

Yvanna Carla de Souza Salgado
(Organizadora)

Patologia: Doenças Parasitárias



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Yvanna Carla de Souza Salgado
(Organizadora)

Patologias: Doenças Parasitárias

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APRESENTAÇÃO

No volume II da coleção Patologia intitulado: Doenças Parasitárias, apresentamos em capítulos, diversos artigos de pesquisas realizadas em diferentes regiões. A temática inclui estudos sobre doenças tropicais, protozooses e parasitoses; dados epidemiológicos, diagnósticos e tratamentos, bem como temáticas correlacionadas e alguns acidentes por animais peçonhentos.

As doenças parasitárias decorrem da presença de macroparasitas (p. ex. helmintos) e/ou microparasitas (p. ex. protozoários), e envolvem em seu ciclo, hospedeiros, isto é, organismos vivos em que os parasitas se desenvolvem. De modo geral, podem ser transmitidas de diferentes formas como: água ou alimentos contaminados, picadas ou fezes de insetos ou outros animais, sexualmente, através de transfusão sanguínea e transplante de órgãos, de mãe para filho durante a gestação; sendo que cada parasitose tem suas características de contaminação. Suas manifestações clínicas são variáveis dependendo do agente etiológico e o local onde se instala, e podem variar de leves e moderadas até graves.

Apesar dos avanços relacionados às medidas preventivas, controle e tratamento, e da diminuição significativa dos níveis de mortalidade; as doenças parasitárias ainda constituem um problema sério de Saúde Pública no Brasil. A incidência das parasitoses tem relação direta com as condições socioeconômicas, com hábitos alimentares e de higiene, crescimento populacional, com saneamento básico, aspectos climáticos, educação, entre outros. No intuito de aprofundar o conhecimento acerca das parasitoses, este volume traz informações de estudos regionais sobre as doenças parasitárias mais conhecidas.

A obra é fruto do esforço e dedicação das pesquisas dos autores e colaboradores de cada capítulo e da Atena Editora em elaborar este projeto de disseminação de conhecimento e da pesquisa brasileira. Espero que este livro possa somar conhecimentos e permitir uma visão crítica e contextualizada; além de inspirar os leitores a contribuírem com pesquisas para a promoção de saúde e bem estar social.

Yvanna Carla de Souza Salgado

SUMÁRIO

CAPÍTULO 1 1

ESTUDO EPIDEMIOLÓGICO DA OCORRÊNCIA E VIAS DE TRANSMISSÃO DA DOENÇA DE CHAGAS NA REGIÃO NORTE E NORDESTE DO BRASIL NO PERÍODO DE 2009 A 2016

Kamilla Peixoto Bandeira
João Ancelmo dos Reis Neto
João Vitor de Omena Souza Costa
Priscilla Peixoto Bandeira
Renata Valadão Bittar
Monique Carla da Silva Reis
José Edvilson Castro Brasil Junior

DOI 10.22533/at.ed.9781918031

CAPÍTULO 2 8

TAXA DE MORTALIDADE PELA DOENÇA DE CHAGAS NA REGIÃO NORDESTE DO BRASIL E NA BAHIA DE 2010 À 2015

Edna Moura de Santana Brito
Mithaly de Jesus Teixeira
Paulo José dos Santos Matos
Marla de Jesus Teixeira
Jorge Sadao Nihei
George Mariane Soares Santana

DOI 10.22533/at.ed.9781918032

CAPÍTULO 3 16

DOENÇA DE CHAGAS NA AMAZÔNIA: UM ESTUDO EPIDEMIOLÓGICO NA CIDADE DA MAIOR USINA HIDRELÉTRICA GENUINAMENTE BRASILEIRA

Ana Caroline de Oliveira Coutinho
Aira Beatriz Gomes Pompeu
Erielson Pinto Machado
Rafael Vulcão Nery
Raimundo Batista Viana Cardoso
Silvio Henrique dos Reis Júnior

DOI 10.22533/at.ed.9781918033

CAPÍTULO 4 25

AUMENTO DA DISTRIBUIÇÃO GEOGRÁFICA DE *Rhodnius stali* E *Rhodnius montenegrensis*: PRIMEIRO RELATO NA REGIÃO DO VALE DO JURUÁ, ACRE, BRASIL

Adila Costa de Jesus
Fernanda Portela Madeira
Madson Huilber da Silva Moraes
Adson Araújo de Moraes
Gilberto Gilmar Moresco
Jader de Oliveira
João Aristeu da Rosa
Luis Marcelo Aranha Camargo
Dionatas Ulises de Oliveira Meneguetti
Paulo Sérgio Bernarde

DOI 10.22533/at.ed.9781918034

CAPÍTULO 5 35

ESPÉCIES DE TRIATOMÍNEOS OCORRENTES NOS ESTADOS DO ACRE E RONDÔNIA, AMAZÔNIA OCIDENTAL, BRASIL

Gabriela Vieira de Souza Castro
Mariane Albuquerque Lima Ribeiro
Leandro José Ramos
Janis Lunier Souza
Simone Delgado Tojal
Jader de Oliveira
João Aristeu da Rosa
Luis Marcelo Aranha Camargo
Dionatas Ulises de Oliveira Meneguetti

DOI 10.22533/at.ed.9781918035

CAPÍTULO 6 48

UMA ABORDAGEM INTEGRAL AO PORTADOR DE DOENÇA DE CHAGAS: RELATO DE EXPERIÊNCIA

Jadianne Ferreira Da Silva
Aguyda Naiara De Lima Pereira Bento
Allana Regina De Lima Silva
Cassandra Barros Correia De Moura
Ericka Azevedo Dos Santos
Ericka Vanessa De Lima Silva
Manuela De Souza Calado

DOI 10.22533/at.ed.9781918036

CAPÍTULO 7 55

ANTITRYPANOSOMAL ETHNOPHARMACOLOGY IN THE BRAZILIAN AMAZON

Dionatas Ulises de Oliveira Meneguetti
Adila Costa de Jesus
Fernanda Portela Madeira
Romeu Paulo Martins Silva

DOI 10.22533/at.ed.9781918037

CAPÍTULO 8 73

ASPECTOS EPIDEMIOLÓGICOS DA LEISHMANIOSE VISCERAL NO NORDESTE BRASILEIRO (2007-2017)

Ana Maria Fernandes Menezes
Kaic Trindade Almeida
Maryana de Moraes Frota Alves
Kelle Araújo Nascimento Alves
Ana Karla Araujo Nascimento Costa

DOI 10.22533/at.ed.9781918038

CAPÍTULO 9 85

ASPECTOS EPIDEMIOLÓGICOS E SOCIODEMOGRÁFICAS DA LEISHMANIOSE VISCERAL HUMANA NO MUNICÍPIO DE OURICURI, PERNAMBUCO, BRASIL, NO PERÍODO DE 2013 A 2017

