JOÃO PAULO ASSOLINI

EFEITO LEISHMANICIDA E IMUNOMODULADOR DOS COMPOSTOS DIETILDITIOCARBAMATO, 4-NITROCHALCONA LIVRES E NANOPARTICULADOS

Londrina
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Tese apresentada ao Programa de Pós Graduação em Patologia Experimental da Universidade Estadual de Londrina, como requisito parcial para obtenção do título de Doutor.

Orientador: Prof. Dr. Wander Rogério Pavanelli

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ASSOLINI, João Paulo. **Efeito leishmanicida e imunomodulador dos compostos Dietilditiocarbamato, 4-Nitrochalcona livres e nanoparticulados**. 2019. 116 f. Tese (Doutorado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2019.

RESUMO

A Leishmaniose Tegumentar Americana (LTA), doença causada por protozoários flagelados do gênero *Leishmania*, transmitidos ao homem pela picada de insetos de flebotomíneos, é reconhecida pela Organização Mundial de Saúde (OMS) como uma das mais importantes doenças infecto-parasitárias, devido ao elevado potencial epidêmico e capacidade de produzir deformidades. No Brasil, é amplamente distribuída ocorrendo em todos os estados, possuindo caráter endêmico. As terapias disponíveis têm se baseado no uso de antimônios pentavalentes, todavia, estes apresentam dificuldades de administração, alto custo e importantes efeitos colaterais. Desta forma, compostos naturais e sintéticos, bem como o uso de nanotecnologia têm sido amplamente explorados como alternativas terapêuticas para esta parasitose. O presente estudo teve como objetivo avaliar *in vitro* o efeito leishmanicida e imunomodulador de Dietilditilcarbamato (DETC), 4-Nitrochalcona sobre formas promastigotas; e encapsulados em nanopartículas lipídicas sólidas sobre amastigotas de *L. amazonensis*, bem como elucidar os possíveis mecanismos de morte. Assim, DETC e 4-Nitrochalcona livres apresentam atividade antipromastigota, causando despolarização da membrana mitocondrial, aumento da produção de espécies reativas de oxigênio (EROs), aumento da exposição de fosfatidilserina e também a perda da integridade de membrana plasmática, além de alterar a morfologia e reduzir o volume celular do parasito. Ao avaliar a toxicidade destes compostos, foi demonstrado que DETC e 4-Nitrochalcona livres apresentam toxicidade, todavia o uso de nanopartículas lipídicas sólidas foi capaz de reduzir a toxicidade destes compostos em macrófagos murinos. Além disso, DETC e 4-Nitrochalcona encapsulados exerceram efeito leishmanicida sobre amastigotas intracelulares, modulando a resposta imunológica e microbicida dos macrófagos infectados. DETC encapsulado foi capaz de aumentar a produção das citocinas TNF-α, lL-6 e reduzir IL-10, aumentando a produção de EROs. Já as nanopartículas lipídicas sólidas carregadas com 4-Nitrochalcona induziram a síntese de TNF-α, lL-6, IL-10 e também de EROs e óxido nítrico. Em conclusão, DETC e 4-Nitrochalcona apresentam efeito leishmanicida sobre promastigotas de *L. amazonensis* através de mecanismos apoptose-*like*. A encapsulação destes compostos reduziu a toxicidade sobre macrófagos. Além disso, DETC e 4-Nitrochalcona encapsulados apresentam atividade anti-amastigota em baixas concentrações, ativando mecanismos microbicidas dos macrófagos como EROs, NO e modulação de citocinas (TNF-α, lL-6, IL-10).

Palavras-chave: *Leishmania*. Chalconas. Citocinas. Imunomodulação. Macrófagos. Espécies reativas de oxigênio (EROs). Nanotecnologia.

ASSOLINI, João Paulo. **Leishmanicidal and immunomodulatory effect of the compounds Diethyldithiocarbamate, 4-Nitrochalcone free and nanoparticulate**. 2019. 116 p. Thesis (Doctorate in Experimental Pathology) – State University of Londrina, Londrina, 2019.

ABSTRACT

American Cutaneous Leishmaniasis (ACL), a disease caused by flagellate protozoa of the genus *Leishmania* transmitted to man by insect bite from phlebotomines, is recognized by the World Health Organization (WHO) as one of the most important infectious and parasitic diseases due to the high epidemic potential and ability to produce deformities. In Brazil, it is widely distributed occurring in all states, and in Paraná it has an endemic character. Currently the available therapies have been based on the use of pentavalent antimonials, however, these present difficulties of administration, high cost and important side effects. In this way, natural and synthetic compounds, as well as the use of nanotechnology have been widely explored as therapeutic alternatives for this parasitosis. The objective of the present study was to evaluate in vitro the leishmanicidal effect of Diethyldithiocarbamate (DETC), 4-Nitrochalcone free on promastigote forms; and encapsulated in solid lipid nanoparticles on *L. amazonensis* amastigotes, as well as to elucidate the possible mechanisms of action. Thus, free DETC and 4-Nitrochalcone exhibit antipromastigote activity, causing depolarization of the mitochondrial membrane, increased production of reactive oxygen species (ROS), increased phosphatidylserine exposure and also loss of plasma membrane integrity, as well as altering cell morphology and volume of the parasite. In evaluating the toxicity of these compounds, it was demonstrated that free DETC and 4-Nitrochalcone showed toxicity, however the use of solid lipid nanoparticles was able to reduce the toxicity of these compounds in murine macrophages. In addition, encapsulated DETC and 4-Nitrochalcone exert leishmanicidal effect on intracellular amastigotes, modulating the immunological and microbicidal response of infected macrophages. Encapsulated DETC was able to increase the production of cytokines TNF-α, IL-6 and reduce IL-10, increasing the production of ROS. Already the solid lipid nanoparticles loaded with 4-Nitrochalcone induced the synthesis of TNF-α, IL-6, IL-10 and also of ROS and nitric oxide. Em conclusão, DETC e 4-Nitrochalcona apresentam efeito leishmanicida sobre promastigotas de *L. amazonensis* através de mecanismos apoptose-like. The encapsulation of these compounds reduced toxicity to macrophages. In addition, encapsulated DETC and 4-Nitrochalcona have anti-amastigote activity in low concentrations, activating microbicidal mechanisms of macrophages such as ROS, NO and cytokine modulation (TNF-α, IL-6, IL-10).

Key-Words: *Leishmania*. Chalcones. Cytokines. Immunomodulation. Macrophages. Reactive oxygen species (ROS). Nanotechnology.

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LISTA DE ABREVIATURAS E SIGLAS

4-NC	4-Nitrochalcona
ACL	*American Cutaneous Leishmaniasis*
ATP	Adenosina Trifosfato
Bcl-2	*B-cell lymphoma 2*
C3b	Componente 3b do complemento
C3bi	Componente 3b inativo do complemento
C5	Componente 5 do complemento
$CaCl_2$	Cloreto de Cálcio
CCCP	*Carbonyl cyanide m-chlorophenylhydrazone*
CL	*Cutaneous Leishmaniasis*
cm	Centímetro
CO_2	Dióxido de Carbono
CR3	Receptor do Complemento 3
DETC	Dietilditiocarbamato
DMSA	Ácido dimercaptosuccínico
DMSO	Dimetilsulfóxido
DNA	Ácido desoxirribonucleico
ELISA	*Enzyme-linked immunosorbent assay*
ERN	Espécies Reativas de Nitrogênio
ERO	Espécies Reativas de Oxigênio
FBS	*Fetal bovine serum*
FSC-H	*Forward SCatter Height*
g	grama
gp63	Glicoproteína de 63 kDa
GTP	Guanosina Trifosfato
h	Hora
H_2DCFDA	*2', 7'-dichlorofluorescein diacetate*
H_2O_2	Peróxido de hidrogênio
HCl	Ácido Clorídrico
IC50	*Inhibitory concentration 50%*
IFN-γ	Interferon gama
IL-10	Interleucina 10

IL-13	Interleucina 13
IL-17	Interleucina 17
IL-1β	Interleucina 1 beta
IL-21	Interleucina 21
IL-22	Interleucina 22
IL-4	Interleucina 4
IL-6	Interleucina 6
IL-8	Interleucina 8
iNOS	Óxido Nítrico Sintase induzível
LCD	Leishmaniose cutânea difusa
LCL	Leishmaniose cutânea localizada
LMC	Leishmaniose mucocutânea
LPG	Lipofosfoglicano
LPS	Lipopolissacarídeo
LTA	Leishmaniose Tegumentar Americana
LV	Leishmaniose visceral
MDC	*Monodansylcadaverine*
min	minuto
mL	mililitro
mM	milimolar
MTT	*3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide*
NFκB	*Nuclear Factor kappa B*
nm	Nanômetro
nM	Nanomolar
NO	Óxido Nítrico
NO_2	Nitro
NOS	Óxido Nítrico Sintase
NOX	NADPH oxidase
Nps	*Nanoparticles*
Nrf2	*Nuclear factor erythroid 2-related factor 2*
O/W	*Oil/ Water*
ºC	Graus Celsius
OMS	Organização Mundial da Saúde
PBS	*phosphate buffered saline*

PDI	*Polydispersity index*
pg	Picograma
PGE	*polyethylene glycol*
pH	potencial Hidrogeniônico
PI	*Propidium Iodide*
PLA	*Polylactic acid*
PLGA	*poly (D,L-lactic-co-glycolic acid)*
rmp	Rotações por minuto
ROS	*Reactive Oxygen Species*
s	segundo
SEM	*Scanning electron microscopy*
SLN	*Solid lipid nanoparticle*
SOD1	Superóxido Dismutase 1
TEM	*Transmission Electron Microscopy*
TGF-β	*Transforming Growth Fator beta*
Th1	T *helper* 1
Th17	T *helper* 17
Th2	T *helper* 2
TLR	*Toll-like receptor*
TMRE	*Tetramethylrhodamine-ethyl ester*
TNFR	*Tumor necrosis factor receptor*
TNF-α	*Tumor necrosis factor alpha*
Treg	Células T regulatórias
µL	microlitro
µM	micromolar

SUMÁRIO

1 INTRODUÇÃO

1.1 Aspectos Gerais da Leishmaniose

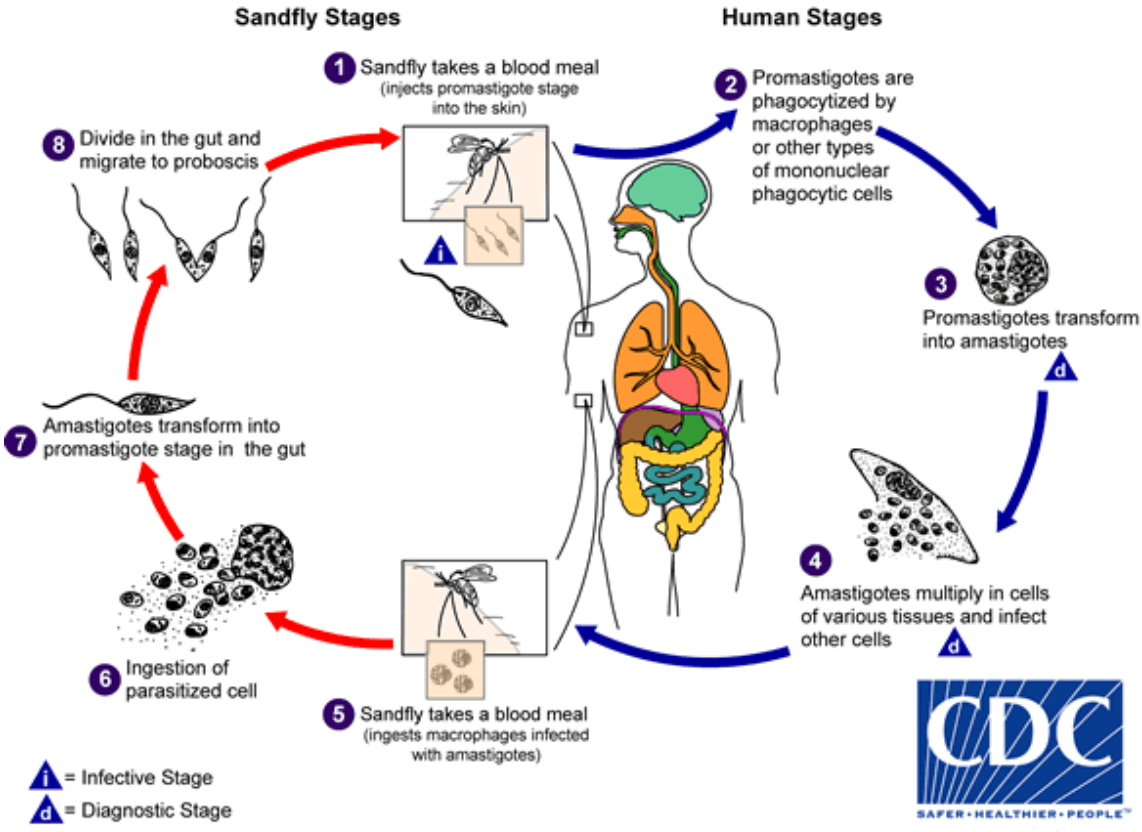
Leishmanioses são um complexo de doenças causadas por mais de 20 espécies de protozoários *Leishmania* (ordem Kinetoplastida, família Trypanosomatidae), os quais são transmitidos ao homem pela picada de insetos fêmeas de flebotomíneos (gêneros *Phlebotomus* no Velho Mundo e *Lutzomyia* no Novo Mundo). O parasito apresenta morfologia variável de acordo com o hospedeiro que infecta. As formas amastigotas são arredondadas e sem flagelo aparente, sendo encontradas principalmente em macrófagos teciduais do hospedeiro vertebrado (HALL; JOINER, 1991), já as promastigotas apresentam formas alongadas, possuem flagelo e são altamente infectantes, encontradas no tubo digestivo do inseto vetor (WALTERS, 1993).

O ciclo biológico (Figura 1) inicia-se quando o inseto vetor infectado, no momento do repasto sanguíneo inocula formas promastigotas metacíclicas juntamente com a saliva na derme do hospedeiro vertebrado.

Com isso, ocorre o recrutamento de células de defesa, principalmente macrófagos que realizam a fagocitose e internalização destes parasitos em vacúolos fagocíticos. Devido ao pH ácido, temperatura de 37º C, restrição de nutrientes dentro do vacúolo, as formas promastigotas se diferenciam em amastigotas, que são mais resistentes. Esta forma inicia um processo de divisão binária até ocorrer a lise do macrófago infectado, e assim são liberadas para o meio extracelular, favorecendo a infecção de novos macrófagos ou podem ser ingeridas por outro flebotomíneo no momento da hematofagia.

Devido as condições encontradas no trato digestivo do inseto vetor, como o aumento do pH e redução da temperatura, as amastigotas se diferenciam em promastigotas procíclica, uma forma curta e levemente móvel que inicia a proliferação no vetor. As promastigotas procíclicas realizam divisão binária e passam por um processo denominado metaciclogênese, tornando-a infectante. As promastigotas metacíclicas são altamente ativas e migram ao longo do intestino anterior até a probóscide onde podem ser transmitidas e dar início à um novo ciclo (BATES; ROGERS, 2004; BATES, 2007; SCOTT; NOVAIS, 2016; SERAFIM et al., 2018).

Figura 1 – Ciclo biológico de *Leishmania* sp.



Fonte: Center for Disease Control and Prevention (2018).

A Leishmaniose é reconhecida pela Organização Mundial de Saúde (OMS) como uma doença tropical negligenciada e a segunda causa de morte relacionada com parasitos depois da malária, sendo que mais de um bilhão de pessoas em 98 países do mundo vivem sob o risco de desenvolver uma das formas clínicas da doença (WHO, 2018).

A doença possui algumas formas clínicas principais: cutânea localizada (LCL), mucocutânea (LMC), cutânea difusa (LCD) e visceral (LV), as quais são determinadas tanto pela espécie do parasito, quanto pela resposta imunológica do hospedeiro (TORRES-GUERRERO et al., 2017).

Nas Américas, a forma cutânea é conhecida como Leishmaniose Tegumentar Americana (LTA), a qual apresenta manifestações que variam desde a formação de úlcera única a formas disseminadas, podendo ou não acometer mucosas (OLGA ZERPA; PADRÓN-NIEVES; PONTE-SUCRE, 2018). A LTA consiste em um problema de Saúde Pública, devido sua alta incidência, ampla distribuição e grande

complexidade, tendo possibilidade do aparecimento de lesões destrutivas, desfigurantes e até mesmo incapacitantes para os indivíduos infectados (OLGA ZERPA; PADRÓN-NIEVES; PONTE-SUCRE, 2018).

A LCL apresenta lesões localizadas, podendo ocorrer de forma única ou múltipla, normalmente no local da picada do inseto vetor. Nesta forma clínica, as lesões são geralmente bem delimitadas, ulceradas, com bordas elevadas e fundo granulomatoso. Estas lesões apresentam poucos parasitos, com predomínio de uma resposta celular do tipo Th1, e podem evoluir para cura espontânea, todavia, também pode ocorrer a forma disseminada, devido a disseminação hematogênica ou linfática, na qual é caracterizada por múltiplas lesões pequenas, ulceradas e distribuídas por todo o corpo, com uma resposta Th1 sobrepondo a Th2 (ANVERSA et al., 2018; BRASIL, 2017).

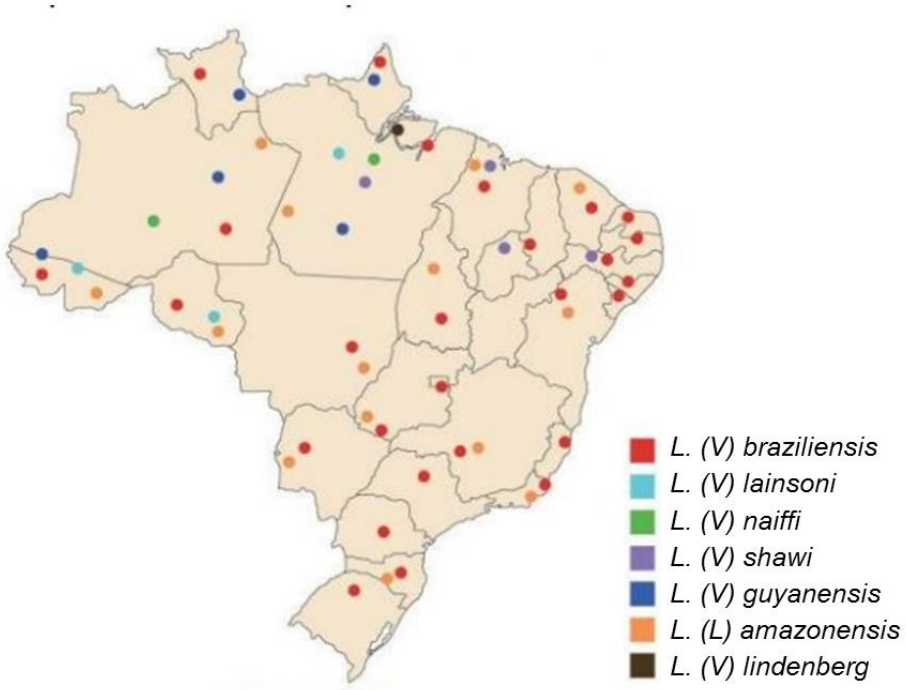
A forma mucosa (LMC) é caracterizada pela sua gravidade, com lesões destrutivas que acometem cartilagens e mucosas, principalmente nas regiões oro-respiratórias, como a mucosa, afetando o septo cartilaginoso, palato, lábios, língua, faringe e laringe, afetando a respiração, deglutição e fala. Esta forma clínica é caracterizada por respostas imunológicas Th1 com grande predomínio de IFN-γ, TNF-α e lL-4, e baixa produção de IL-10 e TGF-β, o que pode explicar a gravidade na destruição tecidual e escassez no número de parasitos nas lesões (ANVERSA et al., 2018; BRASIL, 2017).

A LCD é uma forma rara, com manifestações graves, que ocorre em pacientes considerados anérgicos, ou seja, deficientes em uma resposta imune celular específica para antígenos de *Leishmania*. É caracterizada pela formação de placas infiltradas e múltiplas nodulações não ulceradas, amplamente distribuídas na pele. Estas lesões apresentam um grande número de parasitos, com predomínio de uma resposta do tipo Th2, com elevados níveis de IL-4 e IL-10 e baixos níveis de IFN-γ (ANVERSA et al., 2018; BRASIL, 2017).

A LTA apresenta ampla distribuição no continente americano, com casos registrados desde o sul dos Estados Unidos até o norte da Argentina, no qual o Brasil é considerado uma região endêmica, e um dos países com as mais altas taxas de notificação da doença em todos os estados brasileiros, tendo como as principais espécies circulantes: *Leishmania (Leishmania) amazonensis, Leishmania (Viannia) guyanensis, Leishmania (V.) braziliensis, Leishmania (V) shawi, Leishmania (V) naiffi,*

Leishmania (V) lainsoni e *Leishmania (V) lindenberg* (BRASIL, 2017; GENARO, 2005) (Figura 2).

Figura 2 – Distribuição das espécies de *Leishmania* responsáveis pela LTA no Brasil



Fonte: BRASIL (2017).

No Estado do Paraná, a LTA é endêmica com notificação em 276 dos 399 municípios, principalmente das regiões norte e oeste, apresentando 235 casos confirmados no ano de 2017 (SVS - Sistema de Informação de Agravos de Notificação - Sinan Net, 2019). Apesar da existência de várias espécies de *Leishmania* spp., no Paraná, Silveira et al. (1990) apontam a presença de somente *Leishmania (Viannia) braziliensis* e *Leishmania (Leishmania) amazonensis*. Detoni et al. (2019) identificaram 108 casos de LTA no norte do Paraná no período de 2010 e 2015.

1.2 Interação Parasito-Hospedeiro e Resposta Imune na LTA

O processo de instalação da LTA no hospedeiro é determinado por uma complexa associação, entre os fatores de virulência do parasito e a resposta

imunológica do hospedeiro (GENARO, 2005). As primeiras células a migrarem para o local da picada são os neutrófilos e macrófagos, sendo evidenciado que essas células ativadas são capazes de eliminar os parasitos intracelulares (MENEZES; SARAIVA; ROCHA-AZEVEDO, 2016). Para tanto, dentre as estratégias microbicidas utilizadas por esses fagócitos, principalmente por macrófagos para combater os parasitos, está o desencadeamento do "*burst*" oxidativo, com produção de espécies reativas de oxigênio (EROs) e nitrogênio (ERN), durante o processo de fagocitose (QADOUMI et al., 2002; CUNNINGHAM, 2002; SCOTT; NOVAIS, 2016). Os macrófagos ativados aumentam a expressão de NADPH oxidase (NOX) e Óxido Nítrico Sintase induzível (iNOS), para a produção de ânion superóxido e óxido nítrico (NO), respectivamente.

Apesar da resposta imune do hospedeiro, os parasitos *Leishmania* apresentam vários mecanismos de evasão, devido principalmente a presença de dois fatores de virulência, lipofosfoglicanos (LPG) e glicoproteína de 63 kDa (gp63). Estas moléculas encontradas na superfície de promastigotas conferem resistência à lise mediada pelo sistema complemento (SACKS et al., 2002), uma vez que LPG previne a formação do complexo C5b-9 e gp63 cliva C3b em C3b inativo (C3bi), evitando a formação de C5 convertase. C3bi pode atuar como uma opsonina, facilitando a fagocitose do parasito de maneira "silenciosa" via Receptor do Complemento 3 (CR3) (DOS-SANTOS et al., 2016).

Estes parasitos são capazes de retardar a fusão do vacúolo parasitóforo com o lisossomal, permitindo a diferenciação de promastigota em amastigotas e manter o pH neutro, apesar do ambiente ácido do fagolisossomo (BASU; RAY, 2005). Além disso, a gp63 tem capacidade de degradar enzimas lisossomais presentes no vacúolo, permitindo a sobrevivência e multiplicação do parasito no vacúolo digestivo (CUNNINGHAM, 2002). LPG também protege os parasitos da ação do estresse oxidativo, impedindo a montagem de NADPH oxidase (NOX2) na superfície do fagolisossomo (ROSSI; FASEL, 2017).

