucy Nixdorf[c], Waldiceu A. Verri Jr[a], Idessania Nazareth Costa[a], Ivete Conchon-Costa[a], Wander Rogerio Pavanelli[a]

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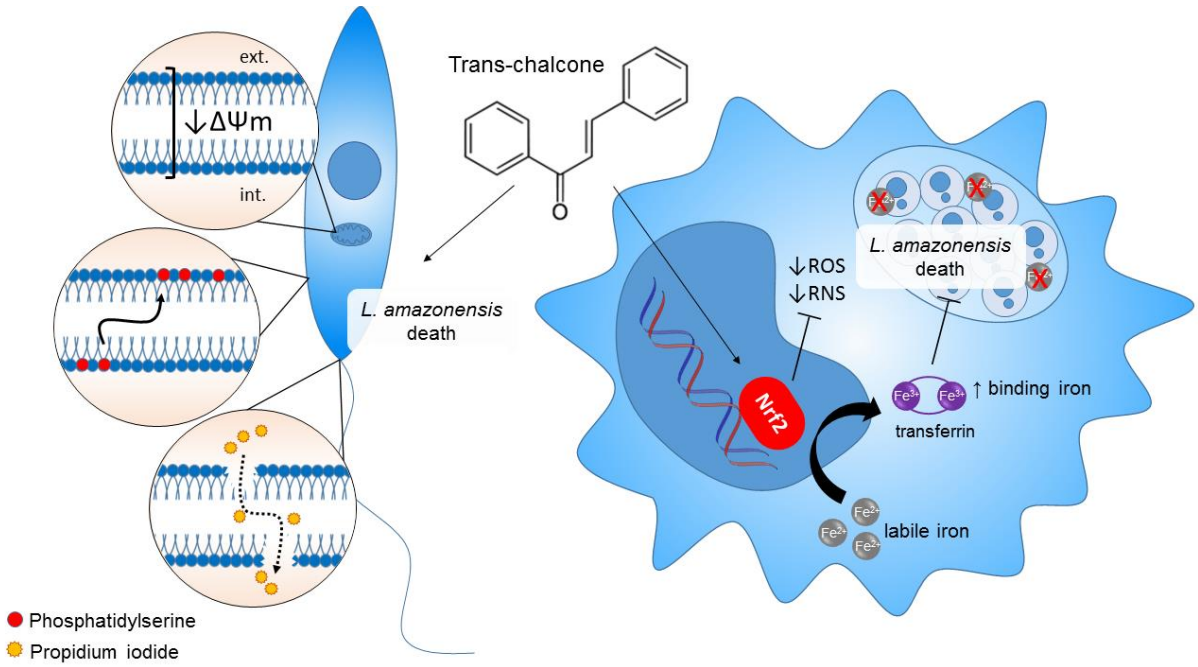
Abstract

Leishmania parasites infect macrophages, causing a wide spectrum of human diseases, from cutaneous to visceral forms. Currently drugs used in leishmaniasis treatment are highly toxic and associated with acquired resistance. In search of novel therapeutic targets, we performed comprehensive *in vitro* study to investigate the action of *trans*-chalcone (TC) against *Leishmania amazonensis* promastigote and amastigote forms. TC is a common precursor of flavonoids, with lack of studies on its pharmacological properties. *In silico* predictions showed good drug-likeness potential for TC showing high oral bioavailability and intestinal absorption. The TC-treatment acted directly on promastigote forms leading to death by late apoptosis-like process due to an increased production of reactive oxygen species (ROS), loss of mitochondrial integrity, phosphatidylserine exposure and damage on membrane. In *L. amazonensis*-infected macrophages, TC-treatment reduced the percentage of infected cells and the number of amastigotes per macrophage, consequently, the number of promastigotes recovered without cytotoxic effects on macrophages, showing selectivity index (SI) for the parasite of 53.8. This leishmanicidal effect was accompanied by a decrease in the levels of TNF-α, TGF-β, IL-10, ROS and NO and upregulation of transcription factor Nrf2 expression, modulating iron metabolism, depleting available iron for parasite replication and survival within macrophages. These results suggested *trans*-chalcone as a good support for further studies and can become a lead molecule for the design of new prototypes of antileishmanial drugs.

Keywords Leishmaniasis, apoptosis-like death, iron metabolism, transcription factor Nrf2

Graphical abstract



Highlights

TC-structure predicts a high human oral bioavailability and intestinal absorption

TC directly affects promastigote and amastigote forms *in vitro*.

TC –treatment of promastigote forms results in late apoptosis-like death.

TC eliminates amastigotes forms via ROS and RNS-independent pathway.

TC upregulates Nrf2 expression interfering in iron metabolism.

1. Introduction

Leishmaniasis is a broad term applied to a group of vector-borne diseases caused by more than 20 species of protozoan parasites of the genus *Leishmania* (Borghi et al., 2017). Infection occurs from the bite of an infected phlebotomine sandfly and depending on the species of the parasite, the host immune response, as well as the genetically determined response by the host a spectrum of diseases ranging from the cutaneous to the visceral forms may occur. The disease is endemic in 102 countries, areas or territories worldwide with more than one billion people living at risk of infection of the disease forms – visceral, cutaneous, mucocutaneous and post kala-azar dermal leishmaniasis – occurring 20.000 to 30.000 deaths annually (Alvar et al., 2012; WHO, 2016).

The factors involved in the relationship between parasite and host are complex. Experimental models have shown that the outcome of *Leishmania* infection is critically $CD4^+$ T cell-dependent and the prevalence of Th2 immune response is associated to host susceptibility and the resistance is associated a Th1-inflammatory profile, leading to macrophage activation, resulting in the increase of reactive nitrogen species (RNS) production, mainly nitric oxide (NO) and reactive oxygen species (ROS) production (Sacks and Noben-Trauth, 2002; Van Assche et al., 2011). However, *Leishmania* parasites have a sophisticated survival mechanism that involves downregulation of microbicidal mechanisms of macrophages and can sequester hosts nutrients, as scavenge labile iron pool and uses it to survival and replication inside macrophages (Cecílio et al., 2014; Das et al., 2009; Liu and Uzonna, 2012; Zaidi et al., 2017). In this way, although the Th1 inflammatory profile is the most beneficial to the natural resolution of leishmaniasis, the chronicity, produced by continuous stimulation of inflammatory cells and mediators of inflammation lead to increased tissue damage and exacerbation of the injury, resulting in a ‘‘immune conflict” because the same immune response that reduces infection worsens the clinical course (Scott and Novais, 2016).

The treatment of leishmaniasis is still problematic because most of the drugs currently used to treat patients are high toxicity and cause serious systemic side effects, besides the expensive cost, invasive route of administration, and long treatment duration (Croft and Olliaro, 2011; Santos et al., 2008). In this way, the search for new therapies well-tolerated, orally acting and short-course treatments are needed.

Chalcones (1,3-diphenyl-2-propen-1-ones) are open-chain precursors for biosynthesis of flavonoids and isoflavonoids being biologically classified as secondary metabolites of low

molecular weight (Sahu et al., 2012; Wong, 1968). The structure of chalcones consists of two aromatic rings joined by a three-carbon unsaturated chain and a carbonyl group and in most of the cases, the isomer *trans*-chalcone (TC) (Figure 1) is more stable, which makes it the predominant configuration among the chalcones (Evranos Aksöz and Ertan, 2011).

Naturally occurring chalcones, several other synthetic chalcones and chalcone-hybrid molecules have been studied as a new class of substances against *Leishmania*. Recently Tajuddeen et al. (2017) reviewed the antileishmanial activity of 278 synthetic and 34 chalcones of plant origin. Chalcones have a simple chemistry which enables a multiplicity of substitutions allowing a large number of derivatives (Gomes et al., 2017). However, these alterations in the chemical composition of the molecule result in important changes in the promising biological activities and mechanism of action often not studied in these works.

The TC leishmanicidal effect was reported by Piñero et al. (2006). In this study, it was shown the *in vitro* direct effect of TC against promastigote forms of *L. braziliensis, L. tropica, L. infantum* and *L. amazonensis* and reported that polylactic-acid (PLA) and poly D,L-lactide-co-glycolide (PGLA) implants of TC at 4 mg/kg promoted the reduction of lesion development in BALB/c mice infected with *L. amazonensis.*

TC also possesses anti-inflammatory and antioxidant effects important in *Leishmania* spp. pathogenesis. Evidence shows that TC has hepatoprotective effect reducing oxidative stress and inflammation in *in vitro* (Sikander et al., 2011a) and *in vivo* model, reducing fibrosis (Karkhaneh et al., 2016; Srivastava et al., 2016). TC blocked ischemia-induced VEGF and ICAM-1 expression due the inhibition of activated STAT3 and NF-κB (Lamoke et al., 2011).

