Oral administration of buparvaquone nanostructured lipid carrier enables in vivo activity against Leishmania infantum

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Highlights

- Miltefosine is the only drug approved for leishmaniasis oral treatment.
- We developed buparvaquone-NLC aiming to contribute it as a therapeutic option.
- Buparvaquone dissolution was improved in simulated intestinal fluids.
- Parasite burden reduction was higher than 80% in the liver.
- Our work brings a new perspective for leishmaniasis oral treatment.

Oral administration of buparvaquone nanostructured lipid carrier enables *in vivo* activity against *Leishmania infantum*

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Abstract

Leishmaniasis, a neglected tropical disease, is prevalent in 98 countries with the occurrence of 1.3 million new cases annually. The conventional therapy for visceral leishmaniasis requires hospitalization due to the severe adverse effects of the drugs, which are administered parenterally. Buparvaquone (BPQ) showed in vitro activity against leishmania parasites; nevertheless, it has failed in *in vivo* tests due to its low aqueous solubility. Though, lipid nanoparticles can overcome this holdback. In this study we tested the hypothesis whether BPQ-NLC shows *in vivo* activity against *L. infantum.* Two optimized formulations were prepared (V1: 173.9 ± 1.6 nm, 0.5 mg of BPQ/mL; V2: 232.4 ± 1.6 nm, 1.3 mg of BPQ/mL), both showed increased solubility up to 73.00-fold, and dissolution up to 83.29%, while for the free drug it was only 2.89%. Cytotoxicity test showed their biocompatibility (CC50 >554.4 µM). Besides, the V1 dose of 0.3 mg/kg/day for 10 days reduced the parasite burden in 83.4% ±18.2% ($p < 0.05$) in the liver. BPQ-NLC showed similar

leishmanicidal activity compared to miltefosine. Therefore, BPQ-NLC is a promising addition to the limited therapeutic arsenal suitable for leishmaniasis oral administration treatment.

Keywords: Buparvaquone, Nanostructured Lipid Carrier, Leishmaniasis, Neglected Diseases.

1. Introduction

Leishmaniases are among the leading neglected tropical diseases (NTDs). New cases occur worldwide from 1.5 to 2 million per year. They are highly associated with poverty and prevalent in 89 countries, in four of the five continents. More than 1 billion people are living in endemic areas, and the estimated number of deaths from visceral leishmaniases ranges from 20,000 to 70,000 per year [1].

As an obligate intracellular parasite, leishmania is shielded from conventional chemotherapy, which does not readily diffuse through the host cellular membrane. The pentavalent antimonial drugs such as meglumine antimoniate or sodium stibogluconate have been the first-line treatment for more than 60 years, with a success rate between 60 and 80%. Moreover, the therapy for visceral leishmaniasis requires hospitalization to monitor severe adverse effects due to the parenteral administration of large and repeated doses, which can last from 20 to 40 days. Additionally, resistance to second-line drugs, such as miltefosine and liposomal amphotericin B, is prone to develop [2,3].

Aiming to discover new drugs, a series of hydroxynaphthoquinones were synthetized in the 1980s. Considering the potential of these molecules for use in the treatment of neglected diseases, they were tested in vitro against *Leishmania donovani*. Buparvaquone (BPQ) showed 100-fold increased activity against amastigotes (intracellular parasite form) compared to other hydroxynaphthoquinones [4,5]. However, in dogs infected with *L. (L.) infantum*, only a negligible effect was observed. The aqueous solubility of BPQ is very low (<1 $mg.L^{-1}$), and therefore it is poorly soluble in biological fluids, such as gastric and interstitial juices. In addition to the low water solubility, drug CYP metabolism by liver enzymes may also explain the drug's low bioavailability and limited *in vivo* efficacy [6].

Approximately 80% of drugs are preferentially administered by the oral route. The most evident benefits include ease of administration, cost-effective manufacturing, less rigid storage conditions, high patient compliance, and more accurate self-administered dose. However, a drug substance must have proper absorption from the gastrointestinal tract (GIT). Stability in the gastric environment and aqueous solubility at GIT pH are imperative for the development of an oral dosage form [7,8].

The development of nanostructured delivery systems is one of the most promising alternatives to conventional treatments in meeting the leishmaniases therapy needs [9]. The advantages of nanostructured systems comprise the enhancement of water solubility of poorly

water-soluble drugs, and the development of modified and site-specific drug delivery systems, which have the potential to increase the therapeutic efficacy and reduce drug toxicity [10,11]. Among the options, nanostructured lipid carriers (NLC), composed of biocompatible lipids, have attracted the attention of formulation scientists as carriers for the modified release of poorly water-soluble drugs [12,13]. These carriers have been introduced as alternatives to conventional colloidal ones, such as liposomes and polymeric nanoparticles, due to improved physical and chemical stability, the viability of industrial scale and lower cost of raw materials [14,15]. Also nanostructured lipids have the potential to reduce drug degradation by liver, owing to selective lymphatic absorption [16].

Hence, considering the potential of nanotechnology-based drug delivery systems and the need for an innovative product to treat leishmaniasis, we tested the hypothesis whether oral administration of affordable and safe BPQ nanostructured lipid carrier presents suitable *in vivo* activity against *Leishmania infantum.* The formulations were developed and optimized using design of experiments (DoE), taking into consideration the factors with high impact over the product quality profile. To the best of our knowledge, this study is the first one to show a proof of concept and *in vitro* immunomodulatory effects of BPQ-NLC for the treatment of leishmaniasis.

2. Materials and methods

2.1. Materials

Softisan® 154 was kindly donated by CREMER Oleo Division (Germany), glyceryl monocaprylate, medium-chain triglycerides (MCT) were kindly donated by Abitec (USA). Kolliphor® P188 was acquired from BASF (Germany) and Tween 80 from Millipore Sigma (Germany). Buparvaquone (purity 99.5%) was donated by Shaanxi King Stone (Xian, China). Buparvaquone analytical standard Vetranal Supelco was purchased from Merck Sigma (Germany). Culture media M199 and RPMI 1640, and MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) were purchased from Merck Sigma (Germany). Pancreatin (*4X USP activity)* was purchased from Merck Sigma (Germany). Purified water was obtained by a Milli-Q system from Merck Millipore (Germany). Organic solvents were HPLC grade, and all other chemicals used were of the at least analytical grade.

