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Copper(II) complex-loaded castor oil-based nanostructured lipid carriers used against *Mycobacterium tuberculosis*: Development, characterisation, *in vitro* and *in vivo* biological assays

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A copper(II) complex-loaded castor oil-based nanostructured lipid carrier was evaluated to enhance the poor water solubility of antimicrobial compounds, improving their biological properties and antimicrobial activity against *Mycobacterium tuberculosis*. Nanostructured lipid carriers were composed of the castor oil, polyoxyethylene 40 stearate and caprylic/capric triglyceride, poloxamer 407, cetyltrimethylammonium bromide and three different copper(II) complexes. The systems were ultrasonicated at an amplitude of 8% for 20 min and an ice bath was used throughout the procedure. The blank nanostructured lipid carrier (F5) and nanostructured lipid carriers loaded with copper(II) complex 1, 2 and 3 (F5.1, F5.2 and F5.3, respectively) for 45 days presented values of mean diameter, polydispersity index and zeta potential ranging from 186 to 199 nm, 0.14 to 0.2 and 24 to 30 mV, respectively. Atomic force microscopy indicated that the nanostructured lipid carriers were distributed at the nanoscale, corroborating the mean diameter data. Differential scanning calorimetry determined the melting points of the constituents of the nanostructured lipid carriers. The antimicrobial activity of copper(II) complex-loaded F5 against *M. tuberculosis* H_{37}Rv showed better anti-tuberculosis activity than the free complexes. *In vivo* biological assays of complex-loaded F5 demonstrated reduced toxicity. Our results suggest that nanostructured lipid carriers could be a potential nanotechnological strategy to optimise tuberculosis treatment.

1. Introduction

Tuberculosis (TB) is currently one of the deadliest infectious diseases in the world. According to the World Health Organisation, around 10 million people worldwide developed TB and there were approximately 1.3 million deaths in 2017 (WHO 2018).

The main obstacles to eradicating TB include the long period of treatment, its adverse effects and the development of multidrug-resistant strains. Moreover, alarming data reinforce the necessity and search for new therapeutic models against *Mycobacterium tuberculosis* (Adediji et al. 2013).

Related to TB treatment, studies have shown that metal-based complexes are biologically active molecules capable of enhancing antimicrobial activity (De Freitas et al. 2014). In this context, copper(II) complexes have shown promising results against *M. tuberculosis*. However, their poor solubility in water is a limiting characteristic of these complexes (Da Silva et al. 2015).

For the purpose of improving the physicochemical characteristics of a drug, its biological properties and its aqueous solubility, nanotechnological strategies for controlled-release drug delivery systems have been investigated (Kaur et al. 2016).

First and second generation nanoparticles, such as solid lipid nanoparticles and nanostructured lipid carriers, are highly promising systems because of their differentiated lipid composition and the formation of matrices (Naseri et al. 2015).

Solid lipid nanoparticles (SLN) are colloidal drug carrier systems obtained from solid lipids that form highly organised structures. Nanostructured lipid carriers are produced by a mixture of solid and liquid lipids at room temperature, forming imperfect lipid particle matrices (Wu et al. 2016).

Thus, matrix imperfections allow the nanostructured lipid carriers to compartmentalise drug molecules, encapsulate lipophilic drugs and achieve physical-chemical stability. They are non-toxic and resistant to degradation, meaning that lower dosages are required and fewer adverse effects are seen. In addition, the lipids used are biocompatible and biodegradable (Singhal et al. 2011; Kolenyak-Santos et al. 2015).

The use of different lipids and the ability to incorporate lipophilic drugs allows nanostructured lipid carriers to improve the biopharmaceutical properties of drugs and solve problems of water/oil solubility, degradation and drug toxicity (Shastri 2017).

Among the lipids employed in the literature, castor oil (the main constituent is ricinoleic acid, around 87-90%) is composed of a fatty acid containing a double bond and a hydroxyl group. This provides interesting chemical properties such as solubility and stability at different conditions of pressure and temperature (Rowe et al. 2011).

