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(Organizadora)

Patologia: 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 critica 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 17

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.ml¹ 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.ml¹ 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 I INTRODUCTION

Leishmaniases are anthropozoonoses 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 I MATERIAL AND METHODS

2.1 Materials

For the development of chalcone-containing nanoemulsions Poloxamer 188 (Kolliphor P188 micro, BASF, USA); ,oy 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).

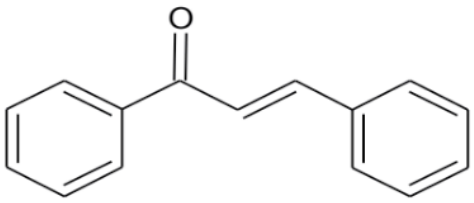

Figure 1: Trans-chalcone chemical structure: $C_{15}H_{12}O$ and molecular weight 208.26 g/mol.

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
Lecitin	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 ul-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 C_{18} reverse phase column (FR-Thermo Scientific, 250 mm x 4.6mm, particle size 5um, 30ºC) 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 ug/ml; y= 2,193, r^2=0,999) all analyses were performed in triplicate and the results were expressed by mean ± 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 μg/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 ($1x10^4$ 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 100ug/ml). After 72 h, 100 μl fresh medium and 10 μl MTT (final concentration 5mg/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 λ = 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 ± standard deviation.

3 I 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 t 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.

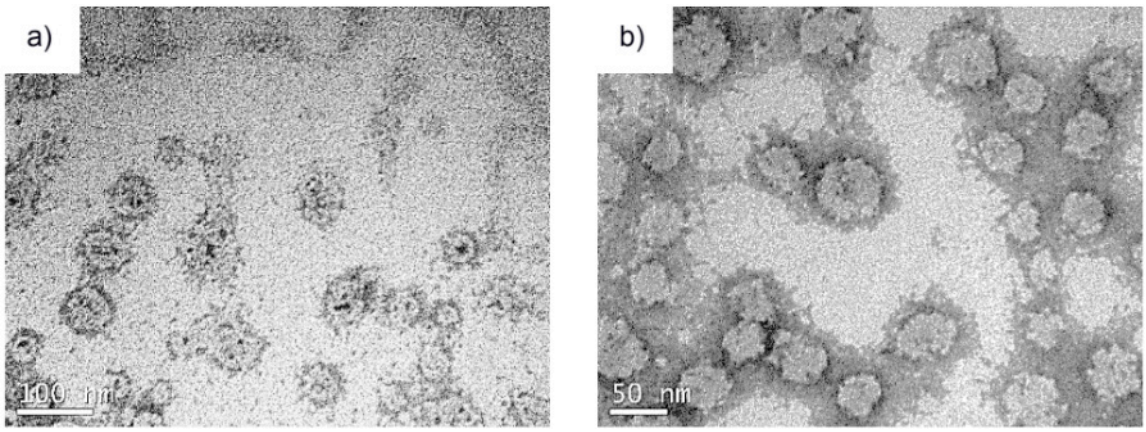

Figure 2: TEM micrographs of trans-chalcone nanoemulsion.

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 µg/ml to 300 µg/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 µg/ml for *trans*-chalcone nanoemulsion, as higher contents showed to be toxic after exposure by 24, 48 and 72 h. Concentrations above 50 µg/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 µg/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 ug/ml (± 3.45) and has fallen to 3.42 ug/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 ug/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.

