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Potential of the association of dodecyl gallate with nanostructured lipid system as a treatment for paracoccidioidomycosis: *In vitro* and *in vivo* efficacy and toxicity

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ABSTRACT

Paracoccidioidomycosis (PCM) is a systemic mycosis endemic in Latin America, caused by *Paracoccidioides* spp. A limited number of antifungal agents are available and the search for new compounds has increased. Additionally, nanostructured lipid system (NLS) has emmerged as an interesting strategy to carrier compounds for the treatment of mycosis. In this work, the antifungal efficacy and toxicity of dodecyl gallate (DOD) associated with a NLS was evaluated through *in vitro* and *in vivo* tests. DOD showed good *in vitro* antifungal activity and low toxicity in lung fibroblasts and zebrafish embryos, but no antifungal efficacy in infected mice, which may have been a result of low bioavailability. On the other hand, the association of DOD + NLS was beneficial and resulted in lower toxicity in lung fibroblasts and zebrafish embryos. In addition, NLS + DOD promoted a significant reduction in the fungal burden of mice lungs and could be a potential therapeutic option against PCM.

1. Introduction

Paracoccidioides brasiliensis and *Paracoccidioides lutzii* belong to the group of thermo-dimorphic fungi and cause paracoccidioidomycosis (PCM). This human systemic mycosis is endemic in Latin America, in which Brazil, Venezuela and Colombia are the countries with the highest number of cases (Bocca et al., 2013; Shikanai-Yasuda et al., 2006). The habitat of *Paracoccidioides* spp. can be the organic matter present in the soils, especially in areas of coffee and sugar cane cultivation (Arantes and Theodoro, 2016). The infection of these fungi occurs in mycelial form, which differentiates into yeast form after inhalation by the host. This transition is essential to establish the infectious process, in which the lung is the first site of infection and the yeasts can spread to other organs such as liver, spleen, mucous membranes, skin and central nervous system (Lacaz, 1994; Shikanai-Yasuda et al., 2006).

PCM requires prolonged treatment, which can occur for more than 1 year. Patients with this mycosis are commonly treated with amphotericin B (AmB), azoles and sulfonamides (Hahn et al., 2002). AmB is mainly used in severe cases of PCM and there are few instances of fungal resistance to this drug. The oral administration of this drug is not possible due to characteristics such as poor membrane permeability, low aqueous solubility, and instability at the low pH of the stomach (Mistro et al., 2012; Volmer et al., 2010). Thus, the conventional AmB (formulated in sodium deoxycholate) is administered intravenously and significant adverse effects may sometimes require the discontinuation of therapy. Injection usually results in chills, fever, tinnitus, headache, and vomiting. However, the most common and serious adverse effect is nephrotoxicity (Kauffman, 2006; Rang and Dale, 2007). A liposomal formulation was developed to minimize this effect, but the high cost has limited its use (Botero Aguirre and Restrepo Hamid, 2015). In addition, the use of new carrier systems such as poly (lactic-co-glycolic acid) nanoparticles, solid lipid nanoparticles (SLNs) and carbon nanotubes has been investigated (Benincasa et al., 2011; Chaudhari et al., 2016; Verma et al., 2011).

Azole, especially itraconazole, has been used in mild or moderate cases of PCM. Itraconazole is lipophilic and higher levels of this drug are found in different tissues and organs than serum, except in the cerebrospinal fluid, where the level of this antifungal is limited (Marwaha and Maheshwari, 1999). This class of drugs can be

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administered orally and have mild adverse effects (Rang and Dale, 2007). However, in addition to the inhibition of cytochrome P450 enzymes in fungi, azole inhibits the enzymes responsible for the hepatic metabolism of drugs in humans. As a consequence, azoles interact with various classes of antihistaminic, antineoplastic, steroid, antimicrobial, antiretroviral, opioid, barbiturate, cardiovascular, psychotropic and oral contraceptive drugs (Bates and Yu, 2003); moreover, they are teratogenic. In addition, Hahn et al. (2003) described the occurrence of ketoconazole resistant isolates of *P. brasiliensis* in patients with PCM. Since the similarity between fungal and mammalian cells makes the discovery and development of effective and safe antifungal agents a challenge, there are a limited number of antifungal agents and they present numerous disadvantages; in recent years, interest in the study of new compounds with antifungal potential and nanoparticle carriers for drugs and compounds has increased (Derengowski et al., 2009; Ostrosky-Zeichner et al., 2010; Petrikkos and Skiada, 2007; Scorzoni et al., 2017; Voltan et al., 2016).