Taken together, nanostructured lipid carrier-based drug delivery systems have considerable potential for the treatment of TB (Patil and Deshpande 2018). Moreover, they can be delivered by pulmonary, intravenous and oral routes (Weber et al. 2014). Among these, the oral route is the most commonly used for the treatment of TB, because it allows the patient more convenient drug adminis-

tration, is less painful, is more reliable and is economically feasible (Costa-Gouveia et al. 2017).

The aim of this study was to develop nanostructured lipid carriers for oral administration comprised of caprylic/caprylic triglycerides and castor oil as the liquid lipids and polyoxyethylene 40 stearate as solid lipid; these were combined with copper(II) complexes $[CuCl_2(INH)_2]\cdot H_2O$, $[Cu(NCS)_2(INH)_2]\cdot 5H_2O$ and $[Cu(NCO)_2(INH)_2]\cdot 4H_2O$ in the system. These copper(II) complex-loaded castor oil-based nanostructured lipid carriers were tested against *M. tuberculosis* using *in vitro* and *in vivo* biological assays.

2. Investigations and results

To prepare copper(II) complex-loaded castor oil-based nanostructured lipid carriers, and to characterise and evaluate them, *in vitro* and *in vivo* biological assays against *M. tuberculosis* have been proposed in this study.

The nanostructured lipid carriers were prepared by ultrasonication, according to Sato et al. (2017), with some modifications. Six formulations were developed containing fixed concentrations of the lipids polyoxyethylene 40 stearate, caprylic/capric triglyceride and castor oil and cetyltrimethylammonium bromide. The concentration of the surfactant poloxamer 407 was varied at 1, 2, 2.5, 3, 3.5 and 4% to prepare F1, F2, F3, F4, F5 and F6, respectively. The components and concentrations of nanostructured lipid carriers used in this study are listed in Table 1.

Table 1: Composition of nanostructured lipid carriers

	Composition (%)					
Nanostructured lipid carriers	E-40	CCTG	CO	P-407	CTAB	Ultrapure water*
F1	2.07	2.05	0.88	1	0.50	qs
F2	2.07	2.05	0.88	2	0.50	qs
F3	2.07	2.05	0.88	2.5	0.50	qs
F4	2.07	2.05	0.88	3	0.50	qs
F5	2.07	2.05	0.88	3.5	0.50	qs
F6	2.07	2.05	0.88	4	0.50	qs

*Quantity sufficient to make 2 mL of formulation

The methodology and concentration of surfactant are essential parameters to ensure a homogeneous and stable system (Uner 2006). The ultrasonication process is fast, highly reproducible and frequently used to disperse two immiscible phases, such as lipid and water. Ultrasound is based on the cavitation process, in which cavitational bubbles of the inner phase are generated. The turbulence and high speed of the ultrasound enable better dispersion of the oil phase in the aqueous phase (Patil and Pandit 2007). Thus, this method is effective for the preparation of reduced, homogeneously distributed and physically stable particles (Gonzalez-Mira et al. 2010).

After sonication, F1, F2, F3, F4, F5 and F6 were visually observed to verify organoleptic modifications and phase separation. Formulations F1, F2, F3, F4 and F6 showed organoleptic modifications when observed visually and subsequent phase separation. The study of Das et al. (2012) reported that high concentrations of surfactants between 3% and 3.5% produce physically stable systems. Similar to our results, the formulation composed of poloxamer 407 at 3.5% (F5) did not present phase separation.

These parameters led to the elimination and non-characterisation of these formulations. F5 was then visually analysed and characterised by dynamic light scattering analysis over 45 days of storage.

The copper(II) complexes $[CuCl_2(INH)_2]\cdot H_2O$, $[Cu(NCS)_2(INH)_2]\cdot 5H_2O$ and $[Cu(NCO)_2(INH)_2]\cdot 4H_2O$, designated as1, 2 and 3, respectively, were incorporated into F5 during the pre-emulsion phase at a concentration of 5 mg/mL to prepare F5.1, F5.2 and F5.3, respectively.

Formulations F5. F5.1, F5.2, F5.3 were analysed by dynamic light scattering to determine the mean hydrodynamic diameter (d.nm) and polydispersity index (Fig. 1) for 1, 15, 30 and 45 days.