Martinez et al. (2017a,b) showed that the systemic administration and topical formulation of TC presented anti-inflammatory and antioxidant mechanisms on ultraviolet (UV) irradiation-induced skin inflammation and oxidative stress in hairless mice. The topical formulation of TC downregulated TNF-α and superoxide anion level and improved antioxidant potential, enhancing the levels of mRNA expression of nuclear factor erythroid 2-related factor (Nfr2) and heme-oxygenase-1 (HO-1) (Martinez et al., 2017b).

These data show that TC is a promising therapeutic flavonoid, but also that there is a limited number of studies addressing the immunomodulatory activities of TC in *Leishmania* model. Therefore, the present study examined the TC theoretical predictions of drug-likeness and investigated the *in vitro* TC-effect on promastigote and 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. Material and methods

2.1 Culture of Leishmania (Leishmania) amazonensis

Promastigote forms of *Leishmania (Leishmania) amazonensis (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), 1M-HEPES buffer, 1%-human urine, 1%-L-glutamine, 10U/mL-penicillin and 10μg/mL-streptomycin (GIBCO, Invitrogen, New York, USA), and-10% sodium bicarbonate. The cell culture was maintained in B.O.D. at 24°C in a 25 cm^2-culture flask. Promastigote forms in the stationary growth phase (5-day culture) were used for all of the experiments.

2.2 trans-Chalcone

Commercial *trans*-chalcone (TC), ≥97% purity, was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA- Catalog Number sc-204681 - Dallas, USA). The stock solution of trans-chalcone was dissolved in 1% dimethyl sulfoxide (DMSO) (GIBCO, Invitrogen, New York, USA). DMSO concentration did not exceed 0.02% in all experiments.

2.3 Animals and Ethics Committee

BALB/c mice were kindly provided from Carlos Chagas Institute /Fiocruz-PR, Curitiba, Brazil. We maintained the animals weighing approximately 25-30 g and aged 6-8 weeks under sterile conditions 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).

2.4 trans-Chalcone in silico study

The TC structure *in silico* study was carried out to evaluate theoretical drug-likeness parameters related to oral bioavailability and absorption, distribution, metabolism, excretion and toxicity (ADMET) properties. The oral bioavailability was performed using Molinspiration properties calculation software (www.molinspiration.com) according to Lipinski's rule of five (Ro5) (Lipinski et al., 1997) followed by the additional rule proposed by Veber et al., (2002). According to Ro5, the parameters evaluated were molecular weight (≤ 500), lipophilicity (Log P) (≤ 5), hydrogen bonding acceptors (≤ 10) and hydrogen bonding donors (≤ 5). According to Veber, were analyzed the number of rotatable bonds (≤ 10), molecular polar surface area (TPSA) (≤ 140 Å) and sum of hydrogen bonding donors and acceptors (≤ 12).

Some ADMET properties predictions were calculated using admet SAR (http://lmmd.ecust.edu.cn/admetsar1/) online database (Cheng et al., 2012). It was predict carcinogenicity (Lagunin et al., 2009), AMES mutagenicity (Hansen et al., 2009), blood-brain barrier penetration, human intestinal absorption (Shen et al., 2010), Caco-2 permeability (Pham The et al., 2011), hERG inhibition (Marchese Robinson et al., 2011), aqueous solubility (Wang et al., 2007) and inhibition of five most important cytochrome P450 isoforms as well as their inhibition promiscuity (Cheng et al., 2011).

2.5 Antipromastigote assay

L. amazonensis promastigote forms (10^6 cells/mL) were treated with different concentrations of TC (2-12µM). Viable promastigote concentration was determined in a CASY model TT cell counter and analyzing system (ROCHE Innovatis, Germany) after 24 and 48 h of treatment. We used as negative controls the *L. amazonensis* promastigote maintained in culture without treatment and DMSO 0.02% vehicle. Amphotericin B (AmB) 1 µM as a positive control. The plates were also inspected under an inverted microscope to assure growth of the controls and sterile conditions.

2.6 Scanning electron microscopy of promastigotes

Scanning electron microscopy (SEM) of promastigotes forms was performed according to Da Silva et al., (2013), where the parasites (10^6) were treated with TC-IC_{50} and incubated for 24 h at 24ºC. The promastigotes were collected, washed with PBS and fixed with 2.5% glutaraldehyde 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. The samples were then dehydrated with graded ethanol baths, CO_2 dry point, gold coated, and observed by SEM (FEI QUANTA 200 scanning electron microscope). DMSO 0.02% vehicle was used as negative control.

2.7 Determination of the parasites cell volume

L. amazonensis promastigote forms (10^6 cells/mL) were treated with TC-IC_{50} and incubated for 24 h at 24ºC, harvested, and washed with PBS. Subsequently, the parasites were analyzed using a BD Accuri™ C5 Plus personal flow cytometer (BD Biosciences, San Jose, CA). Histograms were generated, and FSC-H represented the cell volume. A total of 10000 events were acquired in the region that corresponded to the parasites. DMSO 0.02% vehicle was used as negative control.

2.8 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, the promastigote forms (1×10^6 cells/mL) treated for 24-hour with TC-IC_{50} were washed and incubated with 25 nM of TMRE for 30 min at 25°C, washed once again in PBS, and immediately analyzed in a fluorescence microplate reader (Victor X3, PerkinElmer, Finland) with a 480 nm-excitation wavelength and a 580 nm-emission wavelength. DMSO 0.02% vehicle was used as negative control.

2.9 Determination of ROS generation on L. amazonensis promastigotes

To evaluate the ROS generation in promastigote forms of *L. amazonensis*, 10^6 parasites were pretreated or not with 5 mM-N-acetyl-L-cysteine (NAC) (Sigma, St. Louis, MO, USA) for 1 h at 24°C after incubated with TC-IC_{50} for 24h. Then, they were washed in PBS (pH 7.4) and loaded with 10μM of a permeant probe diacetate 2',7'-dichlorofluorescein (H2DCFDA) (Sigma, St. Louis, MO, USA) diluted in DMSO, 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 in a fluorescence

microplate reader (Victor X3, PerkinElmer, Finland). DMSO 0.02% vehicle was used as negative control.

2.10 Determination of phosphatidylserine exposure

Phosphatidylserine exposure was detected using Annexin-V FITC (Invitrogen, Eugene, USA), a calcium-dependent phospholipid binding protein used as an apoptosis marker. The promastigotes (1×10^6 cells/mL) were submitted to a 24-hour with TC-IC_{50}. Subsequently, the parasites were washed and resuspended in a 100 µL- binding buffer (140 mM NaCl, 5 mM $CaCl_2$, and 10 mM HEPES-Na, pH 7.4) followed by the addition of 5 µL-calcium-dependent phospholipid binding protein Annexin-V FITC for 15 min at room temperature. Subsequently, we added the incubation binding buffer (400 µL). Data acquisition was performed using a fluorescence microplate reader (Victor X3, PerkinElmer, Finland) at a 488 nm-excitation wavelength and a 520 nm-emission wavelength. For a comparison of the different treatments, we normalized the fluorescent values obtained to the respective amount of cells. DMSO 0.02% vehicle was used as negative control.

2.11 Determination of cellular membrane integrity

Promastigotes (1×10^6 cells/mL) submitted to a 24-hour treatment with TC-IC_{50} at 25°C were harvested, washed with PBS and directly followed to a five-minute incubation with propidium iodide (PI) (Sigma, St. Louis, MO, USA) (0.50 µg/mL) according to the manufacturer's instructions. Immediately thereafter, the parasites were analyzed using a fluorescence microplate reader (Victor X3, PerkinElmer, Finland) with a 480 nm-excitation wavelength and a 580 nm-emission wavelength. The fluorescent values obtained were normalized to the total number of cells per treatment. DMSO 0.02% vehicle was used as negative control.

2.12 Co-determination of annexin V and propidium iodide label

Promastigotes (10^6 cells/mL) submitted to a 24-hour treatment with TC-IC_{50} at 25°C 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™ C5

Plus personal flow cytometer (BD Biosciences, San Jose, CA). A total of 10000 events were acquired in the region that was previously established as the one that corresponded to the parasites. DMSO 0.02% vehicle was used as negative control.

2.13 Viability of peritoneal macrophages

The cytotoxic effects of TC on 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, following the description by Mosmann (Mosmann, 1983). The macrophages ($5x10^5$ cells/mL) were recovered from peritoneal cavity with cold PBS supplemented with 3%-FBS and subsequently 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 TC (10 - 10000 µM) or DMSO 0.02% (vehicle) and submitted to 24-hour culture under the same conditions. After this period, the culture was washed with PBS and added with MTT (5 mg/mL), followed by a four-hour incubation. The MTT product (formazan crystals) was diluted in 300 µL of DMSO, transferred to 96 well plates and submitted to a spectrophotometer reading (Thermo Scientific, Multiskan GO) to 550 nm. The results were expressed as a percentage of viability compared to the control group calculated with the following formula: % (viable macrophages) = (samples of the treated TC/sample OD untreated) x 100.