Gallic acid is an example of a compound derived from secondary metabolism in several species of plants such as *Paeonia rockii, Astronium* sp., *Syzygium cumini, Euphorbia lunulata, Labisia pumila, Zingiber officinale, Klainedoxa gabonensis, Nervilia aragoana, Atalantia monophylla, Lawsonia inermis, Ardisia chinensis* and *Alchornea glandulosa*, which can be extracted from the leaves, stems, roots or fruits (Choubey et al., 2015; Santos et al., 2016). Previous studies showed that the gallic acid derivative, dodecyl gallate (DOD), presented *in vitro* activity against human fungal pathogens, including *Candida* spp, *Cryptococcus gattii*, *Histoplasma capsulatum* and *Paracoccidioides* sp. (de Paula e Silva et al., 2014). On the other hand, dodecyl gallate presents low solubility in water, which limits its parenteral administration and release in the bloodstream.

One strategy to increase drug stability and solubility and offer better bioavailability is the use of lipid nanoparticles, as for example, in the case of nanoemulsion for the antifungal itraconazole (Bunjes, 2010; Thakkar et al., 2015). Additionally, lipid nanoparticles could increase the therapeutic index of drugs and compounds by improving their activity and reducing their toxicity. Lipid-conjugated formulations of AmB, for example, were approved in the 1990s and reduced the nephrotoxicity of the drug (Moen et al., 2009; Voltan et al., 2016). Lipid nanoparticles are widely used for various routes of administration including parenteral due to their excellent biocompatibility and low toxicity. Because of these reasons, we evaluated and characterized the association of the DOD in a nanostructured lipid system (NLS) for the treatment of PCM through the use of *in vitro* and *in vivo* antifungal and safety tests. *In vitro* tests were undertaken in fungal (*Paracoccidioides* species) and in mammalian cells (pulmonary fibroblasts). *In vivo* tests were performed in an embryotoxicity model with zebrafish and in a PCM murine model.

2. Materials and methods

2.1. Drugs and compound

Amphotericin B and itraconazole were obtained commercially (Sigma-Aldrich) and dodecyl gallate (DOD) was synthesized according to Morais et al. (2010). To prepare stock solutions, the drugs and compound were solubilized in dimethylsulfoxide, DMSO (Labsynth).

2.2. Association of dodecyl gallate with nanostructured lipid system

The nanostructured lipid system (NLS) was prepared at the following composition: 10% cholesterol (oil phase), 10% mixture of polyoxyethylene (23) lauryl ether (Brij® 35) and soybean phosphatidylcholine (Epikuron® 200) 2:1 (surfactant) and 80% phosphate-buffered saline (PBS - aqueous phase) as described by Formariz et al. (2005) and Bonifácio et al. (2015) with adaptations. The mixture was prepared in an ice bath using a sonicator (Q500 – Qsonica, Newtown, CT, USA) with a potency of 500 W, in discontinuous mode for 10 min with 30 s of incubation every 1 min during the sonication process. DOD (5 mg) was associated to a previously prepared NLS (1 mL) with the aid of the sonicator in rod for 2 min under the same conditions used for the preparation of NLS.

2.3. Characterization of the system

The determination of the diameter of the particle and polydispersity index (PDI) of the free NLS or associated with DOD was performed using dynamic laser scattering at 20 °C. Free NLS or associated with DOD was also characterized for the zeta potential using the electrophoretic mobility. The samples were first diluted (10 μL.mL^{-1}) in aqueous potassium chloride (KCl) solution. All the parameters were evaluated using a Zetasizer Nano NS instrument (Malvern Instruments, Worcestershire, UK). Three determinations of the parameters were carried out.

2.4. Entrapment efficiency

NLS + DOD (1 mL) was centrifuged using a centrifugal filter unit (Amicon Ultra-4, PLGC Ultracel-PL Membrane, 100 kDa; EMD Millipore, Billerica, MA, USA) at 3000 rpm for 20 min at 20 °C. Free drug content (F) in eluent was analyzed by spectrophotometry at 200 nm. The sample was measured in duplicate. The encapsulation efficiency was calculated using the following equation:

% entrapment efficiency = $[(T - F)/T] \times 100$

where, T is the total drug content and F is the free drug content

2.5. In vitro fungal activity of dodecyl gallate associated with nanostructured lipid system

The microdilution susceptibility test was performed as described in document M27-A3 from the Clinical and Laboratory Standards Institute, CLSI (CLSI, 2008), with some modifications (de Paula e Silva et al., 2013). *P. brasiliensis* S1 isolated 18 (chronic PCM/São Paulo, Brazil) and *P. lutzii* Pb01-like-strain ATCC MYA-826 (acute PCM/Goiânia, Brazil) were maintained in Fava-Netto medium at 37 °C for 4 days. The inoculum was prepared in PBS and further dilutions were prepared in sterile RPMI 1640 medium (Sigma-Aldrich) to get about 5×10^3 cells.mL^{-1}. DOD dissolved in DMSO or associated with NLS was diluted in RPMI to obtain final concentrations of 0.015–250 mg/L. AmB and itraconazole were used as control drugs. The solvent DMSO and the NLS were tested at concentrations corresponding to those of the compound. The plates were incubated with shaking (150 rpm) at 37 °C for 48 h. After this period, the indicator Alamar Blue (BioSource International) was added and the plates were incubated with shaking (150 rpm) at 37 °C for 24 h. The absorbance was read on a microplate reader at 570–600 nm and the minimum inhibitory concentration (MIC) was determined. Three independent experiments were performed.

2.6. Cytotoxicity of dodecyl gallate associated with nanostructured lipid system

MRC5 (pulmonary fibroblasts) cell line (Banco de Células do Rio de Janeiro - BCRJ, Federal University of Rio de Janeiro, Brazil) was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 2% of a solution of antibiotics. For the assay, cell suspensions were seeded into each well of a 96-well plate (5×10^5 cells per well), which was incubated for 24 h at 37 °C in an atmosphere of 5% CO_2 to allow cell adhesion, obtaining at least 80% confluence. DOD dissolved in DMSO or associated with NLS at concentrations of 0.015–250 mg/L were added to the wells. The solvent DMSO and the NLS were tested at concentrations corresponding to those of the compounds. The cells were exposed to the compounds for 24 and 48 h. Subsequently, 10 μL of 0.01% resazurin (Sigma-Aldrich)

was added and the plates were incubated for 6 h. After incubation, the absorbance was read on a microplate reader at 570–600 nm and the 50% inhibitory concentration (IC_{50}) was determined. Three independent experiments were performed.

2.7. Toxicity of dodecyl gallate associated with nanostructured lipid system in zebrafish embryos

Wild type zebrafish (*Danio rerio*) were kept in a temperature controlled aquarium (28 ± 0.5 °C) in a laboratory with a 14 h light/10 h dark cycle. Adult fish were placed for mating (1:1 or 1:2 or 2:1 male/female ratio) and the embryos collected in the breeder. Embryos were washed with embryonic medium (10 mM NaCl, 0.34 mM KCl, 0.66 mM $CaCl_2{\cdot}2H_2O$, 0.66 mM $MgCl_2{\cdot}6H_2O$) supplemented with 0.00003% methylene blue and transferred to 96-well plates (2 embryos/well). Different concentrations of free DOD or associated with NLS were added (0.015–125 mg/L). The plates were incubated at 28 °C and malformation phenotypes such as coagulation of fertilized eggs, lack of somite formation, lack of tail detachment and lack of heart rate were observed at 5, 24, 48 and 120 hpf (OECD, 2013). Three independent experiments were performed (24 embryos/concentration).

2.8. Evaluation of dodecyl gallate associated with nanostructured lipid system in mice

The use of male Balb/c mice was approved by the Research Ethics Committee of UNESP-Araraquara (Protocol CEUA/FCF/CAr n° 21/2013). Before and during the experiment, they were maintained in a 12 h light/dark cycle with *ad libitum* acess to water and standard rodent chow. Male Balb/c mice with 6-week-old and about 25 g were anesthetized intramuscularly (im) with 80 mg/kg of ketamine and 10 mg/kg of xylazine. *P. brasiliensis* 18 was grown in Fava-Netto solid medium for 7 days at 37 °C. The inoculum preparation was performed in PBS and 50 μL of fungal suspension with 3×10^5 cells was inoculated intratracheally. One day after infection, the animals were divided into six groups administered the following treatments: PBS; itraconazole 5 mg/kg/day; DOD 10 mg/kg/day; NLS; NLS + DOD 10 mg/kg/day; in addition, uninfected animals treated with PBS were used as a control. The treatments were administered intraperitoneally (ip) for 20 consecutive days. At the end of the experiment, the mice were euthanized and their lungs removed for CFU count. The organ was ground in PBS with the aid of a glass Potter homogenizer and 100 μL were plated in BHI solid medium supplemented with 4% fetal bovine serum, 5% filtered of Pb339 and gentamicin 40 $mg.L^{-1}$ (Granzoto et al., 2013). The plates were incubated at 37 °C for 10 days. Two independent experiments were performed, with 3–4 animals/group in each experiment.

