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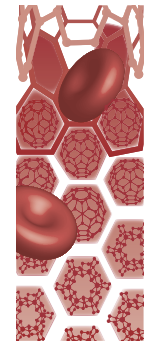


Licochalcone A-loaded solid lipid nanoparticles improve antischistosomal activity *in vitro* and *in vivo*

Lívia Mara Silva[1], Danielle Gomes Marconato[2], Marcos Paulo Nascimento da Silva[3], Nádia Rezende Barbosa Raposo[1], Gabriela de Faria Silva Facchini[4], Gilson Costa Macedo[4], Fernanda de Sá Teixeira[5], Maria Cecília Barbosa da Silveira Salvadori[5], Priscila de Faria Pinto[2], Josué de Moraes[3], Frederico Pittella[1] & Ademar Alves Da Silva Filho*[,1]

[1]Department of Pharmaceutical Sciences, Faculty of Pharmacy, Federal University of Juiz de Fora, Juiz de Fora - MG, 36036-900, Brazil

[2]Department of Biochemistry, Biological Sciences Institute, Federal University of Juiz de Fora, Juiz de Fora - MG, 36036-900, Brazil

[3]Research Center for Neglected Diseases, Guarulhos University, Guarulhos, 07025-000, SP, Brazil

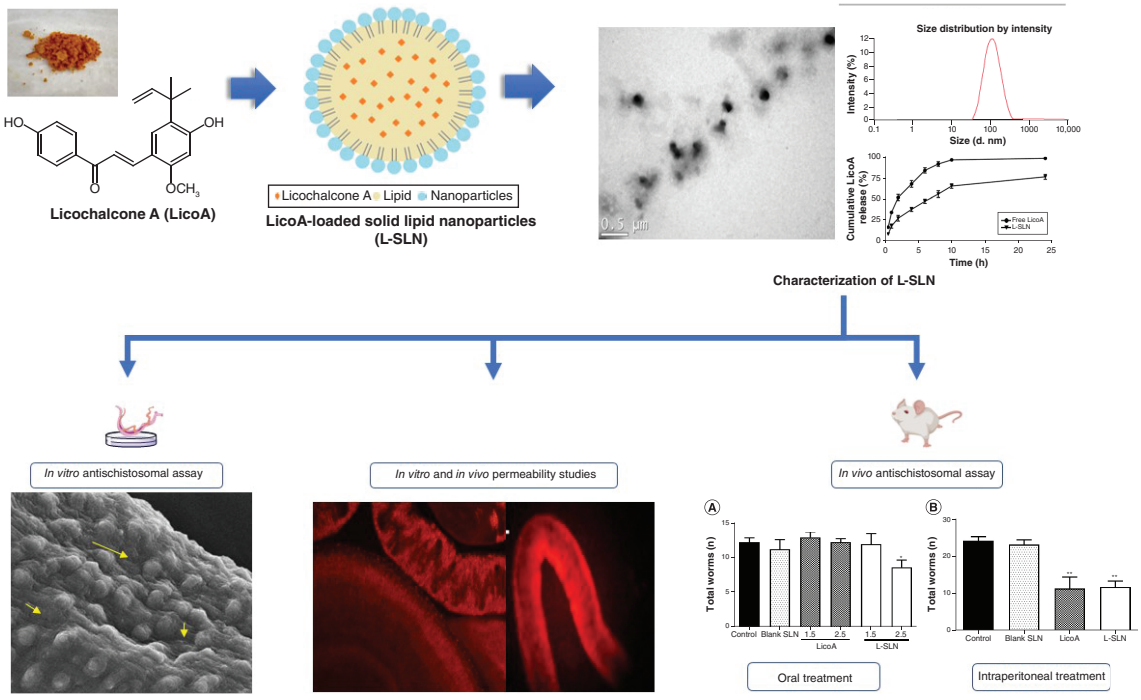
[4]Department of Parasitology, Microbiology & Immunology, Biological Sciences Institute, Federal University of Juiz de Fora, Juiz de Fora - MG, 36036-900, Brazil

[5]Instituto de Física, Universidade de São Paulo, São Paulo, SP, Brazil

*Author for correspondence: Tel.: +55 322 102 3893; ademar.alves@ufjf.edu.br

Aim: To isolate licochalcone A (LicoA) from licorice, prepare LicoA-loaded solid lipid nanoparticles (L-SLNs) and evaluate the L-SLNs *in vitro* and *in vivo* against *Schistosoma mansoni*. **Materials & methods:** LicoA was obtained by chromatographic fractionation and encapsulated in SLNs by a modified high shear homogenization method. **Results:** L-SLNs showed high encapsulation efficiency, with satisfactory particle size, polydispersity index and Zeta potential. Transmission electron microscopy revealed that L-SLNs were rounded and homogenously distributed. Toxicity studies revealed that SLNs decreased the hemolytic and cytotoxic properties of LicoA. Treatment with L-SLNs showed *in vivo* efficacy against *S. mansoni*. **Conclusion:** L-SLNs are efficient in reducing worm burden and SLNs may be a promising delivery system for LicoA to treat *S. mansoni* infections.

Graphical abstract:



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Schistosomiasis, caused by trematode flatworms of the genus *Schistosoma*, is a neglected tropical disease that is present in more than 70 tropical and subtropical countries, harming over 250 million people and threatening 800 million people who are at risk of infection [1]. Currently, only one drug, praziquantel (PZQ), is available for the treatment of schistosomiasis, which unfortunately has a limited effect on granulomatous lesions and is inactive against juvenile *Schistosoma* [2,3].

Furthermore, the exclusive dependence on only one drug, as well as the widespread use of PZQ in both humans and animals, along with the frequent reported cases of reduced susceptibility of field and laboratory worms to PZQ, raise serious concerns about the risk of drug-resistant strains [4]. Therefore, new antischistosomal drugs and technologies are needed to complement or to replace PZQ chemotherapy against *Schistosoma* [3,5].

Many compounds with promising antischistosomal properties have been identified in recent years, especially from natural sources [6,7]. Among them, licochalcone A (LicoA, Figure 1A) is a chalcone found in the roots of licorice (*Glycyrrhiza inflata*, Leguminosae) that exhibits remarkable biological activities, such as anti-inflammatory [8,9], anticancer [10], antibacterial [11], anti-*Toxoplasma gondii* [11] and antimalarial properties [12,13]. Also, a recent study evaluated the *in vitro* effects of LicoA on adult *S. mansoni* worms, showing high mortality in the schistosomes and pronounced damage to female and male teguments [14]. However, LicoA has not been yet evaluated *in vivo* against *Schistosoma* spp.

Although it may be a promising lead compound against several diseases, LicoA has some characteristics that may limit its therapeutic use, such as poor solubility in water and possible hemolytic effects [15,16]. To overcome this restriction, technological alternatives can be developed to improve the biopharmaceutical properties of LicoA, such as the use of nanoparticles as drug carriers [17–19]. For example, solid lipid nanoparticles (SLNs) have emerged as lipophilic drug-delivery system and have gained huge attention in the biopharmaceutical field [18,20]. The use of SLNs has been shown to improve drug protection, physical stability, as well as the capability to control and target the drug delivery [18,20,21]. As fatty acids are the major components of SLNs that have been prepared using naturally balanced seed butter, they may promote the oral absorption of poorly water-soluble drugs [19,20,22], as well as extend drug release after intraperitoneal administration [20].