Sarah Mourão de Sá
Ana Maria Parente de Brito
Marília Rabelo Pires
José Alexandre Menezes da Silva

DOI 10.22533/at.ed.9781918039

CAPÍTULO 10 91

DISTRIBUIÇÃO EPIDEMIOLÓGICA DA LEISHMANIOSE VISCERAL (CALAZAR), NO PERÍODO DE 2013 A 2018, NO MUNICÍPIO DE TUCURUÍ - PA

Juliane da Silva Barreiros
Isabelle Guerreiro de Oliveira
Letícia Sousa do Nascimento
Thays Queiroz Santos
Daniele Lima dos Anjos Reis
Kátia Simone Kietzer
Anderson Bentes de Lima

DOI 10.22533/at.ed.97819180310

CAPÍTULO 11 98

URBANIZAÇÃO DA LEISHMANIOSE TEGUMENTAR AMERICANA NO MUNICÍPIO DE TUCURUÍ, PARÁ, BRASIL

Ingridy Lobato Carvalho
Juliane Moreira de Almeida
Gabriel Costa Vieira
Hiandra Raila Silva da Costa
Tatiana Menezes Noronha Panzetti

DOI 10.22533/at.ed.97819180311

CAPÍTULO 12 109

LEISHMANIOSE VISCERAL CANINA NO MUNICÍPIO DO IPOJUCA - PE/BRASIL

Hallysson Douglas Andrade de Araújo
Jussara Patrícia Monteiro Vasconcelos
Eduardo José da Silva
Josinaldo Leandro dos Santos
Jackson José dos Santos
Roseane Cabral de Oliveira
Odilson Bartolomeu dos Santos
Andrea Lopes de Oliveira
Juliana Carla Serafim da Silva

DOI 10.22533/at.ed.97819180312

CAPÍTULO 13 111

ESTUDO COMPARATIVO DA RESPOSTA TERAPÊUTICA À ANFOTERICINA B LIPOSSOMAL NA LEISHMANIOSE VISCERAL EM ADULTOS COM E SEM HIV

Marcello Bertoldi Sanchez Neves
Bruna Thais Raiter
Keli Balduino de Ramos
Luiz Felipe Espindula Beltrame
Igor Valadares Siqueira
Matheus Marques Rodrigues de Souza
Mauricio Antônio Pompílio
Anamaria Mello Miranda Paniago
Angelita Fernandes Druzian

DOI 10.22533/at.ed.97819180313

CAPÍTULO 14 120

LEISHMANIOSE VISCERAL NA MACRORREGIÃO DO VALE DO SÃO FRANCISCO E ARARIPE, PERNAMBUCO – 2001-2015

Cesar Augusto da Silva
Tathyane Trajano Barreto

Artur Alves da Silva

Luiz Carlos Lima da Silva Junior

DOI 10.22533/at.ed.97819180314

CAPÍTULO 15 128

ANÁLISE DE BIÓPSIAS CUTÂNEAS E PERFIL DE PACIENTES ATENDIDOS NO SERVIÇO DE DERMATOLOGIA DE UM HOSPITAL PÚBLICO COM SUSPEITA CLÍNICA DE LEISHMANIOSE TEGUMENTAR

Caroline Louise Diniz Pereira

Cynthia Pedrosa Soares

Fábio Lopes de Melo

Milena Lima Rodrigues

Silvania Tavares Paz

Selma Giorgio

Francisca Janaína Soares Rocha

DOI 10.22533/at.ed.97819180315

CAPÍTULO 16 134

ANÁLISE DOS RESULTADOS DAS AÇÕES INTEGRADAS DE VIGILÂNCIA E ASSISTÊNCIA NA MELHORIA DA OPORTUNIDADE DO DIAGNÓSTICO E TRATAMENTO DOS CASOS DE LVH NA REGIÃO DO SERTÃO DO ARARIPE, PERNAMBUCO, BRASIL DE 2014 A 2017

Sarah Mourão de Sá

Ana Maria Parente de Brito

Marília Rabelo Pires

José Alexandre Menezes da Silva

Regina Coeli Ferreira Ramos

DOI 10.22533/at.ed.97819180316

CAPÍTULO 17 141

NANOEMULSIONS CONTAINING CHALCONE: DEVELOPMENT, OPTIMIZATION AND ANALYSIS OF *IN VITRO* CYTOTOXICITY AGAINST AMASTIGOTA FORM OF *Leishmania amazonensis*

Daniela Sousa Coelho

Letícia Mazzarino

Beatriz Veleirinho

Ana Paula Voytena

Thaís Alberti

Elizandra Bruschi Buzanello

Milene Hoehr de Moraes

Mário Steindel

Rosendo Yunnes

Marcelo Maraschin

DOI 10.22533/at.ed.97819180317

CAPÍTULO 18 155

MALÁRIA GRAVE IMPORTADA E SEPSE POLIMICROBIANA ASSOCIADA A CATETER VASCULAR: RELATO DE CASO NO RIO DE JANEIRO

Isabelle Christine de Moraes Motta

Dirce Bonfim de Lima

Paulo Vieira Damasco

DOI 10.22533/at.ed.97819180318

CAPÍTULO 19	160
A IMPORTÂNCIA EM PROMOVER MEDIDAS PROFILÁTICAS CONTRA MALÁRIA EM PAÍSES SUBDESENVOLVIDOS	
<i>Bruno Vinícios Medeiros Mendes</i>	
DOI 10.22533/at.ed.97819180319	
CAPÍTULO 20	167
PROMOÇÃO DA SAÚDE ACERCA DA MALÁRIA JUNTO AOS AGENTES COMUNITÁRIOS DA UNIDADE BÁSICA DE ILHAS DA REGIÃO AMAZÔNICA	
<i>Márcia Ribeiro Santos Gratek</i>	
<i>Eloise Lorrany Teixeira Benchimol</i>	
<i>Leandro Araújo Costa</i>	
<i>Ana Salma Laranjeira Lopes Pires</i>	
<i>Lindolfo Cardoso Nunes</i>	
DOI 10.22533/at.ed.97819180320	
CAPÍTULO 21	171
JOGOS EDUCATIVOS COMO UMA ESTRATÉGIA PARA O CONTROLE DA MALÁRIA EM UMA ÁREA DE ALTA ENDEMICIDADE NO MÉDIO RIO NEGRO, AMAZONAS, BRASIL	
<i>Jessica de Oliveira Sousa</i>	
<i>José Rodrigues Coura</i>	
<i>Martha Cecília Suárez-Mutis</i>	
DOI 10.22533/at.ed.97819180321	
CAPÍTULO 22	186
TOXOPLASMOSE CEREBRAL EM PACIENTE HIV NEGATIVO RELATO DE CASO DIAGNOSTICADO EM AUTÓPSIA	
<i>Paula Regina Luna de Araújo Jácome</i>	
<i>Kátia Moura Galvão</i>	
<i>Mariana de Albuquerque Borges</i>	
<i>Agenor Tavares Jácome Júnior</i>	
<i>Roberto José Vieira de Mello</i>	
DOI 10.22533/at.ed.97819180322	
CAPÍTULO 23	192
EFEITO OVICIDA E LARVICIDA DO ÉTER METIL DILAPIOL (EMD) EM <i>Aedes aegypti</i> , MANAUS-AM	
<i>Junielson Soares da Silva</i>	
<i>Ana Cristina da Silva Pinto</i>	
<i>Luiz Henrique Fonseca dos Santos</i>	
<i>Míriam Silva Rafael</i>	
DOI 10.22533/at.ed.97819180323	
CAPÍTULO 24	205
ASPECTOS EPIDEMIOLÓGICOS, CLÍNICOS E TERAPÊUTICOS DAS ENTEROPROTOZOSES NO BRASIL: UMA REVISÃO SISTEMÁTICA	
<i>Raimundo Diego Ferreira Amorim</i>	
<i>Ionara Bastos de Moraes</i>	
<i>José Denilson Ferreira Amorim</i>	
<i>Iago Sávyo Duarte Santiago</i>	
<i>Pedro Walisson Gomes Feitosa</i>	
<i>Diogenes Pereira Lopes</i>	
<i>Maria do Socorro Vieira Gadelha</i>	
DOI 10.22533/at.ed.97819180324	