The blank nanostructured lipid carrier (F5) and nanostructured lipid carriers loaded with copper(II) complexes 1 and 2 showed no

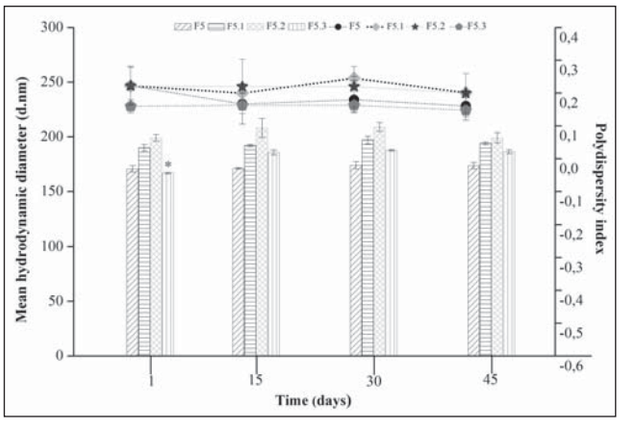

Fig. 1: Mean hydrodynamic diameter (d.nm) and polydispersity index determined by dynamic light scattering analysis. Values are the mean±standard deviation (n = 3). *P < 0.05 represents statistically significant difference among means.

statistically significant differences in mean hydrodynamic diameter over 45 days. F5.3 showed a significant difference in mean diameter on 1 day; however, it remained stable for 45 days. F5 exhibited a mean diameter of 173±3 nm, which was smaller than F5.1, F5.2 and F5.3 and presented mean diameter values at 45 days of 193±1, 199±5 and 186±2 nm, respectively.

According to these results, the particle hydrodynamic diameter was influenced by the addition of the copper(II) complexes into F5, indicating that the complexes were incorporated.

The polydispersity index values of F5, F5.1, F5.2 and F5.3 were not significantly different, i.e. 0.16±0.1, 0.2±0.1, 0.2±0.1 and 0.14±0.1, respectively. This parameter is essential to measure the particle size distribution in relation to the standard deviation. The results indicate that the particles were homogeneously distributed in the nanostructured lipid carrier dispersions (Nemen and Lemos-Senna 2011).

Our results were similar to the study of Da Silva et al. (2015) in which the copper(II) complexes 1-, 2- and 3 incorporated into the microemulsion presented values from 158±1 to 212±1 nm and were larger than the blank nanostructured lipid carriers at 125±2 nm; all formulations presented polydispersity index values of 0.2.

The zeta potential analysis of F5. F5.1, F5.2, F5.3 was performed by dynamic light scattering for 1, 15, 30 and 45 days (Fig. 2).

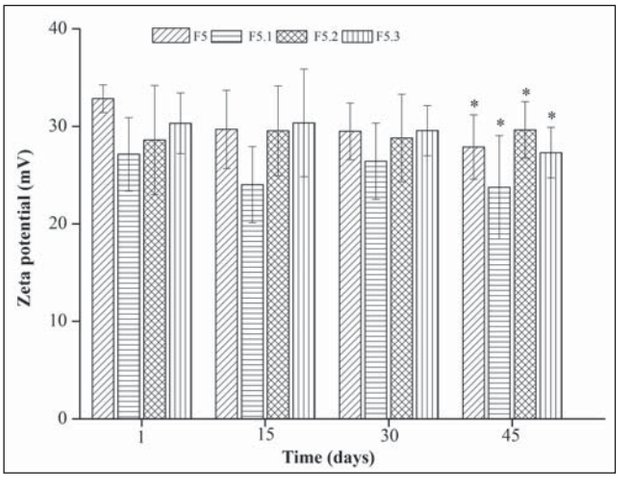

Fig. 2: Zeta potential (mV) measurements by dynamic light scattering analysis. Values are the mean ± standard (n = 3). *P < 0.05 represents statistically significant difference among means.