Adicionalmente, esses parasitos apresentam uma enzima, chamada arginase que consome arginina, um aminoácido fundamental para a sobrevivência da *Leishmania* sp. e utilizado pela iNOS, na produção de NO, a principal molécula leishmanicida produzida por macrófagos ativados (BOGDAN; ROLLINGHOFF, 1999). Estes parasitos também apresentam mecanismos antioxidantes que auxiliam na proteção contra as ERO e ERN, tem sido descrito que *L. braziliensis* e *L. amazonensis* são capazes de aumentar a expressão de superóxido dismutase 1 (SOD1) no

hospedeiro (KHOURI et al., 2009). A SOD (SOD-Fe) de *Leishmania* spp. é sintetizada nas mitocôndrias e desempenha um papel importante na virulência do parasita, no balanço redox em amastigotas intracelulares e promastigotas axênicas, protegendo contra o estresse oxidativo e participando da sinalização para a diferenciação de formas infectantes mediadas por ROS (KIMA, 2014).

Ademais, este protozoário pode interferir na sinalização celular do hospedeiro, das vias ativadas por receptores tipo *toll* (TLR), modulação em citocinas e apresentação de antígeno (DUQUE, DESCOTEAUX, 2015; GUPTA, SATOSKAR, 2013; PODINOVSKAIA, DESCOTEAUX, 2015; ROSSI, FASEL, 2017).

O estabelecimento do parasito no hospedeiro, associada ao desenvolvimento da resposta imune adaptativa, são fatores que estão intimamente relacionados com o quadro clínico da leishmaniose, na qual tem sido amplamente estudado em modelos murinos (LAUNOIS et al., 1996).

Em modelos murinos, diversos trabalhos já demostraram que camundongos C57BL/6 infectados com *Leishmania (L.) major* são resistentes à infecção, enquanto que 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 produzem principalmente INF-γ e TNF-α, e levam a ativação clássica dos macrófagos, aumento na atividade de iNOS, consequentemente a produção de NO, seguido de eliminação do parasito. Todavia, esse tipo de resposta exacerbada está intimamente relacionado ao desenvolvimento de lesões leishmanióticas e lesão tecidual (PIRMEZ et al., 1993; LESSA et al., 2001; LASKAY et al., 2008). Já o perfil Th2, com produção de IL-4 e IL-13 está relacionado com a suscetibilidade à infecção (LAUNOIS et al., 2002; AWASTHI; MATHUR; SAHA, 2004).

A dicotomia Th1 e Th2 está bem estabelecida na infecção experimental por *L. major*, todavia, em humanos isso não ocorre, resultando em um espectro de manifestações. Além disso, a complexa interação parasito-hospedeiro nas infecções experimentais de diferentes linhagens de camundongos com diferentes espécies de *Leishmania*, diferem em muitos aspectos, como no desenvolvimento de lesões e padrões de resposta imunológica do hospedeiro quando comparado com a infecção experimental por *L. (L.) major* (Tabela 1) (OSORIO Y FORTEA et al., 2007; PEREIRA; ALVES, 2008; SILVEIRA et al., 2009; AFONSO; SCOTT, 1993; LEMOS DE SOUZA *et al.,* 2000; SCOTT; NOVAIS, 2016). Esta correlação da polarização da resposta

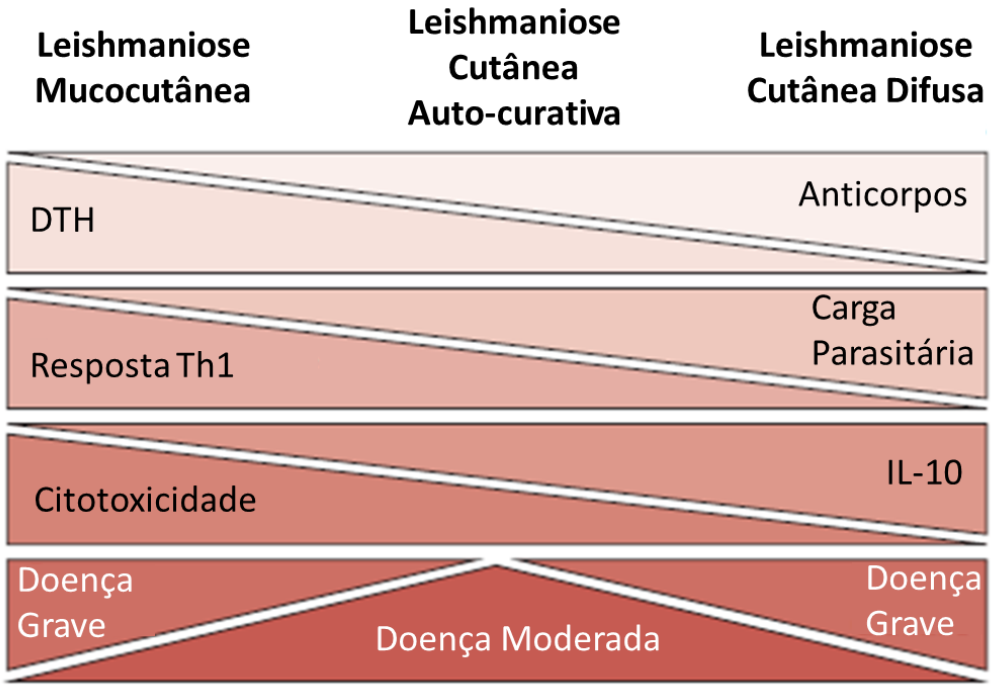
imune e o resultado da infecção levou ao conceito de que o balanço da resposta Th1/Th2 é o que determina o resultado clínico da infecção (ROBERTS, 2006) (Figura 3).

Tabela 1: Doença humana e murina causada por *Leishmania* spp. frequentemente utilizados em estudos experimentais

***Leishmania* spp.**	**Humano**	**Camundongo**			
		C57BL/6		**BALB/c**	
		Tipo de doença	**Resposta imune dominante**	**Tipo de doença**	**Resposta imune dominante**
Leishmania major	Leishmaniose cutânea auto-curativa ou crônica, geralmente causada por uma única lesão cutânea	Auto cura	Th1	Crônico	Th2
Leishmania major Seidman strain	Leishmaniose cutânea crônica	Crônico	Th1	Crônico	Th2
Leishmania amazonensis	Leishmaniose cutânea auto-curativa ou crônica geralmente causada por uma única lesão cutânea, e leishmaniose cutânea difusa	Crônico	Th1 e Th2	Crônico	Th2
Leishmania mexicana	Cura ou leishmaniose cutânea crônica geralmente causada por uma única lesão cutânea, e leishmaniose cutânea difusa	Crônico	Th1 e Th2	Crônico	Th2
Leishmania braziliensis	Cura ou leishmaniose cutânea crônica geralmente causada por uma única lesão cutânea, e leishmaniose mucocutânea.	Auto cura	Th1	Auto cura	Th1

Fonte: SCOTT; NOVAIS (2016)

Figura 3 - Espectro da doença na leishmaniose cutânea em humanos.



Fonte: SCOTT; NOVAIS (2016).

Além do perfil Th1/Th2, tem sido demonstrado nos últimos anos o papel importante das células Th17 e T reguladoras (Tregs) na leishmaniose.

Células Th17 por possuírem propriedades pró-inflamatórias, são diferenciadas na presença de TGF-β e IL-6, e secretam principalmente IL-17, IL-21 e IL-22. Alguns estudos demonstram sua contribuição na resistência ou suscetibilidade à infecção (NASCIMENTO et al., 2014; TERRAZAS et al., 2015). Tem sido descrito que a infecção de camundongos BALB/c com *L. major* induz elevados níveis de IL-17, enquanto a falta de IL-17 levou ao controle da doença (KOSTKA et al., 2009). IL-17 também tem sido detectada em pacientes com LC e LCM (BACELLAR et al., 2009; BOAVENTURA et al., 2010). Gonzalez-Lombana et al. (2013) mostraram que IL-17 na ausência de IL-10 contribui para o agravamento da infecção por *L. major*. Os linfócitos Th17 também atuam indiretamente no recrutamento de neutrófilos pela indução de IL-8, contribuindo diretamente com o desenvolvimento e agravamento da lesão (KOSTKA et al., 2009), devido ao fato evidenciado em alguns estudos em que neutrófilos parasitados podem servir como um “cavalo de Tróia” para a entrada do parasito na sua célula alvo. Isto porque, neutrófilos apresentam um período de vida curto e sofrem apoptose, sendo fagocitados por vias que não conseguem ativar os

mecanismos de defesa dos macrófagos (LASKAY; VAN ZANDBERGEN; SOLBACH, 2008). Com isso, as infecções por parasitos *Leishmania* desencadeiam a diferenciação de células T produtoras de IL-17, que auxiliam na eliminação dos parasitos, todavia esta resposta também está relacionada com a exacerbação da lesão na leishmaniose cutânea (GONÇALVES-DE-ALBUQUERQUE et al., 2017).

Os linfócitos T reg ($CD4^+CD25^+FOXP3^+$) são importantes fontes de citocinas IL-10 e TGF-β que atuam suprimindo células do sistema imune inato e adaptativo (FEHERVARI; SAKAGUCHI, 2004). Tem sido evidenciado a presença de células T reg nas lesões de pacientes com leishmaniose cutânea (BOURREAU et al., 2009; CAMPANELLI et al., 2006). Devido a produção de IL-10, estes linfócitos são capazes de suprimir a atividade de células efetoras, favorecendo a persistência do parasito (BELKAID et al., 2002; YURCHENKO et al., 2006). Em modelos murinos, a transferência de células T reg para camundongos já curados da leishmaniose cutânea causada por *L. major*, levaram a reativação da infecção latente (MENDEZ et al., 2004). De forma diferente, a transferência de células T reg de camundongo infectado para um camundongo naive antes da infecção por *L. amazonensis* reduziu o desenvolvimento da lesão (JI et al., 2005). Mostraram que as lesões de pacientes com leishmaniose cutânea infectados com *L. braziliensis* não estão relacionadas com o comprometimento funcional das células Treg ou a falha em responder, mas, o aumento da ativação de Treg em pacientes com leishmaniose cutânea pode ser prejudicial para a eliminação do parasito, levando à uma infecção crônica (COSTA et al., 2013).

Embora as células T reg possam estar relacionadas com a suscetibilidade à infecção e supressão de uma resposta microbicida, estes subtipos de linfócitos também podem auxiliar no controle de respostas imunopatológicas e progressão da doença. Com isso é difícil definir um tipo de resposta na leishmaniose cutânea, uma vez que a resposta imunológica pode ser influenciada tanto pela espécie do parasito quanto por aspectos genéticos do hospedeiro.

1.3 TRATAMENTO

O tratamento da Leishmaniose é baseado na eliminação de formas amastigotas de *Leishmania* spp. No entanto, a localização intramacrofágica desta forma, dificulta uma atuação mais eficaz dos fármacos (RODRIGUES et al., 2006).

As formas de tratamento têm se baseado no uso de antimônios pentavalentes, como o antimonato de *N*-metil glucamina (Glucantime®) e o estibogluconato de sódio (Pentostam® da Glaxo ou Solustibosan® da Bayer). Embora o mecanismo de ação destes fármacos não seja totalmente conhecido, acredita-se que a forma pentavalente atua como uma pró-droga, sendo convertida para a forma mais tóxica, o antimônio trivalente, podendo atuar na inibição da glicólise e na oxidação de ácidos graxos, redução na produção de ATP e GTP, além de interferir em enzimas antioxidantes do parasito (BALAÑA-FOUCE et al., 1998; CROFT; SEIFERT; YARDLEY, 2006). Apesar do antimônio trivalente apresentar uma atividade leishmanicida importante, esta forma também é tóxica ao paciente, sendo responsável pelos graves efeitos colaterais (LIMA et al., 2007). A administração é via parenteral, sendo que a dose diária padrão é de 20 mg/ kg durante aproximadamente vinte dias. Alguns efeitos colaterais são: artralgia, mialgia, náusea, vômito, acometimento gastrointestinal e cardíaco, prurido, febre, fraqueza, cefaleia, tontura, edema, elevação de enzimas hepáticas, insuficiência renal (LIMA et al., 2007). Junto com isso, existem relatos de que a cura clínica não ser acompanhada de cura parasitológica, pois tem sido observado parasitos na cicatriz de indivíduos após o tratamento (MCGREEVY; MARSDEN, 1986; LUCUMI et al., 1998; RATH et al., 2003; CROFT; BARRETT; URBINA, 2005). Além disso, estes fármacos são contraindicados para pacientes com cardiopatias, nefropatias, doença de chagas, tuberculose pulmonar, hepatopatias. No entanto quando houver necessidade do uso nestes pacientes, será de suma importância a realização de forma rigorosa de avaliações clínicas e exames periódicos.

Outros fármacos como anfotericina B, anfotericina B lipossomal, pentamidina, miltefosine e a paramomicina, têm sido usados como alternativas nos casos de resistência aos antimoniais, todavia, não possuem um índice terapêutico tão favorável e também apresentam várias reações adversas (BRAY et al., 2003; BERMAN, 2006). O mecanismo de ação da anfotericina B se dá por meio da interação com o ergosterol e formação poros da membrana plasmática, levando ao aumento da permeabilidade das membranas, causando a morte celular. Embora o uso da forma lipossomal é capaz de reduzir os efeitos tóxicos da anfotericina B, este medicamento apresenta elevado custo, inviabilizando seu uso por grande parte dos pacientes (LIMA et al., 2007).

De forma geral, o tratamento convencional da LTA representa um grande desafio, pois os fármacos disponíveis até o momento apresentam elevada toxicidade

e não têm demonstrados serem plenamente eficazes. A recidiva, a falha terapêutica em pacientes imunocomprometidos e a resistência aos tratamentos, são fatores que motivam a busca por novos fármacos que apresentem atividade leishmanicida mais eficazes e menos tóxicas ao paciente. Neste contexto, o uso de compostos naturais ou sintéticos, bem como o uso da nanotecnologia podem ser alternativas promissoras para o tratamento da LTA.

1.4 DIETILDITIOCARBAMATO (DETC)

Dietilditiocarbamato (DETC) (Figura 4) é um quelante metálico de cobre, com capacidade de inibir a superoxido dismutase 1 (SOD1), uma enzima que catalisa o ânion superóxido em peróxido de hidrogênio, importante na defesa antioxidante (COCCO et al., 1981).

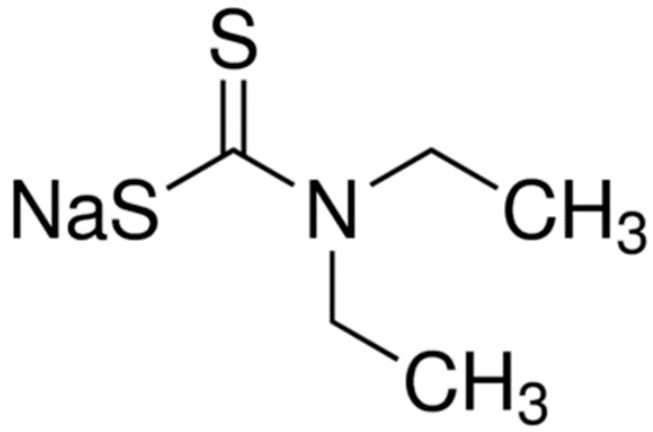
SOD1 desempenha um papel importante no desenvolvimento e progressão da leishmaniose cutânea, devido suas propriedades antioxidantes, proporcionando assim um ambiente propício para a sobrevivência e multiplicação de *Leishmania* spp. em macrófagos. Khouri et al. (2010) investigaram o papel de DETC contra infecção por este parasito, e verificaram que o tratamento com este composto sintético foi capaz exercer efeito leishmanicida contra promastigotas e amastigotas, promovendo alterações ultraestruturais e redução na infecção de macrófagos humanos pelo aumento da produção de ROS, bem como a redução da lesão e do número de parasitos em camundongos BALB/c infectados com *L. amazonensis* (KHOURI et al., 2009). Recentemente, Oliveira et al. (2019), também mostraram que DETC isolado ou associado com outros oxidantes, são capazes de reduzir amastigotas por macrófagos e a porcentagem de macrófagos infectados com *L. braziliensis*. Mazur et al. (2019), mostraram que DETC encapsulado em nanopartículas lipídicas sólidas apresentou efeito anti-promastigota sobre *L. amazonensis*, além de reduzir os efeitos tóxicos sobre macrófagos murinos.

Também já foi descrito que o tratamento com o DETC é capaz de ativar o Nrf2 em cultura de células endoteliais vasculares (FUJIE et al, 2016), aumentar a produção de NO e TNF-α em células de Kupffer de ratos estimulados com LPS (ISHIYAMA; HOGLEN; SIPES, 2000), de aumentar a produção de IL-1β e induzir apoptose e necrose em células de leucemia promielocítica HL-60 (SCHMALBACH et al., 1992; KIMOTO-KINOSHITA; NISHIDA; TOMURA, 2004), além de poder atuar como inibidor

de NFκB e modular óxido nítrico sintase (NOS) (MÜLSCH et al., 1993). Além disso, DETC também apresenta capacidade anti-*Schistosoma mansoni* (FILADELFO, 2009) e anti-*Saccharomyces cerevisiae (*LUSHCHAK et al., 2005*).*

Embora o DETC tenha demonstrado efeito leishmanicida, os mecanismos de morte do parasito e os aspectos imunológicos desencadeados por esse composto ainda são desconhecidos na Leishmaniose Tegumentar Americana.

Figura 4 – Estrutura química de dietilditiocarbamato (DETC)



Fonte: Sigma-Aldrich

1.5 4-NITROCHALCONA

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. Devido sua estrutura simples, é possível realizar diversas alterações, o que pode resultar em mudanças nas atividades biológicas, farmacológicas e no mecanismo de ação.

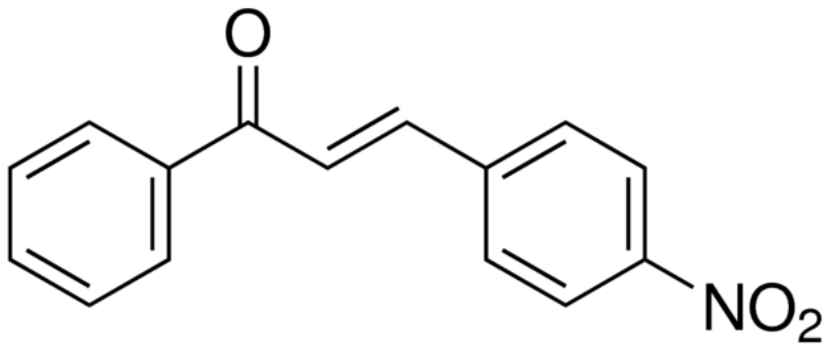
Estes compostos apresentam um amplo espectro de atividades biológicas, como ação anti-bacteriana, antimalárica, anti-helmíntica, trypanocida, antinociceptiva, antioxidante, antitumoral, imunomoduladora, anti-inflamatória, antifúngica, antiviral, antimalárica e leishmanicida entre outras (CHEN et al., 1997; GO et al., 2004; FRISS-MOLLER et al., 2002; TSUKIYAMA et al., 2002; BOECK et al., 2005; BHAT et a., 2005; BATOVSKA et al., 2007; PADARATZ et al., 2009; MAHAPATRA et al., 2015;

SCHIANO MORIELLO et al., 2016; MARTINEZ et al., 2017a; 2017b; ZHOU et al., 2018; PEREIRA et al., 2018; STAURENGO-FERRARI et al., 2018; MIRANDA-SAPLA et al., 2019; DE MELLO et al., 2018; TAJUDDEEN et al., 2018).

A 4-nitrochalcona (Figura 5) é uma chalcona que apresenta radical NO_2 na posição 4 do segundo anel aromático. Essa apresenta atividade anti-proliferativa em linhagens de células tumorais como, JR8 e HL-60 (DALLA VIA et al., 2009), L1210, CEM (DIMMOCK et al., 2002) MCF-7 e T47D (ILANGO; VALENTINA; SALUJA, 2010), atividade antifúngica, contra leveduras de *Candida albicans* (BATOVSKA et al., 2007) e dermatófitos (LÓPEZ et al., 2001) e anti-protozoários, inibindo o crescimento de *Entamoeba histolytica* (WANI et al., 2012).

Embora não tenha pesquisas demonstrando a ação deste composto diretamente em infecções causadas por espécies de *Leishmania*, Boeck et al. (2006) relataram atividade leishmanicida exercida por uma chalcona com radical NO_2 na posição 3, contra *L. amazonensis* e *L. infantum*. No entanto, baseado nas evidências experimentais dos mecanismos de ação de outras chalconas, é possível que a 4-nitrochalcona possa apresentar atividade leishmanicida sobre *Leishmania amazonensis.*

Figura 5 – Estrutura química de 4-Nitrochalcona



Fonte: Sigma-Aldrich

1.6 SISTEMAS NANOPARTICULADOS

Os avanços na nanomedicina estão sendo promissores, possibilitando o uso da nanotecnologia para o tratamento de diversas doenças causadas por microorganismos intracelulares. Destaca-se entre eles o uso de nanomateriais, caracterizados por apresentarem tamanho menor que 1000 nm, podendo ser de

natureza metálica, lipídica ou polimérica (GUTIÉRREZ et al., 2016), além atuar como carreadores de fármacos, reduzir a toxicidade, modular a farmacocinética, aumentar a biodisponibilidade e a liberação direcionada do fármaco no alvo específico (KHALIL et al., 2013).

Nanopartículas metálicas são baseadas em pequenos agregados bem definidos de metais (EDMUNDSON; CAPENESS; HORSFALL, 2014). Vários trabalhos têm demonstrado que nanopartículas de prata, ouro, selênio, zinco, além de nanopartículas de óxido de metal como nanopartículas de dióxido de titânio, óxido de zinco e óxido de magnésio apresentam atividade leishmanicida (ADERIBIGBE, 2017; DE SOUZA et al., 2018). Além disso, algumas nanopartículas metálicas, como a de prata, podem ser sintetizadas de maneira biológica, também conhecida como síntese verde. Fanti et al. (2018) mostraram que nanopartículas de prata produzidas através do fungo *Fusarium oxysporum* apresentam efeito leishmanicida direto sobre formas promastigotas e amastigotas de *L. amazonsensis*.

Nanopartículas poliméricas são sistemas sólidos, que apresentam características de biocompatibilidade e biodegradáveis, onde o agente terapêutico pode ser dissolvido, encapsulado ou adsorvido na matriz do polímero. Esses nanomateriais podem conter polímeros sintéticos, como poly (D,L-lactic-co-glycolic acid)(PLGA), polyethylene glycol (PGE), ou polyester bio-beads, e naturais como alginato, inulina ou quitosana (CHAN et al., 2010).

O interesse pelo uso de carreadores lipídicos como nanoemulsões, nanopartículas lipídicas sólidas e carreadores de lipídios nanoestruturados tem aumentado, devido à sua versatilidade e também pela possibilidade de administração de fármacos hidrossolúveis, além de poder ser adaptada para diferentes tipos de formulações para se adequar com a via de administração ou doença a ser tratada (DE SOUZA et al., 2018). De Souza et al. (2018) realizaram uma revisão sobre o uso de nanopartículas poliméricas para o tratamento da leishmaniose, os autores mostraram o efeito leishmanicida de anfotericina B carregada em nanopartículas de PLGA e de ácido dimercaptosuccínico (DMSA), rifampicina carregada em nanopartículas de quitosana e manose, hidroximetilnitrofurazona carregada em nanopartículas de poli (n-butil cianoacrilato).

As nanoemulsões são formadas por duas fases imiscíveis, lipídios líquidos em água ou água em lipídios, já as nanopartículas lipídicas sólidas apresentam núcleo com lipídios no estado sólido, o que possibilitam controlar a velocidade de liberação

de fármacos ou moléculas (MUKHERJEE; RAY; THAKUR, 2009). Já os carreadores de lipídios nanoestruturados, são considerados a segunda geração de nanopartículas lipídicas, que apresentam uma matriz composta por uma mistura de lipídios sólidos com líquidos, mas se mantém sólidos a temperatura corporal (DE SOUZA et al., 2018). Alguns estudos têm demonstrado o efeito leishmanicida de vários fármacos como Paromomicina, Trifluralina e seus análogos, chalconas, Anfotericina B, Orizalina, Miltefosina, Buparvaquona utilizando estes tipos de nanomateriais lipídicos (DE SOUZA et al., 2018).

Os nanomateriais apresentam grande versatilidade em suas aplicações, podendo atuar não apenas para o tratamento, mas também no diagnóstico e prevenção de diversos tipos de doenças (ASSOLINI et al., 2017). Diante da diversidade de formas, materiais utilizados em nanopartículas, suas características, bem como suas aplicações, a nanotecnologia vem sendo amplamente estudada, apresentando resultados promissores para a aplicação no tratamento da leishmaniose (GUTIÉRREZ et al., 2016).