The aim of this study was to evaluate the schistosomicidal properties of LicoA and LicoA-loaded solid lipid nanoparticles (L-SLNs), formulated using cupuaçu (*Theobroma grandiflorum*) butter. Thus, in the present study, we isolated LicoA from the extract of *G. inflata* and evaluated its *in vivo* antischistosomal properties. In addition, we prepared and characterized L-SLNs and assessed their antischistosomal activities *in vitro* and *in vivo* by using oral and intraperitoneal routes in mice infected with *S. mansoni*.

Materials & methods

Materials

The dried extract of *G. inflata* was provided by Shanghai OpenChem International Co., Ltd. (Shanghai, China). Cupuaçu (*T. grandiflorum*) butter, used as lipid matrix, was acquired from Mercado Ver o Peso in Belém, Pará (Brazil). As surfactant, polyoxyethylene-polyoxypropylene copolymer (Pluronic F-127®) was purchased from Sigma-Aldrich (MO, USA).

Isolation of LicoA

Dried extract of *G. inflata* (10 g) was chromatographed over silica gel using a vacuum liquid chromatography system and hexane: ethyl acetate mixtures in increasing proportions as eluents, furnishing five fractions. Fractions IV (1.26 g) and V (0.75 g) were solubilized in 1.0 M sodium hydroxide basic solution at pH 12.0. After, they were filtered and acidified with 1.0 M hydrochloric acid to pH 3.0, generating a yellow precipitate corresponding to LicoA (0.20 g). Chemical structure of LicoA was established by ^{1}H- and ^{13}C-NMR data analysis in comparison with the literature. The purity of LicoA was more than 95% based on ^{13}C-NMR data and HPLC analysis.

Preparation of solid-lipid nanoparticles

SLNs were prepared by a modified high shear homogenization method, according to Soldati *et al.* [23]. Briefly, cupuaçu (*T. grandiflorum*) seed butter (1% w/v) and LicoA (0.05% w/v) were used as the lipid phase and a

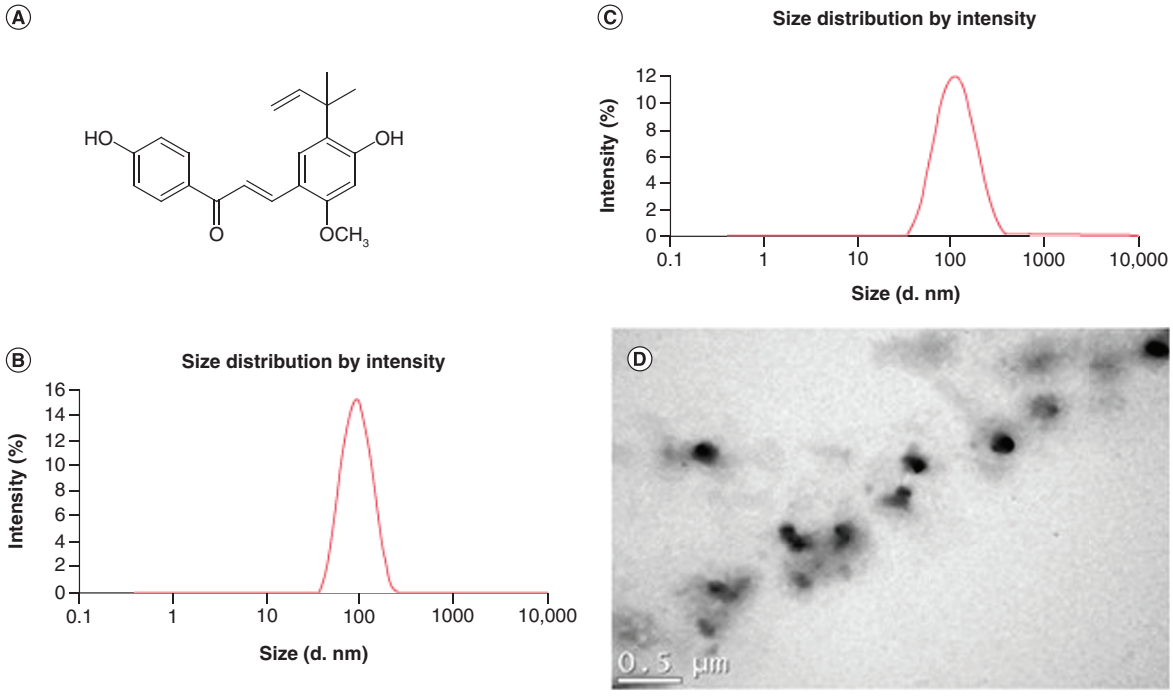

Figure 1. Chemical structure of LicoA and characterization of SLNs. (A) Chemical structure of Licochalcone A. **(B)** Blank-SLN particle size distribution curve by intensity. **(C)** LicoA-loaded solid lipid nanoparticle particle size distribution curve by intensity. **(D)** Transmission electron microscopy image of LicoA-loaded solid lipid nanoparticle.

Table 1. Composition and physicochemical properties of solid lipid nanoparticles.

	Composition % (m/v)			Physicochemical properties				
	CB	F127	LicoA	PS (nm)	PdI	ZP (mV)	EE (%)	pH
Blank SLN	1.0	0.7	–	89.66 ± 1.3	0.23 ± 0.04	-34.8 ± 3.6	–	6.26
L-SLN	1.0	0.7	0.05	101.07 ± 1.8	0.17 ± 0.01	-32.34 ± 2.9	98.33	6.32

Data are expressed as the mean ± SD (n = 3).
Blank SLN: Blank-solid lipid nanoparticle; CB: Cupuaçu seed butter; EE: Encapsulation efficiency; F127: Pluronic F-127; LicoA: Licochalcone A; L-SLN: LicoA-loaded solid lipid nanoparticle; PdI: Polydispersity index; pH: Potential of hydrogen; PS: Particle size; SD: Standard deviation; ZP: Zeta potential.

suspension of Pluronic F-127 (0.7% w/v) as the aqueous phase (Table 1). Both phases were heated to 10°C above the lipid's melting point (45°C). The aqueous phase was added in the organic phase under high shear homogenization at 8000 r.p.m. (Ultra-Turrax®, T 25, IKA, Königswinter, Germany) for 15 min, followed by 5 min of 30% intensity sonication (Vibra Cell, Sonics, CT, USA). The obtained nanodispersions were maintained at room temperature to recrystallize the lipid core toward the formation of L-SLNs.

Physicochemical characterization of solid lipid nanoparticles & colloidal stability

The mean particle size and polydispersity index (PdI) were measured by dynamic light scattering at fixed angle of 25°C and Zeta potential was determined by electrophoretic mobility (Zetasizer NanoZS, Malvern Instruments, Malvern, Worcestershire, UK). The dispersion was diluted 1:100 in purified water before analysis and the measurements were performed in triplicate [23,24]. Considering the *in vitro* antischistosomal evaluation of SLNs, the influence of the RPMI 1640 culture medium and 0.5% dimethyl sulfoxide (DMSO) on physicochemical parameters of L-SLNs was also evaluated. In addition, the stability of SLNs, containing LicoA, upon storage at 4 and 25°C was assessed for 30 days. Particle size, PdI and Zeta potential were evaluated [23,24].