2.2. Development of BPQ-NLC

Lipids were selected as described in our previous work [17]. The buparvaquone nanostructured lipid carrier (BPQ-NLC) formulations were prepared by dissolving free BPQ into the melted lipid phase for 15 minutes. The aqueous phase (70°C) was added to the lipid phase and

mixed for 5 minutes. The BPQ encapsulation efficiency was 99% of the amount added in the beginning of the process. Pre-homogenization was performed using a high-performance disperser (8,000 RPM for 5 min) (T25 digital ULTRA-TURRAX, IKA, Staufen, Germany). Afterward, the emulsion was passed through a high-pressure homogenizer (Nano DeBEE 45-2, Bee International, South Easton, MA, USA) at 600 bars for five cycles. The solid lipid, liquid lipid, and the surfactant were Softisan 154, MCT (capric/caprylic triglyceride) (1:2), and poloxamer 188, respectively.

Factors with high risk or impact over the quality of preparations were selected: liquid and solid lipid ratio, surfactant concentration, and lipid phase amount. A full factorial 2^3 design (Table 1) was carried out to evaluate the influence of the factors in Z-average. The preparations were performed in random order to minimize systematic error. Statistical analysis was performed (α = 0.05) using Minitab 19 (Stage College, Pennsylvania). The high performance liquid chromatography (HPLC) method (supplementary data) was described in our previous work to quantify BPQ in NLC [17].

Table 1. Formulation design space of buparvaquone nanostructured lipid carrier. Low and high levels determined by preliminary tests.

Factor	Abbreviation	Low level	High level
The ratio of solid and liquid lipids	SL:LL	0.5	3.0
Poloxamer 188 (% w/w)	% POL	1.0	4.0
Lipid phase (% w/w)	% LP	5.0	15.0

2.3. Determination of the Z-average, polydispersity index, and zeta potential

The Z-average, polydispersity index (PDI), particle size distribution, and zeta potential of BPQ-NLC were determined immediately after homogenization and periodically for the stability study (supplementary data). The method used was photon correlation spectroscopy (PCS) using Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK) at 25 °C and 90 °C angle (n = 10). The measurements were carried out in purified water (n = 3), with conductivity adjusted to 50 $\mu S.cm^{-1}$ by the addition of NaCl 0.1% w/w, aiming to avoid fluctuations in ZP. The pH was adjusted to 6.5 ± 0.2 by the addition of 0.01 M HCl or 0.01 M NaOH solution.

2.4. Drug loading and entrapment efficiency

Drug loading (DL) and entrapment efficiency (EE%) were determined after homogenization and periodically during the stability study (supplementary data) by the HPLC method cited in section 2.2. For DL, the amount of BPQ (mg) in 1 mL of preparation was determined as follows: aliquots were diluted to a volumetric flask to a final BPQ concentration of 0.005 $mg.mL^{-1}$ and the volume was filled with the mobile phase. The previous preparations were filtered with PVDF

membrane, 0.45 µM pore size (MilliporeSigma, Germany). Each BPQ-NLC preparation was evaluated in triplicate. The drug load was previously evaluated, and the lipid matrix could accommodate 1% of drug by weight.

EE (%) of BPQ-NLCs was calculated by determining the amount of free drug using ultrafiltration technique. The free BPQ (mg) in 1 mL of preparation was determined as follows: an aliquot of each formulation was placed in EMD Millipore Amicon™ centrifuge filter units, 100 Kd MWCO (MilliporeSigma, Germany). The samples were centrifuged 14,000 g at 20 °C for 30 minutes. The BPQ-NLCs were evaluated in triplicate. EE was calculated using Equation 1 and DL using Equation 2:

$$\%EE = (Wtotal - Wfree)/(Wtotal)\ x\ 100 \qquad \text{Equation 1}$$

$$DL\% = (Wtotal - Wfree)/(Wlipid)\ x\ 100 \qquad \text{Equation 2}$$

where W_{total} is the weight of initial BPQ added, W_{free} is the weight of free drug detected in the filtrate after centrifugation, and W_{lipid} is the weight of lipid at the formulation.

2.5. Morphology by transmission electron microscopy (TEM)

BPQ-NLCs images were acquired using a Morgagni 268 transmission electron microscope with Gatan Digital Camera (Philips/FEI, Hillsboro, Oregon, USA). The samples were diluted in purified water in the ratio of 1:20. The diluted samples were placed over conventional transmission electron microscopy (TEM) grids and allowed to set for 15 seconds. The background was stained with phosphotungstic acid solution 10.0% w/w (Sigma-Aldrich, St. Louis, MO, USA) for an additional 15 seconds.

2.6. Thermal analysis

BPQ crystallization behavior in NLC was carried out by using differential scanning calorimetry (DSC). Free BPQ, V1 and unloaded V1 were characterized in a DSC 4,000 Perkin Elmer cell (Perkin Elmer Corp., Norwalk, CT, USA), under a dynamic N_2 atmosphere (50 $mL.min^{-1}$), using sealed aluminum capsules with about 2 mg of samples. DSC curves were obtained at heating rate of 10 $°C.min^{-1}$ in the temperature range from 25 to 290 °C. An empty sealed pan was used as reference.

2.7. Saturation solubility evaluation

Free BPQ and BPQ-NLC solubility were evaluated in simulated gastric fluid (pH 1.2) and the following pharmacopoeial buffers: pH 4.5; 6.8; 7.4. Also, FaSSIF (Fasted State Simulated Intestinal Fluid) and FeSSIF (Fed State Simulated Intestinal Fluid) were prepared as described previously [18], phosphate buffer 0.05 M (pH 7.4) with sodium dodecyl sulfate (SDS) (1.0% w/w); and phosphate buffer 0.05 M (pH 7.4) with Tween 80 (0.07% w/w) were evaluated.

The shake flask method was applied as follows: after shaking the BPQ saturated media for 24 hours at 37 °C, the samples were filtered through PVDF 0.1 µm pore size membranes and diluted with the mobile phase at least 2-fold. The quantification of BPQ in the samples was performed with HPLC, as cited in section 2.2.