1.7 Morte Celular em Tripanossomatideos

Morte é um processo irreversível caracterizado por uma sequência de eventos que culminam no cessamento de todas as atividades biológicas. Os principais processos de morte celular são classificados de acordo com suas características morfológicas e bioquímicas em: apoptose, necrose e autofagia (SEKERDAG; SOLAROGLU; GURSOY-OZDEMIR, 2018). Esses eventos são bem descritos em metazoários, entretanto, em protozoários como *Leishmania*, os mecanismos de morte não estão totalmente elucidados.

Em organismos multicelulares, a apoptose é essencial para muitos processos biológicos, incluindo a remoção de células não funcionais ou danificadas em todos os tecidos (GREEN, 2003). Apoptose-*like* é o termo que representa a morte celular presente tripanossomatídeos, devido algumas semelhanças com a apoptose em metazoários (ARAMBAGE et al., 2009; DAS; MUKHERJEE; SHAHA, 2001). A identificação deste fenótipo em tripanossomatídeos como *Leishmania*, inclui a fragmentação de DNA, exposição de fosfatidilserina, perda do potencial de membrana mitocondrial, liberação do citocromo c, encolhimento celular e formação de blebs na membrana plasmática (MENNA-BARRETO, 2019). Além disso, nestes protozoários,

a mitocondria é uma organela central nas vias de morte celular, e a geração de EROs pode causar danos mitocondrial levando a morte celular. Tem sido descrito também que algumas condições como calor, agentes oxidantes e fármacos podem induzir à morte por apoptose-*like* (MENNA-BARRETO; DE CASTRO, 2015). No entanto, estes organismos não possuem a mesma maquinaria apoptótica, ausentando-se de membros da família do Bcl-2, TNF-α/ TNFR e caspases (MENNA-BARRETO; DE CASTRO, 2015).

A necrose é considerada uma morte celular acidental que envolve principalmente a perturbação da homeostase da membrana plasmática e do cálcio, liberação de hidrolases lisossomais e a vacuolização citoplasmática (ZONG; THOMPSON, 2006). Durante a necrose, há aumento do volume celular, agregação da cromatina, desorganização do citoplasma, perda da integridade da membrana plasmática e consequente ruptura celular (KROEMER et al., 2009). O conteúdo celular liberado neste processo pode desencadear uma resposta inflamatória, uma das principais diferenças entre a necrose e a apoptose. Em protozoários, a necrose ocorre devido a um estímulo mecânico ou químico e é caracterizada pela formação de vacúolos citoplasmáticos seguida de ruptura da membrana plasmática, levando à lise (ZONG; THOMPSON, 2006).

A autofagia é considerada o principal mecanismo celular que realiza a degradação e reciclagem de organelas e moléculas não funcionais, com a finalidade de manter a homeostase em células eucarióticas (REGGIORI; KLIONSKY, 2005). Em metazoários este processo está envolvido em diversas situações fisiológicas, como no desenvolvimento, crescimento e diferenciação celular. Além disso, a autofagia está relacionada com a sobrevivência celular em condições de estresse como falta de nutrientes, hipóxia, temperatura elevada e pH ácido (HE; KLIONSKY, 2009).

Em tripanossomatideos, de maneira diferente de apoptose e necrose que são processos típicos de morte celular, a autofagia é geralmente um processo que promove a sobrevivência celular, todavia, quando a degradação dos componentes citoplasmático é ininterrupta, pode resultar em morte celular (BRENNAND et al., 2011; KROEMER et al., 2009). A principal alteração estrutural observada é a formação de fagóforos que irão atuar na formação do autofagossomo, que posteriormente irá se fundir às estruturas semelhantes a lisossomos, formando o autofagolisossomo. Nos autofagossomos encontram-se proteínas autofágicas denominadas Atgs (relacionadas à AuTophaGy), associadas em diferentes eventos autofágicos

(DUSZENKO et al., 2011). Em *Leishmania* spp., o processo de autofagia participa do processo de metaciclogênese, e também ocorre na diferenciação de promastigotas metacíclicas em amastigotas quando submetidos à pH baixo, temperatura alta e privação nutricional (BESTEIRO et al., 2006; WILLIAMS et al., 2006).

1.8 JUSTIFICATIVA

A atual quimioterapia para LTA não é satisfatória, devido à toxicidade dos fármacos utilizados, dificuldade de administração, longo tempo de tratamento, e o surgimento de cepas resistentes. Portanto, sabendo da gravidade desta doença, de suas complicações anatomofisiológicas, associado 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 LTA.

É neste sentido que alguns pesquisadores têm investigado e demonstrado o efeito leishmanicida de chalconas e DETC sobre as formas de *Leishmania* spp em experimentos *in vitro* e *in vivo*.

Além do uso desses compostos naturais e sintéticos, o uso de nanomateriais vem sendo amplamente explorado para o tratamento da leishmaniose e outras doenças causadas por patógenos intracelulares, pois esses são formulados para atuarem de forma específica, devido ao controle de liberação do fármaco, reduzindo assim a toxicidade. Essa nanotecnologia também possibilita o uso de diversos tipos de nanopartículas, podendo atuar como carreadores de um ou mais fármacos, possibilitando assim, um sinergismo entres compostos e/ou fármacos, como evidenciado por Gupta et al. (2015), que mostraram o efeito leishmanicida de nanoemulsões contendo óleo de copaíba e anfotericina B, com redução dos efeitos tóxicos.

Baseados nessas evidencias, é possível que 4-Nitrochalcona, DETC, bem como o uso desses compostos encapsulados em nanopartículas, possam exercer atividade leishmanicida. Além disso, devido à escassez de estudos, esse trabalho propõe avaliar parâmetros imunomoduladores eventualmente induzidos por esses compostos sobre macrófagos, o que possibilitará o melhor entendimento dos mecanismos e vias da ação leishmanicida.

2 OBJETIVOS

2.1 Objetivo Geral

Avaliar *in vitro* o efeito leishmanicida de Dietilditilcarbamato (DETC), 4-Nitrochalcona livres e encapsulados em nanopartículas lipídicas sólidas, bem como clarear os possíveis mecanismos de morte.

2.2 Objetivos Específicos

Avaliar a atividade direta de DETC e 4-Nitrochalcona livres sobre formas promastigotas de *L. amazonensis*.

Caracterizar a atividade leishmanicida, bem como os mecanismos envolvidos na morte, durante a ação direta destes tratamentos sobre as formas promastigotas.

Avaliar a citotoxicidade de DETC e 4-Nitrochalcona livres e encapsulados em nanopartículas lipídicas sólidas sobre macrófagos murinos e eritrócitos de carneiro.

Verificar a atividade anti-amastigota dos compostos livres e encapsulados em macrófagos infectados com *L. amazonensis*.

Investigar o efeito imunomodulador dos fármacos livres e nanoparticulados sobre macrófagos infectados com *L. amazonensis*, através de dosagem de citocinas, óxido nítrico e espécies reativas de oxigênio.

3. ARTIGO CIENTÍFICO 1

Diethyldithiocarbamate encapsulation reduces toxicity and promotes leishmanicidal effect through apoptosis-like mechanism in promastigote and ROS production by macrophage

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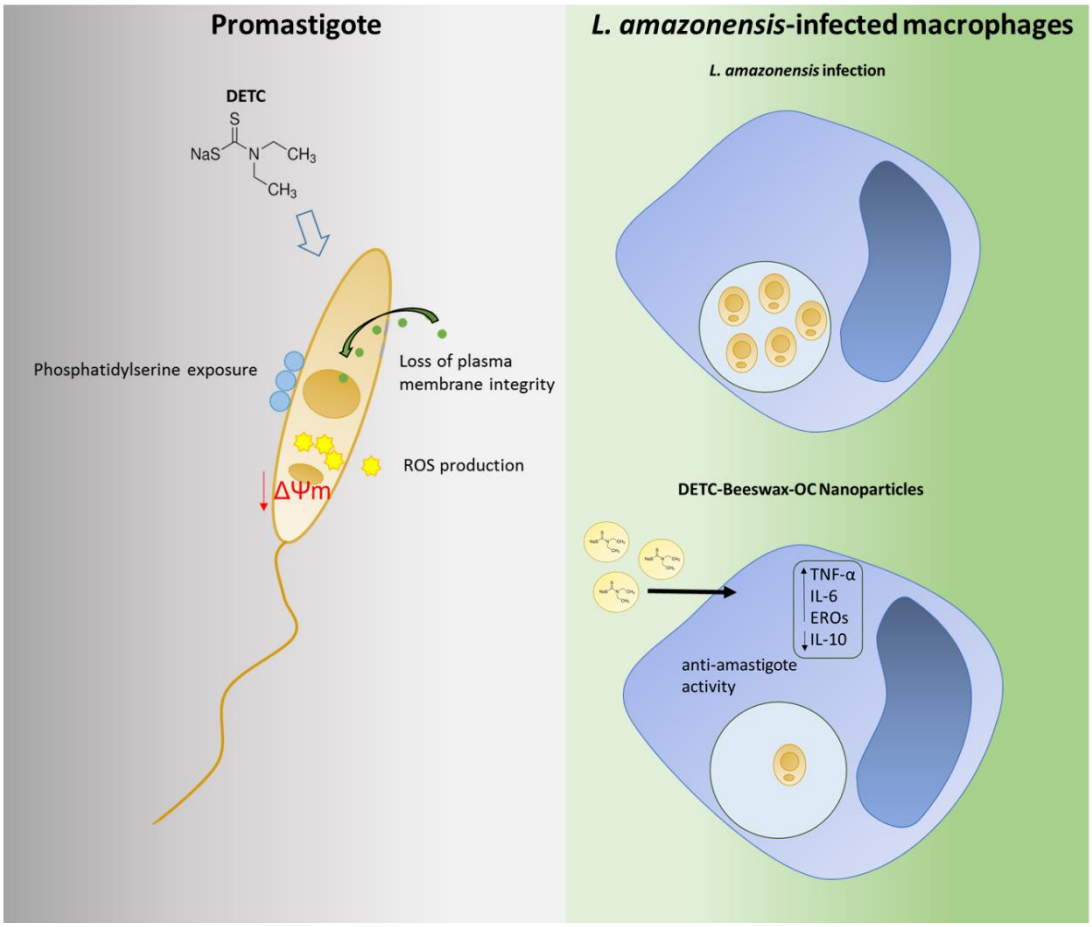
Graphical abstract



Abstract

American Cutaneous Leishmaniasis (ACL) is caused by the protozoan *Leishmania* spp. Currently available treatment consists of pentavalent antimonials, amphotericin B and pentamidine; however, these drugs are unsatisfactory causing significant side effects, besides the high costs and parasite resistance. The use of compounds from natural or synthetic sources, as well nanotechnology may be alternatives for the development of new drugs for the leishmaniasis treatment. Thus, the objective of this study was to evaluate *in vitro* the leishmanicidal effect of free Diethydithiocarbamate (DETC) and DETC loaded in beeswax-copaiba oil nanoparticles (DETC-Beeswax-CO Nps) on *L. amazonensis* forms, as well as to elucidate the possible mechanisms involved in the parasite death. DETC-Beeswax-CO Nps presented size below 200 nm, spherical morphology, negative zeta potential and high encapsulation efficiency. Free DETC was able to reduce the viability of promastigotes (IC_{50} = 0.33 μM), and increased ROS production, reduced the mitochondrial membrane potential, caused phosphatidylserine exposure and increased plasma membrane permeability, as well as promoted morphological changes in the parasite. In assessing toxicity to murine macrophages, free DETC was toxic, however encapsulation of this compound was able to reduce these toxic effects on macrophages. DETC-Beeswax-CO Nps exerted anti-amastigote effect, increasing the production of total ROS, superoxide anion, TNF-α, IL-6 and reduced IL-10 in macrophages. In this way, free DETC induces antipromastigote effect by apoptosis-like; and DETC-Beeswax-CO Nps exerted anti-leishmanial effect due to pro-oxidant and pro-inflammatory response, without affecting the viability of host cells.

Keywords: Leishmaniasis; Solid lipid nanoparticle; cytokine; Reactive Oxygen Species

1 Introduction

American Cutaneous Leishmaniasis (ACL) is a neglected tropical disease caused by protozoa of *Leishmania* genus, transmitted by the bite of phlebotomine insects [1]. Different clinical manifestations may occur, from the formation of a single ulcer to disseminated forms, being able to progress to mucous forms. In the epidemiological context, this disease has a wide distribution in the American continent, being *Leishmania amazonensis* and *L. brasiliensis* the main species that cause ACL in Brazil [2]. These parasites present two morphological forms: the promastigote forms, characterized by being elongated, flagellated and highly infective, found in the digestive tract of the insect vector [3]; and the amastigote forms, which have rounded morphology, no apparent flagella and are found mainly in the parasitophorous vacuoles of tissue macrophages of the vertebrate host [3,4].

The current treatment for cutaneous leishmaniasis is based on the use of pentavalent antimonials, as well as second-choice drugs such as amphotericin B and pentamidine. Despite efforts, these treatments have high toxicity, causing several adverse effects, in addition to having high cost, administration difficulties, and consequently drug-resistance [5-7]. In this way researches that seek alternative treatments against this disease, through the use of new compounds that perform leishmanicidal activity with more effective and fewer toxic effects for the patients, are of great importance.

Diethydithiocarbamate (DETC) is a copper metal chelator with the ability to inhibit superoxide dismutase 1 (CuZnSOD/ SOD1), an enzyme that catalyzes the superoxide anion to hydrogen peroxide, important in antioxidant defense [8]. Elevated levels of SOD1 in plasma may be indicative of failure to treat cutaneous leishmaniasis [9], thus, SOD1 plays a deleterious role in cutaneous leishmaniasis due to its antioxidant properties, which provides an environment conducive to the survival and multiplication of *Leishmania* spp. in macrophages [10]. Some *in vitro* and *in vivo* studies have shown that DETC has leishmanicidal effect against promastigotes and amastigotes forms of *Leishmania* suggesting a potential use as treatment for this disease [11-14].

The advances in nanomedicine are promising, allowing the use of nanotechnology for the treatment of various diseases caused by intracellular

microorganisms, such as *Leishmania*, whose effect is based on the elimination of the amastigotes forms, acting in intra-macrophagic parasites [13, 15, 16]. It stands out among them, the use of nanomaterials that can act as drug carriers, reducing toxicity, modulating pharmacokinetics, increasing bioavailability and the delivery of the drug into the specific target [17, 18]. Besides that, some works have shown that solid lipid nanoparticles (SLNs) have good characteristics to enable their use in the development of new treatments for leishmaniasis [13, 19, 20].

Some studies have shown the leishmanicidal effect of DETC through superoxide anion production by macrophages [11-14], however, additional action mechanisms in promastigotes and action of the encapsulated compound in SLN in intracellular amastigotes, as well as immunomodulation in infected macrophages has not been fully elucidated to date. Thus, the present study aimed to evaluate *in vitro* the anti-*Leishmania amazonensis* effect of free DETC and DETC loaded in beeswax-copaiba oil nanoparticles (DETC-Beeswax-OC Nps), as well as to elucidate the possible mechanisms involved in parasite death.

2 Materials and methods

2.1 Compound

The compound sodium diethyldithiocarbamate trihydrate (DETC) (Figure 1) was purchased from Vetec Química Fina (Duque de Caxias, RJ, Brazil).

2.2 Preparation of DETC loaded in beeswax-copaiba oil nanoparticle (DETC-Beeswax-CO Nps)

The beeswax lipid nanoparticles containing DETC were prepared by the double emulsion/melt technique as previously described by Mazur et al. [13]. In the first step, a water in oil emulsion was prepared by the emulsification of 0.3 mL of aqueous phase, containing 0.030 $g.mL^{-1}$ of DETC, in a lipid phase composed of Crodamol (0.3g), copaiba oil (0.1g) and beeswax (0.45 g), melted at 60 °C in the presence of the surfactant lecithin (0.045 g). In sequence the course emulsion was sonicated at 45 % amplitude for 15 s (Fischer Scientific, Ultrasonic Dismembrator Model 500) to form a water in oil miniemulsion. Subsequently, this first miniemulsion was added to 9 mL of the aqueous solution with the second surfactant Tween 80 (0.090 g) and kept under magnetic stirring (300 rpm) for 10 min at 60 °C. The second emulsion was sonicated

at 60 % amplitude for 60 s, forming a double emulsion (water/oil/water). To promote the rapid lipid solidification, the double emulsion was added to 35 mL of water at 2 °C under magnetic stirring (400 rpm) for 10 min. The beeswax nanoparticles without DETC were used as blank (Beeswax-CO). For freeze-drying, nanoparticles were centrifuged, the supernatant was removed and nanoparticles were redispersed in 10% Trehalose solution. Nanoparticles were frozen (-80 °C) and then *freeze*-dried (L101, Liotop, Brazil).

2.3 Characterization

The size and morphology of the nanoparticles was evaluated using transmission electron microscopy (model JEM 2100F, 80Kv). The samples were prepared by dropping the diluted dispersion (0.5% solids content) onto the copper grid (300 mesh) with a Formvar/carbon film and dried. The copper grid was coated with carbon and the imagens were recorded. The intensity average particle diameter and polydispersity index were determined by dynamic light scattering (Nanosizer, Malvern Instruments, U.K). The surface charge of the nanoparticles was obtained by zeta potential measurements (Zetasizer, Malvern Instruments, U.K.). All samples were analyzed five times (pH 7.4). Results are shown as mean ± standard deviation (mean ± SD). The encapsulation efficiency (EE%) of DETC encapsulated in beeswax nanoparticles was determined by UV–Vis spectrophotometry, model U-1900, Hitachi [13]. Nanoparticles were centrifuged using Amicon® Ultra centrifugal filter (Millipore, 100 kDa) at 13400 rpm for 30 min and the supernatant was removed. DETC concentrations were determined at 281 nm and the EE was calculated using the following equation (1):

$$EE\% = \frac{(C_1 - C_2)}{C_1} \times 100 \qquad (1)$$

where C_1 is the total concentration of DETC at the beginning of the process and C_2 is the DETC concentration in the supernatant.

2.4 *In vitro* leishmanicidal activity analysis on promastigote forms of *L. amazonensis*

2.4.1 *L. amazonensis* maintenance

Promastigote forms of *Leishmania (L.) amazonensis* (MHOM / BR / 1989 / 166MJO) from the State University of Maringá were maintained in culture medium 199 (GIBCO Invitrogen) supplemented with 10% fetal bovine serum (FBS) (GIBCO invitrogen), 10 mM Hepes, 1% Human Urine, 1% L-glutamine, streptomycin with penicillin (10U/ml-10µg/ml, GIBCO Invitrogen) and 10% sodium bicarbonate. The culture was maintained in an incubator at 24°C in a 25cm^2 culture flask for five days (stationary growth phase). All promastigote forms were used in the stationary growth phase.

2.4.2 Antipromastigote assay

The antipromastigote activity was performed according to Gonçalves et al. [21]. Promastigote forms of *L. amazonensis* (10^6 cells/mL) were treated with 0.1, 1, 10 and 100 µM of free DETC. The parasites were counted in a Neubauer chamber after 24 h of treatment. Untreated promastigotes were used as controls. From the results of the DETC-antipromastigote effect, the minimum concentration capable of inhibiting 50% of the viable parasites (IC_{50}) by non-linear regression was calculated.

2.4.3 Reactive Oxygen Species (ROS) production in promastigotes forms of *L. amazonensis*

In order to evaluate ROS production in *L. amazonensis*, promastigote forms (10^6 cells/mL) were treated with free DETC IC_{50} (0.33 µM) for 24h, then the parasites were washed with phosphate buffered saline (PBS) and incubated with probe diacetate 2', 7'-dichlorofluorescein (H_2DCFDA) (Sigma, St. Louis, MO, USA) 10µM for 45 min at 24°C.

ROS were measured as an increase in fluorescence caused by the conversion of non-fluorescent dichlorohydrofluorescein to the highly fluorescent 2,7-dichlorofluorescein, with an excitation wavelength of 488 nm and emission of 530 nm in fluorescence microplate reader (Victor X3, PerkinElmer, Finland). Hydrogen peroxide (H_2O_2) was used as a positive control. In order to compare the treatment, the fluorescence values obtained were normalized to the respective number of parasites.

2.4.4 Determination of mitochondrial membrane potential

The analysis of mitochondrial membrane potential was performed by tetramethylrhodamine-ethyl ester (TMRE) (Sigma, St. Louis, MO, USA). For this, promastigote forms of *L. amazonensis* (10^6 cells/mL) were treated with free DETC IC_{50} (0.33 µM) for 24 h. Subsequently the parasites were washed with PBS and incubated with 25 nM TMRE for 30 min at 24°C, followed by washing with PBS and immediately analyzed on a fluorescence microplate reader (Victor X3, PerkinElmer, Finlândia). Carbonyl cyanide m-chlorophenylhydrazone (CCCP) 100µM was used as a positive control.

2.4.5 Determination of phosphatidylserine exposure

Phosphatidylserine exposure was detected using Annexin-V FITC (Invitrogen, Eugene, USA). Promastigotes forms of *L. amazonensis* (10^6 cells/mL) were treated with free DETC IC_{50} (0.33 µM) for 24 h at 24°C. The parasites were then washed and resuspended in 100 µL of binding buffer (140 mM NaCl, 5 mM $CaCl_2$, and 10 mM HEPES-Na, pH 7.4), followed by the addition of 5 µL of Annexin-V FITC for 15 min at room temperature. After incubation, it was added 400µL of binding buffer. Camptothecin (Sigma, St. Louis, MO, USA) (10 µM) was used as positive control. The data acquisition was performed using a fluorescence microplate reader (Victor X3, PerkinElmer, Finland) at excitation wavelength of 488 nm and emission of 520 nm. In order to compare the treatment, the fluorescence values obtained were normalized to the respective number of parasites.

2.4.6 Determination of cell membrane integrity

Promastigotes forms of *L. amazonensis* (10^6 cells/mL) in 24-well plates were treated with IC_{50} (0.33 µM) of DETC for 24h at 24°C. The parasites were collected and washed with PBS and directly incubated with propidium iodide (PI) (Sigma, St. Louis, MO, USA) (0.50 µg/mL) for 5 min, according to the manufacturer's instructions. Immediately thereafter, the parasites were analyzed using a fluorescence microplate reader (Victor X3, PerkinElmer, Finland) with excitation wavelength of 480 nm and emission of 580 nm. Digitonin (Sigma, St. Louis, MO, USA) (40.0 µM) was used as a positive control. The fluorescence values obtained were normalized to the total number of cells from the treatment.

2.4.7 Co-determination of annexin V and propidium iodide label

Promastigotes forms of *L. amazonensis* (10^6 cells/mL) were treated with IC_{50} (0.33 µM) of free DETC for 24h at 24°C. Then, parasites were washed and resuspended in 100 µL of binding buffer (Santa cruz Biotechnology), followed by the addition of a mix containing 1 µL of annexin-V FITC (Invitrogen, Eugene, USA) and 5 µL of PI (Santa cruz Biotechnology). Data acquisition and analysis were performed using a BD Accuri™ 6 Plus personal flow cytometer. Cells were analyzed as [22, 23].

2.4.8 Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) of promastigotes forms was performed according to da Silva Bortoleti et al. [24]. Briefly, promastigotes forms of *L. amazonensis* (10^6) were treated with IC_{50} (0.33 µM) of DETC for 24 h. After the incubation time, were collected and subjected to 2.5% glutaraldehyde-fixation in 0.1 M of sodium cacodylate buffer containing 1 mM $CaCl_2$, collected and placed in poly-L-lysine treated coverslips and dehydrated with graded ethanol washing, CO_2 dry point, gold coated, and observed under scanning electron microscopy (FEI QUANTA 200 scanning electron microscope).

2.4.9 Determination of cell volume of promastigotes

Cell volume analysis was performed according to da Silva Bortoleti et al. [23,24]. Briefly, *L. amazonensis* promastigotes (10^6) were treated with IC_{50} (0.33 µM) of DETC and incubated for 24 h at 24 °C. After this time, the parasites were collected and washed with PBS. The parasites were then analyzed using BD Accuri™ C6 Plus personal flow cytometer. Forward scatter height (FSC-H) represents the cell volume, and a total of 10,000 events were acquired in the region corresponding to the parasites.

2.5 Analysis of *in vitro* leishmanicidal and immunomodulatory activity on infected macrophages

2.5.1 Experimental animals

BALB/c mice weighing 25–30 g aged 6–12 weeks were obtained from Carlos Chagas Institute/ Fiocruz-PR, Curitiba, Brazil. The mice were maintained under sterile conditions and used according to the protocol approved by the Ethics Committee for the Use of Animals of the State University of Londrina (nº 24299.2017.66).