2.14 Selectivity index (SI)

The effect of TC on *L. amazonensis* promastigote forms was expressed as minimum inhibition concentration for inhibiting 50% of the parasites (IC_{50}) and on citotoxicity of peritoneal macrophages as the cytotoxic concentration to cause death to 50% of cells (CC_{50}). IC_{50} and CC_{50} were calculated by non-linear regression (GraphPad Software, Inc., USA, 5.00). The degree of TC selectivity was expressed as SI= CC_{50} of TC on peritoneal macrophages/IC_{50} of TC on promastigotes.

2.15 Hemolytic activity

TC treatment has been tested for hemolytic capacity, an important analysis to assess whether treatments can cause adverse effects, such as anemia. Blood from sheep (Ethics Committee for Animal Experimentation of the State University of Londrina: 82862016.60) was collected into a heparinized vacuum tube, and the erythrocytes were washed 3 times with PBS (1000 rpm for 10 minutes). A 2%-red cell suspension was prepared in PBS. The TC-treatment (10 - 10000 µM) was incubated 1: 1 in a total volume of 200 µL, with the suspension of 2% red cells in 96-well plate for 3 hours at 37°C. DMSO 0.02% vehicle was used as negative control and distilled water as the positive control for hemolysis. The plates were centrifuged at 1000 rpm for 10 minutes, and the supernatants collected and analyzed by reading the absorbance at 550 nm.

2.16 Antiamastigote assay

The peritoneal macrophages ($5x10^5$ cells/mL) were cultured in 24-well plates containing 13 mm-glass coverslips and submitted to a two-hour incubation with a 200µL-RPMI 1640 medium at 37°C and 5% CO_2. The adherent macrophages were submitted to a two-hour infection with *L. amazonensis* promastigotes ($1x10^6$); subsequently, the non-internalized promastigotes were removed through a PBS washing and the cells submitted to a 24-hour treatment using TC (10, 20 and 30 µM) or DMSO 0.02% vehicle (control) (37°C, 5% CO_2). Then, the cells were stained with Giemsa (Laborclin, Pines-PR Brazil) and 20 fields analyzed through increased soaking using an optical microscope (Olympus BX41, Olympus Optical Co., Ltd., Tokyo, Japan) (1000x magnification) in order to determine the % of infected macrophages as well as the number of amastigotes per cell after a 24-hour with TC treatment. The supernatant was stored at -80º in order to measure the cytokines and nitric oxide.

2.17 Promastigote recovery test

The promastigotes recovery assay was performed according to the description Da Silva et al., (2013). In brief, peritoneal macrophages were infected with *L. amazonensis* promastigotes and treated with TC under the same conditions of the antiamastigote assay protocol. After the 24-hour treatment, the cell culture was washed with PBS and incubated with a 199-culture medium at 24°C in order to induce the differentiation of intracellular amastigotes in promastigote forms. The recovered promastigotes were counted in a Neubauer chamber after 48, 72 and 96 hours.

2.18 Cytokines measurement

The supernatants obtained in section 2.16 were used to determine the levels of IL-1β, IL-12, TNF-α, TGF-β, and IL-10 using eBioscience commercial kits capture enzyme-linked immune sorbent assay (ELISA) (San Diego, CA, USA). According to the manufacturer's instructions, absorbance was read at 450 nm using a spectrophotometer and the results were expressed in pg/mL based on a standard curve. The sensitivity of the test was 8 pg/mL for IL-1-β, TNF-α and TGF-β, 15 pg/mL for IL-12 p70 and 32 pg/mL for IL-10.

2.19 Determination of nitrite as estimative of NO levels

Nitric oxide (NO) was determined by the Griess method. Briefly, supernatant aliquots (60 μL) obtained in section 2.16 were centrifuged at 5000 rpm for 2 min and a volume of 50 μL the supernatant was recovered, placed in 96-well microplates and added 50 μL of Griess reagent (1% sulfanilamide and 0.1% of naftiletilenodiamino-bicloridrato in orthophosphoric acid (H_3PO_4) 5%). After 10 min incubation at room temperature, the samples were read at 550nm on a microplate reader (Thermo Scientific, Multiskan GO). A calibration curve was made using serial dilutions of $NaNO_2$.

2.20 Reactive Oxygen Species (ROS) generation by macrophages

To evaluate the ROS generation, peritoneal macrophages ($5x10^5$ cells/mL - infected and treated under the same conditions described in the section 2.16) were washed in PBS (pH 7.4) and loaded with 2μM of H_2DCFDA diluted in DMSO, incubated in the dark for 30 min, 37°C, 5% CO_2. 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 an emission wavelength of 530 nm in a fluorescence microplate reader (Victor X3, PerkinElmer, Finland).

2.21 Immunocytochemistry

We performed immunocytochemical assays for Nrf2 on TC treated *L. amazonensis*-infected macrophages (as described at 2.16 section) following the streptavidin-biotin method (Dako Universal LSAB® + kit; DAKO Japan, Kyoto, Japan) with no pre-treatment microwave. Slides were submitted to one-hour incubation with 10%-Triton X-100, washed three times with PBS and replenished with 10%-FBS for 40 min at room temperature. Subsequently, the slides

were incubated overnight at 4°C with primary antibody (anti-NRF2 diluted 1:500, Santa Cruz Biotechnology). Thereupon, with the universal secondary biotinylated antibody (anti-rabbit immunoglobulin anti-mouse and anti-goat LSAB + System-HRP, Dako, Kyoto, Japan) for two hours at room temperature, the peroxidase activity was visualized through a 40”- incubation with H_2O_2 and 3,3'-diaminobenzidine (DAB). Finally, the cells were stained with Harry hematoxylin (Merck, Darmstadt, Germany). The intensity and localization of immunoreactivity against the primary antibody used were determined in all blades via a light microscope (Olympus BX41, Olympus Optical Co., Ltd., Tokyo, Japan). For image analysis, color areas representing photomicrographs (400x magnification) were digitally acquired. For the semi-quantitative score, images were assessed using the IHC profiler plugin in ImageJ software (NIH, USA). The positive immunostained area was calculated as positive labeled area. For all analyses, we used at least 10 representative images per treatment/experiment.

2.22 Determination of iron concentration and total bound iron

The determination of iron concentration in the supernatants obtained in section 2.16 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 is required the Flex® reagent cartridge for implementing the test. The dispensation, shaking and processing of the samples was automatically performed by the Dimension® system. Briefly, in acidic conditions (pH 4.5) and in the presence of a reducing agent (ascorbic acid) occurs the release of iron bound to transferrin. The resulting product (Fe^{2+}) forms a blue complex with 3-(2-pyridyl)-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 sMBles 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.

2.23 Statistical analysis

Data were expressed as a mean ± standard error of the mean. 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 by Student's t-test or one-way ANOVA, followed by Tukey's test for multiple comparisons. Differences were considered statistically significant when $p \leq 0.05$.

3. RESULTS

3.1 Drug-likeness assessment of trans-Chalcone

In order to promote the discovery of safe, efficacious and orally acting of TC, we performed a theoretical analysis of the drug-likeness through *in silico* study. TC satisfied Lipinski's rule of five and presented a high probability of human intestinal absorption, suggesting a good chance of drug-likeness and oral bioavailability (Table 1).

ADMET properties were analyzed through admetSAR and reported in Table 2. The result predicts TC non-carcinogenic, non-mutagenic, weak hERG blockers, aqueous soluble, high human intestinal absorption and $Caco_2$ cell permeability. Otherwise, TC showed some unfavorable properties, such as blood-brain barrier penetration, high promiscuity with the main five CYP isoforms (CYP1A2, CYP2C19, CYP2C9, CYP2D6 and CYP3A4).

3.2 trans-Chalcone exerts leishmanicidal effect against promastigote forms of L. amazonensis in vitro.

Initially, the direct effect of TC on *L. amazonensis* promastigote forms was investigated by determining the proliferation of parasites. We incubated the parasites with varying TC concentrations ranging from 2-12 µM for 48 h. TC treatment decreased *L. amazonensis* promastigote viability in all tested concentrations at 24 h ($p<0.0001$) (Figure 2) being determined the IC_{50} 10.3 µM. At 48 h of treatment, only the parasites treated with TC 12 µM, have continued the inhibitory effect, reducing 75% of promastigotes, similarly as AmB treatment (Figure 2). DMSO 0.02% showed no leishmanicidal effect.