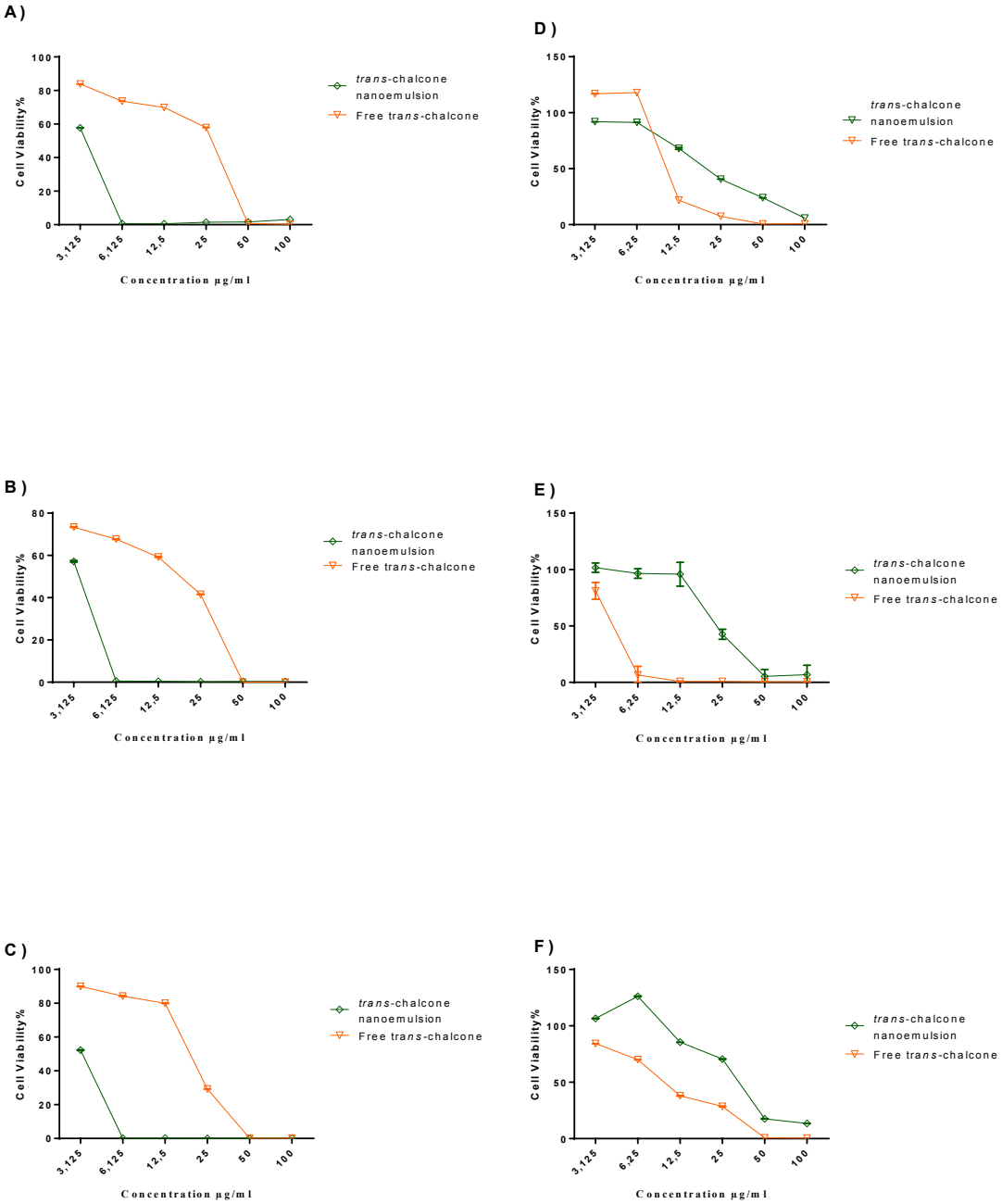

Figure 3: Cell cytotoxicity following treatment with trans-chalcone both in its free and nanoemulsion forms: Charts A, B, and C represent 3T3 fibroblast toxicity curve at 24, 48, and 72h, respectively. Charts D, E, and F represent the HaCat keratinocyte toxicity at 24, 48, and 72h, respectively.

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±).

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 I 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.

REFERENCES

ALMEIDA, O. L. S.; SANTOS, J. B. **Advances in the treatment of cutaneous leishmaniasis of the new world in the last ten years: a systematic review of the literature.** Savior. Anais do Congresso Brasileiro de Dermatologia v. 86, p. 497-506, 2011.

ARANA, B. A. et al. **Randomized, controlled, double-blind trial of topical treatment of cutaneous leishmaniasis with paromomycin plus methylbenzethonium chloride ointment in Guatemala.** Am J Trop Med Hyg, v. 65, p. 466-70, 2001.

ATRUX-TALLAU, N. et al. **Skin cell targeting with self-assemble ligand adressed nanoemultion droplets.** *Int. J. Cosmet. Sci.* 35**,** 310-318, 2013.

BARFOD, L. et al. **Chalcones from Chinese liquorice inhibit proliferation of T cells and production of cytokines.** Intern Immunopharmacol, v. 2, p. 545-555, 2002.

BELLO, M. L. et al. **Trimethoxy-chalcone derivatives inhibit growth of Leishmania braziliensis: synthesis, biological evaluation, molecular modeling and structure-activity relationship (SAR).** Bioorg Med Chem, v. 19, p. 5046-52, 2011.

BOECK, P. et al. **Synthesis of chalcone analogues with increased antileishmanial activity.** Bioorg Med Chem, v. 14, p. 1538-45, 2006.

BOUCHEMAL, K et al. **Nanoemulsion formulation using spontaneous emulsification: solvent,ol and surfatant optimisation.** Int J Pharm, v. 280, p. 241-251, 2004.

CHEN, M. et al. **Purification and enzymatic activity of an NADH-fumarate reductase and other mitochondrial activities of Leishmania parasites.** Apmis, v. 109, p. 801-808, 2001.

CHEN, M. et al. **Licochalcone A, a novel antiparasitic agent with potent activity against human pathogenic protozoan species of leishmania.** Antimicrob Agents Chemother, v. 37, p. 2550-2556, 1993.