2.5.2 Peritoneal macrophage viability analysis by MTT assay

To evaluate the effect of free DETC and DETC-Beeswax-CO Nps on peritoneal macrophages, 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) assay was used as described by Mazur et al. [13]. Briefly, macrophages (5×10^4 cells/mL) were recovered from the peritoneal cavity of BALB/c mice with cold PBS supplemented with 3% of FBS and then cultured in 96-well plates with 200 µL of RPMI 1640 medium (10% FBS) for 2 h (37 °C, 5% CO_2). After this time, the wells were washed with PBS to remove the non-adherent cells. Adherent cells were incubated with 0.1, 1, 10 and 100 µM of free DETC and DETC-Beeswax-CO Nps and cultured for 24 h under the same conditions. Thereafter the supernatants were discarded and each well washed 3 times with PBS to remove potential interferents from the treatments; and MTT (5 mg/mL) was added for 3 h. After this time, the supernatant was removed and 100 µL of dimethyldisulfoxide (DMSO) was added to solubilize the formazan crystals. The absorbances were measured using a spectrophotometer (Thermo Scientific, Multiskan GO) at 550 nm.

2.5.3 Peritoneal macrophage viability analysis by Neutral red assay

To evaluate the effect of free DETC and DETC-Besswax-CO Nps on peritoneal macrophages, the neutral red assay was performed. The treatment was performed equal to item “Peritoneal macrophage viability analysis by MTT assay”. After the treatment time, the supernatant was removed and immediately added the neutral red 50 µg/mL for 2 h. Then the supernatant was removed and a solution of 1% acetic acid and 50% ethanol was added and the reading was performed using a spectrophotometer (Thermo Scientific, Multiskan GO) at 540 nm.

2.5.4 Analysis of hemolytic activity

Sheep blood was collected with heparin (Ethics Committee for Animal Experimentation of State University of Londrina: 82862016.60), and the erythrocytes were washed 3 times with PBS (centrifugation at 1000 rpm for 10 min). A 2% red cell suspension was prepared in PBS. Free DETC and DETC-Beeswax-CO Nps were 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. PBS was used with negative control, and Triton X as a positive control for hemolysis. After this time, the plates were centrifuged at 1000 rpm

for 10 min, and the supernatants collected and analyzed by reading the absorbance at 550 nm in a microplate reader.

2.5.5 Anti-amastigote assay

Peritoneal macrophages $5x10^5$ cells / well were distributed in 24-well plates containing coverslips and incubated for 2 h at 37°C, 5%CO2 for adherence. The phagocytic cells that remained on the coverslips were infected with 2.5 $x10^6$ promastigotes of *L. amazonensis* (5 parasites: 1 macrophage) for 2 h, 37 ° C and 5% CO_2. Subsequently, the wells were washed to remove the non-internalized promastigotes and then 0.1, 1 and 10 µM of DETC-Beeswax-CO Nps were added and incubated for 24 h at 37°C, 5% CO_2. The coverslips containing the cells were fixed with methanol and stained with Giemsa. At least 200 cells were analyzed by immersion optical microscopy to evaluate the percentage of infected macrophages and the number of amastigotes by macrophages. As negative control was used macrophages infected and untreated.

2.5.7 Determination of nitrite levels

The nitrite present in the supernatant of the samples from the anti-amastigote assay was measured as an estimate of nitric oxide (NO) levels. It was added 60 uL of Griess reagent (Reagent I: 50 mg of N-naphthylethylenediamine in 250 mL of distilled water; Reagent II: 5 g of sulfanilic acid in 500 mL of 3M HCl, Sigma) in 60 uL sample. The nitrite concentration of the samples was obtained from the reading of the absorbances of samples and standard curve of nitrite at 550 nm in microplate reader.

2.5.8 ROS production by macrophages

In order to evaluate ROS production, macrophages (10^6 cells/mL) infected with *L. amazonensis* were treated with 0.1, 1 and 10 µM of DETC-Beeswax-CO Nps on black plates for 24 h at 37, 5% CO_2. After this incubation period, the cells were washed with PBS (pH 7.4) and incubated with 10 µM of 2', 7'-dichlorofluorescein diacetate (H_2DCFDA) (Sigma, St. Louis, MO, USA) probe for 30 min at 37°C, 5% CO_2. ROS were measured as an increase in fluorescence caused by the conversion of non-fluorescent dichlorohydrofluorescein to the highly fluorescent 2,7-dichlorofluorescein, with an excitation wavelength of 488 nm and emission of 530 nm in a fluorescence microplate reader (Victor X3, PerkinElmer, Finland).

2.5.9 Superoxide anion production by NBT Reduction

The superoxide anion production was determined by the reduction of the redox dye NBT according to Borghi et al. [25] with some modifications. Peritoneal macrophages (10^6) were infected with $5x10^6$ promastigotes for 2 h, 37ºC, 5% CO_2. After incubation, the wells were washed with PBS to remove non-internalized parasites, and then treated with 0.1, 1 and 10 µM of DETC-Beeswax-CO Nps for 24 h, 37°C, 5% CO_2. Subsequently, the supernatant was removed and added 100 µL of nitro blue tetrazolium solution (1 mg/mL) (NBT, Sigma) to the cells and maintained at 37°C in warm bath for 5 min. The supernatant was removed, and the formazan precipitated was then solubilized by adding 120 µL of 2M KOH and 140 µL of dimeltisulfoxide (DMSO). The optical density was measured using a microplate spectrophotometer reader (Multiskan GO Microplate Spectrophotometer, Thermoscientific, Vantaa, Finland) at 600 nm.

2.5.10 Cytokines production

The supernatants collected from the anti-amastigote assays were used to dose the cytokines TNF-α, IL-6 and IL-10 by means of an enzyme-linked immunosorbent assay (ELISA) using eBioscience Kit (USA) according to the manufacturer's instructions. The concentration of cytokines was determined with reference in the standard curve by serial dilutions, and the optical density measured at 492 nm.

2.6 Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Three independent experiments were performed, each in duplicate. Data were analyzed using the GraphPad Prism statistical software (GraphPad Software, Inc., USA, 500.288). Significant differences between the groups were determined by T test and one-way ANOVA, followed by Tukey s test for multiple comparisons. $p \leq 0.05$ was considered statistically significant.

3 Results

3.1 Physico-chemical characterization of DETC-Beeswax-CO nanoparticles

The DETC-Beeswax-CO Nps obtained by double emulsion/melt technique presented an intensity average particle diameter measured by DLS of 200 nm with polydispersity index (shows particle size distribution homogeneity) of 0.2 (Figure 2A) and relatively high negative zeta potential in terms of absolute values (-44 ± 4), indicating colloidal stability due to electrostatic stabilization. TEM images (Figure 2B) showed that the DETC-Beeswax-CO Nps presented semispherical form [13]. The incorporation of DETC in Beeswax-CO Nps did not modify the physico-chemical properties of the nanoparticles. The DETC encapsulation efficiency in beeswax Nps, measured by UV-Vis spectrophotometry, was 87 ± 5 %.

3.2 Free DETC reduces the promastigote viability by ROS production and mitochondrial damage

The leishmanicidal activity of free DETC on *L. amazonensis* promastigotes was evaluated. After 24 h of treatment, the concentrations of 0.1, 1, 10 and 100 μM, and AmB reduced 31.3%, 72%, 78%, 88.9% and 98.5% the viability of the parasites relative to the control, respectively ($p \leq 0.05$) (Figure 3). From these results the IC_{50} of 0.33 μM was determined.

Knowing that free DETC exerts antipromastigote effect, the mechanism involved in the death of these parasites was investigated. The treatment of promastigotes with free DETC IC_{50}, resulted in increased ROS production ($p \leq 0.05$) (Figure 4A) and led to the depolarization of the mitochondrial membrane ($p \leq 0.05$) (Figure 4B) of the parasite when compared to the control. DETC was also able to increase phosphatidylserine exposure ($p \leq 0.05$) (Figure 4C) and reduced plasma membrane integrity ($p \leq 0.05$) (Figure 4D). In addition, co-labeling with Annexin V and propidium iodide (PI) showed that 17.1% of the treated parasites were doubly labeled (Annexin V^+PI^+), 9.6% were labeled with Annexin V (Annexin V^+PI^-), 0.1% only for PI (Annexin V^-PI^+) and 73.2% were viable (Annexin V^-PI^-), showing that most of the unviable parasites were in the process of late apoptosis-like death. The control group (untreated promastigotes), 99.5% of the parasites were viable (Annexin V^-PI^-) (Figure 4E, 4F). Figure 4F shows a comparative and quantitative analysis of the percentage of Annexin V and PI labeling.

3.3 Free DETC alters morphology and reduces cell volume of promastigotes forms

The evaluation of the morphological changes of promastigotes forms due to the treatment with DETC (IC_{50}) was observed by SEM. The parasites that were not treated (Control) had a typical morphology, with an elongated normal cell body, a prominent flagellum and an integral cell membrane (Figure 5A). DETC treatment led to several morphological changes, such as reduction of the cell body and flagellum, damage to the plasma membrane and flagella, with loss of the integrity of the parasite surface (Figure 5B-D). In addition, to confirm the morphological changes induced by the treatment, the cell volume was evaluated by flow cytometry. Thus, it was observed that the treatment with DETC was able to reduce 55.8% the cellular volume of promastigotes when compared to the control ($p \leq 0.05$) (Figure 5E).

3.4 DETC-Beeswax-CO Nanoparticles protect macrophages from the cytotoxic effect of free DETC

To assess the cytotoxicity of free DETC on macrophages the MTT and the neutral red assays were performed. Free DETC at the concentrations of 1, 10 and 100 µM reduced the viability of macrophages in both tested methodologies ($p \leq 0.05$), that is, only the concentration of 0.1 µM did not alter the cellular viability (Figure 6A e 6C). However, no concentration tested showed hemolytic activity ($p \geq 0.05$) (Figure 6E).

On the other hand, the encapsulation of DETC in Beeswax-CO Nps did not affect the viability of macrophages (Figure 6B and 6D) or erythrocytes (Figure 6F) at tested concentrations ($p \geq 0.05$). Thus, demonstrating that DETC-Beeswax-CO Nps reduced the toxic effects of free DETC on peritoneal macrophages.

3.5 DETC-Beeswax-CO Nanoparticles exerts anti-amastigote activity on infected macrophages

To evaluate the leishmanicidal activity on intracellular amastigotes, the antiamastigote assay was performed. DETC-Beeswax-CO Nps 1 and 10 µM reduced the percentage of infected macrophages (31.66% and 41.9% respectively) ($p \leq 0.05$) (Figure 7A) and the number of amastigotes per macrophage (23.5% and 42.6%, respectively) ($p \leq 0.05$) (Figure 7B). Free DETC 0.1µM showed no anti-amastigote activity (data not shown). Based on these results, the following experiments were performed using only the DETC-Beeswax-CO Nps treatment.

3.6 DETC-Beeswax-CO Nanoparticles induces production of total ROS and superoxide anion

To check whether this anti-amastigote activity would be mediated by microbicidal molecules, it was evaluated the production of NO, ROS and superoxide anion. No concentration of DETC-Beeswax-CO Nps altered NO levels ($p \geq 0.05$) (Figure 8A). DETC-Beeswax-CO Nps 1 and 10 µM increased total ROS and superoxide anion production ($p \leq 0.05$) (Figure 8B-C).

3.7 DETC-Beeswax-CO Nanoparticles induces TNF-α and IL-6 production, and reduces IL-10

To verify whether the treatments induce the production of cytokines, the levels of TNF-α, IL-6 and IL-10 in the culture supernatant of infected, treated and untreated macrophages were measured. DETC-Beeswax-CO Nps 10 µM increased TNF-α and IL-6 levels ($p \leq 0.05$) (Figure 9A and 9B), however treatment with DETC-Beeswax-CO Nps 10 µM reduced IL-10 levels when compared to the control ($p \leq 0.05$) (Figure 9C).

4 Discussion

Due to the difficulties of administration, high cost, resistance and important side effects caused by the drugs used in the current treatment of leishmaniasis [5-7], research aimed at the development of new drugs with potent leishmanicidal activity and reduction of toxicity to individuals are of great relevance. Several natural and synthetic compounds have been studied as possible therapeutic alternatives [21, 23, 24, 26-31].

In this context, the present study provided new perspectives of the DETC mechanism of action for in vitro experimental leishmaniasis model. Data demonstrating that DETC-treatment is effective at low micromolar concentrations against both promastigote and amastigote forms of *L. amazonensis*. The encapsulation reduced the toxicity in the host cell, in addition to increasing the synthesis of ROS, superoxide anion and TNF-α, important for macrophage activation and parasite elimination.

DETC is a copper metal chelator known as an inhibitor of SOD1 enzyme in mammals [8]. *Leishmania* SOD (SOD-Fe) is synthesized in mitochondria, and plays an important role in the virulence of the parasite, in the redox balance in both intracellular amastigotes and promastigote axenic, protecting against oxidative stress,

and participate in signaling to differentiate infectious forms mediated by ROS. Interestingly, DETC can complex with Fe^{2+}, inhibiting the SOD-Fe activity present in *Leishmania* [32-35]

Previous studies have demonstrated leishmanicidal effect of DETC on *L. amazonensis* promastigotes and *L. braziliensis* amastigotes [11-14]. In fact, the present study showed that free DETC exerts an anti-promastigote effect on *L. amazonensis*, exhibiting an IC_{50} = 0.33 µM, causing morphological changes, such as reduction of cellular volume and flagellum size; besides deformations on the surface and membrane of the parasite. These results are are complementary to that observed by Khouri et al [11] who evidenced enlarged mitochondria with reduced electrodensity after 1 h of treatment with DETC.

Trypanosomatids, such as *Leishmania*, have a single large mitochondrion that plays an important role in energy metabolism (ATP production) and cell survival, besides presenting a region rich in kDNA, called kinetoplast [36]. The maintenance of the mitochondrial membrane potential is of great importance to preserve its physiological function and to avoid the high generation of ROS [37]. Mitochondrial damage may lead to increased ROS production in promastigotes, contributing to cell death [38, 39].

When investigating the potential mechanism of death triggered by DETC in promastigote forms, it was evidenced that DETC induces some biochemical alterations, such as increased ROS generation, mitochondrial membrane depolarization, phosphatidylserine exposure and causes plasma membrane disruption. The results also showed that the majority of non-viable parasites were An^+ PI^+ (exhibit phosphatidylserine exposure and increased plasma membrane permeability). Many studies have shown that ROS production, mitochondrial damage, and double labeling for Annexin V and propidium iodide are some important features that suggest late apoptosis-like cell death [21-24, 27, 40].

Knowing the intracellular nature of this parasite, the cytotoxicity of DETC was evaluated on peritoneal macrophages. DETC was toxic at concentrations of 1, 10 and 100 µM. Thus, in order to reduce toxic effects the encapsulation of the drug could be an alternative, in addition to other advantages such as the incorporation of hydrophilic and hydrophobic drugs, controlled release, drug protection, increased amount of encapsulated drug, increased stability and bioavailability, reduction of toxic effects, low

cost and large scale production and having a direct interaction on membrane cell [41-43].

Several studies have reported the advantages of the use of solid lipid nanoparticles (SLNs), since they present less toxicity in comparison to several polymeric materials and can be applied by different routes of administration [42, 44]. Particles with sizes smaller than 200 nm have been linked to increased efficacy [45].

The encapsulated DETC activity on promastigote forms was not evaluated, since the objective was to verify the direct effect of the free compound on the parasites, and the mechanisms of action. In addition, Mazur et al. [13], showed the antipromastigote effect of DETC-Beeswax-CO nanoparticles. It was shown in the present study that DETC encapsulated in Beeswax-CO Nps protected the macrophages from the toxic effects of free DETC. Mazur et al. [13], showed that DETC-Beeswax-CO Nps exerted a leishmanicidal effect against *L. amazonensis* promastigotes and was not toxic to macrophages, improving the selectivity index, however, to date no study has demonstrated the action of DETC-Beeswax-CO Nps on *L. amazonensis*-infected macrophages.

In this way, the leishmanicidal effect of DETC-Beeswax-CO Nps on infected macrophages was investigated. The results of the present study showed that the DETC-Beeswax-CO Nps (1 and 10 μM) were able to reduce the percentage of infected macrophages and the number of amastigotes per macrophage at much lower concentrations. Since the DETC-Beeswax-CO Nps have shown anti-amastigote effect, the possible mechanisms involved in the elimination of these parasite and control of *in vitro* infection were investigated. For this, it was evaluated the production of microbicidal molecules (ROS and NO) and cytokines.

The interaction between macrophage and *Leishmania* is complex [40]. Macrophages are cells of the innate immune system that perform phagocytosis and aim to inhibit proliferation and eliminate the parasites [27]. However, these are also the main cells where parasites reside and multiply [46]. This is due to several mechanisms developed by *Leishmania* to evade the immune system, capture nutrients, and promote their proliferation and survival [47].

The main molecules used by macrophages to control and eliminate *Leishmania* parasites are ROS and NO [1]. However, the parasites are able to reduce the oxidative burst, interfering in the assembly of NADPH oxidase complex in the parasitophorous vacuoles membrane, important for superoxide production [48]. It has also been

described the detrimental role of SOD in leishmaniasis, due to its antioxidant function [9, 10]. *Leishmania* spp. have several antioxidant mechanisms, such as the presence of the enzyme Fe-SOD [49-51]. *L. amazonensis* and *L. brasiliensis* may also increase expression of this enzyme in the host by induction of type I interferons [10]. All these strategies developed or triggered by the parasite provide a favorable environment for its survival within the macrophage.

Knowing the important role of ROS in eliminating these intracellular parasites, the results of this work show that DETC-Beeswax-CO Nps induced the production of total ROS and superoxide anion, but not NO. Similarly, free DETC and DETC-based bacterial cellulose bio-curatives reduced the infection of macrophages by *L. amazonensis* and *L. braziliensis*, respectively, by the increase of superoxide anion, a radical with potent microbicidal activity [11, 12].

In addition to microbicidal molecules like ROS, the cytokines are important mediators in the modulation of the immune response related to the control or susceptibility to pathogens. Exposure of *L. amazonensis*-infected macrophages with DETC-Beeswax-CO Nps led to increased production of TNF-α, IL-6 and reduction of IL-10. Similarly, Celes et al. [12] showed that DETC-based bacterial cellulose bio-curatives induced the production of TNF-α and IL-6 in macrophages infected with *L. braziliensis*, the expression of TNF-α and IL-6 is related to classically activated macrophages [52].

Studies have demonstrated the protective effect of TNF-α on cutaneous leishmaniasis [53-55]. TNF-α is produced by activated macrophages and enhances host antimicrobial defense [56-58], in this sense, this cytokine may induce the production of ROS, and, on the other hand, ROS can also stimulate TNF-α and IL-6 synthesis via NF-κB, leading to a positive feedback loop [59]. In addition, TNF-α can activate the assembly of NADPH oxidase, leading to increased synthesis of superoxide anion [48, 60]. Together, our data are in accordance with the literature, where the axis TNF-α/IL-6/ROS are active and actuating in the intramacrophagic amastigotes elimination. It is important to note that these parasites do not have members of the Bcl-2 family, TNF-α / TNFR and caspases [61].

IL-10, in turn, is related to the persistence and progression of cutaneous leishmaniasis [62, 63]. This anti-inflammatory cytokine plays a role in regulating the immune response by suppressing the microbicidal mechanisms of macrophages [64], resulting in a favorable environment for intracellular *Leishmania* survival. DETC-

Beeswax-CO Nps were able, at the highest concentration, to reduce the levels of this cytokine, generating an ideal environment for the maintenance of the microbicide status, followed by elimination of the parasite.

In conclusion, free DETC exerts antipromastigote effect by ROS generation and mitochondrial depolarization, suggesting late apoptosis-like death. The encapsulation of DETC in Beeswax-CO Nps reduced the toxic effects of the free compound. In addition, DETC-Beeswax-CO Nps have leishmanicidal effect on *L. amazonensis*-infected macrophages through the production of total ROS, superoxide anion, TNF-α, IL-6, and reduces IL-10, favoring an environment for elimination of the intracellular parasite. This research shows the potential of DETC-Beeswax-CO Nps for new *in vivo* studies aiming at alternative treatments of cutaneous leishmaniasis.

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6 Conflict of interest

The authors declared that there is no conflict of interest.

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Figures

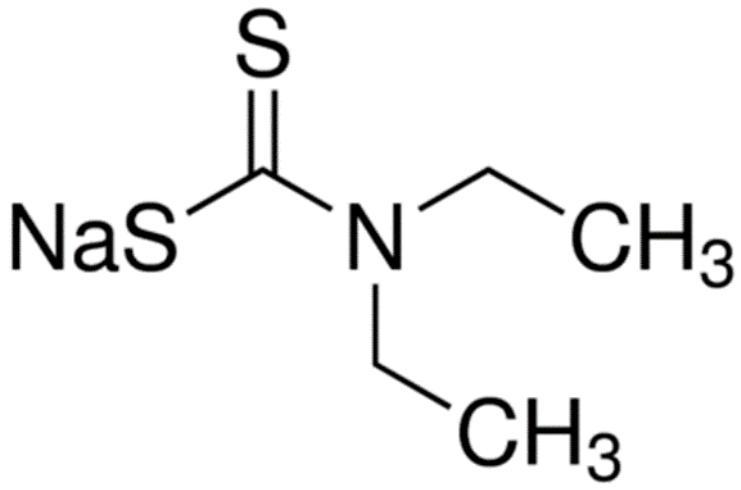

Figure 1. Chemical structure of Sodium diethyldithiocarbamate.

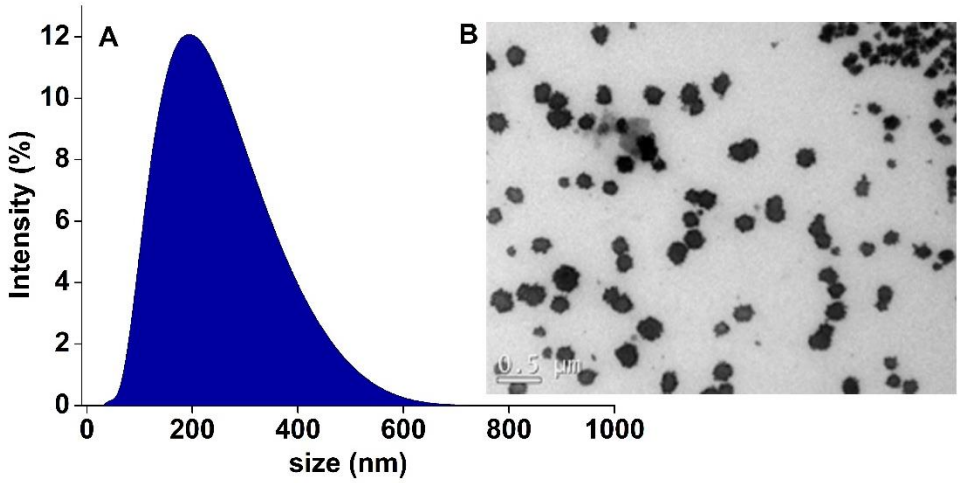

Figure 2. Morphology of DETC loaded in Beeswax-copaiba oil nanoparticles (DETC-Beeswax-CO). A) Particle size distribution measured by DLS, with an average size of 200 nm. (B) Representative TEM image showing the semispherical shape of the nanoparticles.

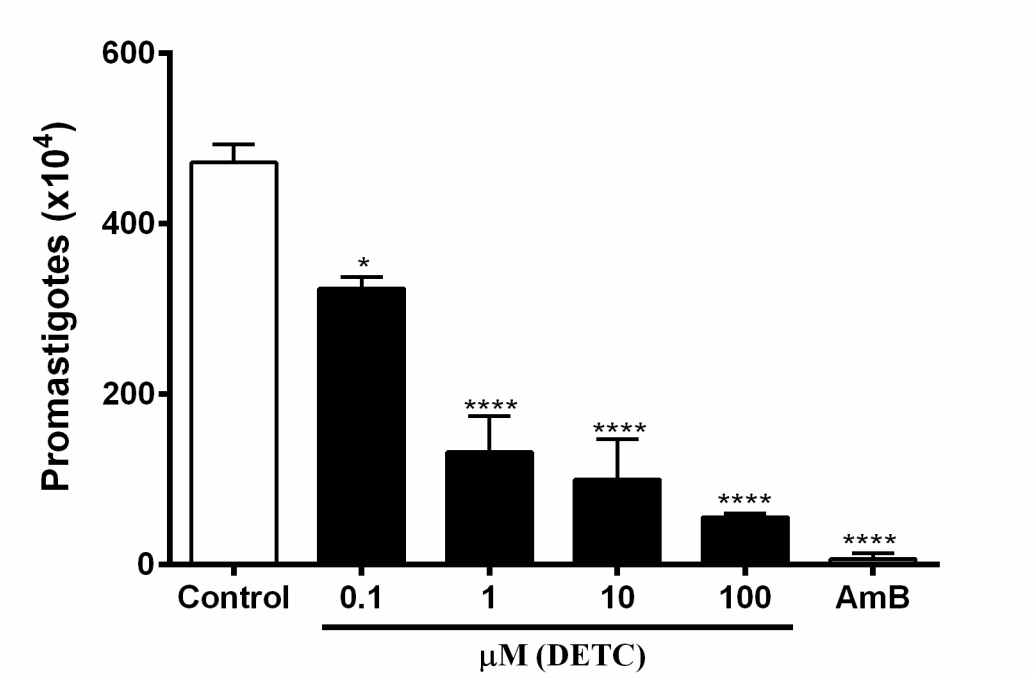

Figure 3. DETC exerts leishmanicidal activity on *L. amazonensis* promastigotes. Promastigotes forms (10^6) were treated with Free DETC (0.1, 1, 10 e 100 µM). After 24 h the parasites were counted in Neubauer's chamber. Untreated parasites were used as control. Amphotericin B (AmB) was used as positive control. The values represent mean ± SEM of three independent experiments performed in duplicate. * significant difference compared to control ($p \leq 0.05$, **** $p < 0.0001$).