3.3 trans-Chalcone induces morphological changes and reduction in the cell volume of promastigote forms

Scanning electron microscopy (SEM) was employed to assess morphological alterations in the *Leishmania* promastigotes induced by TC-IC_{50} treatment for 24h. SEM analysis of the control cells revealed healthy parasites with typical long flagella and slender bodies with unharmed cell surfaces (Figure 3A). At the IC_{50}, TC-treated promastigotes revealed morphological alterations. The cells showed rounding and reduction of the body size of the parasite with multiseptations along the length of the plasma membrane with significant extravasation of the cellular content (Figure 3B). Additional experiments were performed to evaluate cell shrinkage, as shown in Figure 3C, TC-treatment decreased 46.8% of cell volume after 24 h when compared to control.

3.4 trans-Chalcone treatment promotes late apoptotic-like death process in L. amazonensis promastigote forms

In order to elucidate the mechanism by which the parasites were being eliminated, we evaluate mitochondrial membrane potential ($\Delta\Psi m$) using the TMRE, a fluorescent marker that complexes with active mitochondria, to since the significant loss of $\Delta\Psi m$ renders cells exhausted with subsequent death. We found that the TC-treatment (10.3 μM, 24h) decreased the total TMRE fluorescence intensity compared to the control ($p \leq 0.001$), indicating loss of organelle integrity (Figure 4A).

Considering the mitochondrial alterations observed, we evaluated the ROS production to confirm the existence of altered mitochondrial function profile after the promastigotes TC-treatment. We used the H_2DCFDA molecular probe, a good indicator for ROS as it is well retained in the cells and recognizes several oxidant species, such as peroxides, superoxides, and nitric oxide (Gunasekar et al., 2002). The result confirmed the increase on the total ROS production on TC-treated promastigotes when compared with control (Figure 4B). NAC was used as ROS scavengers to confirm the involvement of ROS in H_2DCFDA results.

In addition, we investigated whether the cell death mechanism triggered by the TC-treatment involved apoptosis-like death by marking the externalization of PS, and the involvement of plasma membrane integrity, staining the TC-treated parasites with PI, which diffuses across permeable membranes and binds to nucleic acids (Figure 4C-F). We

differentiated the cell death mechanism in necrotic (PI +), apoptotic (annexin V+) or late-apoptotic (double mark for annexin V+/ PI+) process (Scariot et al., 2017). Our data showed that TC-treated parasites increased PS externalization and PI labeling when compared to the control group (Figure 4C-F). Co-staining of TC-treated promastigotes with PI and annexin V by flow cytometry showed that the percentage of promastigotes annexin V+ was 9.1%, PI+ 4.4% and the annexin V+/ PI+ was 12.2% indicating a predominance of late apoptotic-like death process resulting from TC-treatment (Figure 4E and F).

3.5 trans-Chalcone treatment raises the microbicidal activity of L. amazonensis-infected macrophages

In an attempt to verify the effect of TC-treatment against amastigote forms of *L. amazonensis*, we firstly verified whether the treatment has no toxic effect on murine macrophage and sheep erythrocytes. Assessing the possibility of TC-treatment induces hemolysis, the hemolytic activity was evaluated *in vitro* and only the 10000 µM of concentration caused 2.78 % of erythrocytes lysis (data not shown), not being able to determine the CC50 of erythrocytes. We performed the MTT assay on peritoneal macrophages verifying that concentrations above 1000 µM compromise the viability of this cell type ($p<0.0001$) (Figure 5A), being determined the CC_{50} 555.0 µM which allowed us to determine that TC-treatment has high *in vitro* selectivity index of 53.8.

We performed the antiamastigote assay to evaluate the leishmanicidal effect of TC on *L. amazonensis*-infected macrophages through the percentage of infected macrophages and the number of amastigotes after 24 h of treatment. The results showed that the DMSO 0.02% vehicle did not change the infection parameters of the control macrophages, remaining 70% of the infection rate, as well the number of amastigote/macrophage. All concentrations of TC significantly reduced 74.9%, 82.1 and 95.6% the percentage of infected macrophages for 10, 20 and 30 µM, respectively when compared to the control ($p<0.0001$). Furthermore, TC concentrations significantly decreased the mean number of amastigotes per macrophage (10, 20 and 30 µM reduced 36.3%, 58.7%, 72.8%, respectively) ($p<0.0001$) (Figure 5C). AmB, the standard drug, was used as the positive control reducing 95.5% of the infection rate and 71.4% the number of amastigote/macrophage, similarly as 30 µM TC concentration.

To confirm the reduction of infection by TC-treatment on *L. amazonensis*-infected macrophages, we performed a promastigote recovery assay in which the culture of infected macrophages was submitted to ideal conditions to the differentiation of viable amastigotes in free promastigote forms. It was verified that all tested concentrations promoted at least 60% of reduction in recovery promastigotes forms, after 48, 72 and 96h of culture when compared to the control ($p<0.0001$) (Figure 5D).

3.6 trans-Chalcone treatment down modulates cytokines, NO and ROS levels in L. amazonensis-infected macrophages

In an attempt to determine if TC acted as an immunomodulator to enhance macrophage microbicidal activity, we evaluated the cytokine profile of *L. amazonensis*-infected macrophages treated with TC for 24h. Our results showed that *L.* amazonensis-infection increased up-regulated the TNF-α, IL-10 and TGF-β levels and when these infected macrophages were treated with all TC-concentrations there was a reduction in the synthesis of these cytokines when compared to control group (Figure 6A, B and C). The level of IL-1B and IL-12p70 were below the limit of detection (data not shown).

When evaluated the NO and ROS production, we found the nitrite levels and ROS measurement corroborating with cytokine profile. The TC-treatment of infected macrophages for 24 h reduced the levels of NO at 10 μM ($p\leq0.01$), 20 μM ($p\leq0.001$) and 30 μM ($p\leq0.001$) when compared to control group (Figure 6D) and also reduced the ROS production at 20 μM ($p\leq0.05$) and 30 μM ($p\leq0.05$) when compared to the control (Figure 6F).

3.7 trans-Chalcone treatment upregulates Nrf2 expression enhancing the total bound iron capacity in L. amazonensis- infected macrophages

Although the "classic" immune system's battle against intracellular pathogens death includes the "respiratory burst" via ROS and NO production, Paiva and col. (Paiva et al., 2012) demonstrated the effect of Nrf2, a key regulator of the antioxidant response, suppressing *Trypanosoma cruzi* infection through the up-regulation of multiple genes, including ferritin and HO-1 involved in iron metabolism.

The results showed that TC-treatment of *L. amazonensis*-infected macrophages significantly upregulated the labeling of Nrf2 in all tested concentrations (Figure 7A). Although has been a slight increase on labile iron at 30 μM (Figure 7B), there was an increase in iron

bound to transferrin in all tested concentrations when compared to control (Figure 7C), decreasing the labile bioavailability of this metal.

4. DISCUSSION

The leishmaniasis is classified as neglected tropical diseases (NTDs), and thus not arousing the interest of pharmaceutical groups in relation to research and development of new drugs (Eder et al., 2014; WHO, 2016). The available drugs for the treatment of leishmaniasis are unsatisfactory, possibly because they only target the parasite, which may not alleviate the immunopathological responses that drive disease forms of leishmaniasis (Scott and Novais, 2016). Thus, besides the developing of new drugs to target the parasite, the drug-research should also consider the immunomodulatory action in order to reduce the severity of leishmaniasis-pathology.

In this context, the present study provided new perspectives of the *trans*-chalcone mechanism of action in *in vitro* experimental leishmaniasis model. Our data demonstrated that TC-treatment meets the main *in silico* drug-likeness requirements being effective at low micromolar concentrations against both promastigote and amastigote forms of *L. amazonensis*, modulating the inflammatory response and enhancing the total bound iron capacity through the activation of transcription factor Nrf2 in *L. amazonensis*-infected macrophages.

According to Drugs for Neglected Diseases Initiative (DNDi), ideal leishmaniasis treatment needs to be an oral, safe, effective, low-cost and short-course treatment (Don and Ioset, 2014; Katsuno et al., 2015). A lead drug candidate should be *in vitro* non-cytotoxic (SI>10) and present parasite potency: IC_{50} <10 μM against intracellular *Leishmania* spp.

The prediction of the chemical structure (Figure 1) influence on the oral absorption of a compound through the analysis of pharmacokinetic parameters at the beginning of a research is of extreme importance for the development of a drug candidate (Lipinski, 2004). In relation to orally bioavailable and safe parameters, we performed *in silico* study to evaluate theoretical drug-likeness parameters. Poor ADMET properties are the major reasons for the failure of drug candidates during clinical trials (van de Waterbeemd and Gifford, 2003). TC structure showed to be favorable to the most calculated ADMET properties and presented high oral bioavailability. Since most clinical forms of leishmaniasis present cutaneous symptoms, it is worth mentioning the work of Martinez et al., (2017b), who showed the effectiveness of the TC-application, as a topical formulation through the skin route, showing a further advantage of

using this compound in this important route to the rational design of cutaneous leishmaniasis-treatment. Taken together, these results could be useful to the development of more potent compounds against *Leishmania*-parasites.