2.8. Free BPQ and BPQ- NLC dissolution studies

The dissolution studies of free BPQ and BPQ-NLC were performed in a) phosphate buffer 0.05 M (pH 7.4) with Tween 80 0.07% w/w, with and without pancreatin (0.1% w/w); b) phosphate buffer 0.05 M (pH 7.4) with sodium dodecyl sulfate (SDS) 1.0% w/w, according to USP monograph for fenofibrate capsules test 2, found in the dissolution database [19]. Pancreatin was added aiming to mimic the nanoparticle degradation from intestinal lipases released in the duodenum.

Dissolution settings were: medium volume of 900 mL, apparatus II (paddle), 50 rpm, sample volume of 2.0 mL, with media replacement for 60 or 90 minutes. The samples were filtered with a saturated membrane of PVDF 0.1 µm pore size and diluted with the mobile phase at least 3-fold. For each formulation and medium, the tests were performed in replicates of six. The BPQ quantification was performed by HPLC, as cited in section 2.2.

2.9. Cytotoxicity and leishmanicidal activity

The optmized formulation V1 (402.5 ± 1.2 ug/mL of BPQ) described in the section 3.1 was selected due to the highest surfactant concentration and smallest particle size. Cell viability was determined by the MTT method (3-methyl- [4-5-dimethylthiazol-2-yl] -2,5-diphenyltetrazolium bromide) as described by Monteiro et al., 2017 [20]. For mammalian cells, mouse peritoneum macrophages were incubated for 24 hours at 37 °C (2×10^5). Free BPQ was dissolved in DMSO (1% w/v) and BPQ-NLC V1 was diluted with RPMI to achieve concentrations in the range of 0.2 to 28 µM. Both preparations were added to the wells. After 24 hours, MTT solution (5 $mg.mL^{-1}$) was added, and the plate was incubated at 37 °C, 5% CO_2, 95% RH, for 4 hours. The optical density was measured in a microplate spectrophotometer (λ = 595 nm) (Multiskan™ GO Thermo Scientific™, Finland). Each treatment was evaluated with replicates of six. Cell-free and culture medium containing macrophages were used as a negative and positive control, respectively.

For amastigote test: *L. infantum* infected macrophages ($1x10^6$) were incubated (37°C, 24h) with increased concentrations of BPQ and BPQ-NLC V1. The cells were stained with giemsa and

the number of infected, uninfected cells (n=200) and amastigotes per cell were counted by microscopic examination.

The CC_{50} and IC_{50} values were calculated by non-linear regression analysis using GraphPad Prism version 5.01 (GraphPad Software, Inc., USA). All the experiments have been carried out under the approval of the Institute of Tropical Medicine of São Paulo ethics committee (registration number: CPE-IMT000269A June 5, 2014).

2.10. *In vitro* immunomodulatory effects of BPQ-NLC

The J774A.1 macrophage cell line was cultured in RPMI 1640 medium (Gibco) with 100,000 U/L penicillin and 100 mg/L streptomycin (Sigma), supplemented with 5% Fetal Bovine Serum (Gibco). Cells were grown in T25 culture flasks at 37 ˚C in a 5% CO_2 humidified incubator. Every 2 days, the cells were detached from the culture flask with a cell scraper and sub-cultured. The cells were counted and plated in 24-well plates (Corning) at 5×10^5 cells/well, and incubated overnight. After being washed with RPMI 1640 medium, the cells were treated with NLC or BPQ-NLC at 1, 5, or 10 μM, or stimulated with *Escherichia coli* O111:B4 Lipopolysaccharide (LPS; Sigma) at 1 μg/mL, or left in medium. After 6, 24, 48 and 72 hours, the culture supernatants were harvested and analyzed to determine the cytokine levels. The concentrations of the released mediators IL-1β, IL-6, and TNF-α were determined by ELISA kits (Invitrogen) in accordance with the manufacturer's protocols.

For the comparisons between the effects of different concentrations of NLC and BPQ-NLC (V1) on the levels of cytokines, the one-way ANOVA test was used, with Bonferroni's post hoc test, and for the comparisons between the incubation periods of 6, 24, 48 and 72 hours, the Repeated Measures ANOVA test was used, also with Bonferroni's post hoc test. The analyses were conducted on the GraphPad Prism software (GraphPad Software). Results were presented as mean with SEM, and p values ≤ 0.05 (α = 0.05) were considered statistically significant.

2.11. *In vivo* leishmanicidal activity and DNA quantification by real-time PCR

Gold Syrian hamsters (*Mesocricetus auratus*) is considered the most suitable model to evaluate visceral leishmaniasis disease. It has the closest immunologic features compared to human visceral leishmaniasis among all available animals [21-23]. *L. infantum* (MHOM/BR/1972/LD) was investigated since it is the most relevant and responsible for VL in human and dogs in the New World, especially in Brazil [24]. For the *in vivo* evaluation, BPQ-NLC V1 (402.5 ± 1.2 ug/mL of BPQ) was selected due to the smallest particle size.

Young male Golden hamsters (<110 g) were infected intraperitoneally with *L. (L.) infantum* promastigotes (1×108/animal). Forty days after the infection, the hamsters (n = 5/group) were treated in fasted condition by oral route for 10 consecutive days as follows: BPQ-NLC at 0.3

mg/kg/day (0.9 mol/kg/day), which corresponds to 0.12 mL per animal, miltefosine at 2.0 mg/kg/day (4.9 mol/kg/day) and blank-NLC. Control group was untreated animals. The animals were euthanized 50 days post-infection. The BPQ doses were based in the previous study by Reimão and colleagues (2012) [25]. The miltefosine dose were based in the therapeutic recommendation for dogs.

The spleen and liver were removed, weighed, and the number of DNA copies per mg of tissue was quantified by real-time polymerase chain reaction (RT-PCR). DNA purification was performed using Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germany). Around 20 mg tissue from the liver and 10 mg of spleen were placed into a clean 1.5 mL microcentrifuge tube, then followed manufacturer instructions. The quantity and quality of DNA were determined using NanoDrop Lite Spectrophotometer (Thermo Scientific, USA). Tissues of non-infected animals were used as controls.