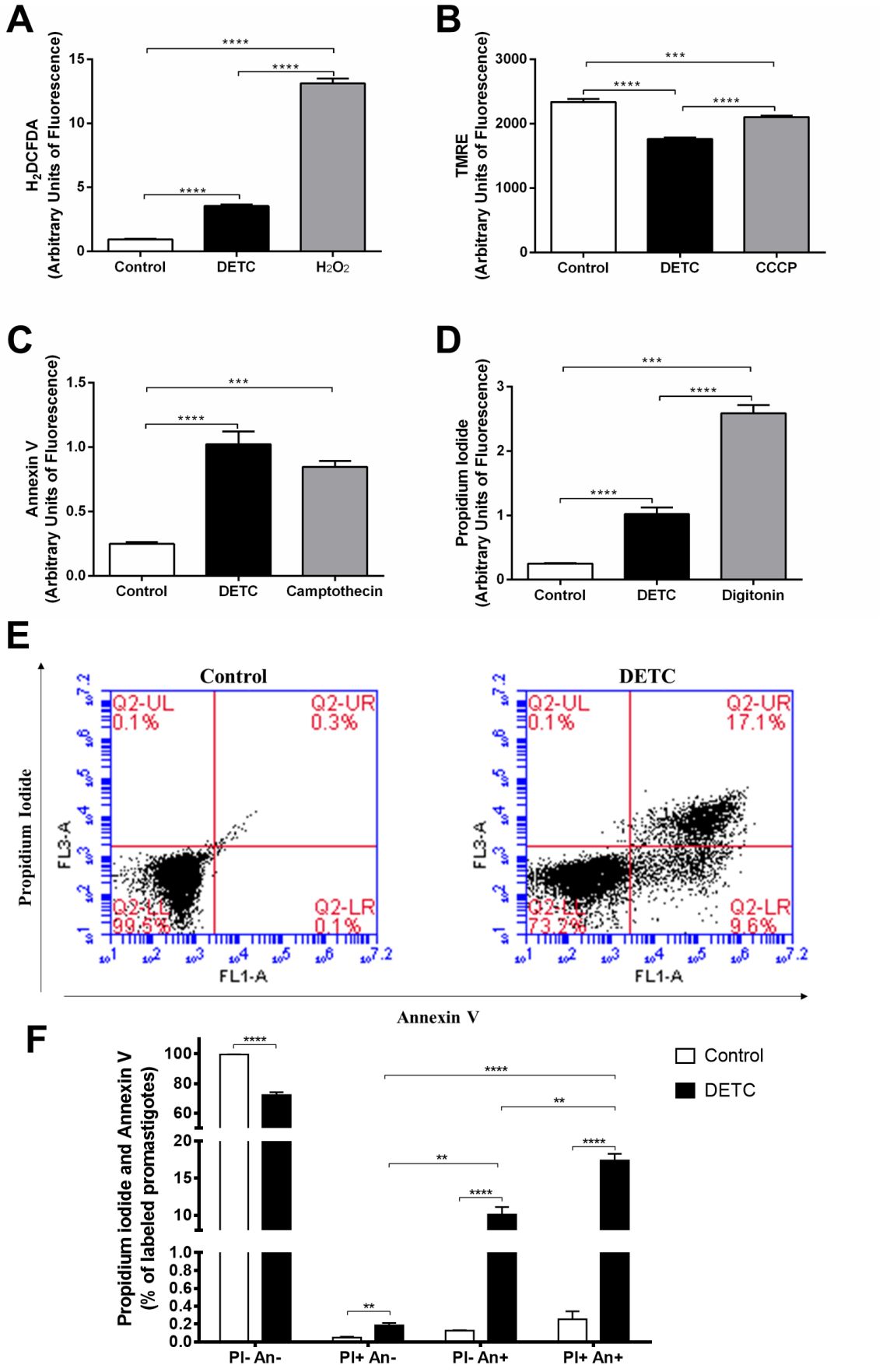
A
H2DCFDA (Arbitrary Units of Fluorescence)
Control
DETC
H2O2
B
TMRE (Arbitrary Units of Fluorescence)
Control
DETC
CCCP
C
Annexin V (Arbitrary Units of Fluorescence)
Control
DETC
Camptothecin
D
Propidium Iodide (Arbitrary Units of Fluorescence)
Control
DETC
Digitonin
E
Control
DETC
Q2-UL 0.1%
Q2-UR 0.3%
Q2-LL 99.5%
Q2-LR 0.1%
Q2-UL 0.1%
Q2-UR 17.1%
Q2-LL 73.2%
Q2-LR 9.6%
Propidium Iodide
FL3-A
FL1-A
Annexin V
F
Propidium iodide and Annexin V (% of labeled promastigotes)
Control
DETC
PI- An-
PI+ An-
PI- An+
PI+ An+

Figure 4. Mechanisms of death triggered by DETC in *L. amazonensis* promastigotes. Promastigotes forms (10^6) were treated with free DETC (IC_{50}) and evaluated the Total ROS production through the H_2DCFDA probe (A), mitochondrial membrane depolarization by TMRE labeling (B), phosphatidylserine exposure by Annexin V labeling (C), membrane integrity by PI labeling (D). Annexin V and PI co-labeling of promastigotes treated with free DETC and analyzed by flow cytometry (E). Typical dot plots of at least three independent experiments are shown (F). P Untreated promastigotes were used as control. Hydrogen peroxide (H_2O_2), Carbonyl cyanide m-chlorophenylhydrazone (CCCP), Camptothecin and Diginitonin were used as positive controls for ROS production, mitochondrial membrane depolarization, phosphatidylserine exposure and plasma membrane permeability, respectively. The values represent mean ± SEM of three independent experiments performed in duplicate. * significant difference compared to control and treatments ($p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****} p < 0.0001$).

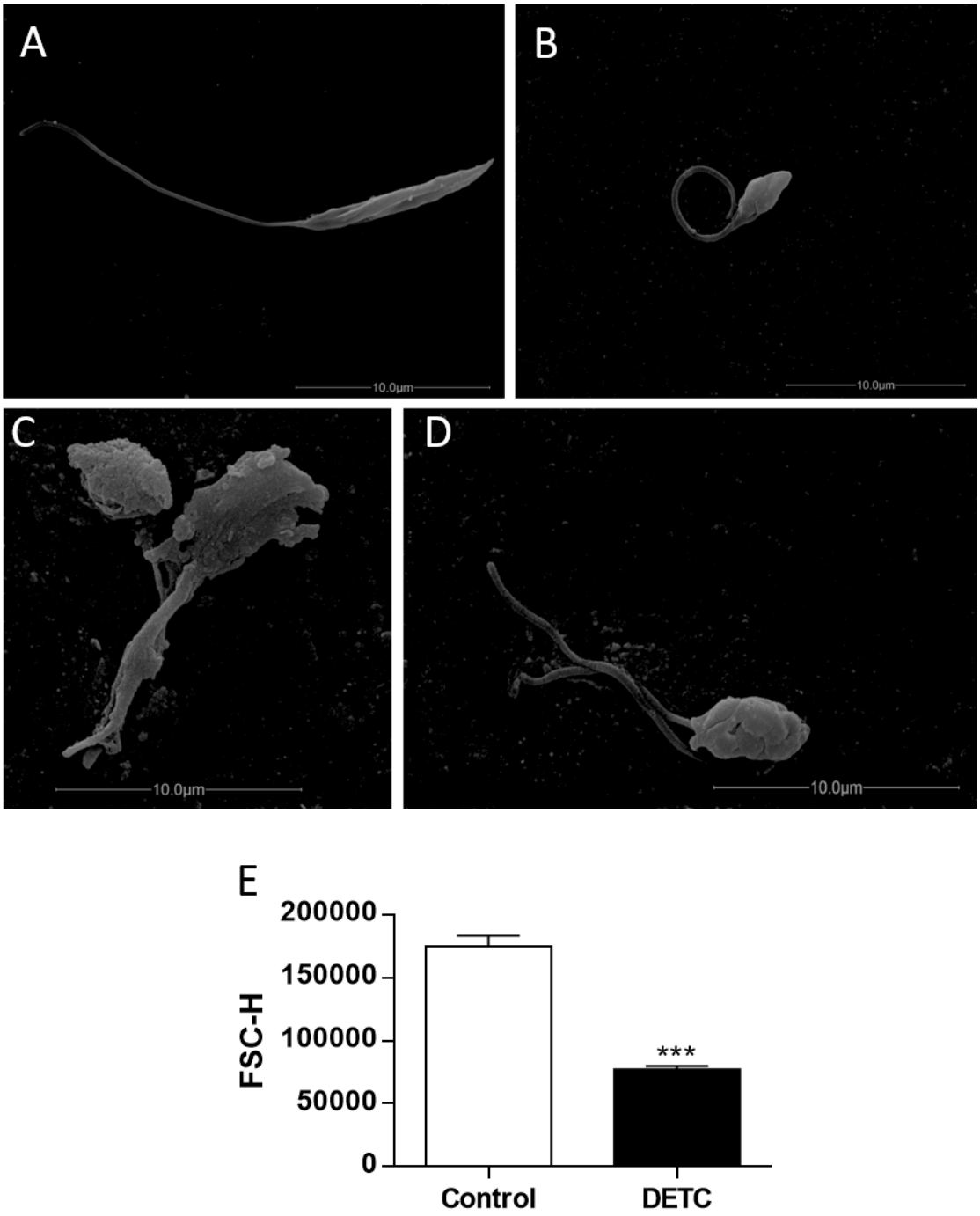

Figure 5. Morphological changes and cell volume of *L. amazonensis* promastigotes treated with DETC. Scanning electron microscopy imagens of promastigotes, Control (A); DETC (IC_{50}) treatment (B-D). Flow cytometry to analyze the cell volume of promastigotes treated with DETC (IC_{50}) (E). FSC-H was considered cell size. The values represent mean ± SEM of three independent experiments performed in duplicate. ***significant difference compared to control ($p \leq 0.001$).

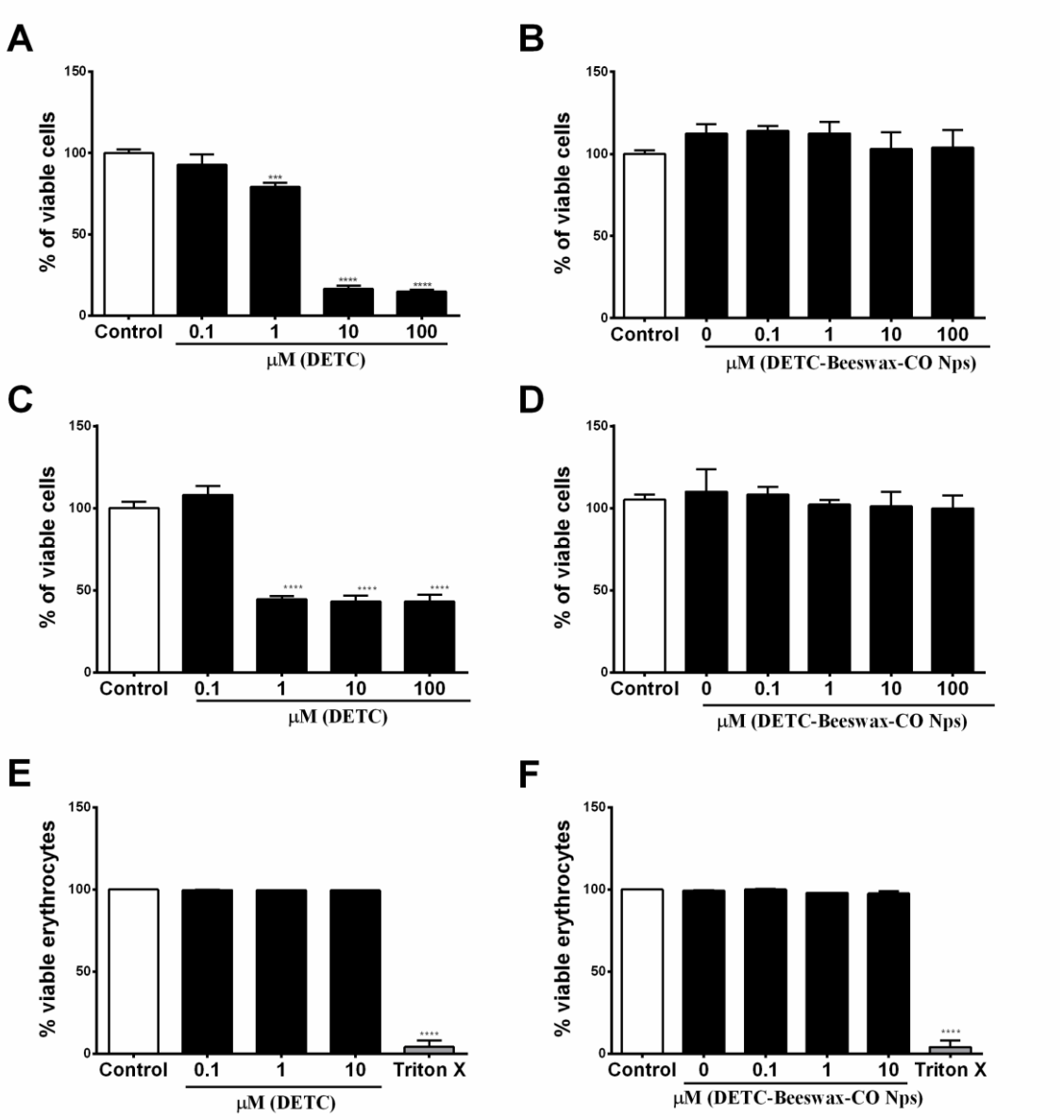

Figure 6. Viability of macrophages and erythrocytes treated with free DETC and DETC-Beeswax-CO Nanoparticles. Peritoneal macrophages were treated with free DETC (A and C) or DETC-Beeswax-CO-Nanoparticles (B and D) and viability was accessed by MTT assay (A and B) and Neutral Red assay (C and D). Untreated macrophages were used as controls. Erythrocytes from sheep were treated with free DETC (E) or DETC-Beeswax-CO Nanoparticles (F) and percentage of viable erythrocytes were determined. PBS was used as non-hemolytic control and Triton X was used as positive control for hemolysis. The values represent mean ± SEM of three independent experiments performed in duplicate. *significant difference compared to control ($p \leq 0.05$, ***$p \leq 0.001$).

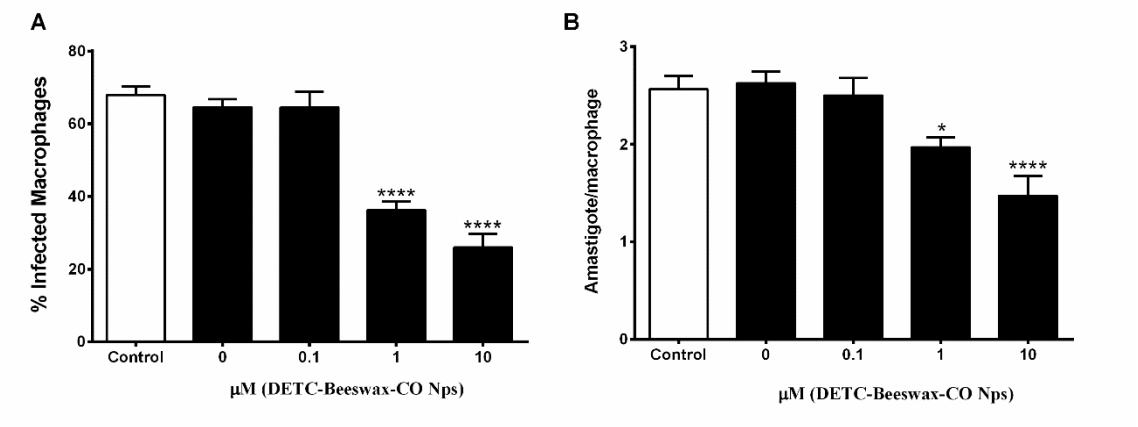

Figure 7. DETC-Beeswax-CO Nanoparticles exerts anti-amastigote effect. Peritoneal macrophages were infected with *L. amazonensis* (1 macrophage: 5 promastigotes) and treated with DETC-Beeswax-CO Nps; and then the percentage of infected macrophages (A) and number of amastigotes per macrophages (B) were determined. Untreated and infected macrophages were used as control. The values represent mean ± SEM of three independent experiments performed in duplicate. *significant difference compared to control ($p \leq 0.05$, ****$p < 0.0001$).

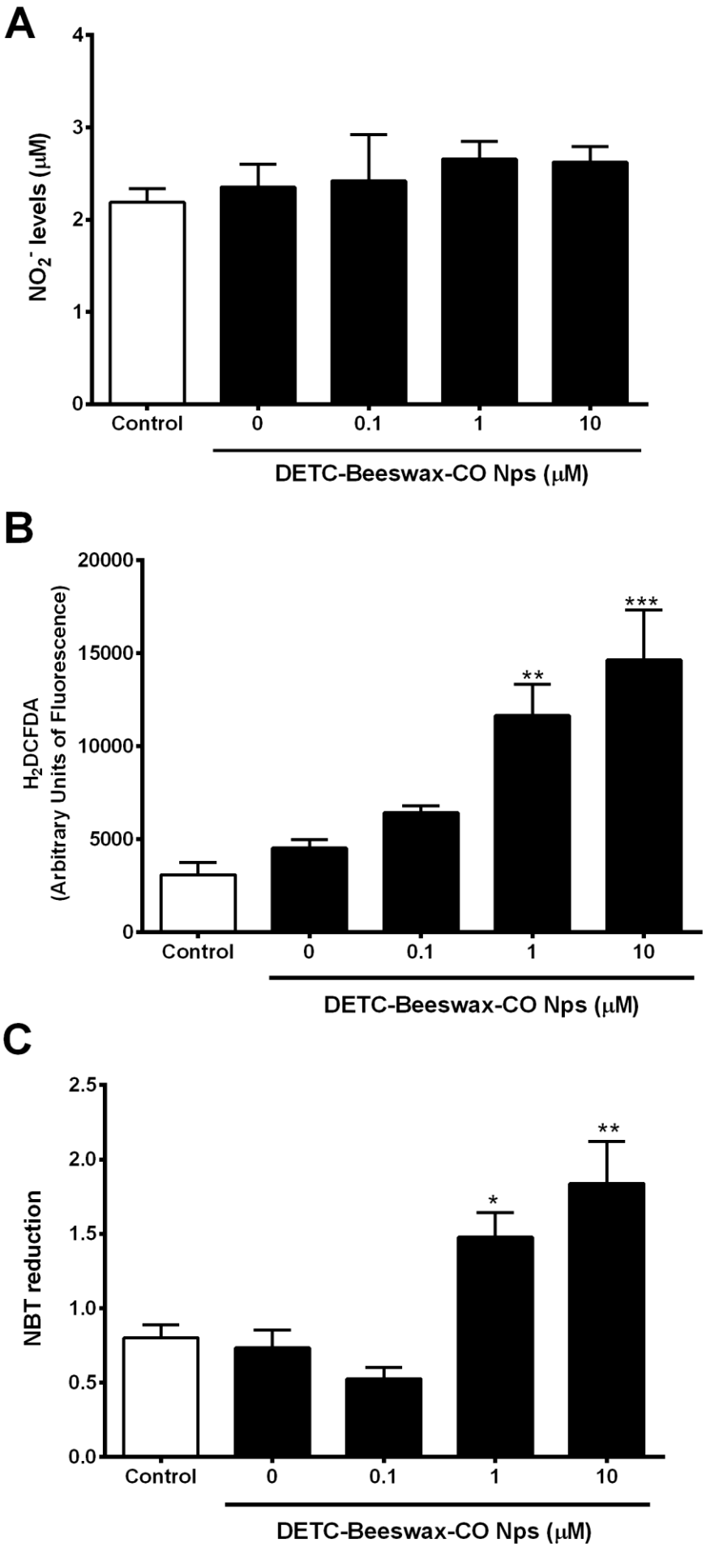
A
NO_2^- levels (μM)
0
1
2
3
4
Control
0
0.1
1
10
DETC-Beeswax-CO Nps (μM)
B
H_2DCFDA
(Arbitrary Units of Fluorescence)
0
5000
10000
15000
20000
**
***
Control
0
0.1
1
10
DETC-Beeswax-CO Nps (μM)
C
NBT reduction
0.0
0.5
1.0
1.5
2.0
2.5
*
**
Control
0
0.1
1
10
DETC-Beeswax-CO Nps (μM)

Figure 8. DETC-Beeswax-CO Nanoparticles increases the production of total ROS and superoxide anion in infected macrophages. Peritoneal macrophages were infected with *L. amazonensis* (1 macrophage: 5 promastigotes) and treated with DETC-Beeswax-CO Nps; the supernatant of the cultures were used to measure the production of nitric oxide (NO) by the Griess method (A), Total ROS through H_2DCFDA probe (B) and superoxide anion by the NBT reduction (C). Untreated and infected macrophages were used as control. The values represent mean ± SEM of three independent experiments performed in duplicate. *significant difference compared to control ($p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$).

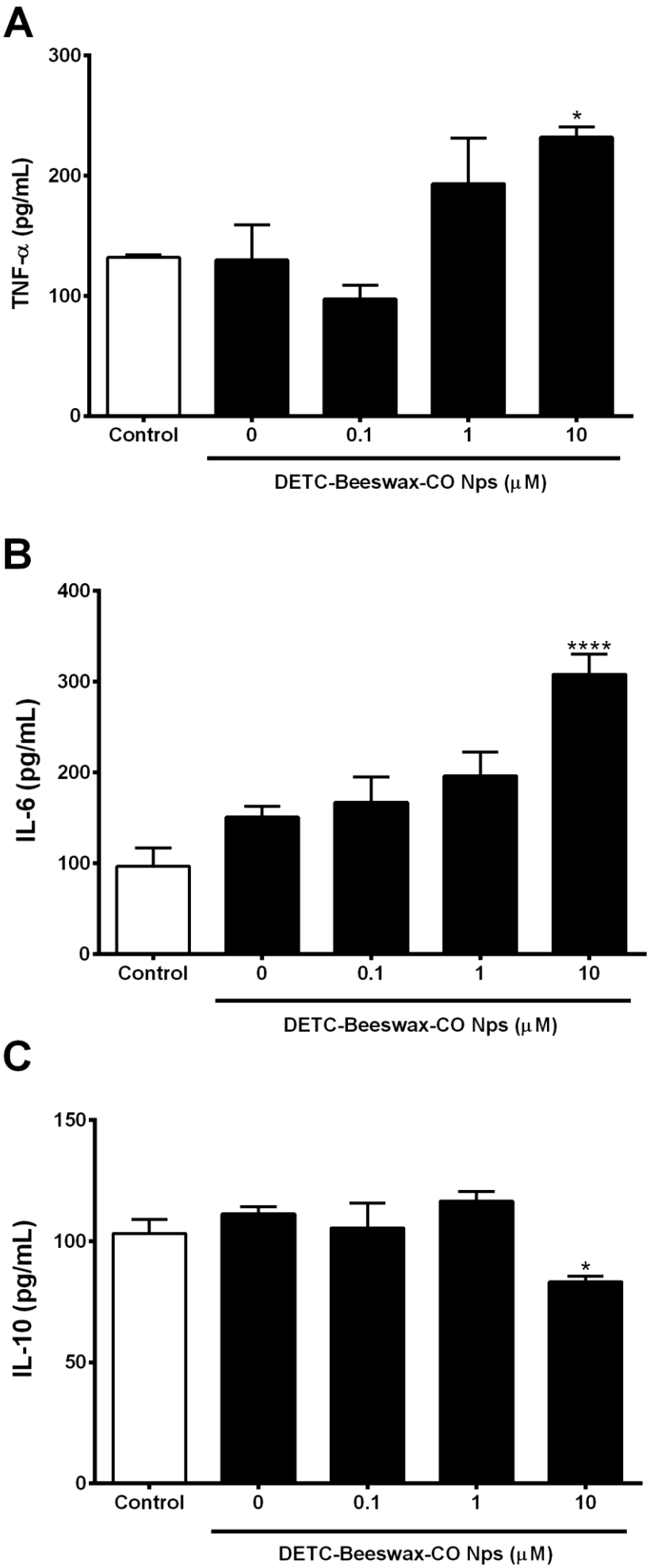
A
TNF-α (pg/mL)
300
200
100
0
*
Control
0
0.1
1
10
DETC-Beeswax-CO Nps (μM)
B
IL-6 (pg/mL)
400
300
200
100
0
****
Control
0
0.1
1
10
DETC-Beeswax-CO Nps (μM)
C
IL-10 (pg/mL)
150
100
50
0
*
Control
0
0.1
1
10
DETC-Beeswax-CO Nps (μM)

Figure 9. DETC-Beeswax-CO Nanoparticles increases the production of TNF-α, IL-6 and reduces IL-10 in infected macrophages. Peritoneal macrophages were infected with *L. amazonensis* (1 macrophage: 5 promastigotes) and treated with DETC-Beeswax-CO Nps; the supernatant of the cultures were used to measure the production of TNF-α (A), IL-6 (B) and IL-10 (C) by ELISA. Untreated and infected macrophages were used as control. The values represent mean ± SEM of three independent experiments performed in duplicate. *significant difference compared to control ($p \leq 0.05$).