Piñero et al., (2006) demonstrated the direct effect of TC against promastigote forms of different *Leishmania* spp. with *L. amazonensis*-IC_{50} determined around 9.84 μM. Our findings were very similarly of this study and beyond the direct reduction in the number of viable parasites we showed physiological and morphological alterations on promastigote forms analyses of the TC-treated parasites, showing loss of volume and cell shrinkage, a hallmarks of apoptotic death.

Apoptosis is a cascade process involving physical and biochemical changes in the cell where mitochondria play a central role in supplying energy through the generation of cellular adenosine triphosphate by oxidative phosphorylation being an easy target for induction of apoptosis under stress conditions (Saudagar and Dubey, 2014). The apoptosis death-induction by TC has already been demonstrated in tumor cell lines MCF-7 through induction of the intrinsic apoptotic by mitochondrial pathway, inducing cytochrome *c* release. TC enhancing the expression of the adapter protein apoptotic protease-activating factor-1 (APAF-1), Bcl-2-associated X protein (BAX) and strong induction of cell death-inducing DNA fragmentation factor alpha-like effector- A (CIDEA) promoter gene and protein, which is related to DNA fragmentation during apoptosis (Bortolotto et al., 2017).

In trypanosomatids, although apoptotic regulators from the Bcl-2 family and the role of metacaspases were not well documented, the release of cytochrome *c* has been related in many species of *Leishmania* in response to several apoptotic stimuli (Gannavaram et al., 2008; Jiménez-Ruiz et al., 2010; Menna-Barreto and de Castro, 2015). For organisms with a single mitochondrion, such as *Leishmania*, the survival depends on the proper functioning of this organelle (Sen et al., 2004). TC-treatment may exert its anti-leishmanial activity by affecting mitochondrial function in the parasite, demonstrated by a decrease in $\Delta\Psi m$, and an increase in ROS production.

Together, our results showed that TC-treatment resulted in late apoptotic-like death mechanism characterized by cell shrinkage, phosphatidylserine exposure, damage in the plasma membrane of the protozoan with significant extravasation of the cellular content after 24h TC-treatment.

Among the major challenges of the search for new drugs for leishmaniasis is the intracellular location of the amastigote forms within macrophages, resulting in additional membranes and pH gradients that drugs must cross before reaching their target (Croft and Olliaro, 2011). Once the effect of the TC on promastigote forms had been proven, we aimed to understand the action of this treatment against intracellular amastigote forms, and we decided to test in this set of experiments concentrations above the IC_{50}. The TC concentrations tested did not show hemolytic effects and deleterious activity to the macrophages, the main host cells of *Leishmania* spp., presenting high selectivity index to protozoan and killing *L. amazonensis* amastigote forms.

Cytokine secretion drives the innate immune response during the early stage of experimental leishmaniasis (Nylén and Gautam, 2010). IL-1β, TNF-α and IL-12 are cytokines which activate macrophages, leading to synthesis ROS and NO, which on the one hand trigger the microbicidal action, but on the other, cause tissue damage and injury progression (Andrews, 2012; Cecílio et al., 2014). Our data showed an immunomodulation of TC on *L. amazonensis*-infected macrophages restoring the levels of the cytokines TNF-α, IL-10 and TGF-β to similar values of the uninfected group. The TC-treatment was also not able to induce the production of ROS, NO and consequent increase in oxidative stress.

This reduction in the synthesis of these immunologic mediators may be due to TC-leishmanicidal action resulting in parasite reduction and, consequently, less activation of the immune response. Besides this, the downmodulation effect of TC on transcription factor NF-κB, essential in inflammation and innate immunity, regulating the expression of proinflammatory genes including IL-1β, TNF-α and iNOS was demonstrated on LPS (Lamoke et al., 2011; Liu et al., 2007), on trinitrobenzenesulfonic acid colits (Park et al., 2012) and cancer cell proliferation models (Shen et al., 2007). The suppression of many inflammatory mediators as TNF-α, NO, lipid peroxidation were also demonstrated (Bortolotto et al., 2017; Hsu et al., 2006; Karkhaneh et al., 2016; Lamoke et al., 2011; Shen et al., 2007; Sikander et al., 2011b; Silva et al., 2016). In addition, TC treatment also reduces the levels of TGF- β in *in vitro* hepatocellular carcinoma (HepG2) cells (Sikander et al., 2011a).

Recently, Martinez et al. (2017b) showed the role of TC in enhancing the levels of mRNA expression of Nrf2 and HO-1 in a model of ultraviolet B radiation skin damage in hairless mice. Nrf2 orchestrates antioxidant responses and cellular protective genes, primarily in response to oxidative stress and genes that contain ARE motifs, upregulating the expression

of ferroportin, ferritin and HO-1 (Alam et al., 1999; Hintze and Theil, 2005; Marro et al., 2010). HO-1 is associated with regulation of labile iron pool, by increasing ferritin, a protective protein that acts sequestering iron (Lin et al., 2008). Increased levels of ferroportin and ferritin are expected to reduce the levels of iron available for intracellular pathogens (Andrews, 2012).

Taylor and Kelly (2010) qualified the iron uptake as an Achilles'heel of the *Leishmania* parasites, suitable for targeting with chemotherapy. The intracellular stage of *Leishmania* are equipped with an iron transport machinery on the surface of the amastigote forms and can also subvert the host's iron uptake systems to their own advantage, utilizing various iron sources within the parasitophorous vacuole such as labile iron or heme, essential for pathogenicity and also for their survival (reviewed in Niu et al., 2016).

Although TC cannot act as iron chelator *per se in vitro* (Martinez et al., 2017a), in our experimental model, TC treatment was also able to increase the expression of Nrf2 and, consequently, modulates the levels of iron bound to transferrin being possible to infer that this iron modulation directly affects the intracellular amastigote forms of *L. amazonensis.*

In conclusion, this work provided new perspectives of the *in vitro* antileishmanial TC-activity acting directly on promastigote forms leading to death by late apoptosis-like process; and in *L. amazonensis*-infected macrophages, modulating the inflammatory response and enhancing the total bound iron capacity through the activation of transcription factor Nrf2. These results associated with the theoretical predictions of drug-likeness suggested *trans*-chalcone as a good scaffold for further studies and can become a lead molecule for the design of new prototypes of antileishmanial drugs.

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Tables

Table 1- Molecular properties of *Trans*- Chalcone based on Lipinski's and Veber's criteria .

		LIMITS	TC
Lipinski's Rules	**MW**	≤500	208.3
	Log P	≤5	3.8
	H-Acc	≤10	1
	H-Don	≤5	0
Veber's Rules	**RB**	≤10	3
	tPSA	≤140$^{o}Å^{2}$	17
	H-Acc + H-Don	≤12	1
Number of Violations			none

MW: molecular weight; Log P: Log of partition-coefficient; H-Acc: number of Hydrogen bond acceptor; H-Don: number of Hydrogen bond donor; RB: number of rotatable bonds; tPSA: molecular polar surface area;

Table 2- ADME/TOX and pharmacological parameter assessment of *trans*-chalcone predicted using admetSAR toolbox

	ADMET Properties	TC	Reference
	Carcinogenicity	Non-carcinogenic	Lagunin et al., 2009
	AMES toxicity	Non-mutagenic	Hansen et al., 2009
	Human intestinal absortion	High	Shen et al., 2010
	Caco-2 permeability	High	Pham The et al., 2011
	Blood-brain barrier penetration	Penetrate	Shen et al., 2010
	Predicted aqueous solubility	Soluble	Wang et al., 2007
	hERG inhibition	Weak inhibitor	Marchese Robinson et al., 2011
CYP 450 subunits	CYP3A4	Non-Inhibitor	Cheng et al., 2011
	CYP2D6	Non-inhibitor	Cheng et al., 2011
	CYP2C19	Inhibitor	Cheng et al., 2011
	CYP2C9	Non-Inhibitor	Cheng et al., 2011
	CYP1A2	Inhibitor	Cheng et al., 2011
	CYP inhibitory promiscuity	High	Cheng et al., 2011

Predictions based on: admetSAR online database developed by Cheng et al., 2012

Figures

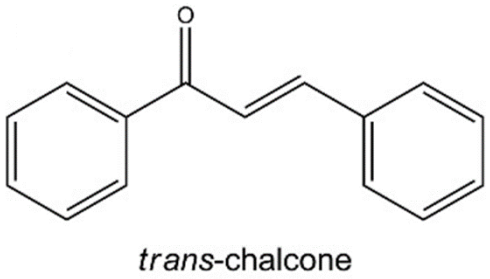

Figure 1- Molecular structure of *trans*-chalcone.