Leishmania HPS70 heat protein shock sequence (234 bp. HSP70F: GGA CGAGATCGAGCGCATGGT. HSP70R: TCCTTCGACGCCTCCTGGTTG) was used for quantification. Sybr Green real-time PCR was set up with Maxima SYBR Green/ROX qPCR Master Mix (2X), 100 nM final concentration of forward and reverse primers, 5 µL of DNA of the sample, control (tissue of non-infected animal) or cloned DNA (quantification curve from10^{-1} to 10^{6} DNA copies), in a total volume of 20 µL. Amplification was performed in a real-time instrument (StepOne Real-Time PCR System, Thermo Fisher/Applied Biosystems) with the following conditions: polymerase activation at 95 °C for 5 min, followed by 40 cycles of 94 °C for 25 s, 60 °C for 20 s, and 72 °C for 30 s. Followed by a holding stage of 72 °C for 2 minutes. The melting curves (for the amplification specificity) were determined at 70 °C (45 s) and an increase in the temperature to 95 °C.

Statistical analysis was performed by ANOVA (α = 0.05) and Dunnett's Post Hoc Test, using Minitab 19. All the experiments have been carried out under the approval of the Institute of Tropical Medicine of São Paulo ethics committee (registration number: 000417A, November 25th, 2019).

3. Results and discussion

3.1. Development of BPQ-NLC

Previous screening experiments showed only one critical process parameter. When the number of cycles is three or less, the drug could not be accommodated in the lipid structure, and precipitation occurred. Therefore, all preparations were performed using five cycles of homogenization. Table 2 shows the design matrix and the experimental results of Z-average, PDI and zeta potential for each preparation. The influence of the factors (SL:L, % poloxamer 188, % lipid phase) and their interactions in the Z-average are shown in the contour plots (Fig. 1).

Table 2. Experimental design matrix, Z-average (Z-ave), polydispersity index (PDI) and zeta potential (ZP) of BPQ-NLC

SO	Level			SL:LL	% POL	% LP	Z-ave (nm)	PDI	ZP (mV)
	SL:LL	% POL	% LP						
F11	0	0	0	1.75	2.5	10.0	231.8	0.164	-22.1
F9	0	0	0	1.75	2.5	10.0	235.5	0.155	-12.8
F8	+1	+1	+1	3.00	4.0	15.0	229.3	0.147	-15.3
F1	-1	-1	-1	0.50	1.0	5.0	239.9	0.158	-20.4
F5	-1	-1	+1	0.50	1.0	15.0	366.9	0.241	-30.6
F4	+1	+1	-1	3.00	4.0	5.0	198.1	0.192	-19.3
F7	-1	+1	+1	0.50	4.0	15.0	208.3	0.164	-24.0
F6	+1	-1	+1	3.00	1.0	15.0	390.6	0.250	-43.7
F10	0	0	0	1.75	2.5	10.0	232.6	0.143	-27.0
F2	+1	-1	-1	3.00	1.0	5.0	267.7	0.223	-28.2
F3	-1	+1	-1	0.50	4.0	5.0	180.6	0.180	-25.3

SO: standard order; SL:LL: solid lipid to liquid lipid ratio; % POL: percentage of poloxamer 188 (w/w); % LP: percentage of lipid phase (w/w).

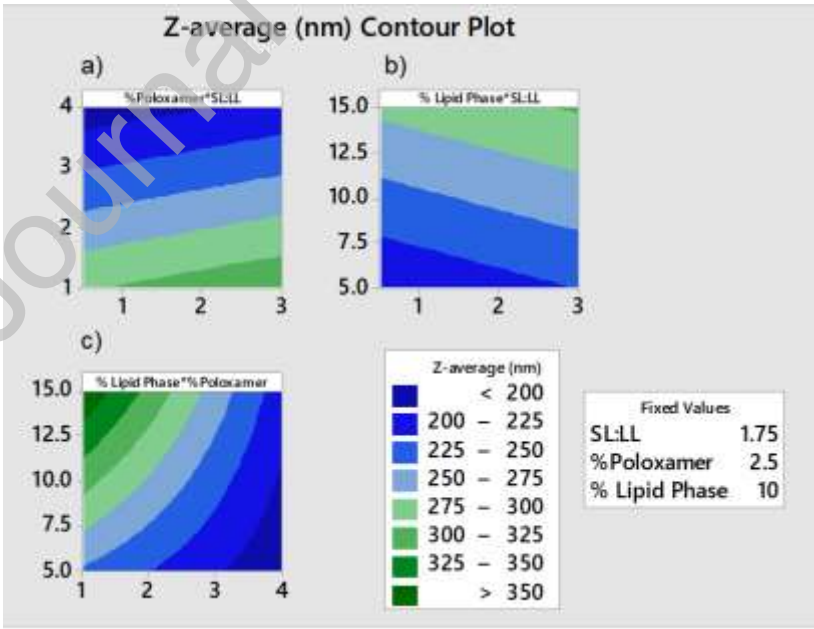


Figure 1. Contour plot of Z-average from buparvaquone nanostructured lipid carrier development using the design space approach.

The lipid phase and poloxamer 188 have a nonlinear relationship, and BPQ-NLCs with Z-average lower than 220 nm can be achieved with a combination of lipid phase lower than 10.0% w/w and poloxamer 188 higher than 3.5% w/w (Fig. 1C). The negligible impact of SL:LL in the Z-average is observed when the % lipid phase is lower than 7.5% w/w (Fig. 1B). The ratio can be changed from 0.5 to 3, but the Z-average only varies from 200 to 225 nm. The absence of the <200 nm zone (Fig. 1C) indicates that nanoparticles in this range are only possible to prepare when poloxamer 188 is above 2.5%.

The variables % lipid phase ($p < 0.05$; $\alpha = 0.05$) and % poloxamer 188 ($p < 0.05$; $\alpha = 0.05$) showed significant effect on the output (Z-average). SL:LL has a low impact in the Z-average, as revealed by the non-significant p value (>0.05) (suplementary data). The goodness-of-fit indexes, the lack-of-fit (p value >0.05; $\alpha = 0.05$), and R^2 of 96.37% and adjusted-R^2 (93.95%) corroborate the suitability of the model, which means that the factors explain most of the variability. The mathematical model to predict Z-average is described in Equation 3:

$$\mathrm{Z-ave} = 173.8 + 9.0\ \mathrm{SL{:}LL} - 5.6\ \%\mathrm{POL} + 15.7\ \%\mathrm{LP} - 3.2\%\mathrm{POL\ x\ \%\ LP} \qquad \text{Equation 3}$$

Where Z-ave: Z-average (nm); % POL: % w/w of Poloxamer 188; % LP: % w/w of lipid phase.