The measurements indicate the degree of repulsion among the charged particles in the nanostructured lipid carrier dispersion and its surface charge. Zeta potential values of approximately ± 30 mV exhibit repulsive forces, indicating physically stable systems.
F5, F5.1, F5.2 and F5.3 did not present significant differences over 30 days, but showed significant differences at 45 days, 28±3, 24±5, 30±3 and 27±3 mV, respectively (Fig. 2).
The zeta potential of the positively charged nanostructured lipid carriers is related to the cationic surfactant cetyltrimethylammonium bromide, which is formed of a quaternary ammonium. The development of cationic nanostructured lipid carriers is an interesting strategy since it allows for better interaction with the wall of *M. tuberculosis* (negatively charged). The cationic surface may also influence encapsulation of the drug, decreasing its expulsion (Stokes et al. 2004; Ayala-Torres et al. 2014).
Therefore, the stability study over 45 days showed that the nanostructured lipid carriers were nanometers and were comprised of monodisperse positively charged particles. These attributes are mainly related to the use of castor oil, which contains hydroxyl groups in its chemical structure, contributing to the stability of the systems and the solubility of copper(II) complexes in the lipid phase (Costa et al. 2004).
The atomic force microscopic photomicrographs were used to analyse the morphology and surface mean size of the nanostructured lipid carriers (Fig. 3).

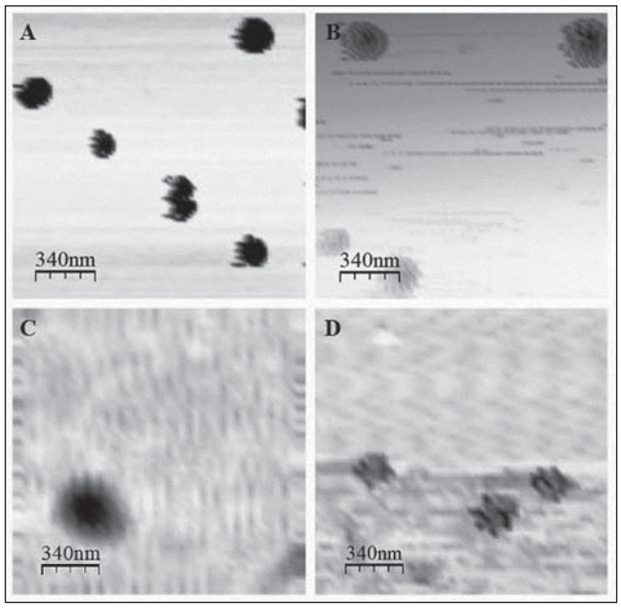

Fig. 3: Atomic force microscopic photomicrographs of the F5 (A), F5.1 (B), F5.2 (C) and F5.3 (D).

The photomicrographs in panels A, B, C and D showed particles with spherical morphology. The particle sizes of both A and B were approximately 150-300 nm. The photomicrographs in panels C and D showed particles of approximately 150-400 nm and 180-300 nm, respectively. These variations may have been caused by the drying process, resulting in the formation of agglomerates with a consequent increase in the size of the spheres (Dubes et al. 2003).
The use of atomic force microscopy allowed for an evaluation of the morphology and particle size, indicating that the ultrasonic processes used in the development of the system were efficient. The particle sizes were distributed in the nanosize range, corroborating the dynamic light scattering data.
The differential scanning calorimetry analysis of the individual components used in the development of nanostructured lipid carriers (polyoxyethylene 40 stearate, caprylic/capric triglyceride, castor oil, cetyltrimethylammonium bromide and poloxamer 407) was reported by Sato et al. (2017).
The melting point values of components agree with previous findings. In addition, the differential scanning calorimetry results for castor oil indicated an endothermic event, related to a melting process at -5.65 °C, which was confirmed by the manufacturer. Copper(II) complexes 1, 2 and 3 did not have a melting peak, indicating that the structure of the complexes is amorphous (Cides et al. 2012).