Transmission electron microscopy

Morphology of L-SLNs was examined by transmission electron microscopy using JEOL, JEM-1011 Electron Microscope (Tokyo, Japan). Before analysis, L-SLNs were sprayed onto copper grids [23]. After drying with nitrogen flow, particles were visualized at 50,000-times magnification at 80 kV.

Encapsulation efficiency

The encapsulation efficiency (%EE) assay was carried out after the separation of SLNs by ultrafiltration. The encapsulation rate was calculated indirectly by determining the concentration of free LicoA (i.e., nonencapsulated) in the aqueous phase of ultrafiltrate. Briefly, 2.0 ml of L-SLN dispersion was added to Amicon device (Millipore®, MWCO 30 KDa), and centrifuged for 10 min at 4000× *g*. The Amicon membrane used in the experiment allows the flow of molecules smaller than 30 KDa through the pores and prevents the escape of larger molecules. Thus, SLNs were trapped in the device, while the released components cross the barrier. Then, LicoA was quantified in the ultrafiltrate by HPLC. HPLC with DAD detector (Waters 2998, Waters Instruments, MA, USA), binary HPLC pump (Waters 1525) and an autosampler (Waters 2707) were used. The analytical column used was a SunFire C_{18} column (5 µm particle size, 4.6 × 250 mm, Waters) with a SunFire C_{18} precolumn (5 µm particle size, 4.6 × 20 mm, Waters). The isocratic mobile phase used was methanol: water (65:35 v/v), acidified with 0.1% (v/v) acetic acid, with injection volume of 50 µl, flow of 1 ml/min, column temperature of 40° C and UV detection at 382 nm. LicoA concentration was calculated using calibration standards. The analyses were done in triplicate. The coefficient of determination (R^2) exceeded 0.99 with excellent linearity.

In vitro release studies

The LicoA release assay was performed by dialyzing the suspension at 37°C, under 100 r.p.m. A 0.9% w/v sodium chloride solution, containing 10% (v/v) ethanol to maintain the sink condition, was used as receptor solution [25]. Samples of L-SLNs were distributed in dialysis tubing (Servapor®, 12,000–14,000 MWCO), previously hydrated and properly closed, and submerged in the receptor solution [25]. Aliquots of receptor solution were collected, with medium replacement, at time intervals of 0, 1, 2, 4, 6, 8, 10 and 24 h and quantified by UV spectroscopy (Spectrophotometer SP 1105, Bel Photonics®, Italy), with detection at 382 nm. Cumulative amounts of released LicoA were plotted as a function of time to determine the release profile.

In vitro cytotoxic & hemolytic activities

Cytotoxicity of free LicoA, L-SLNs and blank SLNs was determined in macrophages (strain J774), using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, according to previous report [26], using three independent experiments in duplicate. The values of cytotoxicity concentration to reduce 50% of viable cells (CC_{50}) were obtained using GraFit Version 5 software.

Hemolytic activity was evaluated for samples of blank SLNs, L-SLNs and free LicoA, according to the method described by Wang *et al.* [27] with modifications. Fresh samples of red blood cells were separated by centrifugation (Refrigerated Centrifuge NT 815, Nova Tecnica®, Brazil) at 2000× *g* for 5 min, washed three times with 0.9% (w/v) sodium chloride solution and suspended in 10 ml of saline buffer (red cells corresponding to 1 ml of blood). Aliquots of L-SLNs and free LicoA (prepared in 1.5% glycerol solution) were added to 10% (v/v) erythrocyte suspensions. Positive control was prepared with addition of purified water and negative control with addition of 0.9% (w/v) sodium chloride solution. Samples were incubated for 1 h at 37°C and centrifuged for 5 min at 2000× *g*. The supernatant was removed and analyzed by UV/Vis spectrometry at 415 nm (Spectrophotometer SP 1105, Bel Photonics). The percentage hemolysis rate was obtained by the formula:

$$\%\ \text{Hemolysis} = [(\text{ABS sample} - \text{ABS negative})/(\text{ABS sample} - \text{ABS positive})] \times 100$$

GC–MS analysis of cupuaçu (*T. grandiflorum*) seed butter

GC–MS analysis of cupuaçu was conducted according to the methods in the Supplementary Information.

Antischistosomal assays

Maintenance of the S. mansoni life cycle

S. mansoni (BH strain) was maintained by passage through *Biomphalaria glabrata*, as the intermediate host, and Swiss female mice (Anilab, São Paulo, Brazil) as definitive host as previously described [28]. Both mice and snails

were kept under environmentally controlled conditions (temperature, 25°C; humidity, 50%; standard food and water *ad libitum*).

In vitro antischistosomal assay

Seven weeks postinfection *S. mansoni* were removed from the hepatic portal system and cultured in RPMI 1640 culture medium supplemented with 5% inactivated fetal calf serum and 100 U/ml penicillin and 100 µg/ml streptomycin (Vitrocell, Campinas, São Paulo, Brazil) at 37°C in an atmosphere of 5% CO_2 until usage. Parasites were transferred to 24-well culture plates containing one pair per well in RPMI 1640 medium, supplemented with 10% fetal bovine serum (Vitrocell) and incubated in 37°C and 5% CO_2 atmosphere. Free LicoA, L-SLNs and blank SLNs were diluted in DMSO and used to obtain the equivalent concentrations of 100, 50, 25 and 12.5 µM of LicoA. Blank SLNs were diluted similarly to L-SLNs to obtain equivalent volumes. All samples were tested in triplicate with one worm of each sex placed into each well. Wells with the highest concentration of DMSO in medium (0.5%) served as controls. RPMI 1640 medium plus 0.5% DMSO and RPMI 1640 medium were used as negative control groups, while PZQ (2 µM) served as a positive control. RPMI 1640 medium plus 0.5 % DMSO was used as vehicle. Parasites were kept for 72 h (37°C, 5% CO_2) and their viability and motor activity were assessed in real time using a light microscope (Leica Microsystems, Wetzlar, Germany) [28,29]. Changes in worm motor activity were assessed qualitatively as slight or significant in comparison with the negative control groups [30].

Scanning electron microscopy studies

Tegumental alterations in adult *S. mansoni* worms were performed for LicoA and L-NLS using SEM, according to method described previously [31]. After death, parasites (three schistosomes per experimental group) were fixed in 1 ml of 2.5% glutaraldehyde for 3–24 h at room temperature. Specimens were air-dried, mounted on stubs and metalized with gold using a Sputter Coater. Samples were then visualized using a high-resolution SEM accelerating voltage of 20 kV (Jeol-JSM-6460LV).

In vivo antischistosomal studies after oral & intraperitoneal treatments

Female Swiss mice (4–7 weeks), weighing approximately 20 g, were obtained, and maintained in Schistosomiasis Laboratory of the René Rachou Research Center and Adolfo Lutz Institute. Mice were housed under controlled conditions (22°C; 50% relative humidity; 12/12 h light/dark cycle; standard food and water *ad libitum*). Each mouse was infected subcutaneously with approximately 70 *S. mansoni* cercariae (LE strain). In oral administration experiment, 63 animals were used (seven per group), while in the intraperitoneal treatment, 20 animals were used (five per group).

For oral gavage administration, groups of seven infected mice received LicoA (1.5 or 2.5 mg/kg) or L-SLNs (with the same equivalent doses of 1.5 or 2.5 mg/kg of LicoA) in double doses on days 45 and 52 postinfection. The first group (control group) received no treatment, while the second group received blank SLNs.