CAPÍTULO 25 223

FATORES SOCIOAMBIENTAIS E CLÍNICOS DA ESQUISTOSSOMOSE MANSONI NA ZONA DA MATA DE PERNAMBUCO

Claudinelly Yara Braz dos Santos
Paula Carolina Valença da Silva
Aline Vieira da Silva
Letícia Moura Vasconcelos
Ilana Brito Ferraz de Souza
Taynan da Silva Constantino
Antônio José de Vasconcelos Neto
Florisbela de Arruda Camara E Siqueira Campos

DOI 10.22533/at.ed.97819180325

CAPÍTULO 26 235

ESQUISTOSSOMOSE EM PERNAMBUCO: ANÁLISE PRÉ E PÓS IMPLANTAÇÃO DO PROGRAMA SANAR PARA ENFRENTAMENTO DE DOENÇAS NEGLIGENCIADAS

Monique Oliveira do Nascimento
Rebeka Maria de Oliveira Belo
Alyson Samuel de Araujo Braga
Cindy Targino de Almeida
Tamyres Millena Ferreira
Hirla Vanessa Soares de Araújo
Karyne Kirley Negromonte Gonçalves
Simone Maria Muniz da Silva Bezerra

DOI 10.22533/at.ed.97819180326

CAPÍTULO 27 245

QUAL IMPACTO DA COBERTURA DA ESTRATÉGIA SAÚDE DA FAMÍLIA NAS INTERNAÇÕES E ÓBITOS POR DOENÇAS INFECCIOSAS E PARASITÁRIAS?

Valdecir Barbosa da Silva Júnior
Maria Tatiane Alves da Silva
Danilson Ferreira da Cruz
Amanda Priscila de Santana Cabral Silva

DOI 10.22533/at.ed.97819180327

CAPÍTULO 28 256

ESQUISTOSSOMOSE: UMA DOENÇA NEGLIGENCIADA NO ESTADO DE ALAGOAS

Nathalia Lima da Silva
Luana Carla Gonçalves Brandão Santos
Gisélia Santos de Souza
Larissa Suzana de Medeiros Silva
Carolayne Rodrigues Gama
Bárbara Melo Vasconcelos
Lorena Sophia Cadete de Almeida Lemos Vilela
Karol Bianca Alves Nunes Ferreira
Raíssa Fernanda Evangelista Pires dos Santos
Thycia Maria Cerqueira de Farias
Alessandra Nascimento Pontes
Hulda Alves de Araújo Tenório
Mariana Gomes de Oliveira
Tânia Katia de Araújo Mendes
Keila Cristina Pereira do Nascimento Oliveira
Maria Luiza de Azevedo Garcia
Beatriz Santana de Souza Lima
Luciana da Silva Viana

Marilucia Mota de Moraes

DOI 10.22533/at.ed.97819180328

CAPÍTULO 29 261

UM TEMPO ONDE A CIÊNCIA FAZ HISTÓRIA E AS DOENÇAS PARASITÁRIAS AINDA SÃO MARCADORES DAS MAZELAS SOCIAIS

Randyston Brenno Feitosa

Maria Alexandra De Carvalho Meireles

Rovilson Lara

DOI 10.22533/at.ed.97819180329

CAPÍTULO 30 263

DOENÇAS TROPICAIS NEGLIGENCIADAS: ESTADO DA ARTE DAS PUBLICAÇÕES CIENTÍFICAS

Leonardo Pereira Tavares

Hellen Lima Alencar

Pedro Paulo Barbosa Oliveira

Maria do Socorro Vieira Gadelha

DOI 10.22533/at.ed.97819180330

CAPÍTULO 31 266

ANÁLISE DA EPIDEMIOLOGIA DE ACIDENTES ESCORPIÔNICOS NO NORDESTE

Hellen Lima Alencar

Leonardo Pereira Tavares

Pedro Paulo Barbosa Oliveira

Maria do Socorro Vieira Gadelha

DOI 10.22533/at.ed.97819180331

CAPÍTULO 32 270

ASPECTOS CLÍNICO-EPIDEMIOLÓGICOS DOS ACIDENTES ESCORPIÔNICOS REGISTRADOS EM UM MUNICÍPIO DO INTERIOR DA AMAZÔNIA: UM CORTE DE UMA DÉCADA

Edson Jandrey Cota Queiroz

Alexandre Vasconcelos Dezincourt

Ana Paula Costa Diniz

Everaldo de Souza Otoni Neto

Emanuel Roberto Figueiredo da Silva

Tyala Oliveira Feitosa Gomes

Caroline Gomes Macêdo

DOI 10.22533/at.ed.97819180332

CAPÍTULO 33 283

INJÚRIA CAUSADA POR ARRAIA DE ÁGUA DOCE (*Potamotrygon* SP.) NO MUNICÍPIO DE AFUÁ, ILHA-DE-MARAJÓ, PARÁ, BRASIL (2017)