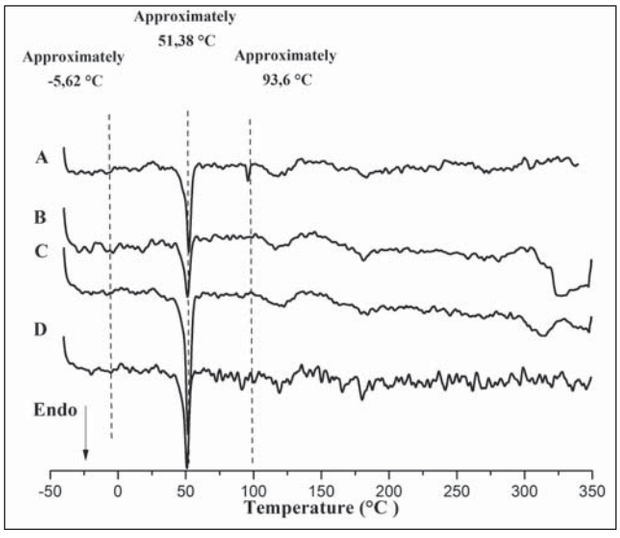

Fig. 4: Differential scanning calorimetry scans of the F5 (A), F5.1 (B), F5.2 (C) and F5.3 (D).

The differential scanning calorimetry scans of the blank nanostructured lipid carriers (F5) and complexes 1-, 2-, and 3-loaded F5 (F5.1, F5.2 and F5.3, respectively), where endo is endothermic (Fig. 4).
The differential scanning calorimetry scans of A, B, C and D showed endothermic events corresponding to the melting point of individual components, such as caprylic/capric triglyceride and castor oil at approximately -5.62 °C, polyoxyethylene 40 stearate and poloxamer 407 at approximately 51.38°C and cetyltrimethylammonium bromide at approximately 93.6 °C, different from the pure melting points at 103.93 °C. These endothermic events were similar to those presented in a previous study (Sato et al. 2017).
This melting temperature variation may occur due to the potential rearrangement of the crystalline structure of the material in the presence of other nanostructured lipid carrier components, which could be an indication of the formation of imperfect crystals. Secondly, Doktorovová et al. (2010) reported these change to occur due to the interaction between surfactants and solid and liquid lipids.
It is worth mentioning that when carried in the nanostructured lipid carriers, the copper(II) complexes did not present a modification in the structure (polymorph).

Table 2: Results of antimicrobial activity of the copper(II) complexes and those loaded nanostructured lipid carriers

Minimum inhibitory concentration (µg/mL)	
Free copper(II) complex and nanostructured lipid carriers	*M. tuberculosis**
Free complex1	104±37
Free complex 2	63±36
Free complex 3	81±38
F5	5±5
F5.1	2±0.1
F5.2	2.5±1
F5.3	3±0.4
Rifampicin	0.4±0.01

*Mean of the results ± standard deviation (n=3)

F5.1, F5.2, F5.3 and complexes 1, 2 and 3 solubilised in dimethyl-sul[hoxide were tested against *M. tuberculosis* H37Rv (ATCC 27294) using the resazurin microtitre assay (Table 2).

The minimum inhibitory concentration (lower concentration capable of inhibiting 90% of the growth of *M. tuberculosis*) of F5 presented a good value compared to other compounds used in the treatment of tuberculosis, like pyrazinamide (50-100 µg/mL).

When carried with the copper(II) complexes, the minimum inhibitory concentration values of the F5.1, F5.2, F5.3 were considerably better (Table 2), demonstrating that the incorporation of the copper(II) complexes improved the inhibitory capacity of the nanostructured lipid carriers.

The classical median lethal dose method, denoted as LD_{50}, was performed in Swiss mice to evaluate the safety profile of copper(II) complex-loaded castor-oil based F5. The acute toxicity assay was followed according to the Organisation for Economic Cooperation Development with modifications (OECD 2011). The percentage survival of groups of animals over 14 days after the single administration of the control groups sunflower oil, F5, free complexes 1, 2 and 3, and F5.1, F5.2 and F5.3 *via* gavage at a dose of 1,000 mg/kg bodyweight was assessed (Fig. 5).

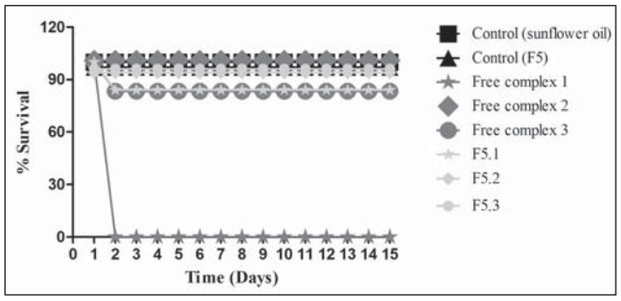

Fig. 5: Results of percentage survival of the acute toxicity assay versus time in days of groups of animals after of single administration of nanostructured lipid carriers and sunflower oil.