In the intraperitoneal treatment, animals received intraperitoneal multiple doses (five daily doses) of L-SLNs (equivalent doses of 5 mg/kg of LicoA) or LicoA (5 mg/kg), which was injected in 1% DMSO plus 1% Tween 80 in saline. All treatments were done on days 49–53 postinfection. Surviving schistosomes residing in the mesenteric veins and the liver were counted and sexed as previously described [28]. Additionally, the assessment of the therapeutic efficacy was also based on the technique of qualitative and quantitative oograms [28].

Randomization & blinding

Animals were randomly assigned to the experimental groups, and pharmacological *in vivo* treatments were counterbalanced randomly as well. The animals were euthanized in a random manner inside a group and all parameters were conducted by different people, done by two different investigators. Therefore, operators of experiments were not the same as the data analysts, to eliminate bias in interpretation [28].

In vitro & in vivo permeability studies with SLNs in the tegument of S. mansoni

The permeability of the SLN system in adult *S. mansoni* worms was studied using SLNs labeled with Nile Red (NR), a lipophilic fluorescent pigment [32]. Preparation of NR-solid lipid nanoparticles (NR-SLNs) was performed as previously described to L-SLNs, by replacing the LicoA with NR.

To determine the *in vitro* permeability, NR-SLNs (equivalent NR concentrations of 50 µM) were diluted with RPMI-1640 medium and added to wells containing schistosomes. After 2 h of incubation at 37°C and 5% CO_2

atmosphere, adult schistosomes were washed in RPMI 1640, and observed under florescence microscopy at 552 nm (excitation) and 578 nm (emission) [32]. Blank SLNs were used as a negative control.

In addition, the *in vivo* permeability of the SLN system was determined in Female Swiss mice (4–7 weeks), weighing approximately 20 g, which were housed under controlled conditions (22°C; 70% relative humidity; 12/12 h light/dark cycle; standard food and water *ad libitum*). Each mouse was infected subcutaneously with approximately 70 *S. mansoni* cercariae (LE strain). On day 45 postinfection, 2 h before euthanasia, mice received an intraperitoneal dose of NR-SLNs and the animals were sacrificed. Parasites were collected after perfusion, washed and examined by fluorescence microscopy at 552 nm (excitation) and 578 nm (emission) [32]. Images were taken using an inverted fluorescent microscope (Axio Scope, A1 ZEISS, Jena, Germany) equipped with a monochrome camera. Pictures were recorded setting the camera integration time to 10 ms [32].

Ethics statement

All experiments were conducted in conformity with the Brazilian Law for Guidelines for Care and Use of Laboratory Animals (Law 11790/2008). All experiments were carried out by the Animal Care Ethics Committee of the Adolfo Lutz Institute (São Paulo, Brazil) and René Rachou Research Center – Fiocruz (Minas Gerais, Brazil), in accordance with nationally and internationally accepted principles for use and care of laboratory animals (CEUA, 05/2015; 031/2017; LW21/16). Animal studies are reported in compliance with the ARRIVE guidelines.

Statistical analysis

All statistical analyses were performed using Graph Pad Prism software 5.0 (Graphpad software Inc., CA, USA). For *in vivo* experimental analysis, a parametric Dunnett's multiple-comparison test was used to analyze the statistical significance of differences between mean experimental and control values. p-values < 0.05 were considered significant. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology [28,31].

Results

Isolation of licoA

LicoA (Figure 1A) was obtained from the dried extract of *G. inflata* by a few steps of chromatographic fractionation. Using ^{1}H and ^{13}C NMR data analysis (Supplementary Figures 1 & 2) in comparison with the literature [33], the identification of LicoA was confirmed in high purity, as indicated by ^{13}C NMR and HPLC analysis.

Preparation, characterization & colloidal stability of solid lipid nanoparticles

LicoA was encapsulated in SLNs by the high shear homogenization method using cupuaçu seed butter and Pluronic F-127 as surfactant. As shown in Table 1 and in the DLS size distribution curve (Figure 1B & C), L-SLNs exhibited mono dispersed characteristic with a Z-ave of 101.1 ± 1.8 nm and PdI of 0.17 ± 0.01, with narrow distribution. Blank SLNs, used as control, presented a size of 89.6 ± 1.3 and PdI of 0.23 ± 0.04. Also, NR-SLNs, used to determine the permeability of SLNs in adult *S. mansoni*, presented a size of 81.28 ± 0.5 and PdI of 0.15 ± 0.02. Also, GC–MS analysis showed that the cupuaçu seed butter composition was composed mainly of palmitic and oleic acids (Supplementary Table 1 & Supplementary Figure 3).

The morphology of L-SLNs was also evaluated by transmission electron microscopy (Figure 1D). It was observed that the SLNs were rounded and homogenously distributed, which agree with measurements by DLS. Also, L-SLNs and blank SLNs presented Zeta potential values of -32.34 ± 2.9 and -34.8 ± 3.6 mV, respectively. In fact, L-SLNs retained their physicochemical characteristics after storage for 30 days at both 4 and 25°C (Supplementary Table 2). No significant alterations ($p > 0.05$) were observed in Zeta potential, PdI and particle size values of blank SLNs and L-SLNs. Besides, the influence of the RPMI 1640 culture medium and DMSO on L-SLNs was evaluated. After incubation of L-SLNs with RPMI 1640 medium and 0.5% DMSO at 37°C, no changes were observed in Zeta potential, particle size and PdI, as well as in the culture medium (data not shown). Additionally, %EE exceeded 98.0% in L-SLN formulations (Table 1), with an average drug payload greater than 0.43 mg/ml.

Moreover, the release profiles of L-SLNs and free LicoA are shown in Figure 2. Free LicoA diffused rapidly through the dialysis membrane, completing 97% of the drug concentration in 10 h. L-SLNs presented a non-linear profile, with an initial burst release of 27.94% ± 0.60 in 2 h, followed by a sustained release. The process completed 76.97% ± 1.54 of LicoA release in 24 h.

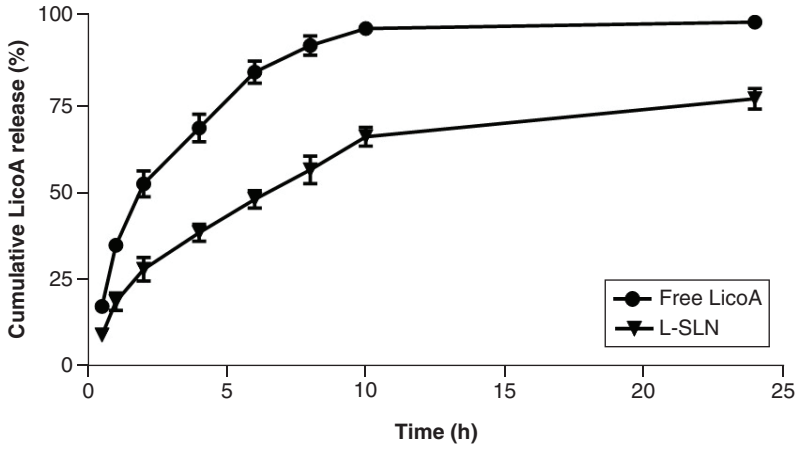

Figure 2. *In vitro* release profiles of loaded solid lipid nanoparticles in 0.9% w/v saline solution for 24 h at 37°C.
Data are expressed as the mean ± standard deviation (n = 3).
LicoA: Licochalcone A; L-SLN: LicoA-loaded solid lipid nanoparticle.