Elder Oliveira da Silva

Ednaldo Bezerra Galvão Filho

Pedro Pereira de Oliveira Parda

Suelen dos Santos Ferreira

Pasionaria Rosa Ramos Ruiz Diaz

DOI 10.22533/at.ed.97819180333

CAPÍTULO 34 296

DOENÇAS DE VEICULAÇÃO HÍDRICA: ESTRATÉGIAS DE EDUCAÇÃO EM SAÚDE

Nathalia Lima da Silva

Luana Carla Gonçalves Brandão Santos
Gisélia Santos de Souza
Larissa Suzana de Medeiros Silva
Carolayne Rodrigues Gama
Bárbara Melo Vasconcelos
Lorena Sophia Cadete de Almeida Lemos Vilela
Karol Bianca Alves Nunes Ferreira
Raíssa Fernanda Evangelista Pires dos Santos
Thycia Maria Gama Cerqueira
Alessandra Nascimento Pontes
Hulda Alves de Araújo Tenório
Mariana Gomes de Oliveira
Tânia Katia de Araújo Mendes
Keila Cristina Pereira do Nascimento Oliveira
Maria Luiza de Azevedo Garcia
Beatriz Santana de Souza Lima
Luciana da Silva Viana
Marilucia Mota de Moraes
Uirassú Tupinambá Silva de Lima

DOI 10.22533/at.ed.97819180334

CAPÍTULO 35 301

ASPECTOS EPIDEMIOLÓGICOS, CLÍNICOS E TERAPÊUTICOS DAS HELMINTÍASES NO BRASIL:
UMA REVISÃO SISTEMÁTICA

Ionara Bastos De Moraes
Raimundo Diego Ferreira Amorim
José Denilson Ferreira Amorim
Iago Sávyo Duarte Santiago
Pedro Walisson Gomes Feitosa
Diogenes Pereira Lopes
Marcos Antônio Pereira De Lima
Maria Do Socorro Vieira Gadelha

DOI 10.22533/at.ed.97819180335

SOBRE A ORGANIZADORA..... 315

NANOEMULSIONS CONTAINING CHALCONE: DEVELOPMENT, OPTIMIZATION AND ANALYSIS OF *IN VITRO* CYTOTOXICITY AGAINST AMASTIGOTA FORM OF *Leishmania amazonensis*

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RESUMO: Leishmaniose é uma doença parasitária com diferentes formas clínicas, variando entre a visceral (maior letalidade) e a cutânea (maior prevalência). Assim, é de suma importância o desenvolvimento de tratamentos eficazes, não invasivos e com baixos efeitos colaterais. Chalconas são compostos apolares, com capacidade leishmanicida. Nanoemulsões são sistemas de liberação que contribuem à penetração de compostos ativos na pele e, portanto, a incorporação de chalconas em nanoemulsões parece ser promissora ao desenvolvimento de formulações ao tratamento tópico da leishmaniose cutânea. O objetivo deste trabalho é desenvolver e caracterizar nanoemulsões contendo *trans*-chalcona, visando aplicação cutânea para tratamento de leishmaniose. Foram desenvolvidas nanoemulsões por emulsificação espontânea, utilizando-se concentrações de surfactante

lecitina de soja e co-surfactante poloxamer nas proporções de 0,1, 0,2 e 0,4% (p/p), na presença ou ausência de glicerol 0,4% (v/v). Foram determinadas as variáveis de tamanho, índice de polidispersão, potencial zeta e morfologia dos nanoderivados e eficiência de encapsulação. Os ensaios leishmanicidas foram realizados em cepas de *Leishmania amazonensis*. A dispersão coloidal apresentou tamanho de partícula inferior à 200 nm, distribuição monodispersa com índice inferior a 0,3 e potencial zeta inferior a -30 mV, com teor de 0,96 mg.ml⁻¹ de chalcona e 99,87% de eficiência de encapsulação. A citotoxicidade *in vitro* contra *L. amazonensis* apresentou IC₅₀ de 24,42 (± 6,76) ug.ml⁻¹ para a forma livre, enquanto a nanoemulsão *trans*-chalcona apresentou 9,09 (± 1,24) ug.ml⁻¹. Assim, o composto foi incorporado com sucesso pelo método e a formulação apresentou-se estável. Além disso, a nanoemulsão *trans*-chalcona foi mais efetiva que o composto livre contra o parasita. Portanto, essas nanoemulsões podem ser consideradas uma estratégia promissora como agente leishmanicida.

PALAVRAS-CHAVE: Leishmaniose, nanoemulsão, *trans*-chalcona, citotoxicidade

ABSTRACT: Leishmaniasis is a parasitic disease with different clinical forms, ranging from visceral (more lethal) to cutaneous (more prevalent). Thus, the development of effective, non-invasive treatments with low side effects is of paramount importance. Chalcones are apolar compounds, with leishmanicidal capacity. Nanoemulsions are release systems that increase the penetration of compounds into the skin and, therefore, the incorporation of chalcones into nanoemulsions seems to be promising for the development of topical formulations to treat cutaneous leishmaniasis. The objective of this work is to develop and characterize *trans*-chalcone-containing nanoemulsions for the treatment of leishmaniasis via cutaneous application. Nanoemulsions were developed by spontaneous emulsification by varying concentrations of surfactant (soy lecithin 0.1, 0.2 and 0.4% w/w), co-surfactant (poloxamer 0.1, 0.2 and 0.4% w/w) in the presence or absence of 0.4% glycerol (v/v). The following nanoemulsion's parameters were evaluated: size, polydispersity index, zeta potential, morphology and encapsulation efficiency. Leishmanicidal assays were performed on *Leishmania amazonensis* strains. The colloidal dispersion showed a mean particle size of 200 nm, monodisperse distribution, with polydispersity index lesser than 0.3 and zeta potential lower than -30 mV, containing 0.96 mg.ml⁻¹ chalcone and 99.9% encapsulation efficiency. *In vitro* cytotoxicity assay against *L. amazonensis* presented IC₅₀ of 24.42 (± 6.76) ug.ml⁻¹ for the free form, while the nano-*trans*-chalcone showed 14.08 (± 1.23) ug.ml⁻¹. Therefore, the compound was successfully incorporated by the employed method, and the formulation was stable. In addition, the nano-*trans*-chalcone was more effective than the free compound against the parasite. Therefore, these nanoemulsions may be considered a promising strategy as a leishmanicidal agent.

KEYWORDS: Leishmaniasis, nanoemulsion, *trans*-chalcone, cytotoxicity

1 | INTRODUCTION

Leishmaniasis are anthroponoses caused by protozoan parasites

(Trypanosomatidae) belonging to the genus *Leishmania* (MARLOW, 2013). According to the World Health Organization (WHO), it is estimated that 350 million people are at risk, and two million new cases of different clinical forms per year are expected to occur (WHO, 2015). Leishmaniasis can be classified as cutaneous/tegumentary, cutaneous-mucous, and visceral, and these different clinical manifestations depend on the parasite species and of the patient's immunological conditions (PINERO et al., 2006; GUPTA et al., 2014). Clinical symptoms of cutaneous leishmaniasis (LC) are single or multiple erythematous papules located at the exposed region of the integument, that develop to ulcers with raised borders, regular contours and gross granulations, covered or not by sero-purulent exudate.

The currently used leishmanicidal therapies are uncomfortable for the patient, involving daily injections for prolonged periods of time, requiring hospitalization and, consequently, increasing treatment costs. These factors contribute to the abandonment of the treatment, which can increase the risk of resistance of the parasite (ALMEIDA and SANTOS, 2011; DE MELLO et al., 2015).