In the control groups, sunflower oil and F5 did not induce death in animals. However, 24 h after administration of the dose, the loss of 100% of the mice was observed, demonstrating that the complex was toxic. Therefore, the median lethal dose of free complex 1 could not be determined in the group receiving a dose of 1,000 mg/kg bodyweight.

The animal group that received free complex 2 at the dose of 1,000 mg/kg body weight presented 100% survival; similarly, the group that received complex 3 at the same dose obtained 83.3% survival. Thus, it is not necessary to determine median lethal dose since it is greater than 1,000 mg/kg.

In the group that received F5.1, the death of only one animal was observed after 1 day of administration, resulting in 83.3% survival. In addition, the groups F5.2 and F5.3 showed 100% survival at a dose of 1,000 mg/kg bodyweight. When comparing the value of F5.1 with the value of the group that received free complex 1 solubilised in sunflower oil, we found that the nanostructured lipid carriers reduced the toxicity of free complex 1. Thus, in this group the survival rate was greater than 50%, making it impossible to calculate the median lethal dose.

These results suggest that incorporation of copper(II) complexes in castor oil-based F5 reduced their toxicity, since none of the groups receiving complex 1, 2 or 3- loaded F5, respectively, showed a greater than 50% loss of animals.

To investigate potential liver damage or toxicity in the liver, the liver transaminases aspartate aminotransferase and alanine aminotransferase were measured in the plasma (Fig. 6).

The liver is the organ responsible for metabolising most drugs. The overall aim of hepatic drug metabolism is to produce a more water-soluble compound to facilitate the excretion of the drug. In this sense, measuring the activity of liver enzymes is important in order to verify liver toxicity.

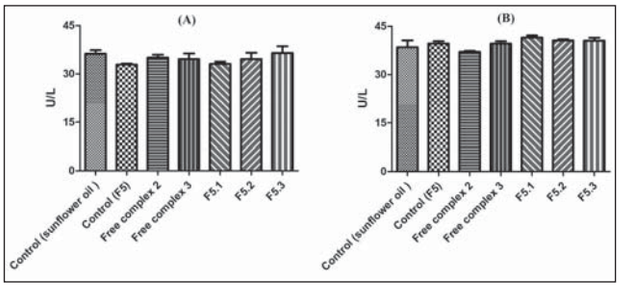

Fig. 6: Results of plasma levels of the transaminases aspartate aminotransferase (A) and transaminases alanine aminotransferase (B) in Swiss mice (units per liter) 14 days after administration of the complexes.

Regarding the results, no statistically significant differences ($P > 0.05$) were observed between sunflower oil and F5 and the groups treated with free complexes 2 and 3 or F5.1, F5.2, and F5.3. The results are in accordance with those of Lopes et al. (2016), although the plasma transaminases aspartate aminotransferase and alanine aminotransferase levels were slightly elevated or close to those of the control groups (sunflower oil and F5), the values are within the acceptable limit. Based on the results of ANOVA and Dunnett's test, the differences were not statistically significant ($P > 0.05$).

These results suggest that Swiss mice treated with free complexes 2 and 3 or F5.1, F5.2 and F5.3 for over 14 days did not suffer from liver toxicity, so it can be inferred that the castor oil used in the nanostructured lipid carriers is safe and biocompatible.