For the mathematical model verification two preparations were obtained: V1 (predicted Z-average: 175.3 nm, SL:LL 1.0, 4.0% w/w poloxamer 188, and 5.0% w/w lipid phase) and V2 (predicted Z-average: 253.3 nm, SL:LL 0.5, poloxamer 188 3.0% w/w and lipid phase 15.0% w/w). The predicted and observed Z-average values for both formulations were in the range of the model (95% CI), confirming the equation suitability. They showed zeta potential of -17.3 mV for V1 and -29.2 mV for V2, low PDI (<0.3) and monomodal particle distribution. Hence, V1 and V2 were further evaluated.

The poloxamer 188 concentration showed an inverse relation effect in the Z-average; the lowest Z-averages were achieved using the highest concentration of the surfactant. This performance can be explained by the reduction in the interfacial tension between the dispersed and continuous phases. With the increase in the content, more surfactant is available for adsorption in the newly developed surface of the particles, during the homogenization process [26]. The opposite performance was found in the % lipid phase, which can be supported by the same mechanism. With an increase in the amount of the lipid phase, more surfactant is required for packing on the particle surface [27]. Another possible mechanism was previously described: the increase in the particle size due to the increment in the amount of lipids can be explained by the reduction in dispersion energy available per unit of lipid, using the high-energy method [28].

The structure of NLC depends on the amount of liquid lipid, being the main factor for reducing the crystallinity of the lipid matrix. The SL:LL factor showed that the increase in the

amount of LL does not affect the Z-average in the range of 0.5 to 3, the lowest and highest levels, respectively (Table 1). Therefore, the lower ratio was applied in V1 and V2 preparations, aiming to increase the drug loading and to reduce the possibility of crystallinity changes during the storage. A similar result was described in the preparation of NLC with different ratios of Precirol, as a solid lipid, and Capryol 90, as a liquid lipid [29]. They showed that an increase of liquid lipid up to 50% of the total lipid phase did not change the particle size or PDI of the preparations. Besides, in the development of NLC prepared with cetyl palmitate and Miglyol 812, the Z-average was not significantly affected, with an increase of Miglyol 812 also up to 50% [30]. It was claimed that the viscosity of the lipid phase, in the homogenization process temperature (approximately 80 °C), of different oils content, should be similar, and the shear rate should have the same effect in the lipid phase breaking down. The non-linear relationship between % lipid phase and % poloxamer is described in the mathematical model (-3.2% POL × % LP). The negative coefficient suggests a synergistic interaction in the reduction of the Z-average. The possible mechanism is the effect of poloxamer P188 on lipids. Their interaction forms a tight and high orderly structure [31,32].

The components used for BPQ-NLC development are commercially available and applied in pharmaceutical products [33]. This drug delivery system has the potential to provide affordable medicines due to the low cost of raw materials, comparing with the phospholipids used in liposomes. However, NLCs remain an expensive formulation due to the cold-transport chain which significantly impacts on cost of therapy, similarly to COVID-19 vaccines. This is especially relevant considering that the leishmania has strong and complex links with poverty. Hence, it is mandatory a public policy to assure the treatment to this vulnerable population.

Besides high-pressure homogenization technology is well-established, reproducible, and shows scale-up feasibility [17]. Although leishmaniasis is prevalent in tropical countries, the stability was not considered a limiting factor since the formulations showed stability at 4°C (supplementary material). In addition, other leishmaniasis medicines are commercialized in lower temperatures, i.e., liposomal amphotericin B.”

3.2. Morphology by transmission electron microscopy (TEM)

The morphology of V1 and V2 formulations by transmission electron microscopy are shown in Fig. 2. The photon correlation spectroscopy (PCS) technique is an indirect method for nanoparticle size evaluation. Therefore, a complementary method must be applied to confirm size and morphology. The diffusion coefficient (D) is used in the Stokes-Einstein equation for the hydrodynamic size calculation. Anisotropic particles have different D value than round ones. As a result, particles with the same size, but distinct morphologies will not have the same results in PCS analysis [34]. Figure 2 shows the round shape of V1 (173.9 ± 1.6 nm) and V2 (232.4 ± 1.6 nm) in the nanometric range. Thus, Z-average results from V1 and V2 can be considered reliable.

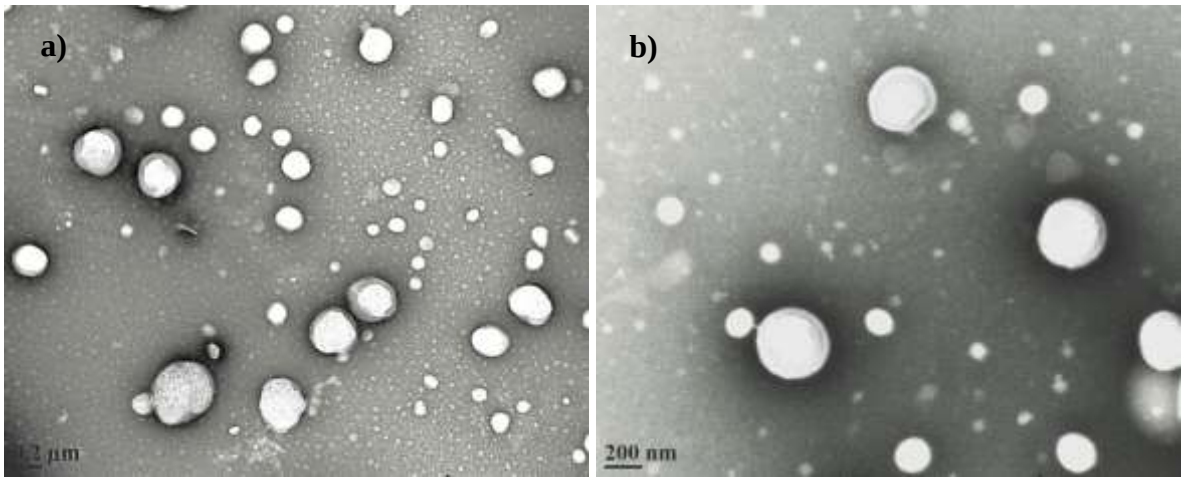


Figure 2. Transmission electronic microscopy of formulation: a) V1; b) V2. Magnification 36kx.