2.9. Animal weight and biochemical analysis

The blood of animals was collected by cardiac puncture and centrifuged at 3500 rpm for 10 min at 25 °C. The serum was analyzed for hepatic parameters, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and renal parameters, urea and creatinine. These biochemical analyses were performed at the Núcleo de Atendimento à Comunidade (NAC) of the Faculty of Pharmaceutical Sciences of UNESP, Araraquara, by dry chemical analysis using Vitros 250 (Orto Clinical Diagnostics - Johnson & Johnson Company®, São Paulo, SP, Brazil). The animals were also weighed at the end of the experiment.

2.10. Statistical analysis

The statistical analysis of the results was performed in the Graph Pad Prisma 5 program (La Jolla, CA, USA). The survival curves were plotted by the Kaplan-Meyer method and analyzed by Log-rank (Mantel-Cox). The fungal burden and biochemical parameters were analyzed by ANOVA with Bonferroni post-test. The p value $< .05$ was considered statistically significant.

Table 1
Mean values and standard deviation of the particle sizes, PDI and zeta potential for the nanostructured lipid system (NLS), as well as for the compound dodecyl gallate associated with system (NLS + DOD).

	Size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
NLS	117.9 ± 2.196	0.153 ± 0.002	−2.610 ± 0.300
NLS + DOD	154.4 ± 2.829	0.255 ± 0.008	−2.731 ± 0.310

3. Results

3.1. Properties of NLS

As depicted in Table 1, the mean particle size of the NLS was 117.9 ± 2.196 nm. The association of DOD caused an increase in particle size (154.4 ± 2.829 nm), which is a strong indication that the incorporation of the compound into the NLS was successful. The polydispersity index (PDI) shows the relative homogeneity between the particle sizes in the sample. The PDI presented values of 0.153 ± 0.002 and 0.255 ± 0.008 for the NLS and NLS + DOD, respectively. These data showed that the samples have homogeneity. The results of the NLS and NLS + DOD for the zeta potential showed that both presented a similar surface charge of approximately 2.7 mV. Additionally, the entrapment efficiency assay was performed and it showed that most of the DOD (99.78%) was associated to the NLS.

3.2. In vitro action

Following CLSI protocols (CLSI, 2008; de Paula e Silva et al., 2013), Table 2 shows the minimum inhibitory concentration (MIC) values for DOD dissolved in DMSO or associated with NLS. The MIC of the DOD was 0.12 mg/L for both *Paracoccidioides* species. When incorporated into the NLS, there was an increase in the MIC value of the compound to 0.24 mg/L and 0.49 mg/L for *P. brasiliensis* and *P. lutzii,* respectively. DMSO and NLS showed no antifungal activity at the equivalent DOD concentrations. AmB had a MIC of 0.03 mg/L for both species and itraconazole presented a MIC of 0.008 mg/L for *P. brasiliensis* and 0.015 mg/L for *P. lutzii*.

An *in vitro* assay was also performed to address whether NLS + DOD exerted toxicity on non-fungal eukaryotic cells (lung fibroblasts). Through the Fig. 1 and the inhibitory concentration 50% (IC_{50}) values (Table 2), it is possible to observe a toxic effect of the compound free or associated to nanoparticle more pronounced at 48 h compared to 24 h of treatment on the cells. A reduction in the viability of the cells occurs with increasing concentration of dodecyl gallate (0.49 to 250 mg/L).

The association with NLS contributed substantially to a toxicity reduction of DOD in the lung fibroblasts, (Fig. 1, C and D). The IC_{50} value of DOD increased with the incorporation to the NLS from 103.9

Table 2
Minimal inhibitory concentration (MIC) against *Paracoccidioides* species and inhibitory concentration 50% in lung fibroblasts (MRC5) of free dodecyl gallate (DOD) and associated wiyh nanostructured lipid system (NLS + DOD).