Although the existing drugs for the treatment of leishmaniasis cause a rapid regression of the clinical and hematological manifestations of the disease, the low dosages and the discontinuity of the treatments have led to failures in the therapy (ALMEIDA and SANTOS, 2011). The pentavalent antimonials, sodium stibogluconate and N-methylglucamine antimoniate are the drugs currently in use, while amphotericin and pentamidine are secondary chemotherapy agents. In addition, most of the drugs currently in use were developed several decades ago, show variable efficacy, have serious side effects, are expensive, can require long-term treatment, may have low activity in immunosuppressed patients, and present and/or induce resistance in parasites (BELLO et al., 2011; KEVRIC et al., 2015; SHOWLER and BOGGILD, 2015). Miltefosine, a chemotherapeutic agent, has been considered the only oral treatment with efficacy against the clinical manifestations of leishmaniasis. This treatment, however, has serious toxic effects such as nephrotoxicity, hepatotoxicity, and teratogenicity (GUPTA et al., 2014; KEVRIC et al., 2015). Paromomycin, a drug used in the treatment of enteric parasites and the topical treatment of leishmaniasis, showed a cure rate of lesions between 17 % and 86 %. The most common adverse effects associated with this drug were application site irritation and pruritus, *i.e.*, mild symptoms that did not lead to treatment discontinuation (ARANA et al., 2001; MATTOS et al., 2012). Due to these factors, it is of utmost importance to develop new treatments that are more effective, non-invasive, and with fewer side effects, to which a very promising approach is to use chalcones.

Chalcones are apolar chemical compounds belonging to the flavonoid family and are reported to have a broad spectrum of biological activity including leishmanicidal ability. Several natural and synthetic chalcones present a wide range of pharmacological and therapeutic profiles, such as anti-inflammatory activity (NOWAKOWSKA, 2007), tripanomicide (LUNARDI et al., 2003), antibacterial (BOECK et al., 2006), antiviral (WU

et al., 2003), and antileishmaniasis (BOECK et al., 2006). Among the chalcones with antileishmania action, the most studied is licochalcona A, and its action mechanism occurs through the inhibition of certain enzymes of the parasite's cellular metabolism and changes in the morphology of its mitochondria (CHEN et al., 1993; ZHAI et al., 1995; CHEN et al., 2001). However, licochalcone A also affected human cells, inhibiting the proliferation of lymphocytes and the production of cytokines, decreasing the patient's response to the parasite (BARFOD et al., 2002; BOECK et al., 2006).

Based on this, a series of synthetic derivatives of chalcones are being developed, aiming towards a greater leishmanicidal activity, but without altering the viability of the body's immune cells. Despite the potential for synthetic chalcone derivatives in the treatment of CL, previous studies have shown that the application of these compounds using simpler presentation forms, *e.g.*, ointments, creams, and gels, has not been effective in combating the disease (ARANA et al. 2001). The low power of penetration through the lipid layers of the skin seems to be the main reason for such. An increase in cellular permeability can be achieved through structural modifications of the compounds under study. The development of innovative materials that act as vehicles and enable the maximum action of these drugs is a determinant strategy to the consolidation of this class of compounds as treatment of CL. To employ these chalcones in the treatment of patients affected by CL, it is therefore necessary to use appropriate delivery systems of these compounds, in reduced dosages when compared to conventional treatments. Nanoemulsions are drug delivery systems that increase the penetration power of compounds through the skin and offer controlled drug release. Furthermore, it can be prepared through the incorporation of chalcones into their formulation, making it an excellent tool for the intended purposes.

In this context, the use of controlled drug release techniques with nanoemulsions may be an interesting alternative to develop a novel topical form CL treatment.

Besides allowing the addition of hydrophobic compounds to aqueous systems, nanoemulsion systems facilitate the intracellular passage of drugs due to their rather small size, providing penetration of the compounds between the layers of the dermis (region in which the parasite is internalized in the macrophages), and it is an essential prerequisite for the treatment of CL by improving the efficacy of these compounds (TEIXEIRA, 2002; MATTOS et al., 2012).

2 | MATERIAL AND METHODS

2.1 Materials

For the development of chalcone-containing nanoemulsions Poloxamer 188 (Kolliphor P188 micro, BASF, USA); soy lecithin (Lipoid S75-3N, Lipoid GmbH, USA), absolute ethyl alcohol (Vetec, Brazil), and medium chain triglycerides (Miglyol 812, Germany) were used. For the *in vitro* assays, cell culture medium and fetal bovine serum were purchased from LCG biotechnology (São Paulo, Brazil), penicillin/streptomycin

antibiotics from Life Technologies (Carlsbad, USA), and dimethyl sulfoxide (DMSO) from Merck (Darmstadt, Germany). *Trans*-chalcone (purity grade 97 %, CAS number 614-47-1, Figure 1), trifluoroacetic acid (TFA) (Tedia, Fairfield, Ohio, USA), methanol (Merck, Darmstadt, Germany), and all other reagents used were purchased from Sigma-Aldrich (St. Louis, MO, USA).

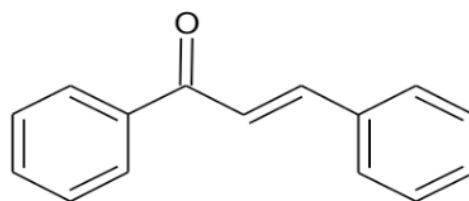


Figure 1: *Trans*-chalcone chemical structure: C₁₅H₁₂O and molecular weight 208.26 g/mol.

2.2 Nanoemulsions

2.2.1 Preparation of nanoemulsion

Eighteen blank formulations containing medium chain triglycerides (MCT), ethanol, and water, with different concentrations of surfactants (lecithin/poloxamer) and co-surfactant (glycerol) were prepared by the spontaneous emulsification technique (Table 1).

The organic phase was slowly added to the aqueous phase and subjected to moderate magnetic stirring. The nanoparticle suspensions were formed instantly and then evaporated under reduced pressure to remove the organic solvent and to achieve the desired concentration. Subsequently, they were filtered on 14 µm filter paper.

These formulations were developed in order to select the most optimized nanoemulsion to associate the *trans*-chalcone with increased anti-leishmania activity (BOUCHEMAL et al., 2004). Subsequently, 1 mg/ml of *trans*-chalcone was added in the formulation that presented the best characteristics.

Composition	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14
MTC	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Lecithin	0.2	0.2	0.2	0.4	0.4	0.4	0.1	0.1	0.1	0.2	0.2	0.2	0.4	0.4
Glycerol	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	-	-	-	-	-
Ethanol	50	50	50	50	50	50	50	50	50	50	50	50	50	50
Poloxamer	0.4	0.2	0.1	0.4	0.2	0.1	0.4	0.2	0.1	0.4	0.2	0.1	0.4	0.2
Water	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Table 1: Nanoemulsion composition (% , w/w).