4. ARTIGO CIENTÍFICO 2

4-nitrochalcone exerts leishmanicidal effect on *L. amazonensis* promastigotes and intracellular amastigotes, and the 4-nitrochalcone encapsulation in beeswax copaiba oil nanoparticles reduces macrophages cytotoxicity

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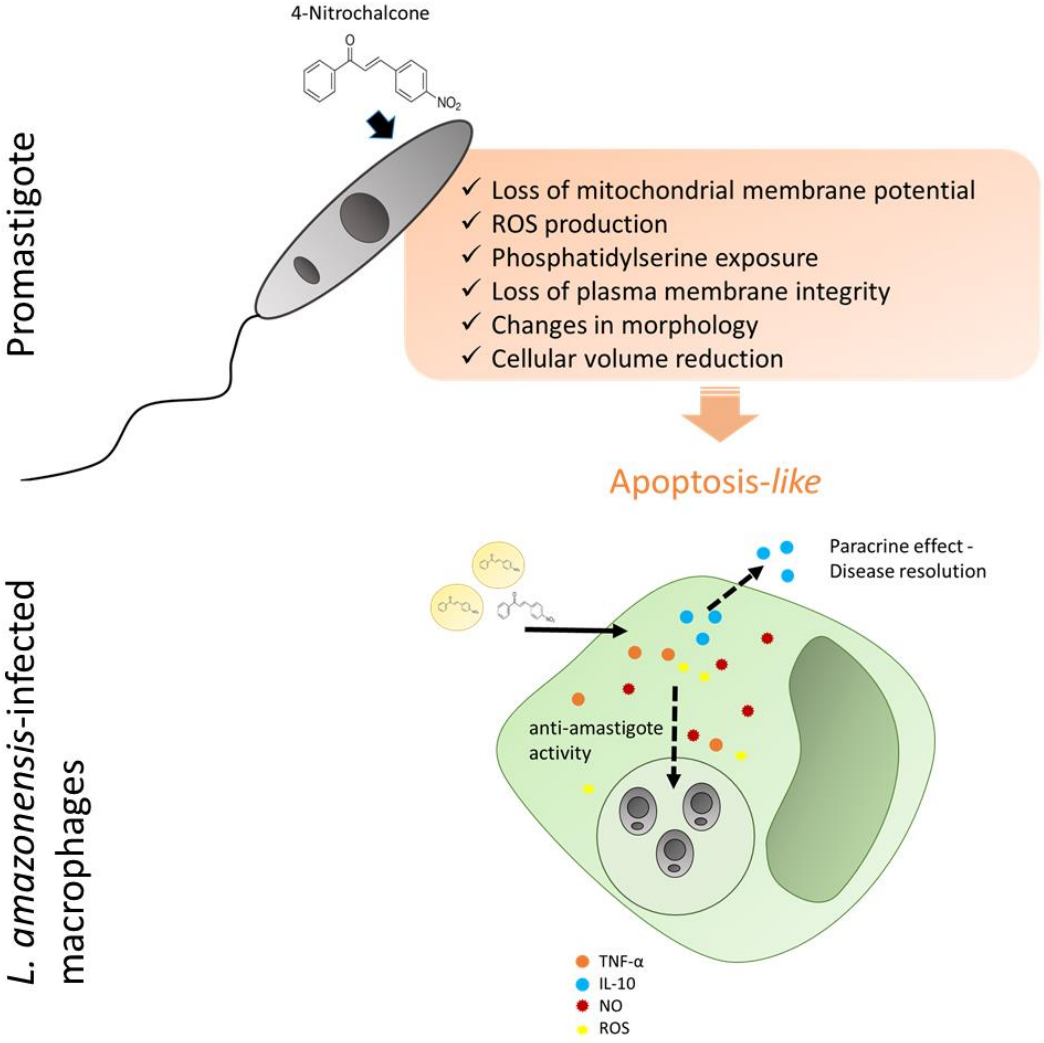
Graphical abstract



Abstract

The current treatment of Leishmaniasis presents several difficulties, such as high toxicity, variable efficacy and high cost. In this way the research of new therapeutic alternatives for this disease becomes very important. In the past years the use of natural compounds, such as chalcones, and nanotechnology has gained great importance in the drug discovery scenario. The aim of this study was to evaluate the *in vitro* leishmanicidal activity of free and encapsulated 4-Nitrochalcone (4NC) on *L. amazonensis*, as well as the mechanisms of action. Free 4NC was able to reduce the viability of promastigotes, induce ROS production, reduce mitochondrial membrane potential, increase plasma membrane permeability and exposure of phosphatidylserine. The treatment also altered the morphology and reduced the cellular volume of the parasite. The treatment with encapsulated 4NC in beeswax-copaiba oil nanoparticles (4NC-beeswax-CO Nps) did not alter the macrophages viability. In addition, 4NC-beeswax-CO Nps reduced the percentage of infected macrophages and number of amastigotes by macrophages, increasing the production of ROS, NO, TNF-α and IL-10. Thus, free 4NC exerts anti-promastigote effect; and 4NC-beeswax-CO Nps showed leishmanicidal effect on *L. amazonensis*-infected macrophages, activating the macrophage microbicidal machinery.

Keywords: Leishmaniasis; Solid lipid nanoparticle; 4-Nitrochalcone; Nanoencapsulation.

Introduction

Leishmaniasis is a complex of diseases caused by protozoa of *Leishmania* genus. It is transmitted by the bite of female sandfly insects (STEVERDING, 2017). Leishmaniasis can manifest in several clinical forms, such as visceral, localized cutaneous, diffuse cutaneous and mucocutaneous forms (WHO, 2019). Cutaneous leishmaniasis (CL) is the most prevalent clinical form in the world (HEPBURN, 2003). In the Americas, the cutaneous form is widely distributed and known as American Cutaneous Leishmaniasis (ACL), with cases reported from the southern United States to northern Argentina. Brazil is considered an endemic region, and it is one of the countries with the highest rates of disease notification, consisting of an important public health problem (MAIA-ELKHOURY et al., 2016).

The current treatment of ACL represents a great challenge, since the available drugs (pentavalent antimonials: first choice drugs; amphotericin B, pentamidine: second choice drugs) present variable efficacy, high patient toxicity, high cost and administration difficulties (SUNDAR; CHAKRAVARTY, 2015; ARONSON; JOYA 2019). Due to all the difficulties faced in the treatment of leishmaniasis, the use of natural and synthetic compounds are being targets of research for new therapeutic alternatives that have a potent leishmanicidal activity, with no side effects.

Chalcones are open-chain flavonoids with two aromatic rings attached by a three-carbon enone portion. These compounds have a broad spectrum of biological activities, such as anti-bacterial, anthelmintic, trypanocidal, antinociceptive, anti-oxidant, anti-tumor, immunomodulatory, anti-inflammatory, antifungal, antiviral, antimalarial and leishmanicidal among others (CHEN et al., 1997; FRISS-MOLLER et al., 2002; TSUKIYAMA et al., 2002; BOECK et al., 2005; BHAT et a., 2005; BATOVSKA et al., 2007; PADARATZ et al., 2009; MAHAPATRA et al., 2015; SHIANO MORIELLO et al., 2016; MARTINEZ et al., 2017a; 2017b; ZHOU et al., 2018; PEREIRA et al., 2018; STAURENGO-FERRARI et al., 2018; MIRANDA-SAPLA et al., 2019).

In addition, chemical changes in the chalcones molecules can result in significant changes in the biological effects and pharmacological activity. Although some studies have demonstrated the analgesic and also the leishmanicidal effect of nitrosylated chalcones (ROCHA et al., 2018; SOUSA-BATISTA et al., 2018), few studies describe the biological effects of 4-Nitrochalcone (4NC). This compound has

anti-proliferative activity in tumor cell lines such as, JR8 and HL-60 (DALLA VIA et al., 2009), L1210, CEM (DIMMOCK et al., 2002) MCF-7 and T47D (ILANGO et al.,2010), HeLa (ARÉVALO et al), antifungal activity against *Candida albicans* yeasts (BATOVSKA et al., 2007) and dermatophytes (LÓPEZ et al., 2001), as well as anti-protozoa activity, in which it inhibited the growth of *Entamoeba histolytica* (WANI et al., 2012).

Solid lipid nanoparticles (SLNs) are nanometer-sized materials that have solid-state lipids core that can act as drug and molecule carriers (MUKHERJEE; THAKUR, 2009). These nanomaterials present important characteristics that allow improving development of new treatments against intracellular pathogens and reducing the toxic effects of free drugs (ASSOLINI et al., 2017; MAZUR et al., 2019).

Although several chalcones have shown leishmanicidal effect, to the best of our knowledge, the evaluation of the anti-leishmanial effect of 4NC has not been addressed before. In this way, the present study aimed to evaluate *in vitro* the anti-promastigote activity of free 4NC and anti-amastigote of 4-Nitrochalcone-beeswax-copaiba oil nanoparticles (4NC-beeswax-CO Nps), and to elucidate possible mechanisms of death.

Materials and methods

Compound

Commercial 4-Nitrochalcone (4NC) (Figure 1), ≥99% purity, was obtained from Sigma Aldrich (Darmstadt, Germany, Catalog Number - 157481). The stock solution of 4NC was dissolved in 1% dimethyl sulfoxide (DMSO) (GIBCO, Invitrogen, New York, USA). DMSO concentration did not exceed 0.05% in all experiments.

Production of solid lipid nanoparticles loaded with 4NC

The encapsulation of 4NC in SLNs was performed by a combined melt dispersion and Oil/ Water (O/W) double emulsion technique using a similar procedure to that described elsewhere (MAZUR et al., 2019), that does not require the use of organic solvents. In the first step, white beeswax (450 mg) was melted at 70° and 0.42 mL of Crodamol GTCC (Alpha Química) containing 10 mg/mL of 4NC was added to this lipid phase under magnetic stirring (50 rpm). In sequence, 9 mL of the aqueous solution containing Tween 80 (Vetec) (90 mg) was added to the organic phase and

kept under magnetic stirring (400 rpm) for 10 min at 70 °C. The O/W emulsion was sonicated at 70 % amplitude for 60 s in a pulsed regime (15 s on, 10 s off), forming O/W nanodroplets. To promote the rapid lipid solidification, the emulsion was immediately added to 35 mL of water at 7 °C and kept under magnetic stirring (400 rpm) for 5 min. Blank SLNs (Pure SLNs) were prepared without 4NC in the lipid phase.

SLNs were centrifuged (MiniSpin, Eppendorf, Germany) at 13,400 rpm by 30 min, the supernatant was removed and centrifuged again under the same conditions. The recovered nanoparticles were then redispersed in 10% D-(+)-trehalose dehydrate solution and frozen at -80 °C by 48h. The freeze-drying process was conducted by 48h (L101, Liotop, Brazil).

Light scattering and zeta potential

The z-average particle size and polydispersity index (PDI) of SLNs were determined by dynamic light scattering (Nanosizer, Malvern Instruments, U.K). The surface charge of dispersed SLNs was verified by zeta potential measurements (Zetasizer, Malvern Instruments, U.K.). The characterization was performed before and after freeze-drying. Results are shown as mean ± standard deviation (n=3).

Encapsulation Efficiency (EE%)

The EE% of O/W technique was indirectly determined by quantification of non-encapsulated 4NC. SLNs loaded with 4NC were centrifuged (EPPENDORF, MiniSpin, Germany) using Amicon® Ultra centrifugal filter (Millipore, 100 kDa) at 13,400 rpm for 30 min. The supernatant was removed, and the absorbance quantified by UV-Vis spectrophotometry (U-1900, Hitachi) at 324 nm. The efficiency was calculated by the equation:

$$EE\% = \frac{(C_1 - C_2)}{C_1} \times 100 \qquad (1)$$

Where C_1 is the total concentration of drug added to the formulation and C_2 is the drug concentration in the supernatant. The analyses were performed in triplicate at 25°C and the results are shown as mean ± standard deviation.

In vitro leishmanicidal activity analysis on promastigote forms of *L. amazonensis*

L. amazonensis promastigote maintenance

Promastigote forms of *Leishmania (L.) amazonensis* (MHOM/ BR/ 1989/ 166MJO) from the State University of Maringá were maintained in culture medium 199 (GIBCO Invitrogen) supplemented with 10% fetal bovine serum (FBS) (GIBCO Invitrogen), 10 mM Hepes, 1% Human Urine, 1% L-glutamine, streptomycin with penicillin (10U/ml-10µg/ml, GIBCO Invitrogen) and 10% sodium bicarbonate. The culture was maintained in an incubator at 24°C in a 25 cm^2 culture flask for five days (stationary growth phase). All promastigote forms were used in the stationary growth phase.

Antipromastigote activity

The antipromastigote activity was performed according to Bortoleti et al. (2019). Promastigote forms of *L. amazonensis* (10^6 cells/mL) were treated with 0.1, 1, 10, 15, 20, 25, 30, 50 and 100 µM of free 4NC. The parasites were incubated at 24ºC in a B.O.D. and growth/proliferation was estimated by counting in Neubauer chamber after 24 h of treatment. Untreated promastigotes were used as controls. DMSO concentration did not exceed 0.05 %/ well.

From the results of the 4NC-antipromastigote action, the minimum concentration capable of inhibiting 50% of the viable parasites (IC_{50}) by non-linear regression was calculated (GraphPad Software, Inc., USA, 500, 288).

Reactive Oxygen Species (ROS) production in promastigotes

In order to evaluate ROS production in *L. amazonensis* promastigote forms ($1x10^6$ cells/mL) were treated with free 4NC IC_{50} (21.2 µM) for 24h at 24ºC in a B.O.D., then the parasites were washed with phosphate buffered saline (PBS) and incubated with probe diacetate 2', 7'-dichlorofluorescein (H_2DCFDA) (Sigma, St. Louis, MO, USA) 10µM for 45 min at 24°C.

Reactive oxygen species were measured as an increase in fluorescence caused by the conversion of non-fluorescent dichlorohydrofluorescein to the highly fluorescent 2,7-dichlorofluorescein, with an excitation wavelength of 488 nm and emission of 530 nm in a fluorescence microplate reader (Victor X3, PerkinElmer,

Finland). Hydrogen peroxide (H_2O_2) was used as a positive control. In order to compare the treatment, the fluorescence values obtained were normalized to the respective number of parasites.

Determination of mitochondrial membrane potential

The analysis of mitochondrial membrane potential was performed by tetramethylrhodamine-ethyl ester (TMRE) fluorescent staining (Sigma, St. Louis, MO, USA). For this, promastigote forms (1×10^6 cells/mL) were treated with free 4NC IC_{50} (21.2 µM) for 24 h. Subsequently, the parasites were washed with PBS and incubated with 25 nM TMRE for 30 min at 24°C, followed by washing with PBS and immediately analyzed on a fluorescence microplate reader (Victor X3, PerkinElmer, Finlândia) at excitation/emission wavelength of 480/580 nm. Carbonyl cyanide m-chlorophenylhydrazone 100 µM was used as a positive control.

Determination of phosphatidylserine exposure

Phosphatidylserine exposure was detected using Annexin-V FITC (Invitrogen, Eugene, USA). Promastigotes (1×10^6 cells/mL) were treated with free 4NC IC_{50} (21.2 µM) for 24 h at 24°C. The parasites were then washed and resuspended in 100 µl of binding buffer (140 mM NaCl, 5 mM $CaCl_2$, and 10 mM HEPES-Na, pH 7.4), followed by the addition of 5 µL of Annexin-V FITC for 15 min at room temperature. After incubation, it was added 400µL of binding buffer. Camptothecin (Sigma, St. Louis, MO, USA) (10 µM) was used as a positive control. The data acquisition was performed using a fluorescence microplate reader (Victor X3, PerkinElmer, Finland) at excitation wavelength of 488 nm and emission of 520 nm. In order to compare the treatment, the fluorescence values obtained were normalized to the respective number of parasites.

Determination of cell membrane integrity

Promastigotes (1×10^6 cells/mL) in 24-well plates were treated with free 4NC IC_{50} (21.2 µM) for 24h at 24°C. The parasites were collected and washed with PBS and directly incubated with propidium iodide (PI) (Sigma, St. Louis, MO, USA) (0.50 µg/mL) for 5 min, according to the manufacturer's instructions. Immediately thereafter, the parasites were analyzed using a fluorescence microplate reader (Victor X3, PerkinElmer, Finland) with an excitation wavelength of 480 nm and emission of 580 nm. Digitonin (Sigma, St. Louis, MO, USA) (40.0 µM) was used as a positive control.

The fluorescence values obtained were normalized to the total number of cells from the treatment.

Co-determination of annexin V and propidium iodide label

Promastigotes (1×10^6 cells/mL) were treated with free 4NC IC_{50} (21.2 µM) for 24h at 24°C. Then, parasites were washed and resuspended in 100 μL of assay buffer (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™ 6 Plus personal flow cytometer. Cells were analyzed as (DOROODGAR et al., 2016; TOMIOTTO-PELLISSIER et al., 2018a).

Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) of promastigotes forms was performed according to Bortoleti et al. (2019). Briefly, promastigotes (10^6) were treated with 4NC IC_{50} (21.2 µM) for 24 h. After the incubation time, the parasites were collected and subjected to 2.5% glutaraldehyde-fixation in 0.1 M of sodium cacodylate buffer containing 1 mM $CaCl_2$, collected and placed in poly-L-lysine treated coverslips and dehydrated with graded ethanol washing, CO_2 dry point, gold coated, and observed under SEM (FEI QUANTA 200 scanning electron microscope).

Determination of cell volume of promastigote

Cell volume analysis was performed according to Gonçalves et al. (2018). Briefly, promastigotes (10^6) were treated with 4NC IC_{50} (21.2 µM) and incubated for 24 h at 24 °C. After this time, the parasites were collected and washed with PBS. The parasites were then analyzed using BD Accuri™ C6 Plus flow cytometer. Forward scatter-height (FSC-H) represents the cell volume, and a total of 10.000 events were acquired in the region corresponding to the parasites.

Analysis of *in vitro* leishmanicidal and immunomodulatory activity on infected macrophages

Experimental animals

BALB/c mice weighing 25–30 g aged 6–12 weeks were obtained from Carlos Chagas Institute/ Fiocruz-PR, Curitiba, Brazil. The mice were maintained under sterile conditions and used according to the protocol approved by the Ethics Committee for the Use of Animals of the State University of Londrina (nº 24299.2017.66).

Peritoneal macrophage viability analysis by MTT assay

To evaluate the effect of free 4NC and 4NC-Beeswax-CO Nps on peritoneal macrophages, 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) was used assay as described by Mazur et al. (2019). Briefly, macrophages (5×10^4 cells/mL) were recovered from the peritoneal cavity of BALB/c mice with cold PBS supplemented with 3% of FBS and then cultured in 96-well plates with 200 µL of RPMI 1640 medium (10% FBS) for 2 h (37 °C, 5% CO_2). After this time, the wells were washed with PBS to remove the non-adherent cells. Adherent cells were incubated with 1, 5 and 10 µM of free 4NC and 4-Nitrochalcone-Beeswax-CO Nps and cultured for 24 h under the same conditions. Thereafter the supernatants were discarded and each well washed 3 times with PBS to remove potential interferents from the treatments; and MTT (5 mg/mL) was added for 3 h. After this time, the supernatant was removed and 100 µL of DMSO (dimethylsulfoxide) was added to solubilize the formazan crystals. The plates were read using a spectrophotometer (Thermo Scientific, Multiskan GO) at 550 nm.

Peritoneal macrophage viability analysis by Neutral red assay

To evaluate the effect of free 4NC and 4NC-Beeswax-CO nanoparticles on peritoneal macrophages, the neutral red assay was performed. The treatment was performed equal to item “Peritoneal macrophage viability analysis by MTT assay”. After the treatment time, the supernatant was removed and immediately added the neutral red 50 µg/mL for 2 h. Then the supernatant was removed and a solution of 1% acetic acid and 50% ethanol was added and the reading was performed using a spectrophotometer (Thermo Scientific, Multiskan GO) at 540 nm.

Analysis of hemolytic activity

Sheep blood was collected with heparin (Ethics Committee for Animal Experimentation of State University of Londrina: 82862016.60), and the erythrocytes were washed 3 times with PBS (centrifugation at 1000 rpm for 10 minutes). A 2% red

cell suspension was prepared in PBS. Free 4NC and 4NC-Beeswax-CO Nps (1, 5 and 10 µM) were 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. PBS was used with negative control, and Triton X as a positive control for hemolysis. After this time, the plates were centrifuged at 1000 rpm for 10 minutes, and the supernatants collected and analyzed by reading the absorbance at 550 nm in a microplate reader.

Anti-amastigote assay

Peritoneal macrophages $5x10^5$ cells/well were distributed in 24-well plates containing coverslips and incubated for 2 h at 37°C, 5%CO_2 for adherence. The phagocytic cells that remained on the coverslips were infected with 2.5 $x10^6$ promastigotes of *L. amazonensis* (5 parasites: 1 macrophage) for 2 h, 37 °C and 5% CO_2. Subsequently, the wells were washed to remove the non-internalized promastigotes and then 1, 5 and 10 µM of 4-Nitrochalcone-Beeswax-CO Nps; 1 and 5 µM of free 4NC were added and incubated for 24 h at 37°C, 5% CO_2. After the treatment period, the supernatant was collected for cytokine and nitric oxide (NO) measurement. The coverslips containing the cells were fixed with methanol and stained with Giemsa (Laborclin, Pines306 PR Brazil). At least 200 cells were analyzed by immersion optical microscopy (Olympus BX41, Olympus Optical Co., Ltd., Tokyo, Japan) (1000x magnification) to evaluate the percentage of infected macrophages and the number of amastigotes by macrophages. As negative control was used macrophages infected and untreated.

Determination of nitrite levels

The nitrite present in the supernatant of the samples from the antiamastigote assay was measured as an estimate of nitric oxide (NO) levels. It was added 60 uL of Griess reagent (Reagent I: 50 mg of N-naphthylethylenediamine in 250 mL of distilled water; Reagent II: 5 g of sulfanilic acid in 500 mL of 3M HCl) in 60 uL sample. A calibration curve was made using serial dilutions of $NaNO_2$. The nitrite concentration of the samples was obtained from the reading of the absorbances of samples and standard curve of nitrite at 550 nm in a microplate reader (Thermo Scientific, Multiskan GO).

Reactive Oxygen Species (ROS) production by macrophages

In order to evaluate ROS production, macrophages ($1x10^6$ cells/mL) infected with *L. amazonensis* were treated with 1, 5 and 10 µM of 4NC-Beeswax-CO Nps; 1 and 5 µM of free 4NC on black plates for 24 h at 37, 5% CO_2. After this incubation period, the cells were washed with PBS (pH 7.4) and incubated with 10µM of 2', 7'-dichlorofluorescein diacetate (H_2DCFDA) (Sigma, St. Louis, MO, USA) probe for 30 min at 37°C, 5% CO_2. Reactive oxygen species were measured as an increase in fluorescence caused by the conversion of non-fluorescent dichlorodihydrofluorescein to the highly fluorescent 2,7-dichlorofluorescein, with an excitation wavelength of 488 nm and emission of 530 nm in a fluorescence microplate reader (Victor X3, PerkinElmer, Finland).