	P. brasiliensis 18 MIC (mg/L)	*P. lutzii* 01 MIC (mg/L)	MRC5 cells 24 h − IC_{50} (mg/L)	MRC5 cells 48 h − IC_{50} (mg/L)
DOD	0.12	0.12	103.9	63.4
NLS + DOD	0.24	0.49	> 250	> 250
DMSO	> 250	> 250	> 250	> 250
NLS	> 250	> 250	> 250	> 250
AmB	0.03	0.03	–	–
ITC	0.008	0.015	–	–

AmB: Amphotericin B; ITC: Itraconazole.

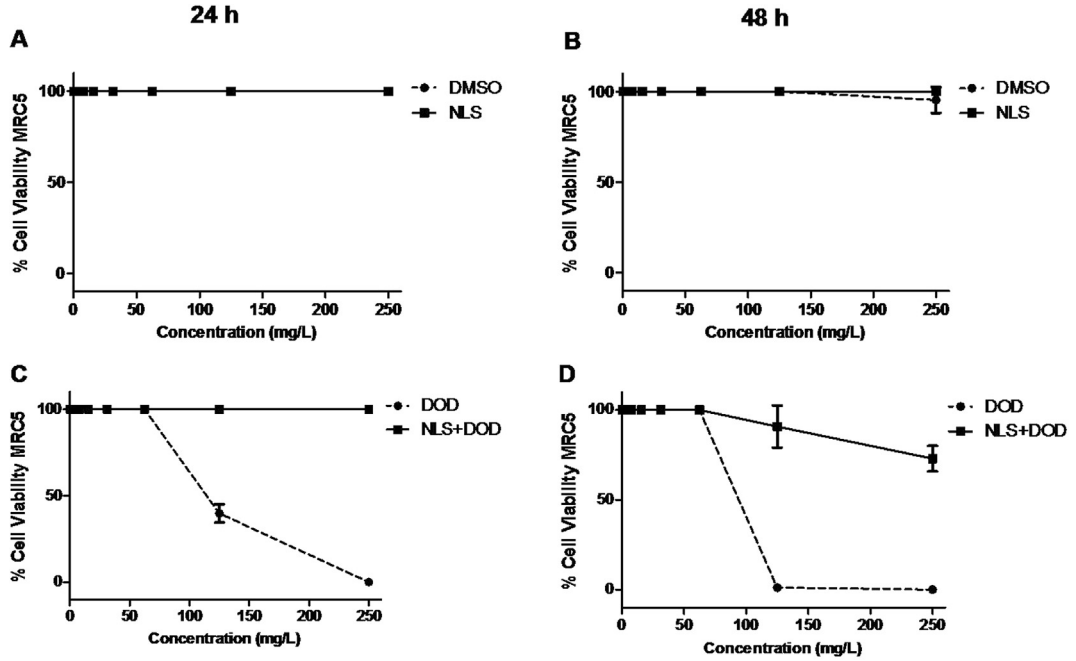

Fig. 1. Cytotoxicity of (A) the vehicle dimethylsulfoxide (DMSO); (B) the nanostructured lipid system (NLS); (C) free dodecyl gallate (DOD); and (D) DOD in association with NLS (NLS + DOD) in lung fibroblasts (MRC5).

to > 250 mg/L after 24 h of treatment and from 63.4 to > 250 mg/L with 48 h of treatment (Table 2). NLS and DMSO showed no toxic effect at the concentrations tested (Fig. 1, A and B). Thus, the IC_{50} values found for the free compound and when associated with NLS in lung fibroblasts were substantially higher than the MIC values shown above.

3.3. NLS reduces the toxicity of DOD in zebrafish embryos

The DOD associated with NLS was also tested in zebrafish embryos at some concentrations previously used for the susceptibility and cytotoxicity tests (0.015, 0.03, 0.12, 0.49, 1.95, 7.8, 31.25, 62.5 and 125 mg/L). Ther toxicity was directly proportional to the concentration of the compounds (Fig. 2). The lethal concentration 50% (LC_{50}) found at 120 hpf for DOD was 16.58 $\mu g.mL^{-1}$ (Table 3). The association with NLS substantially reduced the toxicity of the compound and the LC_{50} was determined as > 125 mg/L. The treatment with NLS alone showed no toxicity in the zebrafish embryos at the tested concentrations.