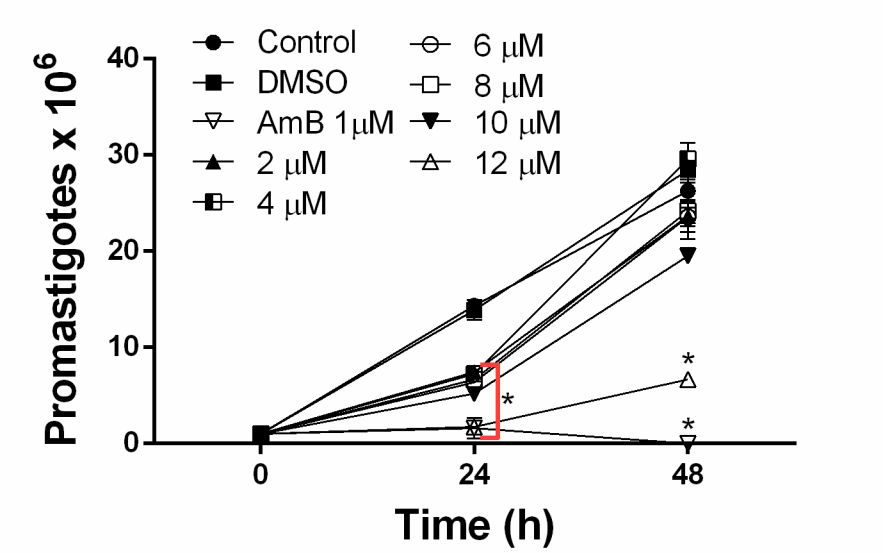

Figure 2 – Antipromastigote effect of *trans*-chalcone. *L. amazonensis* promastigotes forms were subjected to TC treatment (2-12 μM). The parasite viability was assessed at 0, 24 and 48h by CASY model TT cell counter. As control was used non-treated parasites, as vehicle control was used DMSO 0.02% and as a positive control was used amphotericin B (AmB) 1 μM. The values represent the mean ± SEM of three independent experiments performed in duplicate. * Significant difference compared to control group ($p < 0.0001$).

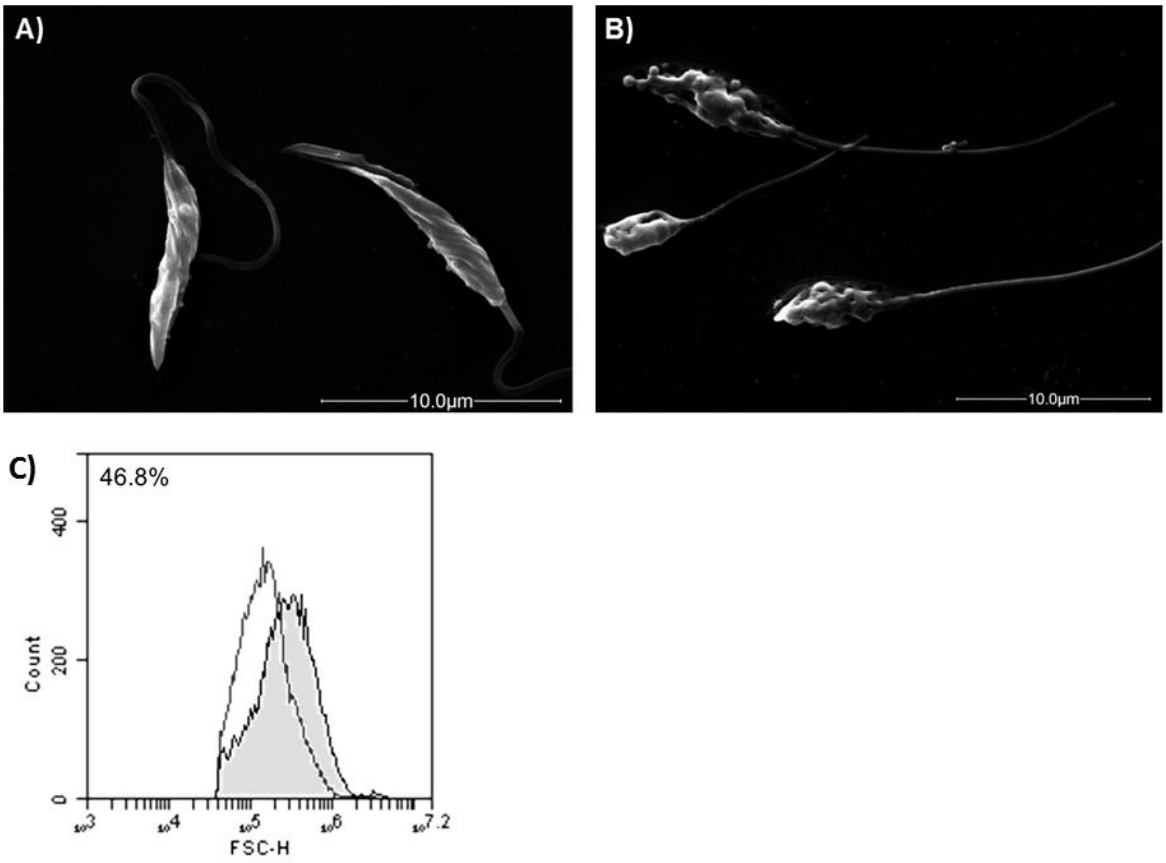

Figure 3 - Morphological changes of *trans*-chalcone-treated promastigote forms. Scanning electron microscopy of *L. amazonensis* promastigote forms of control promastigotes (DMSO 0.02%-vehicle) (A) and promastigotes treated for 24 h at 25ºC with TC concentration corresponded to the IC_{50} (10.3 µM) (B). (A) The protozoan presented typical characteristics, with an elongated shape and free flagellum. (B) TC treatment induced alterations in the shape and size of the protozoan. Scale bar = 10 µm. Cell volume in TC-treated promastigote forms (C). FSC-H was 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.