Cytokines production

The supernatants collected from the antiamastigote assays were used to dose the cytokines TNF-α and IL-10 by means of an enzyme-linked immunosorbent assay (ELISA) using eBioscience Kit (USA) according to the manufacturer's instructions. The concentration of cytokines was determined with reference in the standard curve by serial dilutions, and the optical density measured at 492 nm.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Three independent experiments were performed, each in duplicate. Data were analyzed using the GraphPad Prism statistical software (GraphPad Software, Inc., USA, 500.288). Significant differences between the groups were determined by T-test and one-way ANOVA, followed by Tukey's test for multiple comparisons. $p \leq 0.05$ was considered statistically significant.

Results

Physical-chemical characterization of 4-Nitrochalcone–Beeswax-copaiba oil particles

The 4NC-Beeswax-CO Nps obtained by double emulsion/melt technique presented predominantly size of 229.6 ± 7.1 nm and negative zeta potential (-38.4 ± 2.3). The 4NC encapsulation efficiency in beeswax nanoparticles was of 99.4 %.

4NC induces promastigotes death by late apoptosis-like

The 4NC antipromastigote effect against *L. amazonensis* was evaluated. Free 4NC reduced the viability of promastigotes after 24 hours of treatment at all tested concentrations (0.1-100 μM) compared to the control ($p \leq 0.0001$) (Figure 2), with an IC_{50} of 21.2 μM. DMSO 0.05% showed no leishmanicidal effect.

Since 4NC reduced the viability of *L. amazonensis* promastigotes, it was investigated the possible mechanism involved in the death of these parasites. Free 4NC IC_{50} led to an increase in ROS generation ($p \leq 0.0001$) (Figure 3A), mitochondrial membrane depolarization ($p \leq 0.0001$) (Figure 3B), increased phosphatidylserine exposure ($p \leq 0.0001$) (Figure 3C) and loss of plasma membrane integrity ($p \leq 0.0001$) (Figure 3D) when compared with control,. When evaluating the co-labeling with Annexin V and propidium iodide (PI), we differentiated the cell death mechanism in necrotic (PI^+), apoptotic (annexin V^+) or late-apoptotic (double mark for annexin V^+/PI^+) processes (Scariot et al., 2017). Our data showed that 23.9% of the treated parasites were doubly labeled (Annexin V^+ PI^+), 6.92% were labeled with Annexin V only (Annexin V^+ PI^-), 7.43% were labeled with PI only (Annexin V^- PI^+) and 61.8% were not labeled (Annexin V^- PI^-) (Figure 2E). It was also verified that 97.9% of untreated promastigotes (control group) were viable (Annexin V^- PI^-) (Figure 2E). Treatment with free 4NC significantly increased the labeling with Annexin V, PI and the co-labeling Annexin+PI compared to control (Figure 2F).

4NC causes morphological changes and reduction of the cellular volume of promastigotes

In order to confirm the apoptosis death mechanism, it was evaluated morphological changes and cell volume induced by 4NC treatment by scanning electron microscopy (SEM) and flow cytometry, respectively. Untreated promastigotes (control) showed typical morphology, elongated body, prominent flagellum and intact membrane (Figure 4A). Free 4NC caused rounding of the cell body, changes in the size of the flagellum and changes in the surface/ membrane of the parasite (Figure 4B). In addition, it was also observed that treatment with free 4NC reduced 35% (± 0.4) the cell volume of promastigotes when compared to the control ($p < 0.0001$) (Figure 4C-D).

The encapsulation of 4NC in Beeswax-CO Nanoparticles does not alter the viability of peritoneal macrophages

To evaluate whether free 4NC and 4NC-Beeswax-CO Nps alters the viability of macrophages, it was performed the MTT (Figure 5 A-B) and neutral red assay (Figure 5 C-D). Free 4NC at concentrations of 1 and 5 μM did not alter the viability of macrophages, whereas 10 μM was cytotoxic ($p \leq 0.01$). In an attempt to reduce toxicity of 4NC, the compound was encapsulated in solid lipid nanoparticles (4NC-Beeswax-CO Nps) and cell viability was assessed. Unlike the free compound, none of the 4NC-Beeswax-CO Nps tested concentrations (0, 1, 5 and 10 μM) were able to alter the viability of peritoneal macrophages. In addition, the free and encapsulated compound had no hemolytic effect at the tested concentrations (Figure 5E-F). Thus, the encapsulation of 4NC in Beeswax-CO Nps protected the macrophages from the toxic effects caused by the free compound. Only non-toxic concentrations were used for the following experiments.

Free 4NC and 4NC-Beeswax-CO Nanoparticles exerts antiamastigote effect in *L. amazonensis*-infected macrophages

To evaluate the leishmanicidal effect of 4NC on intracellular amastigotes, it was conducted an antiamastigotes assay to assess the leishmanicidal effect of 4NC and 4NC-Beeswax-CO Nps on *L. amazonensis*-infected macrophages considering the percentage of infected macrophages and the number of amastigotes. The results showed that 5 μM of free 4NC and 5 and 10 μM of 4NC-Beeswax-CO Nps reduced the percentage of infected macrophages in 49.7 %, 34.4 % and 76.7 %, respectively ($p < 0.0001$) (Figure 6A). Furthermore, the treatments led to a significant reduction in the mean number of amastigotes per macrophage. The 4NC 5 μM and 4NC-Beeswax-CO Nps 5 and 10 μM reduced 33.6 %, 25.8 %, 53.1 %, respectively ($p \leq 0.05$) (Figure 6B) compared to the control. The Beeswax-CO Nps (Unloaded nanoparticle) did not change the infection parameters of the control macrophages. The concentration of 1 μM of free 4NC and 4NC-Beeswax-CO Nps had no anti-amastigote effect.

4NC-Beeswax-CO Nps induces macrophage-production of nitric oxide and reactive oxygen species

To verify the possible microbicidal mechanism involved in the anti-amastigote effect, the production of NO and ROS were evaluated. The infected macrophages

subjected to free 4NC 5 μM induced the production of ROS ($p \leq 0.01$), but not NO. 4NC-Beeswax-CO Nps 5 and 10 μM increased ROS production ($p \leq 0.05$), and the concentration of 10 μM induced NO production ($p < 0.0001$) compared to control (Figure 7 A-B). The Beeswax-CO Nps and the concentration of 1 μM was not able to modulate the production of these molecules with microbicidal potential, corroborating with the antiamastigote assay and showing the importance of these molecules as leishmanicidal product induced by the 4NC.

4NC-Beeswax-CO Nanoparticles enhances the production of TNF-α and IL-10

To verify whether the treatment modulates the cytokines production, TNF-α, and IL-10 levels were measured in infected macrophages. Infected macrophages-treatment with 5 μM of 4NC-Beeswax-CO Nps increased only TNF-α level (388.9 pg/mL) ($p < 0.0001$) (Figure 8A), while the concentration of 10 μM was able to increase levels of all analyzed cytokines (TNF-α = 1436.3 pg/ml; IL-10 = 186.6 pg/mL), when compared to control ($p < 0.0001$) (Figure 7A-B). However, free 4NC did not modulate cytokine production at the concentrations tested.

Discussion

American Cutaneous Leishmaniasis (ACL) is a neglected disease that represents a serious public health problem (MAIA-ELKHOURY et al., 2016). The current treatment presents several difficulties, such as high toxicity, which can often lead to the patient withdrawing from treatment (SUNDAR; CHAKRAVARTY 2005; ARONSON; JOYA 2019). In view of this, the use of natural and synthetic compounds has aroused great interest for the development of new drugs (GONÇALVES et al., 2018; CATANEO et al., 2019; BORTOLETI et al., 2018; 2019; VOLPATO et al., 2013; 2018; MENDES et al., 2016; MIRANDA-SAPLA et al., 2019). The chalcone class is an important example of natural compounds, which by its simple structure allows multiple molecular substitutions, enabling the development of bioactive and non-toxic derivatives (GOMES et al., 2017). Thus, the present study showed the antipromastigote activity of free 4NC and the leishmanicidal effect of 4NC encapsulated in Beeswax-CO nanoparticles on *L. amazonensis*-infected macrophages, investigating the possible action mechanisms.

The *in silico* study of a molecule is of great importance in the search for potential new drugs, thus, 4-Nitrochalcone not violating the Ro5/Veber rules (data not shown) (LIPINSKI et al., 1997; VEBER et al., 2002), otherwise, the drugs used for the treatment of cutaneous leishmaniasis do not comply with all the rules (COIMBRA et al., 2011; GILBERT, 2014).

Several natural, synthetic chalcones and hybrid molecules have been reported to present anti-*Leishmania* effect (DE MELO et al., 2018; TAJUDDEEN et al., 2018, MIRANDA-SAPLA et al., 2019). In the present work, it was evidenced that 4NC inhibited the growth of promastigotes, altered the morphology and reduced the cellular volume of the parasites, corroborating with some studies that evidenced the anti-promastigote effect of nitrosylated chalcones. Miranda-Sapla et al. (2019) showed that trans-chalcone exerts a leishmanicidal effect, altering the morphology and size of the parasite. Knowing the anti-promastigote effect of 4-Nitrochalcone, it was investigated the possible mechanisms involved in the death of these parasites.

For organisms with single mitochondria, such as *Leishmania*, there is no possibility of compensating for mitochondrial damage, since the correct functioning of this organelle is critical for the parasite survival (FIDALGO; GILLE 2011). The treatment of *L. amazonensis* promastigotes with 4-Nitrochalcone resulted in increased ROS production and also caused depolarization of the mitochondrial membrane. Damage to mitochondria can be caused by oxidative stress, since it causes loss of mitochondrial membrane integrity (FONSECA-SILVA et al., 2011). In addition, mitochondrial disruption may trigger a high ROS production, contributing to apoptosis death (ROY et al., 2008). Zhai et al. (1995) e Miranda-Sapla et al. (2019) showed that Licochalcone A and *trans*-chalcone are capable of affecting the mitochondria of *L. major* and *L. amazonensis*, respectively. In addition, the compound increased phosphatidylserine exposure and caused loss of plasma membrane integrity. Thus, according to these morphological and biochemical alterations, it can be suggested that 4-Nitrochalcone induces the death of promastigotes by late apoptosis-like mechanism (DOROODGAR et al., 2016; SHADAB et al., 2017; GONÇALVES et al., 2018; BORTOLETI et al., 2018; 2019; MIRANDA-SAPLA et al., 2019).

Solid lipid nanoparticles are inserted in the growing area of nanotechnology, acting in diverse applications in the fields of clinical medicine, research and drugs development (MUKHERJEE et al., 2009). These nanoparticulate systems have important characteristics in the development or improvement of drugs, such as drug

release control, stability enhancement, possibility of encapsulate lipophilic and hydrophilic drugs, high biocompatibility, high-scale production, application versatility, increased drug loading and reduction of toxicity of conventional drugs (MUKHERJEE et al., 2009; EKAMBARAM, SATHALI and PRIYANKA 2012). In the present research, the use of Beeswax-CO nanoparticles reduced the toxic effects of 4NC in murine macrophages.

In this way, the anti-amastigote effect of free and nanoparticulated 4NC was investigated using non-toxic concentrations to the host. It was evidenced that the free and SLN-encapsulated compound are able to reduce the number of amastigotes by macrophages and the percentage of infected macrophages, however, the use of Beeswax-CO nanoparticles made it possible to use more effective concentrations against the parasite.

Some studies also show that nitrosylated chalcones (CH8) exert an anti-amastigote effect on *L. amazonensis in vitro*, and are able to reduce murine infection by *L. amazonensis* and *L. infantum* (BOECK et al., 2006). In addition, nitrosylated synthetic chalcones loaded onto microspheres of Poly(Lactic-Co-Glycolic Acid) and in nanoemulsions maintained the leishmanicidal effect of the free compounds against amastigotes of *L. amazonensis*, with significantly reduced toxicity to the host cell (DE JESUS SOUSA-BATISTA et al., 2018; DE MATTOS et al., 2015). Torres-Santos et al. (1999) and Piñeiro et al. (2006) showed that encapsulation in polylactic acid (PLA) and poly (D, L-lactide-co-glycolide (PGLA) nanoparticles improves the anti-*Leishmania* effect of 2′, 6′-dihydroxy-4′-methoxychalcone and *trans*-chalcona, respectively (TORRES-SANTOS et al., 1999; PIÑEIRO et al., 2006).

Macrophages are cells of the innate immune system and play a dual role in leishmaniasis, as these cells act to combat the parasite by activating its microbicidal machinery, but also provide a suitable environment for the survival and replication of amastigotes due to the various strategies developed by *Leishmania* parasites to manipulate macrophage signaling pathways, sequester nutrients and evade host immune responses (BASU; RAY, 2005; DUQUE; DESCOTEAUX, 2015; PODINOVSKAIA; DESCOTEAUX, 2015; TOMIOTTO-PELLISSIER, 2018b).

Despite the evasion mechanisms performed by *Leishmania*, it has been described that ROS and NO are the crucial molecules produced by macrophages, incapacitating and destroying these intracellular pathogens (SCOTT; NOVAIS, 2016). In the present research, it was observed that 4NC Beeswax-CO Nps induced the

production of ROS and NO, while the free compound only induced the ROS production. Some chalcones may exhibit pro-oxidant properties, inducing increased ROS or reducing antioxidant mechanisms (GUZY et al., 2010; WANG et al., 2015). De Mello et al. (2014) showed that some synthetic chalcones have a leishmanicidal effect against *L. braziliensis* through NO production (DE MELLO et al., 2014).

Cytokines play a crucial role in the innate immune response, acting to orchestrate the activation, response, and communication of macrophages and other cells, and can determine the outcome of an infectious disease (MURRAY; STOW, 2014). Macrophages can also secrete a range of mediators that can also activate their machinery through different signaling pathways, stimulating the synthesis of microbicidal molecules (TOMIOTTO-PELLISSIER et al., 2018b). 4NC-Beeswax-CO Nps increased the production of TNF-α and IL-10 in *L. amazonensis*-infected macrophages. TNF-α is proinflammatory cytokine that is produced by activated macrophages (TOMIOTTO-PELLISSIER et al., 2018b). TNF-α is a mediator that plays an important role in host resistance against *Leishmania* infection (LIEW et al., 1990), participating in the macrophages activation and inducing the production of ROS and NO (LIU; UZONNA 2012). TNF-α and ROS can also activate NF-κB, leading to the production of proinflammatory cytokines that can act in an autocrine manner (LIU; UZONNA 2012). Activation of NF-κB also enhances iNOS expression and regulates NADPH oxidase by increasing gp91phox expression, inducing increased production of the microbicidal molecules NO and ROS, respectively (ANRATHER; RACCHUMI; IADECOLA, 2006; MORRIS et al., 2003).

Although a proinflammatory response is important to eliminate parasites, an exacerbated response may contribute to the pathogenesis and development of lesions in ACL (CAMPOS et al., 2017). Some studies have linked IL-10 to disease progression (KANE; MOSSER, 2001; BELKAID et al., 2001), in addition, through the production of this cytokine may help in the control of an exacerbated inflammatory response. Gonzalez-Lombana et al. (2013) showed that an inflammatory response mediated by INF-γ and IL-17 in the absence of IL-10 contributes to tissue damage in *L. major* infection (GONZALEZ-LOMBANA et al., 2013). Therefore, the increase of IL-10 induced by 4NC-Beeswax-CO Nps evidenced in the present research may be contributing to avoid possible damages caused by the microbicidal machinery of the host macrophages, since the balance between a pro- and anti-inflammatory response is important to kill the parasite and also for the resolution of the disease (TOMIOTTO-

PELLISSIER et al., 2018b). Interestingly, only 4NC-Beeswax-CO Nps was able to modulate the production of cytokines and NO, so further research is needed to target the participation / interaction of free or nanoparticulate 4NC in the triggering of intracellular signaling pathways.

In conclusion, this study demonstrates for the first time that 4-Nitrochalcone causes morphological and biochemical changes in *L. amazonensis* promastigotes, leading to death by apoptosis-like mechanism. The use of 4NC-Beeswax-CO Nps was able to activate the machinery of macrophages to eliminate intracellular amastigotes through the production of inflammatory and microbicidal mediators, without toxic effects for the host. Interestingly, the production of IL-10 can also help to control inflammation and participate in the disease resolution phase. This research provides important information for the development of new *in vivo* studies for the treatment of ACL using this 4NC-Beeswax-CO Nps

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Conflict of interest

The authors declared that there is no conflict of interest.

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Figures

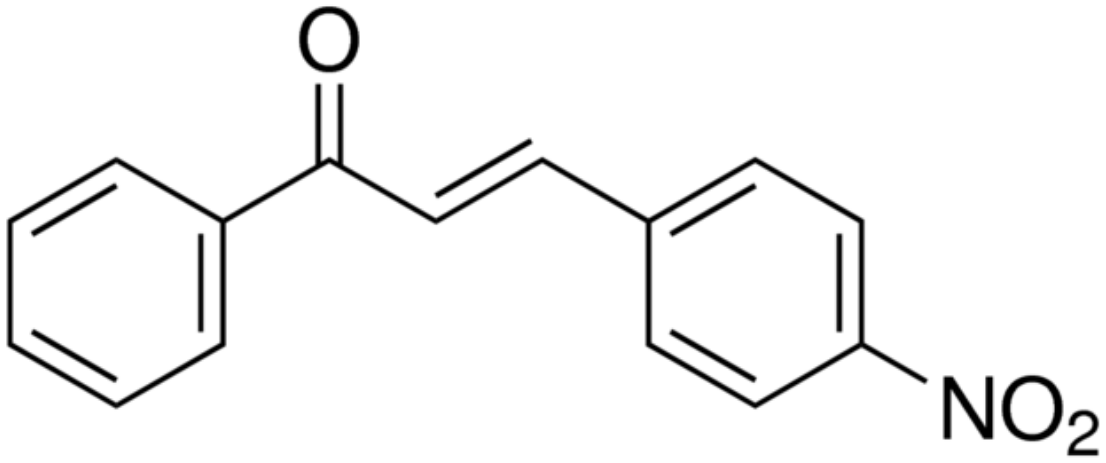


Figure 1. Chemical structure of 4-Nitrochalcone

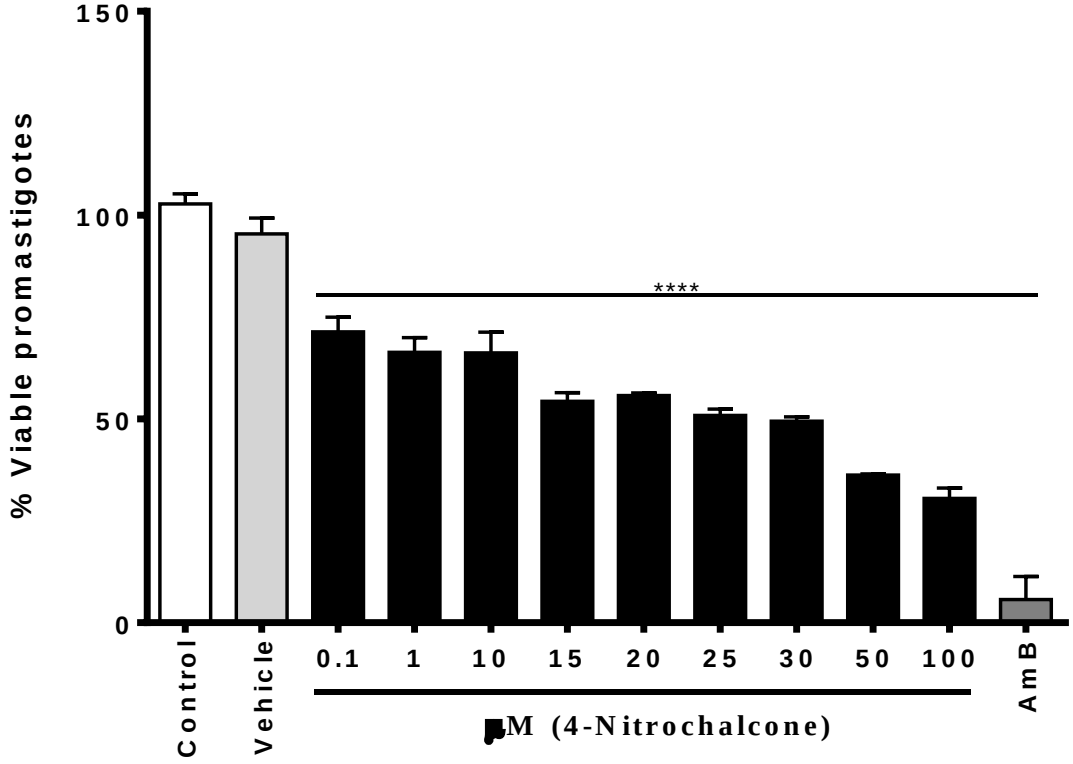


Figure 2. 4NC exerts leishmanicidal activity on *L. amazonensis* promastigotes. Promastigotes ($1x10^6$) were treated with Free 4NC (0.1, 1, 10, 15, 20, 25, 30, 50 e 100 µM). After 24 h the parasites were counted in Neubauer's chamber. Untreated

parasites were used as control. DMSO was used as vehicle. Amphotericin B (AmB) was used as positive control. The values represent mean ± SEM of three independent experiments performed in duplicate. * significant difference compared to control ($p \leq 0.05$, **** $p < 0.0001$).

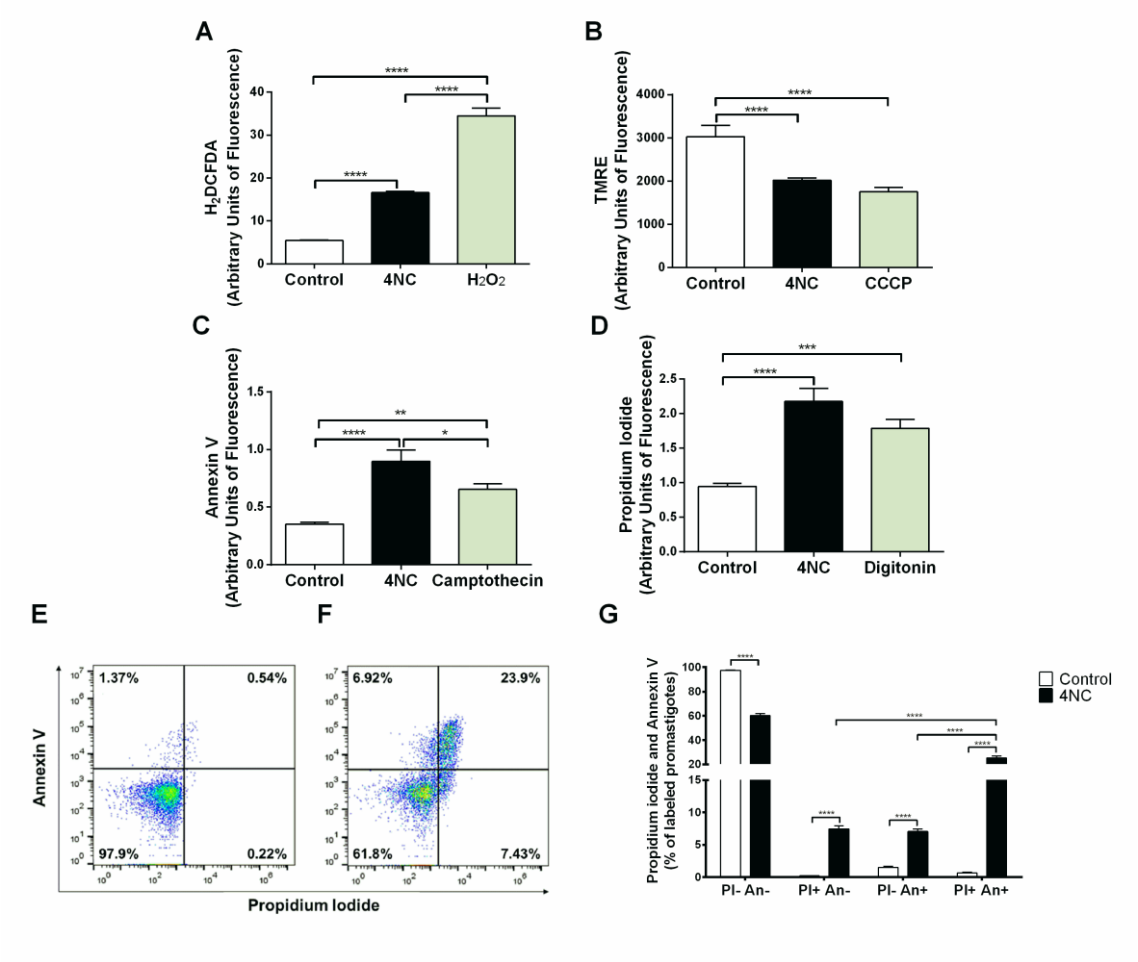


Figure 3. Mechanisms of death triggered by free 4-Nitrochalcone in *L. amazonensis* promastigotes. Promastigotes ($1x10^6$) were treated with free 4-Nitrochalcone (4NC) (IC_{50}) and evaluated total ROS production through the H_2DCFDA probe (A), mitochondrial membrane depolarization by TMRE labeling (B), phosphatidylserine exposure by Annexin V labeling (C), membrane integrity by PI labeling (D). Annexin V and PI co-labeling of promastigotes treated with free 4NC and analyzed by flow cytometry (E-F). Typical dot plots of at least three independent experiments are shown (E-F). Untreated promastigotes were used as control Hydrogen peroxide (H_2O_2), Carbonyl cyanide m-chlorophenylhydrazone (CCCP), Camptothecin and Diginitonin were used as positive controls for ROS production, mitochondrial membrane depolarization, phosphatidylserine exposure and plasma membrane permeability, respectively. The values represent mean ± SEM of three independent experiments performed in duplicate. * significant difference compared to control and treatments ($p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p < 0.0001$).