2.2.2 Characterization of physicochemical properties of nanoemulsions

The particle size, polydispersity index (PDI), and zeta potential of the nanoemulsions were measured by dynamic light scattering (DLS) on a ZetaSizer nano

ZS equipment (Malvern Instruments, Malvern, UK), equipped with a fixed angle of 90°. They were measured at room temperature immediately after sample dilution (40x) with distilled water. To determine the zeta potential, the formulations were transferred to electrophoretic cells, in which an electrical potential of ± 150 mV was applied.

2.2.3 High performance liquid chromatography (HPLC) analysis

The HPLC analysis was performed as described by Mattos et al (2012). For that, a 10 μ l-aliquot of nanoemulsion after extraction of the *trans*-chalcone with methanol (1: 9, v/v) was injected into a Thermo Scientific Ultimate 3000RS UHPLC liquid chromatograph (Thermo Fisher Scientific, California, USA) equipped with a C₁₈ reverse phase column (FR-Thermo Scientific, 250 mm x 4.6mm, particle size 5 μ m, 30°C) and a diode array detector (DAD), operating at 280 nm, 300 nm, 320 nm, and 350 nm. . Analyte elution was performed in an isocratic system with methanol: water (70: 30, v/v), acidified with (TFA, (pH 5.0)) and a flow rate at 0.5 ml.min⁻¹.

2.2.4 Encapsulation efficiency (EE) and *trans*-chalcone content in nanoemulsions

The encapsulation efficiency and the active principle content were determined in samples via reverse phase high performance liquid chromatography (HPLC) coupled to a UV-visible detector. The encapsulation efficiency, as a percentage (%), was estimated to be the difference between the total active principle concentration in the nanoparticle suspensions and the concentration of active principle t in the supernatant, obtained through ultrafiltration/centrifugation (10 min, 5000 rpm) using an Amicon filter device containing an Ultracel-100 filter membrane (100 kDa, Millipore Corp., USA).

For *trans*-chalcona content was calculated using standard curve (0.975-250 μ g/ml; $y = 2,193x$, $r^2=0,999$) all analyses were performed in triplicate and the results were expressed by mean \pm standard deviation (SD).

2.2.5 Morphology of nanoemulsions

Samples were diluted in MILLI-Q® water (10x) and incubated with 2 % (w/v) uranyl acetate solution for 24 h. Afterwards, nanoemulsions were transferred to carbon-coated copper grids (CF300-Cu, TED PELLA INC; USA 300mesh) and analyzed by transmission electron microscopy (TEM) (JEM-1011, Peabody, USA).

2.3 In vitro assays

2.3.1 Cell cultures

Murine fibroblasts (3T3 lineage, clone A 31) and human keratinocytes (HaCat) were obtained from the Rio de Janeiro Cell Bank (BCRJ). Cells were cultured in DMEM culture medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin,

and 100 $\mu\text{g/ml}$ streptomycin. Cultures were maintained in a conditioned oven with a humid atmosphere at 37°C and 5 % CO_2 (v/v).

2.3.2 Citotoxicity test

Cytotoxicity of the free and nanoemulsified forms of chalcone was determined by the colorimetric method 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT). 3T3 and HaCat (1×10^4 cells/well) cells were inoculated in 96-well culture plates and after 12 h exposed to increasing concentrations of free and encapsulated *trans*-chalcone (1.56, 3.125, 6.25, 12.50, 25.50, and 100 $\mu\text{g/ml}$). After 72 h, 100 μl fresh medium and 10 μl MTT (final concentration 5 mg/ml) were added to the cell cultures and incubated for 3 h. The precipitated formazan crystals were dissolved in DMSO. The absorbance was determined at $\lambda = 540$ nm on a microplate reader (Spectramax Paradigm Molecular Device - Sunnyvale, CA, USA). Cell viability was expressed as a percentage, based on the viability of control cells (HEIDARI-KHARAJI et al., 2016).

2.3.3 *Leishmania amazonensis* cultures

The analysis of intracellular leishmanicidal activity of *trans*-chalcone nanoemulsions was performed by a colorimetric assay developed by Tonini, 2003. Adherent and differentiated THP-1 cells were infected with clones of *L. amazonensis* expressing the β -galactosidase enzyme. After 24 h of incubation, to allow the transformation of the promastigotes into amastigotes, the cells were treated with the nanoemulsions and cultured in a CO_2 -enriched atmosphere (5%, v/v) for 72 h, at 34°C. At the end of the incubation, chlorophenol red- β -D-galactopyranoside (CPRG), substrate of the β -galactosidase, was added to the culture medium, allowing the formation of galactose and the red chlorophenol chromophore. The reaction occurs in a period of 4 h, at 37°C, and is monitored spectrophotometrically at 570-630 nm (TONINI, 2013).

2.4 Statistical analysis

Experimental data were collected, summarized, and submitted to statistical analyzes, i.e., analysis of variance (ANOVA) and the *post-hoc* Tukey's test ($p < 0.05$), using the Graph-Pad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA). All experiments were performed in triplicate and the results expressed as mean \pm standard deviation.

3 | RESULTS AND DISCUSSION

3.1 Characterization of the nanoemulsion

Nanoemulsions are heterogeneous, milky-looking systems and generally composed of oil droplets stabilized by surfactants and dispersed in aqueous media. Besides to allowing the addition of hydrophobic compounds to aqueous systems,

such systems facilitate the intracellular transport of drugs (de MELLO et al., 2015; JAISWAL and DUDHE, 2015). Nanoemulsion has been thought to be a promising technique for drug development considering that the efficacy of topical treatment of cutaneous leishmaniasis depends on the penetration of leishmanicidal compounds into the skin layers, i.e., transposition of the dermis, in which the parasite is internalized in macrophages. Since the reduced particle size of nanoemulsions allows increased skin penetration of lipophilic compounds, it improves treatment efficacy (MATTOS et al., 2012).

The literature describes several methods to determine the stability of a nanoemulsion, which can be observed macroscopically due to the absence of phases and maintenance of the original aspect, as well as by determining its physicochemical traits. The proper characterization of nanostructured systems provides a set of information that assists in understanding the release of the encapsulated compound, as well as in the interaction of these systems with the target organ. The composition and technique of preparation are factors that influence the particle size, the polydispersity index (PDI), and the zeta potential of nanoparticles. When the goal is the topical release of a drug, the smaller the particle, the easier the active compound permeates the skin and, therefore, particle sizes between 50 and 200 nm are ideal for retention in the upper layers of the skin (BOCXLAER et al., 2016).

The PDI reflects particle size homogeneity, in which nanostructured systems are considered monodisperse when the PDI values are lower than 0.200. The zeta potential, in turn, translates the surface charge of the particles and high values in module of this variable characterize systems with relevant repulsive forces, avoiding the aggregation of the particles and contributing to a good physicochemical stability of this (SCHAFFAZICK and GUTERRES, 2003).