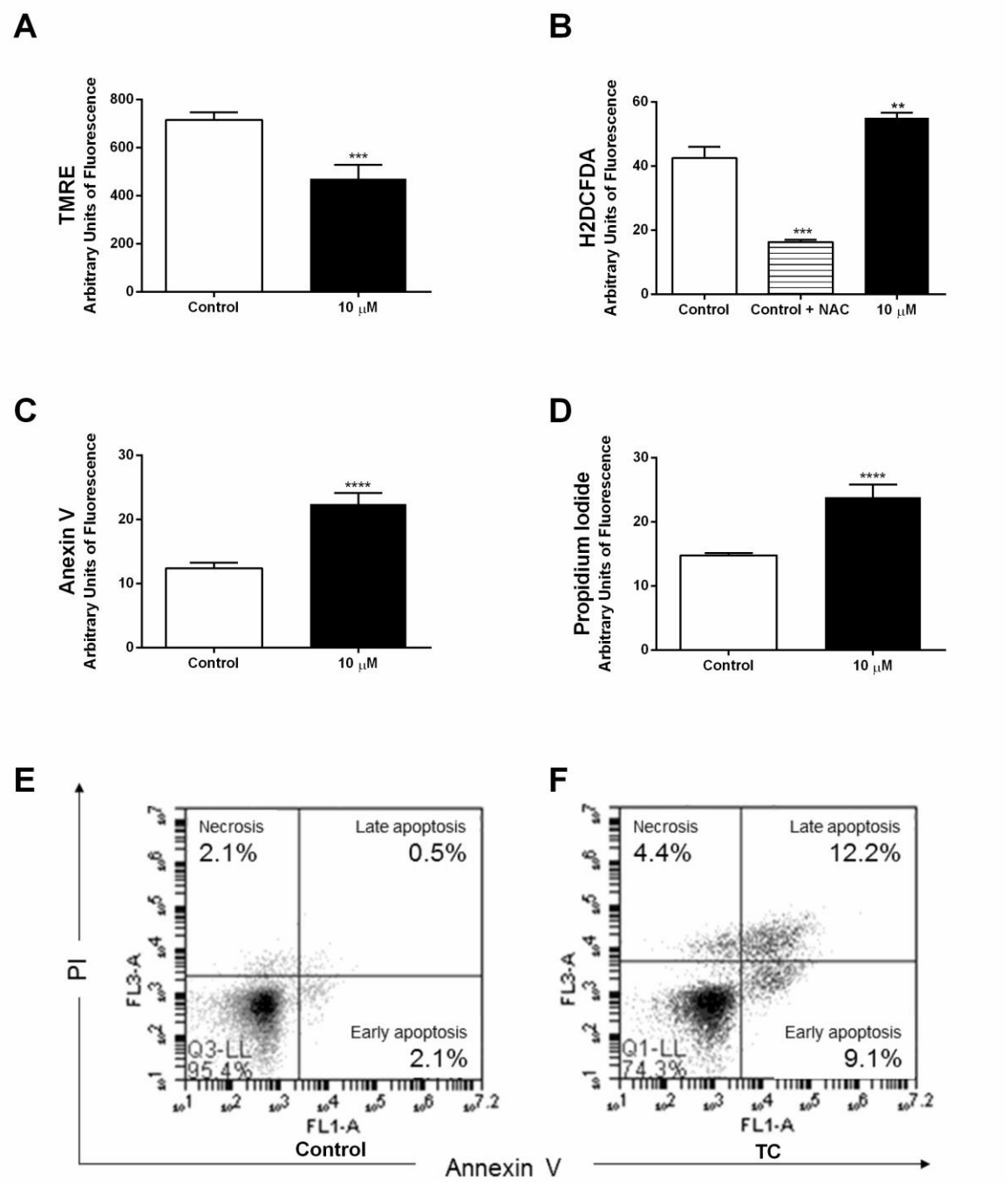

Figure 4 – *Trans*-chalcone induces late apoptotic-like death process in *L. amazonensis* promastigote forms. *L. amazonensis* promastigote forms of control (DMSO 0.02% vehicle) and TC-treated with IC_{50} (10.3 μM) for 24 h at 25ºC were analyzed by the following methods: (A) TMRE assay for fluorimetric analysis of the mitochondrial membrane potential, (B) H_2DCFDA molecular 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. Co-staining of PI and annexin V–FitC analyzed by flow cytometry in (E) Control (untreated parasites) and (F) TC-treated promastigotes. Typical dot plots of at least three independent experiments are shown. Data represent the mean ± SEM of three independent experiments performed in duplicate. ** Significant difference compared to control ($p \leq 0.01$), *** ($p \leq 0.001$), **** ($p \leq 0.0001$).

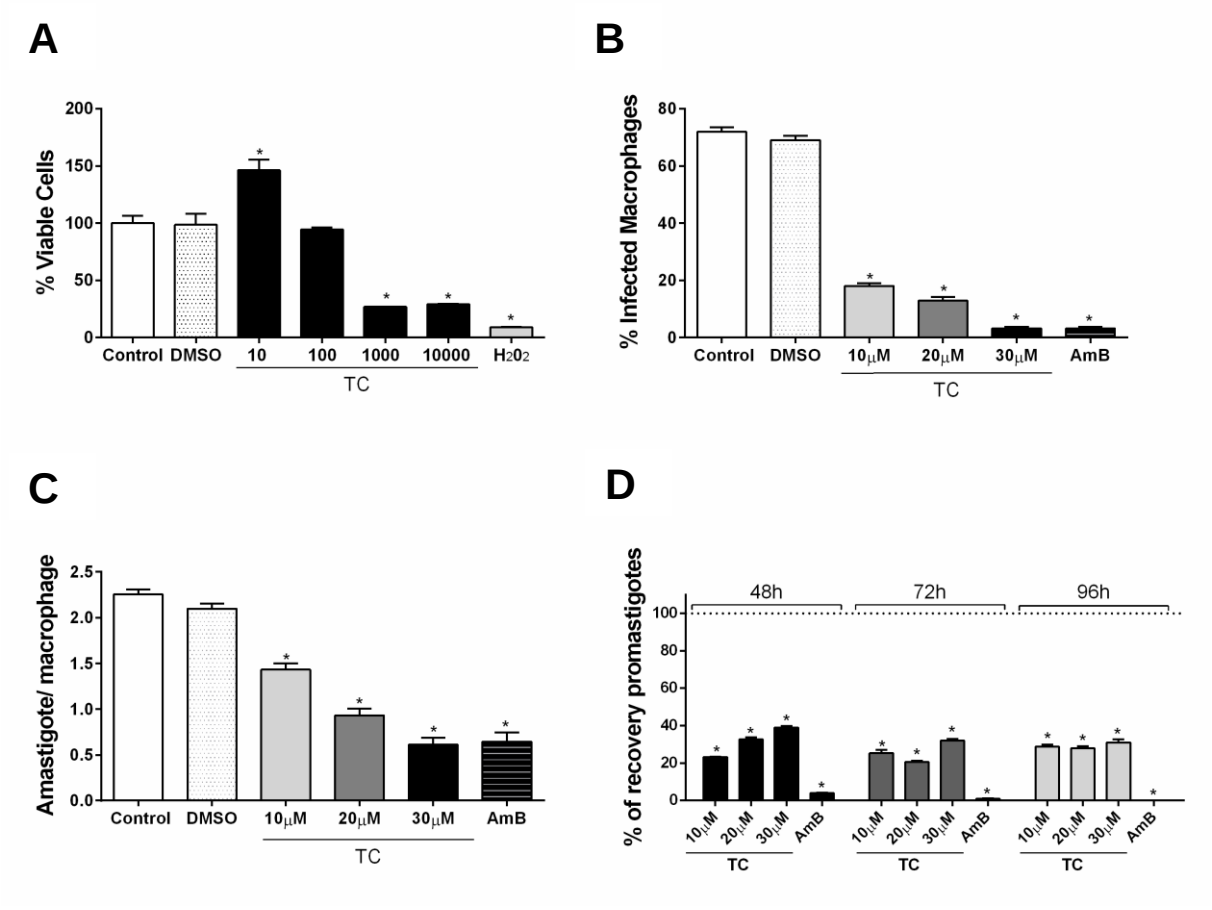

Figure 5 - Effect of *trans*-chalcone on *L. amazonensis*-infected macrophages. (A) Peritoneal macrophages (5 x 10^5/well) of BALB/c mouse were plated in 24-well plates, treated with TC (10,-10000 μM) or DMSO 0.02%-vehicle, incubated for 24h at 37ºC/5% CO_2 and viability analyzed through MTT assay. RPMI 1640 medium and 0.4%-H_2O_2 were used as negative and positive controls, respectively. (B) *L. amazonensis*-infected macrophages submitted to 10, 20 or 30 μM of TC- treatment or DMSO 0.02%-vehicle for 24h at 37ºC/5% CO_2 were assessed as the percentage of infected macrophages and (C) amount of amastigotes per macrophage. RPMI 1640 medium and 1 μM-AmB were used as negative and positive controls, respectively. (D) 48, 72 and 96-hour incubation of the *L. amazonensis*-infected macrophages using medium 199 in B.O.D. A number of recovered parasites was measured in Neubauer chamber and the values converted as %. The dashed line indicates the control group (100%). The values represent the mean ± SEM of three independent experiments performed in duplicate. * Significant difference compared to control ($p < 0.0001$).