3. Discussion

The aim of this study was to develop blank nanostructured lipid carriers and copper(II) complexes 1-, 2- and 3-loaded castor oil-based nanostructured lipid carriers for oral administration. The systems were tested against *M. tuberculosis* using *in vitro* and *in vivo* biological assays. The results demonstrated particle diameters of copper(II) complexes 1-, 2- and 3-loaded nanostructured lipid carriers at the nanometric scales and are physically stable over 45 days, according to the data of dynamic light scattering and microscopy. The antimicrobial activity assay showed that copper(II) complex-loaded nanostructured lipid carriers presented better antimicrobial activity against *M. tuberculosis* than the free complexes. The acute toxicity assay made it possible to observe a lower toxicity of nanostructured lipid carriers when incorporated in the copper(II) complexes In addition, the plasma levels of the transaminases aspartate aminotransferase and alanine aminotransferase underwent no significant changes, confirming the *in vivo* results. These findings show that copper(II) complex-loaded castor oil-based nanostructured lipid carriers presented alterations only in their physicochemical properties, without differences in the biological capacity to inhibit the growth of *M. tuberculosis* compared to previous studies investigating nanostructured lipid carriers composed of polyoxyl 40 hydrogenated castor oil.

4. Experimental

4.1. Chemicals and reagents

Nanostructured lipid carriers were constituted by polyoxyethylene 40 stearate (Sigma-Aldrich, Missouri, USA), caprylic/capric triglyceride (Via Farma, São Paulo, Brazil), castor oil (Pharma Special, São Paulo, Brazil), cetyltrimethylammonium bromide (Sigma-Aldrich, Missouri, USA) and poloxamer 407 (Sigma-Aldrich, Missouri, USA). The copper(II) complexes were synthesised used isoniazid (Sigma-Aldrich, Missouri, USA), sodium thiocyanate and potassium cyanate (Sigma-Aldrich, Missouri, USA). Middlebrook 7H9 broth supplemented with OADC (Becton-Dickinson, NJ, USA), resazurin and rifampicin (Sigma-Aldrich, Missouri, USA) was used to investigate the *in vitro* antimicrobial activity. Ultrapure water (Millipore MilliQ, Massachusetts, USA) was used to prepare all reagents in this study.

4.2. Preparation of nanostructured lipid carriers

A pre-emulsion was formed in that the molten lipid phase was poured onto aqueous phase, which was heated to about 5-10°C above its melting point (70 °C). The formulations were ultrasonically treated (Q700 Sonicator, Qsonica, Sonicator ultrasonic

liquid processors, CT, USA) operated at an amplitude of 8%; $^1/_{16}$ diameter probe for 20 min in an ice bath throughout the procedure and subsequent centrifugation (Spectrafuge™, 16 M Microcentrífuga, Labnet, USA) at 5,000 rpm for 10 min (Agayan et al. 2004; Rigon et al. 2016).

Six nanostructured lipid carriers were prepared (F1, F2, F3, F4, F5 and F6) containing such as components caprylic/capric triglycerides, castor oil, polyoxyethylene 40 stearate, cetrimonium bromide and poloxamer 407. The systems were refrigerated at 4±2 °C.

4.3. Synthesis of copper(II) complexes

Three copper(II) complexes were synthesised according to Da Silva et al. (2015) using as ligands isoniazid (INH), sodium thiocyanate (NaSCN) and potassium cyanate (KCNO): $[CuCl_2(INH)_2]\cdot H_2O$, $[Cu(NCS)_2(INH)_2]\cdot 5H_2O$ and $[Cu(NCO)_2(INH)_2]\cdot 4H_2O$, respectively. These complexes were designated as copper(II) complexes 1, 2 and 3. Employing the same components of nanostructured lipid carriers and following its methodology, the copper(II) complexes were added to F5 at a concentration of 5 mg/mL out during the pre-emulsion phase, preparing F5.1, F5.2 and F5.3.

4.4. Dynamic light scattering analysis

Dynamic light scattering analysis (Zetasizer Nano NS, Malvern Instruments, Malvern, UK) was used to determine the mean hydrodynamic diameter (d.nm) and polydispersity index after 1, 15, 30 and 45 days. Zeta potential (mV) was measured using the same equipment and parameters. Nanostructured lipid carrier dispersions were diluted in ultrapure water (1:100). The analysis was performed in triplicate at 25 °C and results were expressed as mean±standard deviation of the n=3.

4.5. Microscopic analysis

Determination of the morphology of nanostructured lipid carriers was achieved using intermittent-contact mode atomic force microscopy (Dimension Icon, Bruker). A drop of the system was added on the mica, spin-coated at 500 rpm for 30s and 5,000 rpm for 1 min using the spin coater centrifuge (SPI Supplies, Model KW-4A). The photomicrographs were obtained using a frequency of 1 Hz.