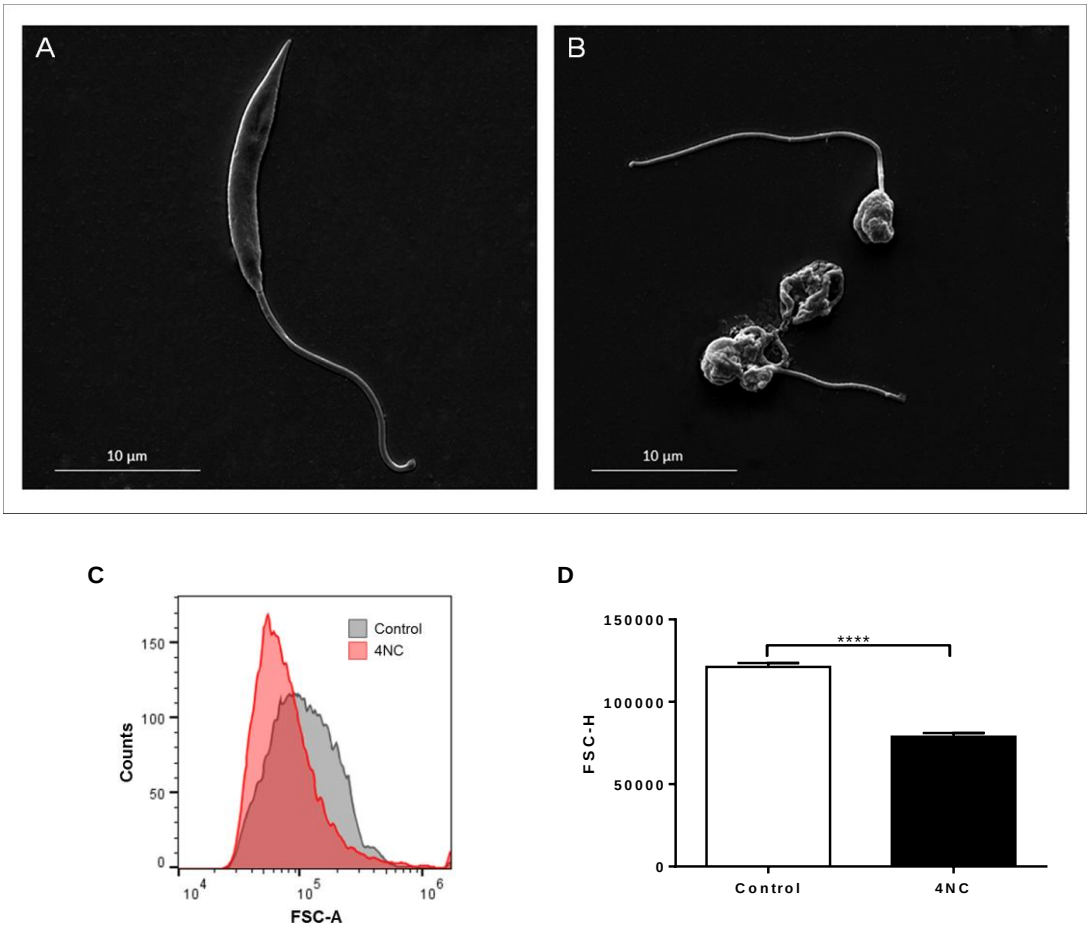


Figure 4. Morphological changes and cell volume of *L. amazonensis* promastigotes treated with free 4-Nitrochalcone by scanning electron microscopy and flow cytometry. Scanning electron microcopy images of promastigotes, Control (A); free 4-Nitrochalcone (4NC) (IC_{50}) treatment (B). Flow cytometry for analysis of cell volume of promastigotes treated with 4NC (IC50) (C-D). FSC-H was considered a function of cell size. The gray area corresponds to the control (untreated parasites) and the red area is the parasites treated with 4NC. Typical histograms are shown (C). The values represent mean ± SEM of three independent experiments performed in duplicate. ***significant difference compared to control ($p \leq 0.001$).

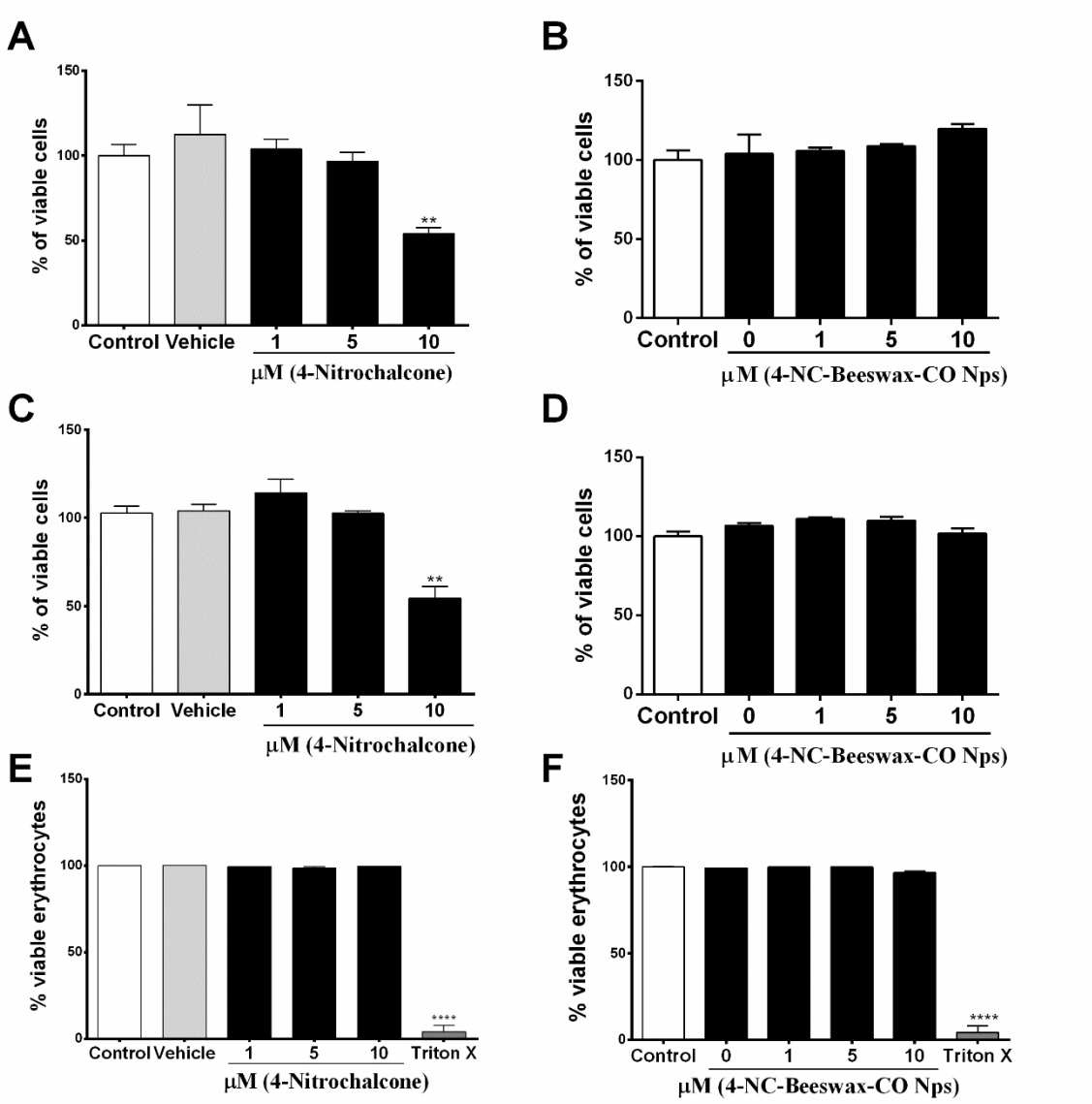


Figure 5. Viability of macrophages and erythrocytes treated with free 4-Nitrochalcone and 4-Nitrochalcone-Beeswax-CO Nanoparticles. Peritoneal macrophages were treated with free 4NC (A and C) or 4NC-Beeswax-CI Nps (B and D) and viability was accessed by MTT assay (A and B) and Neutral Red assay (C and D). Untreated macrophages were used as controls. DMSO was used as vehicle for free compound. Erythrocytes from sheep were treated with free 4NC (E) or 4NC-Beeswax-CO Nps (F) and percentage of viable erythrocytes were determined. PBS was used as non-hemolytic control and Triton X was used as positive control for hemolysis. The values represent mean ± SEM of three independent experiments performed in duplicate. **significant difference compared to control ($p \leq 0.01$, ****$p < 0.0001$).

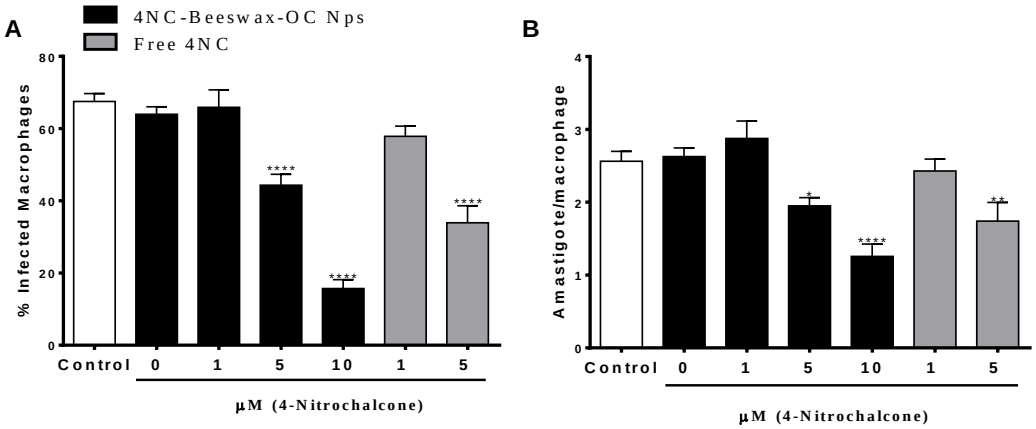


Figure 5. 4-Nitrochalcone-Beeswax-CO Nanoparticles and Free 4-Nitrochalcone and exerts anti-amastigote effect. Peritoneal macrophages were infected with *L. amazonensis* and treated with Free 4NC or 4NC-Beeswax-CO Nps; and then the percentage of infected macrophages (A) and number of amastigotes per macrophages (B) were determined. Untreated and infected macrophages were used as control. The values represent mean ± SEM of three independent experiments performed in duplicate. *significant difference compared to control ($p \leq 0.05$, ****$p < 0.0001$).

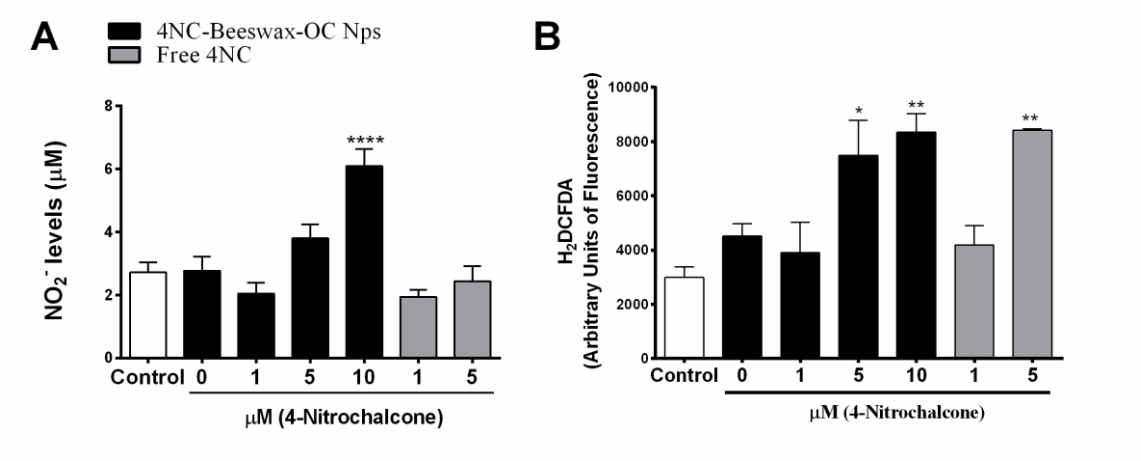


Figure 7. Production of nitric oxide (NO) and reactive oxygen species (ROS) by infected macrophages treated with free 4NC and 4NC-Beeswax-CO Nps. Peritoneal macrophages were infected with *L. amazonensis* (1 macrophage: 5 promastigotes) and treated with 4NC-Beeswax-CO Nps and free 4NC; the supernatant of the cultures were used to measure the production of NO by the Griess method (A) and cells were used to measure total ROS through H_2DCFDA probe (B). Untreated and infected macrophages were used as control. The values represent mean ± SEM of three independent experiments performed in duplicate. *significant difference compared to control ($p \leq 0.05$, **$p \leq 0.01$, ****$p < 0.0001$).

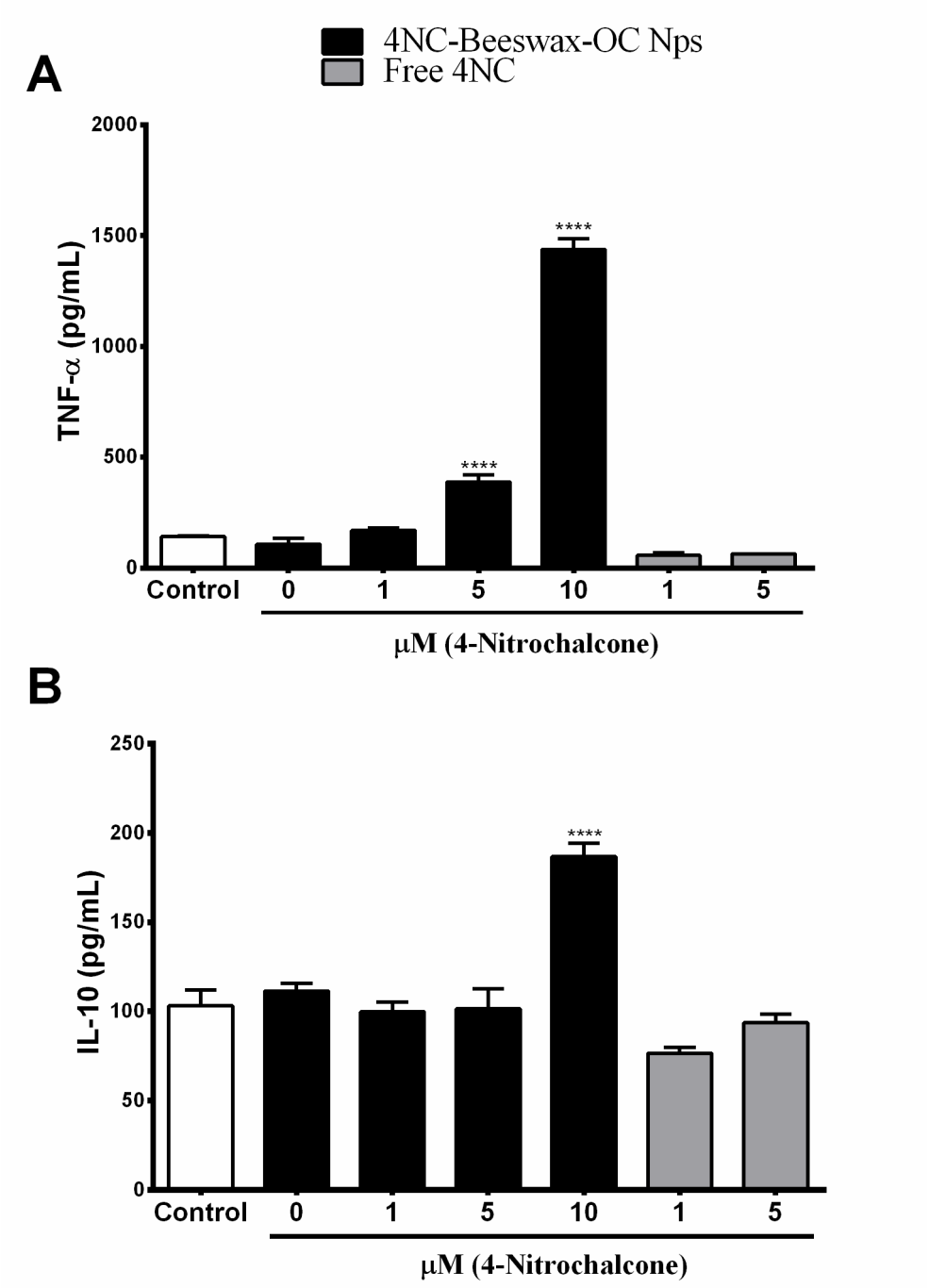


Figure 7. Cytokine produced by infected macrophages and treated with 4NC and 4NC-Beeswax-CO Nps. Peritoneal macrophages were infected with *L. amazonensis* (1 macrophage: 5 promastigotes) and treated with free 4NC and 4NC-Beeswax-CO Nps; the supernatant of the cultures were used to measure the production of TNF-α (A) and IL-10 by ELISA. Untreated and infected macrophages were used as control. The values represent mean ± SEM of three independent experiments performed in duplicate. ****significant difference compared to control ($p < 0.0001$).

5. CONCLUSÃO GERAL

Os compostos DETC e 4-Nitrochalcona livres apresentam efeito leishmanicida sobre formas promastigotas de *L. amazonensis*, devido à perda do potencial de membrana mitocondrial, ao aumento da geração de EROs, aumento da exposição de fosfatidilserina e também a perda de integridade da membrana plasmática de promastigotas, bem como alterações na morfologia do parasito, sugerindo uma morte por apoptose-*like*.

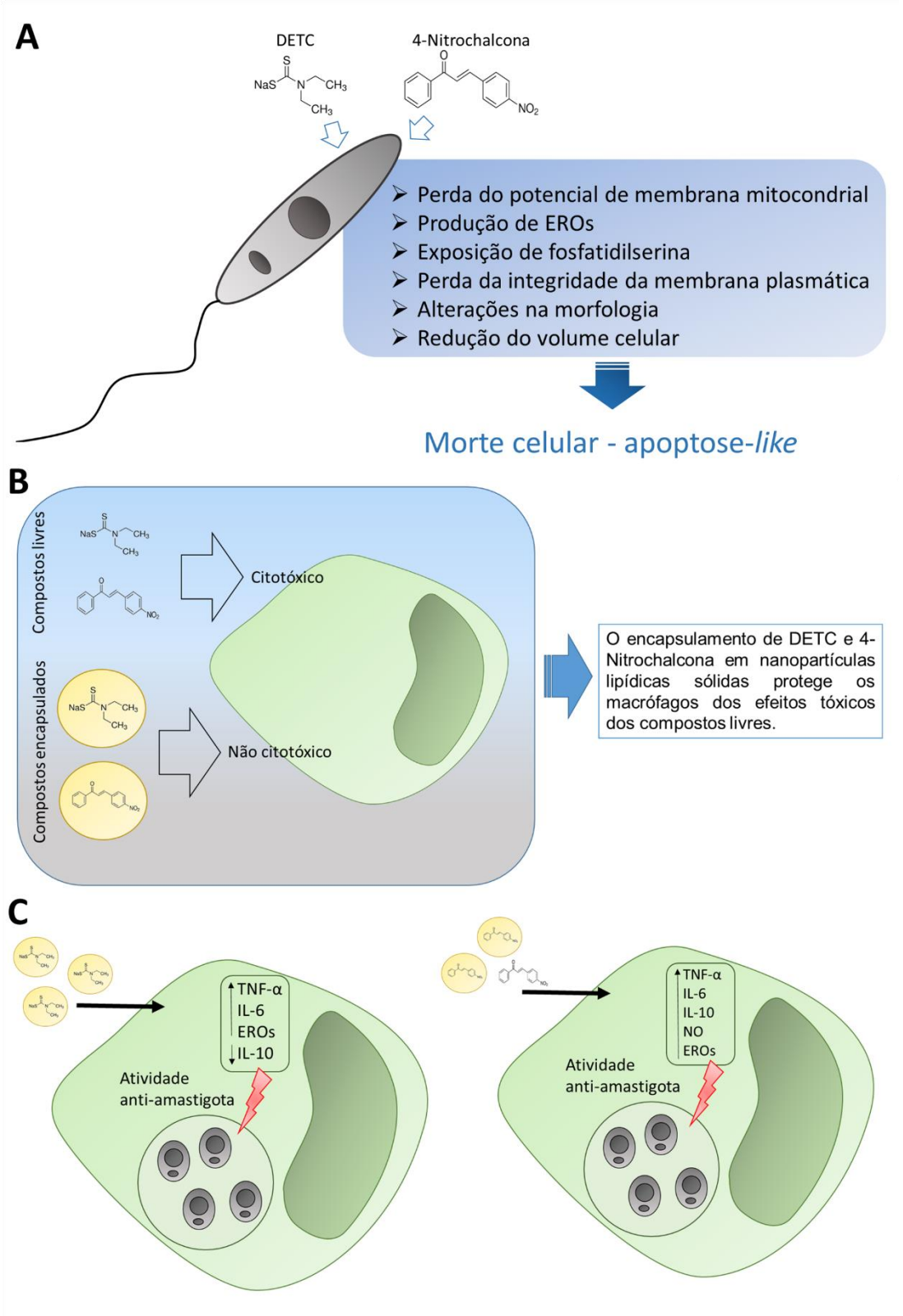
Os compostos livres apresentam toxicidade sobre macrófagos murinos. Por sua vez, o encapsulamento de DETC e 4-Nitrochalcona em nanopartículas lipídicas sólidas protegeu os macrófagos dos efeitos tóxicos dos compostos livres.

4-Nitrochalcona livre na concentração não tóxica, exerce efeito anti-amastigota, mas DETC livre não. Os compostos encapsulados apresentaram efeito leishmanicida sobre amastigotas intracelulares. Além de que o uso de nanopartículas possibilitou o uso de concentrações mais efetivas dos compostos sem afetar a viabilidade das células do hospedeiro.

O efeito anti-amastigota dos compostos nanoparticulados se deve ao seu efeito imunomodulador sobre macrófagos infectados, modulando a produção de citocinas, EROs e óxido nítrico (NO). DETC encapsulado induziu a produção de TNF-α, IL-6 e reduziu IL-10, além disso, aumentou a produção de EROs e ânion superóxido, mas não de NO. Já 4-nitrochalcona encapsulada em nanopartículas lipídicas sólidas, aumentou a produção de TNF-α, IL-6 e IL-10, além de moléculas microbicidas (EROs e NO).

Assim, este trabalho fez possível o uso de DETC e 4-Nitrochalcona encapsulados para novos estudos *in vivo* que visam o desenvolvimento de alternativas terapêuticas para a leishmaniose cutânea, de forma mais efetiva e menos tóxica ao paciente.

Figura 6 – Esquema demonstrando o efeito antipromastigota de DETC e 4-Nitrochalcona livres (A); atividade citotóxica (B) e anti-amastigota (C) dos compostos livres e encapsulados em nanopartículas lipídicas sólidas



Fonte: Próprio autor

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