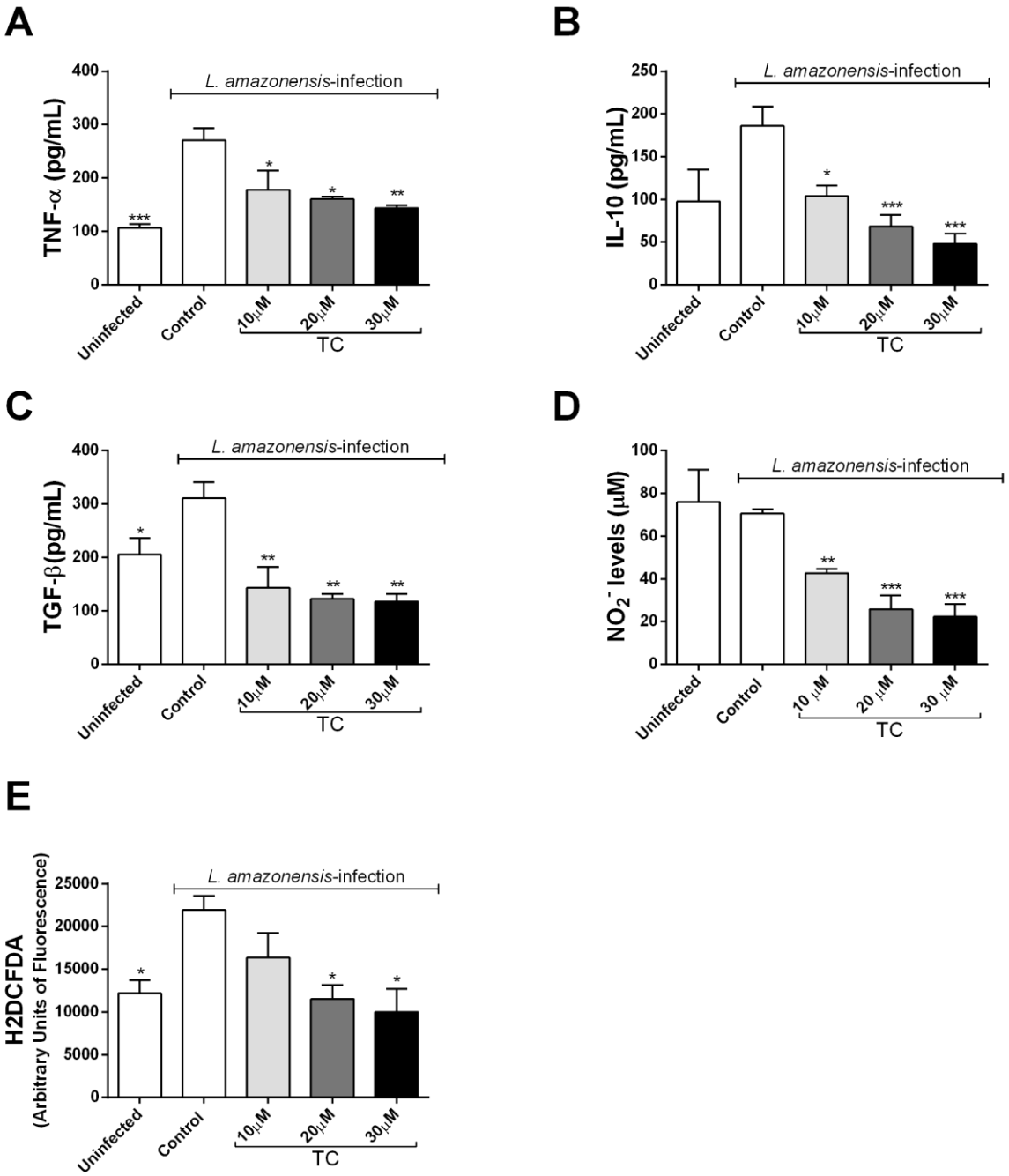

Figure 6 - Intra-macrophage *L. amazonensis* death by *trans*- chalcone treatment is not dependent on NO and ROS. The following methods were used to assess the *L. amazonensis*-infected macrophages submitted TC-treatment for 24h at concentrations of 10, 20 and 30 μM: (A) ELISA assay to measure the TNF-α, (B) TGF-β and (C) IL-10; (D) Griess method for nitrite levels; (E) fluorescent probe H_2DCFDA for reactive oxygen species measurement. The values represent the mean ± SEM of three independent experiments performed in duplicate. * Significant difference compared to control ($p \leq 0.05$), ** ($p \leq 0.01$) *** ($p \leq 0.001$)

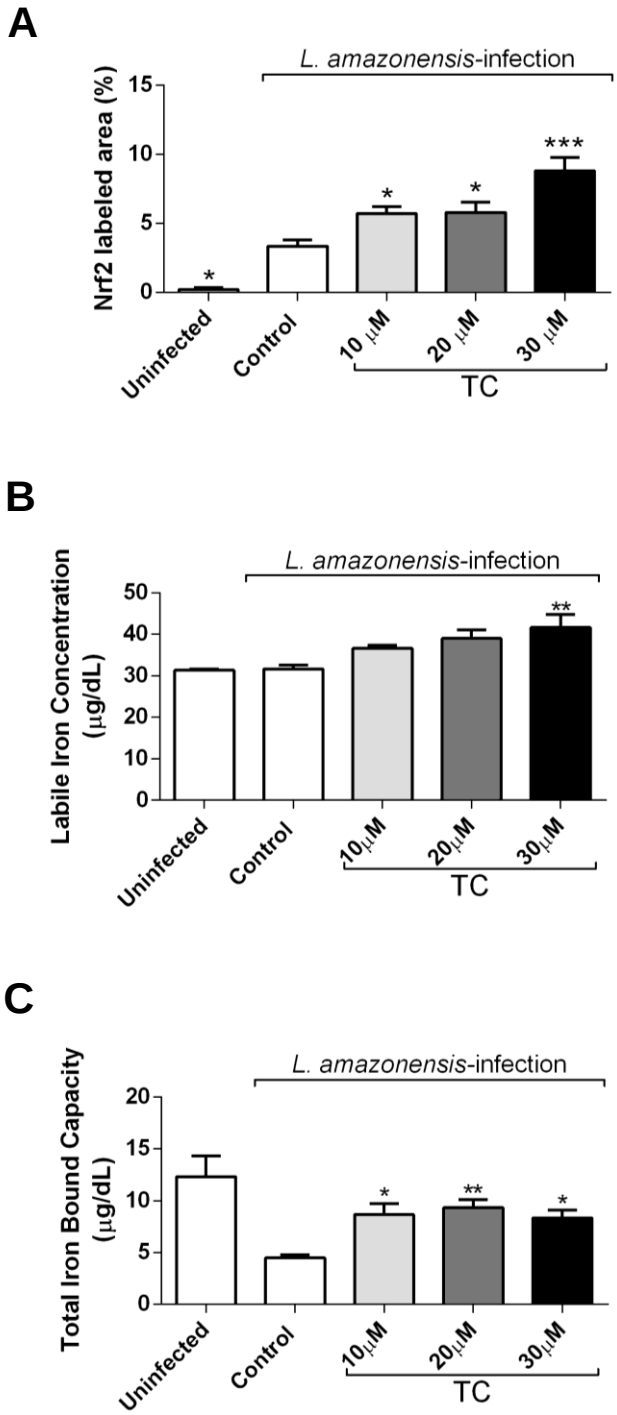

Figure 7 - *Trans*-chalcone treatment upregulates Nrf2 expression enhancing the total bound iron capacity in *L. amazonensis*- infected macrophages. (A) Immunocytochemical labeling for Nrf-2 in peritoneal macrophages infected with *L. amazonensis* and treated with TC (10, 20, and 30 μM) for 24 h. Positive immunolabeling area was calculated as percentage of positive labeled area compared to the total area. The culture recovery supernatants were used to determinate the (B) labile iron concentration and (C) total iron bound capacity utilizing the Dimension® automated system. Data represent mean ± SEM of three independent experiments. * Significant difference compared to control ($p \leq 0.05$), ** ($p \leq 0.01$) *** ($p \leq 0.001$).

CONCLUSÃO GERAL

- O ácido caurenóico (AC) é um diterpeno do tipo caurano com muitas propriedades biológicas. O efeito de AC no modelo *in vitro* de leishmaniose experimental foi inibir a proliferação das formas promastigotas comprovadas pelos ensaios de viabilidade mitocondrial e cinética de proliferação celular. O AC promoveu uma redução significativa na porcentagem de macrófagos infectados, bem como no número de amastigotas por macrófago. Além disso, seu efeito foi associado ao aumentou nos níveis de óxido nítrico (NO) em um mecanismo dependente de cNOS. O tratamento induziu ainda o aumento da da citocina pró-inflamatória IL-1β ativa e expressão do componente ativador de inflammasoma NLRP12. Esses resultados demonstram a capacidade leishmanicida de AC sobre amastigotas intracelulares por um mecanismo NLRP12 / IL-1β / cNOS / NO. Desta forma, esta molécula apresenta potencial terapêutico farmacólogico contra a infecção por *Leishmania.*
- A trans-chacona (TC) é um flavonóide natural com alto indíce de propriedades biológicas favorecendo seus parâmetros farmacológicos. As previsões *in silico* mostraram que TC apresenta bom potencial para fármaco com alta biodisponibilidade oral e absorção intestinal. A TC apresentou efeito antipromastigota, com alterações morfológicas significativas. A morte das formas promastigotas de *L. amazonensis* desencadeada pelo tratamento com a molécula foi por apoptose-*like* tardia, caracterizada pela produção de espécies reativas de oxigênio, despolarização mitocondrial, permeabilização da membrana do parasito, redução do volume celular e exposição de fosfatidilserina. Nas concentrações testadas não foi observado citotoxicidade em macrófagos murinos e eritrócitos ovinos, além disso a TC promoveu a redução na porcentagem de macrófagos infectados e número de amastigotas por macrófagos. Houve diminuição da síntese da citocina inflamatória (TNF-α) e regulatórias (IL-10 e TGF-β) e redução nas moléculas microbicidas clássicas EROs e NO, possivelmente devido a redução dos parasitos e consequentemente, uma menor ativação da resposta imune. O tratamento com TC aumentou a expressão do fator nuclear eritróide 2 (Nrf2) que promove uma proteção celular em relação ao estresse oxidativo, modulando os níveis de ferro

ligado a transferrina colaborando para a eliminação do parasito.TC pode ser uma nova alternativa terapêutica para o tratamento da leishmaniose.

- Os compostos analisados AC e TC apresentaram um perfil pró-inflamatório e anti-inflamatório, respectivamente, os quais foram capazes de atuar inibindo as formas promastigotas e amastigotas por diferentes mecanismos. Esses compostos naturais demonstram ser uma importante abordagem terapêutica para o tratamento da leishmaniose.