The particle size, PDI, and zeta potential of the nanoemulsions studied are shown in Table 2. The glycerol nanoemulsions showed an average size of 210.7 nm, while lower values (201.1 nm) were detected in formulations without glycerol. The mean PDI of the samples was 0.143, and the zeta potential value for all formulations was not less than -43.8, being the negative charges attributed to the use of the anionic surfactant lecithin.

All formulations showed to be stable and homogeneous. The determinant factor for the choice of nanoemulsion formulations for further *in vitro* leishmanicidal assay took into consideration the ones with minimum amounts of additives, low concentrations of surfactants, with smaller nanoparticles, and low degree of polydispersity. Thus, the formulation 6 has been chosen to incorporate *trans*-chalcone. This formulation was further physicochemically characterized, presenting a particle size of 189.2 nm, polydispersity index of 0.210, and zeta potential of -55.7 mV.

Variables	F1	F2	F3	F4	F5	F6	F7	F8	F9
Size	215.6	212.1	210.6	189.8	204.6	189.2	218.7	223.7	232.1
PDI	0.120	0.127	0.096	0.172	0.183	0.210	0.144	0.093	0.089
Zeta potential	-43.8	-45.8	-46.4	-52.8	-51.0	-55.7	-49.7	-49.8	-46.8
Variables	F10	F11	F12	F13	F14	F15	F16	F17	F18
Size	212.8	210.0	201.3	181.0	189.3	178.8	212.3	204.1	220.7
PDI	0.135	0.154	0.162	0.209	0.211	0.184	0.137	0.146	0.143
Zeta Potential	-51.3	-57	-54.4	-51.3	-51.0	-52.7	-50.8	-47.3	-48.3

Table 2: Particle size, polydispersity index, and zeta potential of formulations for further *trans*-chalcone incorporation.

After the optimization of the base formulation (without the active compound), *trans*-chalcone was associated with the nanoemulsion in two concentrations: 1 mg/ml and 10 mg/ml. No significant differences were observed in any physicochemical trait of the formulations (data not shown). However, formulations containing *trans*-chalcone at 10 mg/ml precipitated after 24 h, making unfeasible to use this concentration.

Following *trans*-chalcone encapsulation, the nanoparticle size, PDI, and zeta potential were determined and no significant changes have been recorded, demonstrating that the method used for nanoemulsion development was reproducible and stable. The encapsulation efficiency was greater than 99 % and the *trans*-chalcone concentration in the nanoemulsion was close to 1 mg/ml.

Figure 2 represents the morphology of a typical *trans*-chalcone nanoemulsion visualized by TEM. It is possible to observe isolated, dispersed, and spherical-shaped particles. Furthermore, the size and diameter thereof were compatible with the ones observed by the dynamic light scattering analysis. In addition, homogeneous structures, with slightly irregular borders were observed. The greater contrast among the several particles visualized can be justified by a greater interaction of the active principle with the uranyl acetate dye solution employed in the art.

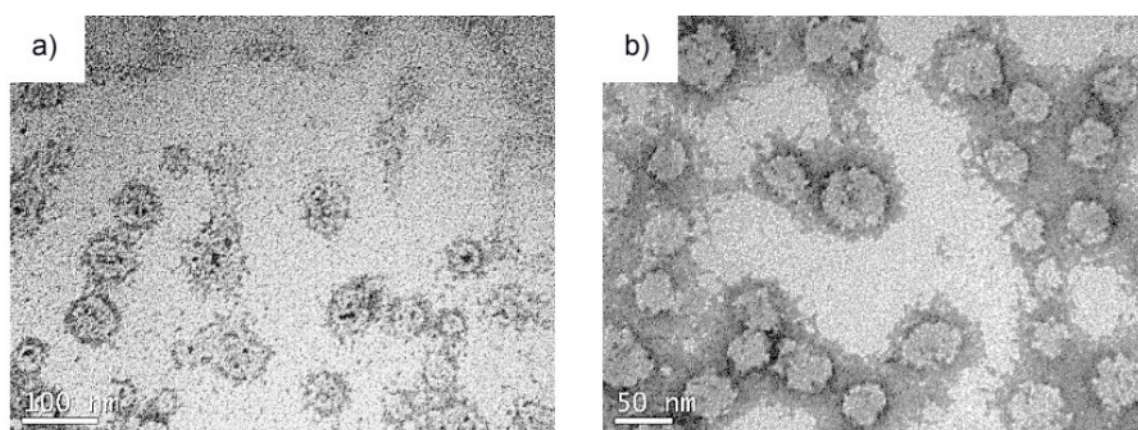


Figure 2: TEM micrographs of *trans*-chalcone nanoemulsion.

3.2 Cell toxicity of free *trans*-chalcone and *trans*-chalcone nanoemulsion

Cell viability of 3T3 fibroblasts and HaCat keratinocytes was assessed by treating cells with concentrations of the bioactive compound ranging from 0.1 $\mu\text{g/ml}$ to 300 $\mu\text{g/ml}$, within a log scale. The compound was considered toxic based on the maximal inhibitory concentration (IC_{50}). The human keratinocytes HaCat cell line did not present a drastic fall in the viability in concentrations lower than 12.5 $\mu\text{g/ml}$ for *trans*-chalcone nanoemulsion, as higher contents showed to be toxic after exposure by 24, 48 and 72 h. Concentrations above 50 $\mu\text{g/ml}$ showed lethal effect on HaCat cells. This toxicity profile was observed for all the experimental times investigated (e.g., 24h, 48h, and 72h). Free *trans*-chalcone displayed higher cytotoxicity than its nanoemulsion form. Free *trans*-chalcone at 12.5 $\mu\text{g/ml}$ reduced cell viability by 50% for all exposure times tested (Figure 3, graphics D, E, and F).

A different cytotoxicity profile was observed in 3T3 fibroblasts, where the *rans*-chalcone-containing nanoemulsions was more toxic than the free form of the drug. The IC_{50} for the free drug was 17.55 $\mu\text{g/ml}$ (± 3.45) and has fallen to 3.42 $\mu\text{g/ml}$ (± 2.18) with *trans*-chalcone nanoemulsion. Interestingly, for all experimental times tested, the IC_{50} of nanoemulsion was constant for cells treated with 17.55 $\mu\text{g/ml}$, as can be seen in figure 3, graphics A, B and C.

The biggest challenge of topical treatment for leishmaniasis is the low permeability of the drugs through skin layer, since it directly affects the cure rate (VAN BOCXLAER et al., 2016). In this study, we have shown that it is feasible to increase the leishmanicidal activity of *trans*-chalcone by nanoencapsulation, without increasing its concentration, avoiding constraints regarding its low solubility in the DMEM culture medium. *Trans*-chalcone and nano-*trans*-chalcone were evaluated on skin cells regarding their cytotoxicity. Typically, lipid nanocarriers toxicity occurs mainly due to the presence of surfactants. Nevertheless, the toxicity of the formulation studied is related to the active compound, given that a blank control, i.e., nanoemulsion lacking *trans*-chalcone was submitted to the same experimental conditions and no toxic effect was observed (data not shown).