3.3. Thermal analysis

The thermal behavior of BPQ, NLC and BPQ-NLC are presented in Figure 3. DSC curve of BPQ showed one sharp endothermic event in the temperature range 170 to 180 °C (T_{onset} = 177 °C; T_{peak} = 183 °C), assigned to the melting process [17]. For BPQ-NLC and NLC, endothermic events were observed at onset temperatures of 40 and 43 °C, whereas for the exothermic events the onset temperatures were 153 and 177 °C, respectively.

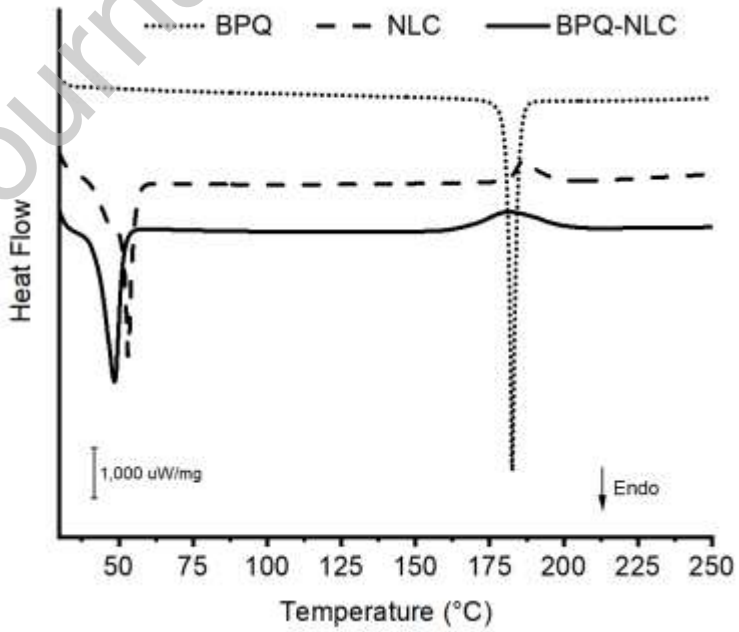


Figure 3. DSC curves of free burpavaquone (PQB), unloaded nanostructured lipid-carrier (NLC) and V1 formulation (BPQ-NLC) obtained under a dynamic N_2 atmosphere (50 mL.min^{-1}) at heating rate of 10 °C.min^{-1}.

DSC analysis was performed to select solid lipids to prepare BPQ-NLC [17]. The assessment was based on the capacity of the melted lipid to solubilize the drug in heating cycles experiments. Softisan® 154 had the best ability to solubilize the drug. Herein, the DSC curves of BPQ-NLC and NCL showed an endothermic event that corresponds to Softisan® 154 melting in the range of 53-58 °C [17]. It is followed by an exothermic event at 177 °C, which might indicate thermal degradation of lipids (Softisan® 154 and MCT). However, the complete absence of the BPQ melting peak in the DSC profile of BPQ-NL indicates that the drug molecule was solubilized in the lipidic matrix, which is congruent with its high lipophilicity [25] and the high entrapment efficiency (~99%) here obtained (supplementary data). Further, both Softisan® 154 melting peak and posterior lipid degradation event are depressed for BPQ-NLC, when compared to the unloaded carrier, which reinforces the good miscibility of BPQ in the lipidic matrix [35].

3.4. Saturation solubility evaluation

The solubility of free BPQ and BPQ released from NLC preparations V1 and V2 are shown in Table 3. Free BPQ showed limited solubility, except in the Fed State Simulated Intestinal Fluid and phosphate buffer pH 7.4 with sodium dodecyl sulfate 1.0% w/w, which revealed the surfactant dependence for BPQ solubilization. Both V1 and V2 showed improved BPQ saturation solubility.

Table 3. Saturation solubility of free BPQ and V1 and V2 preparations of BPQ-NLC (n = 3)

Media	Saturation solubility (μg.mL^{-1})		
	Free BPQ	V1	V2
SGF pH 1.2	0.05 ± 0.01	3.57 ± 1.72	3.64 ± 2.20
PB pH 4.5	0.06 ± 0.04	3.30 ± 0.98	2.78 ± 0.90
PB pH 6.8	0.08 ± 0.02	2.82 ± 1.41	2.77 ± 3.05
PB pH 7.4	0.19 ± 0.10	12.62 ± 1.52	2.16 ± 0.65
FeSSIF pH 5.0	12.53 ± 1.85	24.98 ± 2.04	25.56 ± 1.12
FaSSIF pH 6.5	3.39 ± 0.24	26.30 ± 2.66	2.28 ± 0.85
PB pH 7.4 + SDS 1.0% w/w	11.68 ± 0.78	37.74 ± 10.66	17.08 ± 4.50
PB pH 7.4 + T80 0.07% w/w	3.39 ± 0.30	15.31 ± 7.05	9.19 ± 2.02