4.6. Differential scanning calorimetry

Differential scanning calorimetry was performed using a TA Instruments Model DSC Q100 analyser. Thermal events were done in a high-purity nitrogen gas with a flow rate of 50 mL/min. Here, 5 mg of each lyophilised sample was heated from -50 to 350 °C at a heating rate of 10 °C/min.

4.7. In vitro and in vivo biological assays

4.7.1. Minimum inhibitory concentration

The resazurin microtitre assay was employed to determine the minimum inhibitory concentration of the complexes against *M. tuberculosis*. The complexes were prepared at 5 mg/mL in dimethyl-sulphoxide and the solutions were diluted with Middlebrook 7H9 broth supplemented with OADC (oleic acid, albumin, dextrose, and catalase) obtained by Precision XS (Biotek®, VT, USA), in the drug concentration range from 0.09 to 25 µg/mL. As a standard drug, rifampicin in dimethyl-sulphoxide was used. An inoculum of *M. tuberculosis* H37Rv (ATCC 27294) was cultured in Middlebrook 7H9 broth media supplemented with OADC and 0.05% Tween 80. After 7 days, in a turbidity of McFarland standard Nº. 1, the culture was diluted 1:20 and 100 µL was added to each well of a 96-well microplate with 100 µL of the complexes at the concentrations described. The plates were incubated for 7 days at 37 °C and 5% CO_2. After this time, 30 µL of resazurin, at a concentration of 0.01% dissolved in water was added and the well fluorescence was read using a Cytation 3 (Biotek®, VT, USA). The minimum inhibitory concentration is the lowest concentration that inhibits 90% of the growth of *M. tuberculosis*. The results were obtained as the mean ± standard deviation of three independent experiments (Palomino et al. 2002).

4.7.2. Acute toxicity assay

The National Council for the Control of Animal Experimentation – CONCEA (Federal Constitution Law Nº. 11794/2008) establishes "Develop and review standards for the care and use of animals for educational or scientific purposes, according to the international conventions to which Brazil is a signatory". All animal experiments were carried out accordance with the U.K. Animals (Scientific Procedures) Act, and associated guidelines, EU Directive 2010/63/EU for animal experiments. All animal experiments were approved by the Ethics Committee on the Use of Animals – CEUA Nº. 78/2015, of the Department of Drugs and Medicines, School of Pharmaceutical Sciences, UNESP – State University of São Paulo, Araraquara, Brazil.

The acute toxicity assay followed the Organisation for Economic Cooperation Development with modifications (OECD 2011). Female Swiss mice were purchased from Botucatu Central Biotério, aged 4-8 weeks old, which received 20-40 g food and water ad libitum. Swiss mice were maintained in polycarbonate cages at 23±2 °C, 56±2 % humidity under a 12h light/dark cycle and specific pathogen-free conditions. For the control groups, sunflower oil and F5 was administered. The dose of the complexes was 1,000 mg/kg bodyweight *via* gavage as a single oral dose (n = 6 animals/group). Blood samples (500 µL) were collected from the submandibular vein after 14 days (Lopes et al. 2016). Then, the samples were placed in tubes, centrifuged at 1097.6 × g for 15 min to separate the plasma, stored at -70°C and analysed on the day of collection. The mice were euthanised in CO_2 chambers.

4.7.3. Enzymatic activity assay

In order to investigate indicative changes in the liver, the enzymatic activity of aspartate aminotransferase and alanine aminotransferase was measured in the plasma of Swiss mice 14 days after the administration of complexes. The same methodology described in acute toxicity to collect blood samples were performed (Golde et al. 2005). The enzymes were assayed using kits (Labtest Diagnóstica AS) and analyses followed the guidelines of the kits.

4.8. Statistical analysis

Data were analysed by ANOVA followed by Tukey's post hoc test using GraphPad Prism 6.0 software. *In vivo* biological assays were evaluated by ANOVA followed by Dunnett's test (GraphPad Prism 6.0 software). $P < 0.05$ was considered statistically significant.

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Conflicts of interest: None declared.

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