In vitro antischistosomal, cytotoxic & hemolytic activities of free LicoA & L-SLN

At 25–100 μM, free LicoA exhibited noticeable schistosomicidal activity, causing 100% mortality and significant decrease in motor activity of all schistosomes after 24 h of incubation (Table 2). While L-SLNs (at the same LicoA concentrations) were also able to produce the same schistosomicidal effects, killing all adult worms, this was only after 48 or 72 h of incubation. PZQ (2 μM), used as a reference drug, caused 100% mortality within 24 h, whereas no effects were observed in worms in the blank SLNs, vehicle (RPMI medium plus 0.5 % DMSO) and control groups (RPMI 1640 medium).

Also, as shown in Table 2, free LicoA showed potential cytotoxic effects against J774 macrophages (CC_{50}: 20.7 μM) and hemolytic properties against red blood cells (RBC_{50} <1 mM), while no significant cytotoxic (CC_{50}: 956.2 μM) or hemolytic (RBC_{50} >5 mM) potentials were observed in L-SLNs (Table 2).

In addition to light microscopy, results by SEM (Figure 3) showed that free LicoA caused intense reduction of tubercles and spines and small erosion in tegument of the parasites (Figure 3C & D). Similarly, male adult schistosomes treated with L-SLNs (Figure 3E & F) also showed tegumental damages, with intense desquamation in the tegument, which appeared eroded and deformed, with rupture and/or disintegration of spines along the surface of worms, while blank SLNs (Figure 3B), and nontreated adult worms (Figure 3A) showed intact surfaces, with the tegument covered by tubers and spines.

In vivo antischistosomal properties of free LicoA & L-SLNs

After oral treatment with free LicoA (1.25 or 2.5 mg/kg), no significant worm reductions were found (Figure 4A). On the other hand, the oral treatment with L-SLNs (at double equivalent doses of 2.5 mg/kg of LicoA) showed a significant worm burden reduction of 29.4% ($p < 0.05$) in comparison with infected untreated control group (Figure 4A).

However, after intraperitoneal administration (Figure 4B), the treatment of both free LicoA (5 mg/kg) and L-SLNs (5 mg/kg) caused significant decrease in worm load by 51.2% ($p < 0.01$) and 52.9% ($p < 0.01$), respectively, in comparison with untreated control group (Figure 4B).

Regarding intestinal egg load, all egg stages (immature, mature and dead) were observed in the intestine of infected control and treated mice (Figure 5A & B). The oral administration of both free LicoA and L-SLNs did not reduce eggs or cause alteration in the oogram (Figure 5A). On the contrary, after the intraperitoneal administration of free LicoA and L-SLNs, the number of immature eggs was reduced in 45.5% ($p < 0.001$) and 50.8% ($p < 0.001$), respectively (Figure 5B). In addition, L-SLNs (5 mg/kg of LicoA, i.p.) significantly reduced the number of mature eggs by 38% ($p < 0.05$) (Figure 5B) in comparison with the untreated control group.

In vitro & *in vivo* permeability studies

In assessing the qualitative *Schistosoma* uptake of SLNs, the *in vitro* permeability studies revealed that, after exposure with NR-SLNs, nanoparticles were able to permeate into the tegument of adult schistosomes (Figure 6A), while

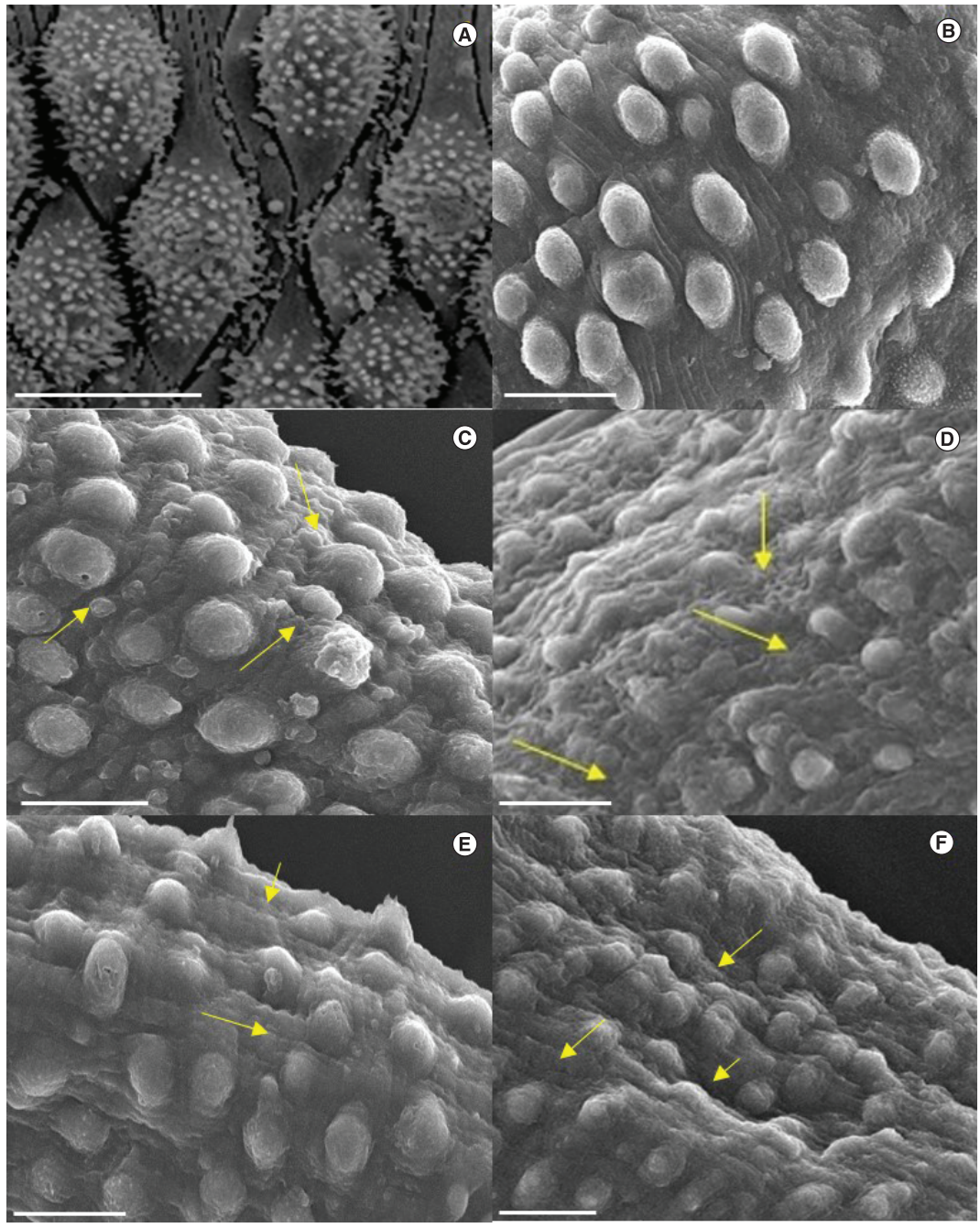

Figure 3. Scanning electron microscopy of adult *Schistosoma mansoni* male worms. The morphological changes described by scanning electron microscopy were observed after the death of the parasites, in other words, 24 or 48 h. Specifically, parasites were fixed after 24 h for 100 μM free Licochalcone A (LicoA) and after 48 h for 100 μM free LicoA, and 50 and 100 μM for LicoA-loaded solid lipid nanoparticles (L-SLN). **(A)** Worms nontreated (control). **(B)** Worms treated with blank-SLN. **(C)** Worms treated with free LicoA (50 μM): tegumental surface showing blebs, swelling and shortening. **(D)** Worms treated with free LicoA (100 μM): tegumental surface showing blebs, swelling and shortening. **(E)** Worms treated with L-SLN (50 μM): dorsal tegumental surface showing swelling, shortening and collapse of the tubercles. **(F)** Worms treated with L-SLN (100 μM): dorsal tegumental surface showing swelling, shortening and collapse of the tubercles. Scale bar 10 μm.