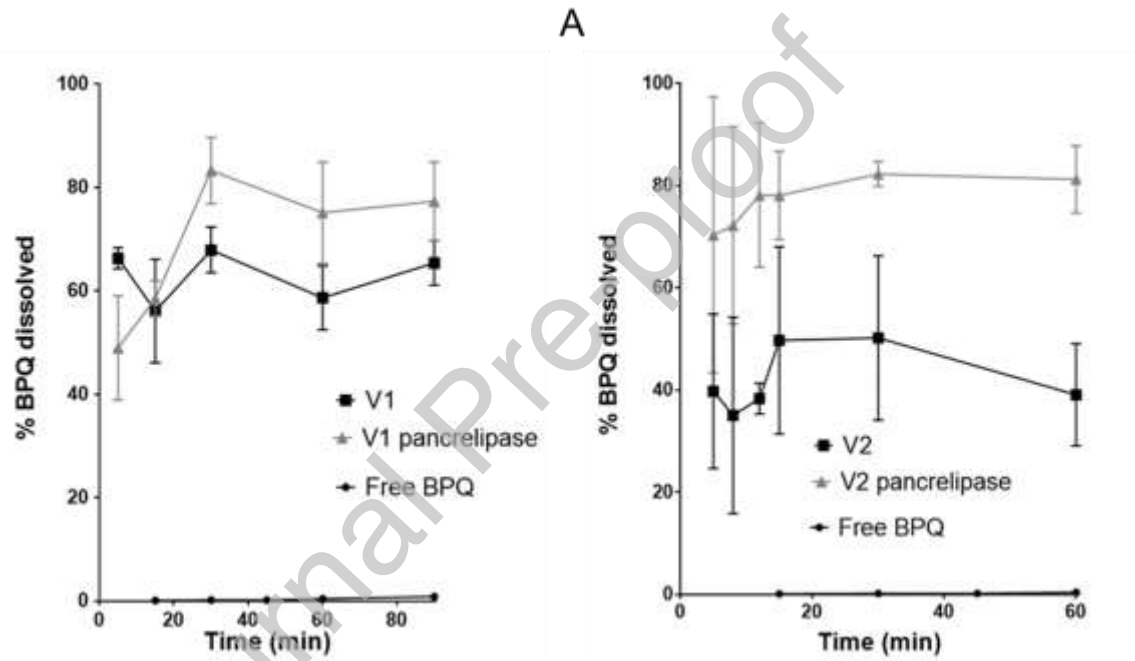
SGF: simulated gastric fluid; PB: phosphate buffer; FeSSIF: fed state simulated intestinal fluid; (FaSSIF): Fasted state simulated intestinal fluid; SDS: sodium dodecyl sulfate; T80: Tween 80.

BPQ-NLC V1 showed an increase in solubility from 2.0 (FeSSIF) to 73.0-fold (pH 1.2) compared to the free drug. For V2 the increase was from 1.5 (pH 7.4 + SDS 1.0% w/w) to 72.8-fold (pH 1.2). The oral administration of BPQ-NLC can be feasible despite the low solubility in the media without surfactant (Table 3) since bile salts and lipases are released in the mammalian

gastrointestinal fluid from the duodenum. These bio-surfactants and enzymes could promote BPQ release from the lipid matrix reducing the drug precipitation; as a result, improving its absorption. Thus, this test allowed selecting the media for the BPQ-NLC dissolution profile study, and it corroborated the potential use of NLCs for improving poor-water soluble drugs.

3.5. Free BPQ and BPQ- NLC dissolution studies

The dissolution profiles of V1 and V2 formulations in phosphate buffer pH 7.4, 0.05 M with Tween 80 (0.07% w/w) with or without pancreatin (0.1% w/w), and in phosphate buffer pH 7.4, 0.05 M with sodium dodecyl sulfate (1.0% w/w) are shown in Figure 4.



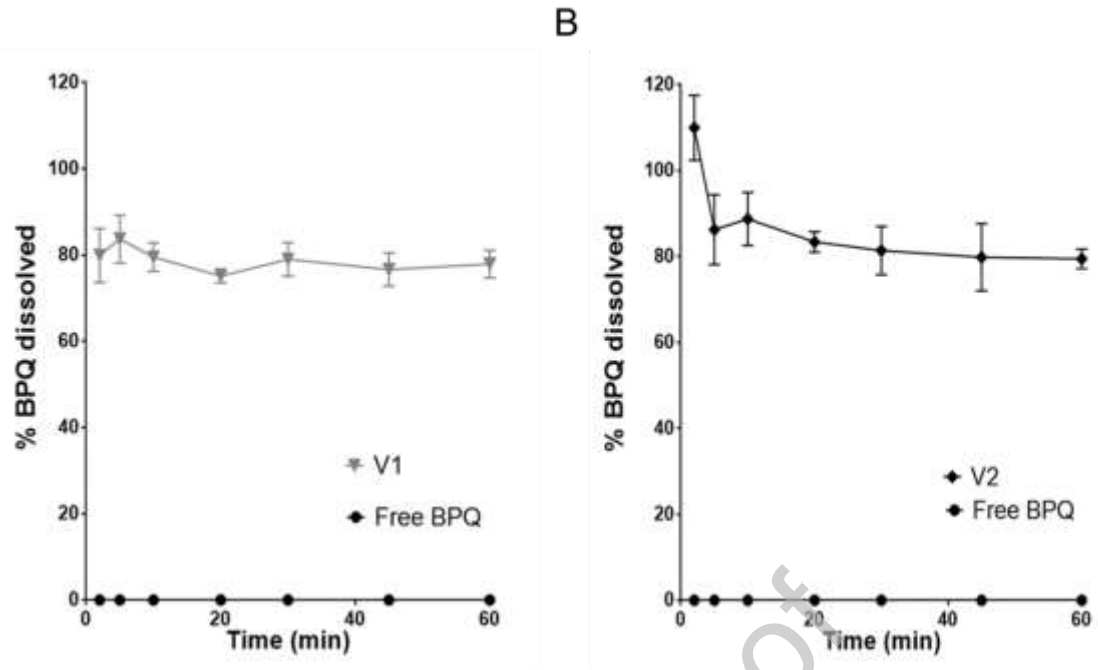


Figure 4. BPQ dissolution of free BPQ (4 mg) (black circles) and BPQ-NLC formulations (containing 4 mg) using USP II apparatus, at 50 rpm, in 900 mL phosphate buffer pH 7.4, 0.05 M with: A) Tween 80 (0.07% w/w) with (gray triangles) and without (black squares) pancrelipase, or B) sodium dodecyl sulfate 1.0% w/w. V1 [Z-average: 173.9 ± 1.6 nm]; V2 [Z-average: 232.4 ± 1.6 nm].

Dissolution profiles of free BPQ and BPQ-NLC were first tested in simulated gastric fluid pH 1.2, phosphate buffer pH 4.5, and pH 6.8. However, no BPQ was dissolved during 24 hours of testing, which shows the potential development of a delayed dosage form. The requirement of a surfactant for drug release was observed, as found in the saturation solubility test. Through oral administration, these BPQ-NLC would avoid drug release and precipitation in the stomach since gastric lipolysis is limited due to low enzyme content and its pH activity profile [36,37].