DE MELLO, T. F. et al. **Activity of synthetic chalcones in hamsters experimentally infected with Leishmania (Viannia) braziliensis.** Parasitol Res, v. 114, p. 3587-600, 2015.

GROGL, Max et al. Drug discovery algorithm for cutaneous leishmaniasis. **The Am J Trop Med and Hyg**, v. 88, n. 2, p. 216-221, 2013.

GUPTA, S. et al. **Synthesis and biological evaluation of chalcones as potential antileishmanial agents.** Eur J Med Chem, v. 81, p. 359-66, 2014.

HEIDARI-KHARAJI, M. et al. **Enhanced paromomycin efficacy by solid lipid nanoparticle formulation against Leishmania in mice model.** Parasite Immunol. v. 38, p. 599-608, 2016. https://doi.org/10.1111/pim.12340.

JAISWAL, M. et al. **Nanoemulsion: an advanced mode of drug delivery system. 3 Biotech**, v. 5, n. 2, p. 123-127, 2015.

KEVRIC, I. et al. **New world and old world leishmania infections: A practical review.** Dermatol Clin, v. 33, p. 579-93, 2015.

LUNARDI, F. et al. **Trypanocidal and leishmanicidal properties of substitution-containing chalcones.** Antimicrob Agents Chemother, v. 47, p. 1449-51, 2003.

MARLOW, M. A. **Molecular epidemiology in American tegumentary leishmaniasis in the state of Santa Catarina, Brazil.** 2013. (PhD thesis). Pos-graduate Program in Biotechnology and Biosciences, Federal University of Santa Catarina, Florianopolis, SC.

MATTOS, C. B. et al. **Development of a stability-indicating LC method for determination of a synthetic chalcone derivative in a nanoemulsion dosage form and identification of the main photodegradation product by LC-MS.** J Pharm Biomed Anal, v. 70, p. 652-6, 2012.

MATTOS, C. B. et al. **Nanoemulsions containing a synthetic chalcone as an alternative for treating cutaneous leshmaniasis: optimization using a full factorial design**. International journal of nanomedicine, v. 10, p. 5529, 2015.

NOWAKOWSKA, Z. **A review of anti-infective and anti-inflammatory chalcones.** Eur J Med Chem, v. 42, p. 125-37, 2007.

PINERO, J. et al. **New administration model of trans-chalcone biodegradable polymers for the treatment of experimental leishmaniasis.** Acta Trop, v. 98, p. 59-65, 2006.

POORRAJAB, F. et al. **Selective leishmanicidal effect of 1, 3, 4-thiadiazole derivatives and possible mechanism of action against Leishmania species.** Experim Parasitol, v. 121, n. 4, p. 323-330, 2009.

RAMÍREZ-MACÍAS, I. et al. **In vitro anti-leishmania evaluation of nickel complexes with a triazolopyrimidine derivative against Leishmania infantum and Leishmania braziliensis**. J Inorg

Biochem, v. 112, p. 1-9, 2012.

SCHAFFAZICK, S. R. et al. **Caracterização e estabilidade físico-química de sistemas poliméricos nanoparticulados para administração de fármacos.** Quím Nova, v. 26, n. 5, p. 726-737, 2003.

SHOWLER, A. J.; BOGGILD, A. K. **Cutaneous leishmaniasis in travellers: a focus on epidemiology and treatment in 2015.** Curr Infect Dis Rep, v. 17, p. 489, 2015.

TEIXEIRA, H. et al. **Preparation and characterization of nanoemulsions containing antisense oligonucleotides by the spontaneous emulsification technique.** Pharmacy Notebook, v. 18, p. 89-93, 2002.

TONINI, M. L. et al. **Desenvolvimento de um teste colorimétrico para triagem da atividade Leishmanicida de compostos utilizando Leishmania amazonensis expressando** à **enzima beta-galactosidase.** Dissertação de mestrado. Universidade Federal de Santa Catarina, Programa de Pós-graduação em Biotecnologia e Biociências p. 1- 121, 2013.

VAN BOCXLAER, K. et al. **Drug permeation and barrier damage in Leishmania-infected mouse skin.** J Antimicrob Chemoth, v. 71, n. 6, p. 1578-1585, 2016.

WHO, **World Health Organization.** Leishmaniasis, 2015.

WU, J. H. et al. **Anti-AIDS agents 54. A potent anti-HIV chalcone and flavonoids from genus *Desmos*.** Bioorg Med Chem Lett, v. 13, p. 1813-5, 2003.

ZHAI, L. et al. **The antileishmanial agent licochalcone A interferes with the function of parasite mitochondria.** Antimicrob Agents Chemoth, v. 39, p. 2742-2748, 1995.

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