3.4. NLS + DOD has activity against P. Brasiliensis in murine model

Twenty days after the treatment of Balb/c mice, the lungs of the animals were removed and the fungal burden was determined through the consideration of weight of the organ. In Fig. 3, the mean of colony-forming unit (CFU)/g of lung in the infected with *P. brasiliensis* 18 (Pb18) and untreated animals was 2.5×10^4. The treatment with 5 mg/kg itraconazole (ITRA) control drug significantly reduced the number of CFU/g in the lungs to 3.6×10^3 ($p < .05$). DOD at 10 mg/kg did not reduce the mean CFU/g in the lungs (7.8×10^3) as compared to the infected and untreated group. However, the incorporation of DOD 10 mg/kg to NLS promoted a significant reduction in the number of CFU/g of lung (4.7×10^3, $p < .05$). NLS also did not demonstrate antifungal activity in the murine model (1.4×10^4 CFU/g lung).

Blood samples were collected from mice for the evaluation of toxicity parameters, ALT, AST, urea and creatinine. AST and ALT are considered biochemical indicators of liver function; their increase may represent the presence of hepatocyte lesions or liver fibrosis. Urea and creatinine are known to be related to renal function and an elevation in their levels may indicate injury or dysfunction of the kidneys. The mean of the four parameters analyzed was not altered in the group infected with *P. brasiliensis* 18 as compared to the non-infected group (control). In addition, the treatment of animals with 5 mg/kg of ITRA and 10 mg/kg of DOD or NLS + DOD did not alter AST, ALT, urea and creatinine levels compared to the control group (Table 4).

The body weight of the mice, which is considered as another indicator of the *in vivo* toxicity of DOD or NLS + DOD, was also analyzed. The animals in all groups were weighed at the end of the experiment and no differences in mean body weight were observed (Table 4).

4. Discussion

Compounds present in plants have antimicrobial properties empirically recognized for centuries and scientifically proven in the last decades (Santos et al., 2016). In this context, several natural products and synthetic derivatives such as maitenine, pristimerine, curcumin, 6-quinolinyl N-oxide chalcone and ajoene showed *in vitro* and *in vivo* activity against *Paracoccidioides* sp. (de Sá et al., 2015; Gullo et al., 2012; Maluf et al., 2008; Martins et al., 2009). In addition, the strategy of using nanoparticles to carry commercially available antifungal agents has been explored (Gupta and Vyas, 2012; Nasti et al., 2006; Voltan et al., 2016). In the present work, we associated a nanostructured lipid system (NLS) with dodecyl gallate (DOD), an antifungal compound derived from gallic acid with low aqueous solubility, and evaluated the association for the treatment of paracoccidioidomycosis (PCM). The NLS and NLS + DOD characterization revealed that the values obtained are in the range of 10–250 nm (100–2500 Å), ideal for the nanostructured lipid system (Formariz et al., 2005). In addition, the negative charge in potential zeta comes from the components of the formulations such as soybean phosphatidylcholine and cholesterol, which have free ester groups [–RCOOR′–] and a free hydroxyl group [–OH], respectively (Silva et al., 2016). In addition, NLS exhibited high entrapment efficiency, which was able to associate the most of the compound (99.78%).

DOD effectively inhibited the growth of *Paracoccidioides* species and the association with NLS slightly increased the MIC values in one or two dilutions. However, the difference between the results for DOD and NLS + DOD was well within the acceptable variation for microdilution