Table 2. *In vitro* schistosomicidal, cytotoxic and hemolytic activities of free licochalcone A and loaded solid lipid nanoparticles.

Groups and concentrations	Incubation period (h)	Dead worms (%)[†,§]	Motor activity reduction (%)[¶]		Cytotoxicity CC_{50} (μM)[#]	RBC_{50} (mM)[††]
			Slight	Significant		
Control[‡]	24–72	–	–	–	–	–
PZQ 2 μM	24–72	100	–	100	–	–
DMSO 0.5%	24–72	–	–	–	–	–
Free Nile Red	24–72	–	–	–	–	–
NR-SLN	24–72	–	–	–	–	–
Blank SLN	24–72	–	–	–	–	–
Free LicoA (μM)						
100	24	100	–	100	20.7	<1
	48	100	–	100		
	72	100	–	100		
50	24	–	–	–		
	48	100	–	100		
	72	100	–	100		
25	24	–	–	–		
	48	–	–	–		
	72	100	–	100		
12.5	24–72	–	–	–		
L-SLN[‡‡] (μM)						
100	24	–	–	–	956.2	>5
	48	100	–	100		
	72	100	–	100		
50	24	–	–	–		
	48	100	–	100		
	72	100	–	100		
25	24	–	–	–		
	48	–	–	–		
	72	100	–	100		
12.5	24	–	–	–		
	48	–	–	–		
	72	50	–	50		

[†]Percentages relative to the 20 worms investigated.
[‡]RPMI 1640.
[§]Incubation period: 24 h.
[¶]Incubation period: 72 h.
[#]CC_{50} values (50% cytotoxicity concentration) on macrophages.
[††]RBC_{50} values (50% hemolytic concentration) on red blood cells.
[‡‡]Equivalent concentrations of LicoA.
DMSO: Dimethyl sulfoxide; LicoA: Licochalcone A; L-SLN: LicoA-loaded solid lipid nanoparticle; NR-SLN: Nile Red solid lipid nanoparticle; PZQ: Praziquantel.

blank SLNs or free NR did not show any fluorescence under the same conditions (Figure 6B). In addition, the most intense fluorescence was observed in the tegument of male and female schistosomes, also reaching the digestive tract of worms (Figure 6A). Also, representative confocal microscopy images of schistosome worms recovered from mice treated with NR-SLNs are shown in Figure 6C & D. When intraperitoneally administered, NR-SLNs were able to reach adult schistosomes *in vivo*, penetrating the tegument of male and female worms (Figure 6C & D).

Discussion

Schistosomiasis is a neglected disease with a considerable and serious impact in public health [2,3]. Recently, due to the urgent need to identify new drugs and formulations, natural and synthetic compounds, as well as their formulations, have been investigated against *S. mansoni* [17,18]. Among the promising compounds, LicoA

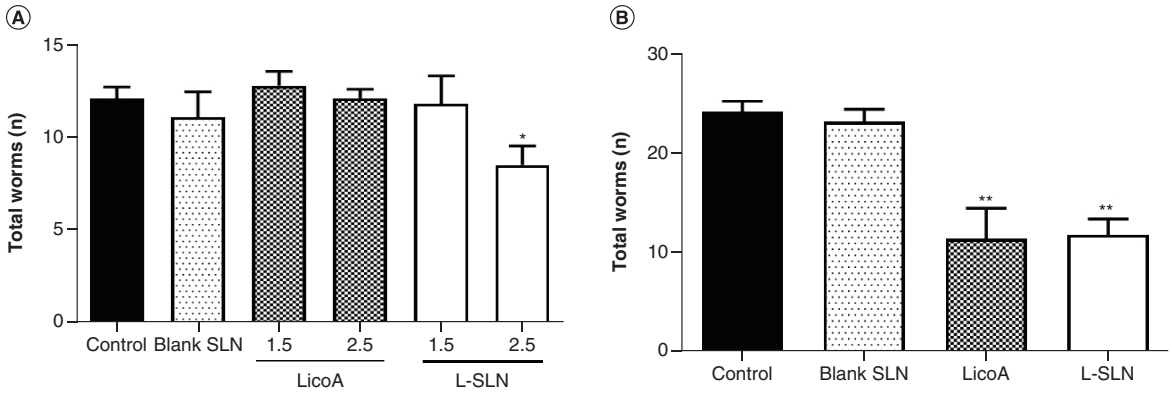

Figure 4. Worm burden in the *in vivo* antischistosomal study. Effects on total worm burden of LicoA, L-SLN and Blank SLN. **(A)** Effect on total worm burden of oral doses of LicoA (1.5 or 2.5 mg/kg), L-SLN (correspondent to 1.5 or 2.5 mg/kg of LicoA) and Blank SLN administered to mice harboring 49-day-old adult *Schistosoma mansoni* infection. **(B)** Effect on total worm burden of intraperitoneal multiple doses of LicoA (5 mg/kg), L-SLN-L (correspondent to 5 mg/kg of LicoA) and blank SLN, administered to mice harboring 49-day-old adult *S. mansoni* infection. Bars represent data from individual mice that were infected and treated with samples or infected and untreated (control). *p < 0.05; **p < 0.01 compared with untreated groups.
LicoA: Licochalcone A; L-SLN: LicoA-loaded solid lipid nanoparticle.

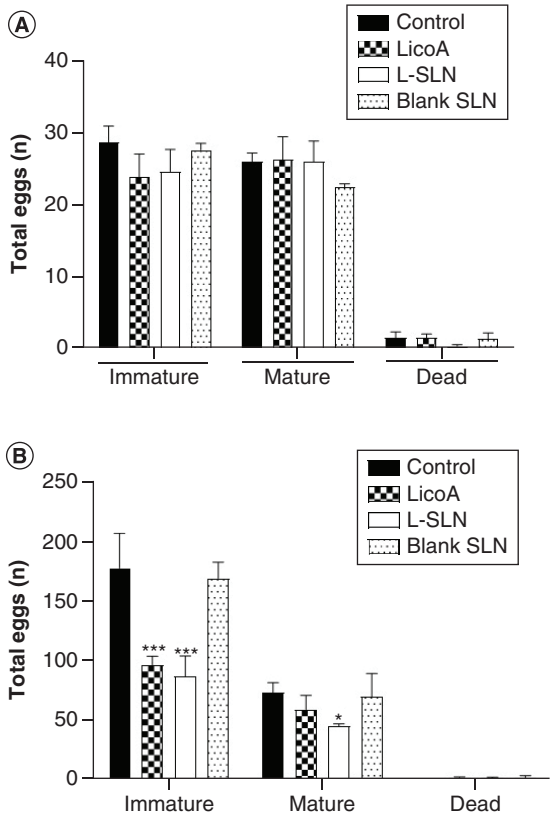

Figure 5. Effects on oograms of LicoA, L-SLN and Blank SLN. (A) Effect on egg development stages (oogram) of oral doses of LicoA (2.5 mg/kg), L-SLN (correspondent to 2.5 mg/kg of LicoA) and blank SLN, administered to mice harboring a 49-day-old adult *Schistosoma mansoni* infection. **(B)** Effect on egg development stages (oogram) of intraperitoneal multiple doses of LicoA (5 mg/kg) L-SLN-L (correspondent to 5 mg/kg of LicoA), and Blank SLN, administered to mice harboring a 49-day-old adult *S. mansoni* infection. Bars represent data from individual mice that were infected and treated with samples or infected and untreated (control).
*p < 0.05; ***p < 0.001 compared with untreated groups.
LicoA: Licochalcone A; L-SLN: LicoA-loaded solid lipid nanoparticle.