For a given treatment with the *trans*-chalcone nanoemulsion, it was found that 3T3 fibroblast cells were about four times more sensitive than HaCat keratinocytes. This finding was also reported by Artrux-Tallau et al., 2013, with normal human fibroblasts.

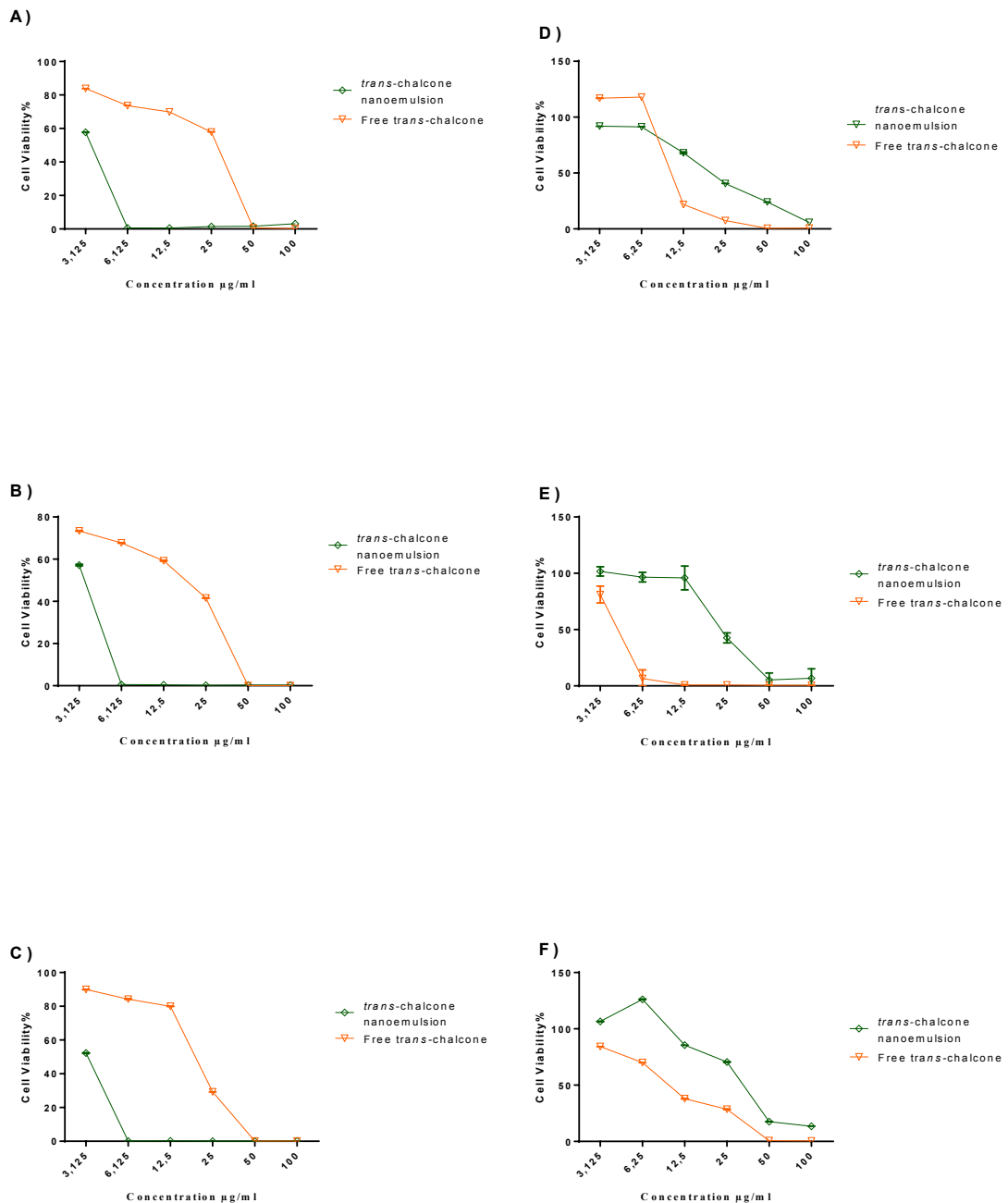


Figure 3: Cell cytotoxicity following treatment with *trans*-chalcone both in its free and nanoemulsion forms: Charts A, B, and C represent 3T3 fibroblast toxicity curve at 24, 48, and 72h, respectively. Charts D, E, and F represent the HaCat keratinocyte toxicity at 24, 48, and 72h, respectively.

3.3 Leishmanicidal potential of free *trans*-chalcone and nano-*trans*-chalcone

The antiparasitic activity of free *trans*-chalcone and its nanoemulsion against amastigotes in THP-1 cell line was evaluated. The free chalcone showed IC_{50} values ranging from 17.66 to 31.18, while the nanoemulsion form exhibited higher leishmanicidal activity ($IC_{50} = 9.09 \pm$).

The highest percentage of inhibition of parasitic growth was observed with *trans*-chalcone nanoemulsion. The ratio of IC_{50} against amastigotas and inhibitory concentration in 50% of THP-1, i.e., the selectivity index (SI), for the free and nano-*trans*-chalcone was 3.18 and 0.65, respectively, which are lower values than the

threshold suggested by Grogl et al., 2013 and Mattos et al., 2015. Nonetheless, some drugs used in the current clinical treatment of cutaneous leishmaniasis showed SI values lower than 0,8 in other species of leishmania (POORRAJAB et al., 2009 & RAMÍRES-MACÍAS et al., 2012).

4 | CONCLUSIONS

Altogether, the results hereby shown allow us to infer that the nanoemulsions containing chalcones (nano-*trans*-chalcone) showed characteristics considered ideal according to the regulatory guides standards. *Trans*-chalcone was successfully incorporated into nanoemulsion droplets using the spontaneous emulsification method. Free *trans*-chalcone was less toxic than its nanoemulsion form.

In human keratinocytes, free *trans*-chalcone showed higher cytotoxicity than its nanoemulsion form. In contrast, in 3T3 fibroblasts, nano-*trans*-chalcone revealed to be more toxic to cells than the free form of the drug. Likewise, the nano-*trans*-chalcone exhibited higher leishmanicidal activity and the highest percentage of inhibition of parasitic growth.

Although nano derivative-based delivery systems are certainly effective, high price can be a disadvantage of this method. Nevertheless, this is avoided by the technique herein applied, since spontaneous emulsification is simple and has a low production cost. Hence, this study could represent a novel approach to a more in-depth investigation of antileishmanial drugs.

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