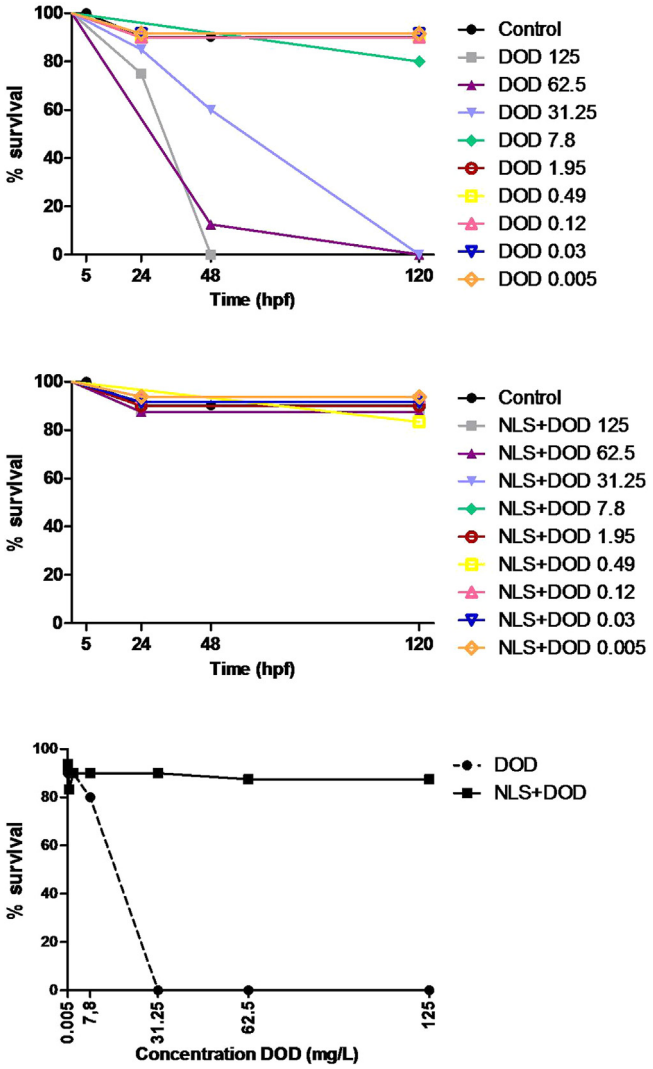

Fig. 2. Survival curve (A and B) and concentration-response curve 120 hpf (C) of zebrafish embryos treated with free dodecyl gallate (DOD) and associated with nanostructured lipid system (NLS + DOD). This experiment was performed in triplicate (2 embryos/well and 24 embryos/concentration).

Table 3
Lethal concentration 50% for free dodecyl gallate (DOD) free and associated with nanostructured lipid system (NLS + DOD) in a zebrafish embryo model.

	LC_{50} (mg/L) − 120 hpf
DOD	16.58
NLS + DOD	> 125
DMSO	> 125
NLS	> 125

methods (Pfaller et al., 2011). The scientific literature reports a variety of effects on nanostructured lipid carriers on the *in vitro* antimicrobial activity. Some drugs, such as miconazole and clotrimazole, had their antifungal effect potentiated when associated with nanostructured lipid systems (Esposito et al., 2013; Mendes et al., 2013; Singh et al., 2016). On the other hand, similar to that observed in the present study, the antimicrobial activity was maintained when tetracycline and ruthenium compounds were associated with these systems (de Freitas et al., 2014; Lin et al., 2013). It may be suggested that different interactions between the drug/compound and the carrier, or between the system and the membrane of the microorganism, may influence the antifungal

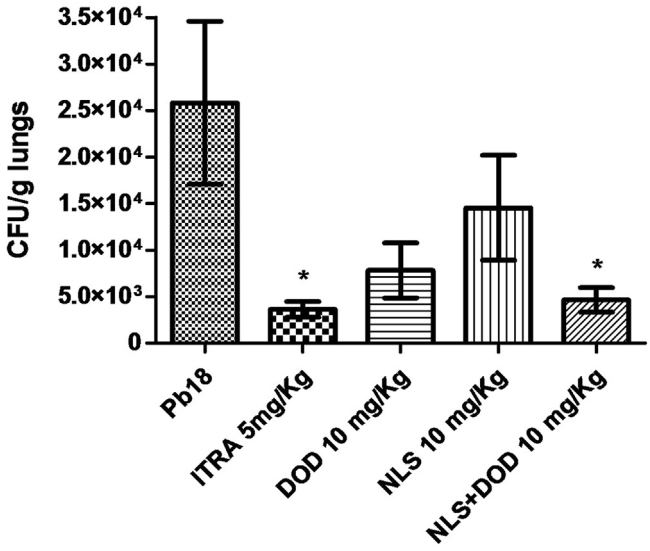

Fig. 3. Fungal lung burden of Balb/c mice infected with *P. brasiliensis* (Pb18) and treated with 5 mg/kg of ITRA, 10 mg/kg of free dodecyl gallate (DOD) and associated with nanostructured lipid system (NLS + DOD). The evaluation was done 20 days after the treatment. Data expressed as mean and standard error mean. $^{*}P < .05$ *vs.* Pb18. N = 6–7/group.

response.

DOD and NLS + DOD were also tested on non-fungal eukaryotic cells. The association with NLS contributed to a substantial reduction in the toxicity of DOD on the lung fibroblasts (MRC5) and the IC_{50} values found were substantially higher than the MIC values, which permitted a wide therapeutic window.