is a chalcone known for its antiparasitic activities, including *in vitro* schistosomicidal [14]. Although the *in vitro* antischistosomal activity of LicoA has been previously described, LicoA has not yet been evaluated *in vivo* against *Schistosoma* infection. Also, LicoA has biological and chemical characteristics that may limit its *in vivo* efficacy [15], including solubility in water. In addition, it has been reported that LicoA may possess a hemolytic potential against human erythrocytes [16]. In this present study, LicoA–loaded SLNs were prepared to overcome these drawbacks.

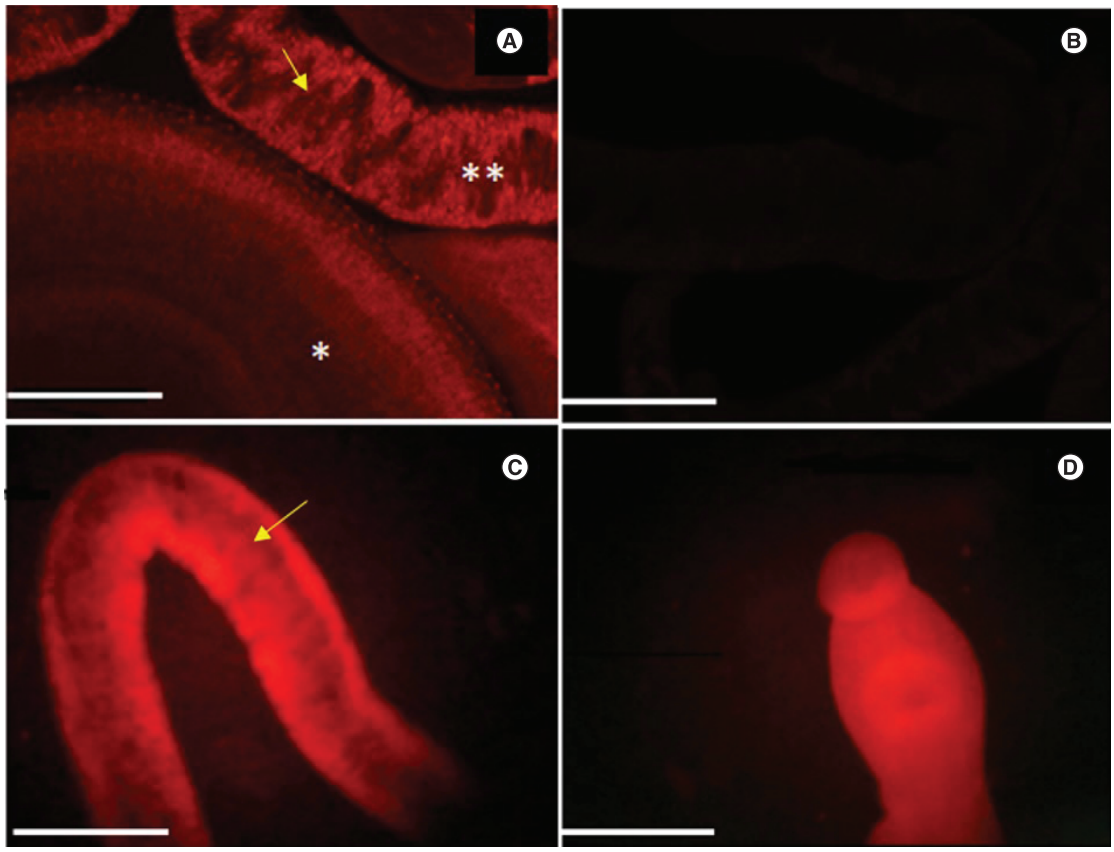

Figure 6. Representative fluorescence microscopy images of *Schistosoma mansoni* male and female worms exposed to *in vitro* incubation with nile red-solid lipid nanoparticles. (A) *In vitro* incubation of adult male (*) and female (**) schistosomes with Nile red-solid lipid nanoparticles (SLN) for 2 h. Scale bar represents 300 μm. **(B)** *In vitro* incubation of adult schistosomes with Blank-SLN for 2 h. Scale bar represents 300 μm. **(C & D)** Female and male adult *S. mansoni* collected from female mice after intraperitoneal application of Nile red-SLN. Scale bar represents 100 μm. The yellow arrows indicate the digestive system of the worms.

First, we isolated LicoA from the dried extract of *G. inflata*. Our present method was faster and consumed less solvents in comparison with a previously reported method [14]. Next, LicoA was encapsulated in SLNs using cupuaçu seed butter and Pluronic F-127 as surfactant. The cupuaçu seed butter composition was composed mainly of palmitic and oleic acids, which is in agreement with a previous report [34].

The size of nanoparticles is an important factor for schistosomicidal activity. Previous studies have shown that, among nanoparticles from 55 to 500 nm, smaller particles produced better antischistosomal results [21,35]. Hypothetically, smaller particles could have better permeation in the parasite tegument, promoting extensive activity. In addition, nanoparticles with average size below 400 nm could be able to cross the intestinal cell barrier when administered orally [21,22]. In this regard, the average size obtained for the L-SLNs in this study, 101.1 ± 1.8 nm, was satisfactory for considering further *in vitro* and *in vivo* assessments.

In addition to the size and PdI, Zeta potential was assessed to clarify the overall surface charge. The resulting negative surface charge may be due to the high number of fatty acids presented in the formulation, which deprotonate in aqueous environment. Also, the high modulus of charge is an indication of possible colloidal stability, as described [19,20].

Also, our results demonstrated excellent storage stability of L-SLNs for 30 days at 4 or 25°C. The stability of nanoparticles involves physicochemical aspects and includes several factors, such as changes in the size and degradation of their constituents [36]. In addition to the high surface charges in modulus, the copolymer F127 may also provide steric stability due to the presence of nonionic poly(ethylene glycol) as outer layer [17,23].

Regarding the EE%, which exceeded 98.0% in L-SLN formulations, values of EE% above 70% indicates a high incorporation of the substances in the nanoparticles [37], rendering satisfactory incorporation for medical purposes.

This high rate of encapsulation can be explained by the fact that the lipid nucleus presents a mixture of different fatty acids from the cupuaçu butter, which causes imperfections in the lipid structure, allowing encapsulation of substances into the nanoparticles [19,20]. Furthermore, SLNs suspensions were formulated in aqueous media, so the hydrophobic active is guided to the lipid matrix. Thus, this assay contemplates the factual condition of the formulation, rather than the movement of the hydrophobic active in sink condition, which was studied in the release kinetic experiments.