Zebrafish (*Danio rerio*) has proven useful for assessing the potential toxic effects of new compounds on embryonic development. The use of zebrafish embryos is advantageous because they are small and can readily absorb the compounds with which they are placed in contact. Other advantages include transparent embryos, which allow the visualization of their development, and the posture of a couple generates up to 200 embryos (Kanungo et al., 2014). The genome of this vertebrate has already been fully sequenced, showing that 71.4% of the zebrafish genes are orthologous with those of humans (Howe et al., 2013). Thus, although mammalian models are still considered gold standard for the study of teratogenic effects, zebrafish has been increasingly accepted as a model for predicting these effects (Kanungo et al., 2014). In this respect, the analysis of the toxicity of nanoparticles and antifungals as azole has been carried out on embryos and larvae of zebrafish individually (de Jong et al., 2011; Kanungo et al., 2014). However, the association of antifungal compound with a nanostructured lipid system has been evaluated for the first time in this work. The DOD presented LC_{50} values in zebrafish greater than 20 times the MIC of these compounds against *Paracoccidioides* sp, that is, they allow a wide therapeutic window. However, the association with the NLS allowed greater safety for the administration of the DOD (LC_{50} 255 times greater than MIC). The low teratogenic potential found is a great advantage for the development of NLS + DOD as new antifungal agent mainly in relation to azoles. This class of drugs has no indication to be used in pregnant women with mycoses, because it has toxic effects to the embryos, causing a variety of malformations, including craniofacial, cardiac, pulmonary, urogenital, spinal, eye and ear (de Jong et al., 2011).

Finally, we tested the DOD and NLS + DOD in conventional murine model of PCM infection. Neither treatment affected the weight of the mice or induced liver or kidney toxicity. DOD at 10 mg/kg did not reduce the fungal burden of mice lungs. However, the incorporation of DOD 10 mg/kg to the NLS showed a significant reduction in fungal burden of mice lungs compared to control group. Previous *in vivo*

Table 4
Body weight and hepatic and renal parameters in the serum of mice treated with free dodecyl gallate (DOD) and associated with nanostructured lipid system (NLS + DOD). Data expressed as mean and standard error mean.

Group	Body weight (g)	AST (U/L)	ALT (U/L)	Urea (mg/dL)	Creatinine (mg/dL)
Control	24.5 ± 0.8	199.0 ± 38.5	55.2 ± 6.6	56.6 ± 2.8	0.18 ± 0.02
Pb18	24.6 ± 0.2	133.3 ± 31.5	58.2 ± 7.1	61.1 ± 2.5	0.15 ± 0.01
ITRA	24.8 ± 0.5	128.4 ± 23.2	49.6 ± 8.0	60.9 ± 4.0	0.16 ± 0.01
DOD	23.3 ± 0.5	204.6 ± 43.2	66.4 ± 3.6	58.3 ± 2.9	0.15 ± 0.01
NLS	24.9 ± 0.5	122.8 ± 50.1	57.8 ± 3.6	61.4 ± 3.0	0.18 ± 0.02
NLS + DOD	24.4 ± 0.4	210.3 ± 67.2	52.7 ± 4.3	55.8 ± 3.0	0.15 ± 0.01

studies also used lipid nanoparticles to carry antifungal drugs. For example, Nasti et al. (2006), used a liposomal formulation to associate anti-fungal nystatin and observed an increase in survival and reduction of fungal load of mice infected with *C. neoformans*. Gupta and Vyas (2012) have shown that the burden of *C. albicans* on a cutaneous infection in mice was lower for lipid nanoparticles carrying fluconazole than free drug.

In summary, *in vitro* and *in vivo* assays were performed to evaluate the efficacy against *Paracoccidioides* sp. and the toxicity of the association of DOD with a NLS. DOD is soluble in alcohols, but has poor solubility in water. Therefore, we selected it for an association with a NLS. The results showed that this association was very beneficial, since it contributed to reduce the toxicity of this compound on lung fibroblasts and on the zebrafish model. This may be attributed to well known propriety of lipid nanostructured system to encapsulate lipophilic drugs/nutrients in their lipid core with a controlled release capacity. In addition, increased antifungal efficacy was observed in the murine model, which may be due to improved bioavailability of DOD from NLS. Thus, in case of NLS + DOD, the compound concentration is maintained for a prolonged time period in the serum and tissues which subsequently enhanced the therapeutic efficacy of DOD against *P. brasiliensis* in mice (Garg and Singh, 2011). Our study demonstrated that NLS is a potential as a delivery system for the use of DOD as a treatment for systemic mycosis.

Declarations of interest

None.

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