Moreover, the release profiles of L-SLNs followed the Higuchi model and is in accordance with Fick's law [38]. This model indicates that the release of substances is controlled by diffusion through the lipid nucleus. The initial burst release is possibly due to the release of LicoA located near the surface of the nanoparticle, while the sustained release is due to the dispersion of LicoA from the lipid matrix after applying the specific temperature [38].

After, LicoA, L-SLNs and blank SLNs were tested against adult *S. mansoni*. Our *in vitro* results showed that both free LicoA and L-SLNs caused changes in motility and pronounced *in vitro* mortality and as expected, L-SLNs showed schistosomicidal effects only after 48 h incubation. The action time observed for the schistosomicidal activity of L-SLNs may be a result of the controlled and prolonged release from solid lipid nanoparticles [19,20], which may generate the late activity compared with the free drug.

Regarding toxicity profiles, while free LicoA showed cytotoxic and hemolytic effects, no significant cytotoxic or hemolytic potential for L-SLNs (at equivalent LicoA concentrations that effectively kill *S. mansoni* worms) was observed. As previously reported [16], LicoA shrinks erythrocytes and causes phosphatidylserine translocation to the erythrocyte surface, causing hemolysis. Moreover, the low hemolytic potential of SLNs suggests that the nanoparticle decreases the interaction of other substances with blood components [35]. Also, studies revealed an enhanced safety profile and biocompatibility of SLN-based formulations [21,22,39]. In this regard, PZQ showed an enhanced safety profile when incorporated in nanostructured lipid carriers [21,40]. A previous study showed that the encapsulation of PZQ into SLNs reduced the PZQ cytotoxicity against HepG2 cells compared with the equivalent concentrations of the free drug [21,40].

In addition, the results by SEM showed that both L-SLNs and free LicoA caused tegumental damages in parasites, which are in accordance with the literature [14]. In this regard, the tegument is pivotal for the survival of *Schistosoma* not only because it is one of the major routes for nutrient absorption, but also because it is important for the protection of the worm, since tegumental changes might result in exposure of parasite antigens to the host immune system [41].

Next, the *in vivo* treatment with free LicoA and L-SLNs was performed in patent *S. mansoni* infection (animals harboring adult schistosomes). In the oral treatment, free LicoA showed no significant worm reductions at the doses tested, while L-SLNs showed a significant worm burden reduction in comparison with the infected untreated control group. However, after intraperitoneal administration, the treatment of both free LicoA and L-SLNs caused significant decrease in worm burden and intestinal egg load. When compared with the oral dose of reference drug PZQ (400 mg/kg), which is known to reduce approximately 90% of the parasitic burden [3], L-SLNs exhibited a moderate antischistosomal efficacy in patent *S. mansoni* infection.

A recent pharmacokinetic study has shown that LicoA is poorly absorbed after oral administration and its blood concentration is affected by the first pass effect in liver or intestine, with a considerable amount of LicoA inactivated or not absorbed after oral administration [15]. Also, a previous report showed that LicoA was active against the parasite *T. gondii* after intraperitoneal administration [11]. In fact, SLNs have been shown to enhance bioavailability and circulation time of orally administered drugs, ultimately improving their pharmacological activity [35,42–44]. Thus, since L-SLNs were more effective in parasitological reduction than the free substance, we hypothesized that the observed activity may be attributed to the advantages of using nanoencapsulated systems. In addition, no antischistosomal activities of blank SLNs were observed in both *in vitro* and *in vivo* studies, reinforcing that the experimental antischistosomal activities of L-SLNs are related to the delivery of encapsulated LicoA in SLNs.

Furthermore, to visualize the permeability of nanoparticles in the tegument of adult schistosomes, NR was incorporated in SLNs as fluorescent indicator, since NR has high affinity for lipids [32]. Blank SLNs were used as a control to ensure the interference of the parasite's autofluorescence and its organelles. *In vitro* permeability studies with NR-SLNs indicated that the SLN system may be able to penetrate the *Schistosoma* parasite and deliver compounds mainly to the tegument and muscle layer of adult male worms. Additionally, the permeation of SLNs was also studied by *in vivo* experiments. In this case, parasites were obtained from mice that received NR-SLNs and evaluated by fluorescence microscopy. It was possible to verify the characteristic fluorescence in the adult worms, indicating that the SLNs had already reached the parasites. This result demonstrates that the nanoparticles

reach the desired target and allow the delivery of substances, which may potentiate the action of schistosomicidal compounds [17,35].

Taken together, permeability studies suggest that SLNs have enhancement capability of penetration in *Schistosoma*'s teguments. Considering that the tegument is one of the most important therapeutic targets in schistosomiasis, SLNs have potential to be used as a delivery system of compounds that cause tegumental damages in *Schistosoma*.

Conclusion

LicoA is a promising natural compound with antiparasitic activities that may display toxic effects on macrophages and red blood cells. In this work, LicoA was isolated from licorice (*G. inflata*) extract and successfully encapsulated in SLNs. The obtained L-SLNs presented a particle size of around 100 nm, satisfactory Zeta potential, high EE, homogeneous distribution and excellent colloidal stability. Comparative toxicity studies revealed that SLNs decrease the hemolytic and cytotoxic activities of LicoA. Permeability studies indicated that SLNs may reach the adult schistosomes *in vitro* and *in vivo*. Both free LicoA and L-SLNs presented *in vitro* and *in vivo* schistosomicidal activity after intraperitoneal administration. Unlike free LicoA, L-SLNs showed *in vivo* efficacy against *S. mansoni* after oral treatment. These results suggest that SLNs may be a promising delivery system for LicoA to treat *S. mansoni* infections. Future studies are in progress to develop SLNs with other promising antischistosomal natural products.

Future perspective

Schistosomiasis is a tropical neglected disease with only a single drug currently used for its treatment. The development of novel nanomedicine would impact the course of the disease and would help health authorities to promote equality for its treatment by using natural sources for drugs and technologies. Improvement of the formulation to higher encapsulation rate in order of increasing the *in vivo* efficacy and the evaluation of pharmacokinetics should be the next steps in the development of this nanomedicine. We believe that this manuscript may expand the search for new drugs and therapies against *S. mansoni*, based on natural products and nanotechnology.

Summary points

- Licochalcone A (LicoA)-loaded solid lipid nanoparticles (L-SLNs) were prepared and characterized.
- L-SLNs showed optimum physicochemical properties and good colloidal stability.
- L-SLNs possess lower cytotoxic and hemolytic properties compared with free LicoA.
- L-SLNs improved *in vivo* antischistosomal effects compared with LicoA.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/nnm-2021-0146

Author contributions

LM Silva contributed in conceptualization, investigation, formal analysis, writing – original draft. DG Marconato contributed in investigation, formal analysis, writing – original draft. MPN da Silva, NRB Raposo, G de Faria Silva Facchini, GC Macedo and Priscila de Faria Pinto contributed in investigation, formal analysis. FS Teixeira and MC Salvadori contributed in investigation. J de Moraes, F Pittella contributed in conceptualization, investigation, formal analysis, writing – original draft, funding acquisition. AA Da Silva Filho contributed in conceptualization, investigation, formal analysis, writing – original draft, resources, project administration, funding acquisition.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations.

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