Free BPQ dissolution in phosphate buffer pH 7.4, 0.05 M with Tween 80 was limited (Fig. 4A). After 60 minutes, 0.43% of the 4.0 mg of the free drug was dissolved. Even after four hours, only 2.89% was dissolved. In the dissolution profiles with SDS (Fig. 4B), the free drug could not be dissolved despite the increased surfactant content. At the end of 60 minutes, the % dissolved was below the detection limit of the HPLC method. The interaction with the anionic surfactant can explain the poor drug dissolution. The BPQ ionization was simulated by the software Chemicalize^{TM} (ChemAxon, Hungary). Above pH 6.0, the drug is dissociated with an anionic charge; it was supposed to dissolve due to a reduction in the interfacial tension, but precipitation occurred when BPQ ions interacted with the charges of SDS. The solubility of the compound PG-300995 in different pH and concentrations of SDS was also assessed. The drug precipitated in pH >7.0 where both molecules are ionized due to an insoluble salt formation [38].

In contrast, the dissolution profile of V1 in phosphate buffer pH 7.4 with Tween 80 without pancreatin could reach 58.66% of the 4.0 mg drug dose after 60 minutes (Fig. 4A). With the addition of the enzyme, 75.15% of BPQ was dissolved. However, it is important to note that in this condition, it was observed drug precipitation. After 30 minutes, the dissolution with and without pancreatin was 83.29% and 67.93%, respectively. In contrast, SNEDDS dissolution profile in the simulated gastric fluid showed a burst in the first 10 minutes and reached a plateau up to 50 min. A complete release of BPQ was observed within 30 min after the pH was increased to 6.8. The authors emphasized that avoiding precipitation may improve BPQ oral absorption [6].

V2 showed a release comparable to V1 in media containing Tween 80 with the enzyme, but slower release in medium without pancrelipase (Fig. 4A). From V2, after 60 minutes, 81.25% and 39.07% of 4.0 mg dose were dissolved, with and without enzyme, respectively. The higher V2 Z-average can explain the release in the medium without pancreatin compared with V1 (Fig. 4A). With the reduction in the particle size, the surface area is increased; thus, improved BPQ solubility and dissolution are expected [39].

Targeting the intestines, the drug must be dissolved only when it enters the duodenum, where bile salts and pancreatic fluids are released in response to lipid ingestion. These fluids contain multiple lipases, such as pancreatic lipase, pancreatic lipase-related protein 2, carboxyl ester lipase and phospholipase A2 [40]. Hence, we hypothesized that this physiological mechanism may allow the BPQ release from NLC throughout GIT (pH >6.4).

BPQ dissolution from both NLCs preparations in medium with SDS was increased. V1 and V2 avoided the drug precipitation, as compared with free BPQ, despite the precipitation by the interaction with SDS charges. The delayed BPQ release showed the potential for BPQ intestine delivery. A similar performance in *in vitro* dissolution of NLC containing vitamin D3 was found [41]. In gastric conditions, less than 4.0% of the vitamin was released after seven hours of testing. Still, the release in the simulated intestinal fluid was 90% after eight hours due to lipase activity.

3.6. Cytotoxicity and leishmanicidal activity

The cytotoxicity against mammalian cells of free BPQ and BPQ-NLC, resulted in a CC_{50} of 554.4 µM (95% CI: 252.2–1090.0) and of 583.4 µM (95% CI: 362.6–938.6) for free BPQ and V1, respectively. The IC_{50} against *L.infantum* amastigotes from free BPQ was 456.5 nM (95% CI: 332.4 - 627.0). From BPQ-NLC, the IC_{50} was 229.0 nM (95% CI: 190.5 - 275.2). Selective index of free BPQ and BPQ-NLC were 1149 and 2548, respectively.

Reimão et al (2012) [25] found BPQ IC50 using *L. infantum chagasi* amastigotes of 1.50 µM (1.41-1.62) or 1500 nM, while Croft and colleagues (1992) [4] found IC50 of 50 nM for *L. donovani* amastigotes. Therefore, we considered that these discrepancies (50, 456.5 and 1,500 nM) may be explained by the methodology difference and parasite sensitivity. Thus, we consider that due this

variability direct comparison with literature were not possible. However, Reimão and colleagues (2012) [25] and Thapa and colleagues (2021) [42] showed the safety and high selective index (>10) of free BPQ. The present results corroborated the previous studies and showed increased selective index of BPQ in nanostrucutured systems as found by Thapa and colleagues (2021). Their study also showed an increase of 13-fold in the selective index of nanostructured BPQ against amastigotes of L. donovani when compared to free BPQ.

3.7. *In vitro* immunomodulatory effects of BPQ-NLC

The *in vitro* immunomodulatory effects of BPQ-NLC were assessed by evaluating the cytokines levels in cultures of J774A.1 cells treated with different concentrations of NLC or BPQ-NLC (V1). As expected, the positive control of LPS stimulation resulted on high levels of IL-1β, IL-6, and TNF-α (data not shown). There were no detectable levels of IL-1β and IL-6 with the treatment with either BPQ-NLC or NLC. On the other hand, TNF-α was detected on cultures of cells treated with 1 or 5 μM of BPQ-NLC or NLC, and at similar levels to those observed on cells in Medium alone (Fig. 5). The 10 μM dosage of both formulations induced a significant decrease of TNF-α, especially after 6 and 24 h of incubation.

The absence of higher TNF-α level on NLC or BPQ-NLC treated cells compared with cells in Medium showed that these formulations do not stimulate an unspecific immune response. Similar results were observed with the nanoliposomal buparvaquone treatment of murine peritoneal macrophages [43]. Furthermore, the highest dosage of both NLC and BPQ-NLC decreased the secretion of this cytokine, which is particularly important during visceral leishmaniasis. The overexpression of TNF-α was associated with higher proliferation of parasites and chronic infection [44]. The lack of IL-1β and IL-6 production after treatment with NLC or BPQ-NLC in the present study may also be beneficial. *Leishmania* spp. are known to induce the production of these cytokines in the host cells with protective effects, along with TNF-α. On the other hand, disease severity is associated with an uncontrolled inflammation caused by excessive amounts of IL-1β, IL-6, and TNF-α [45].

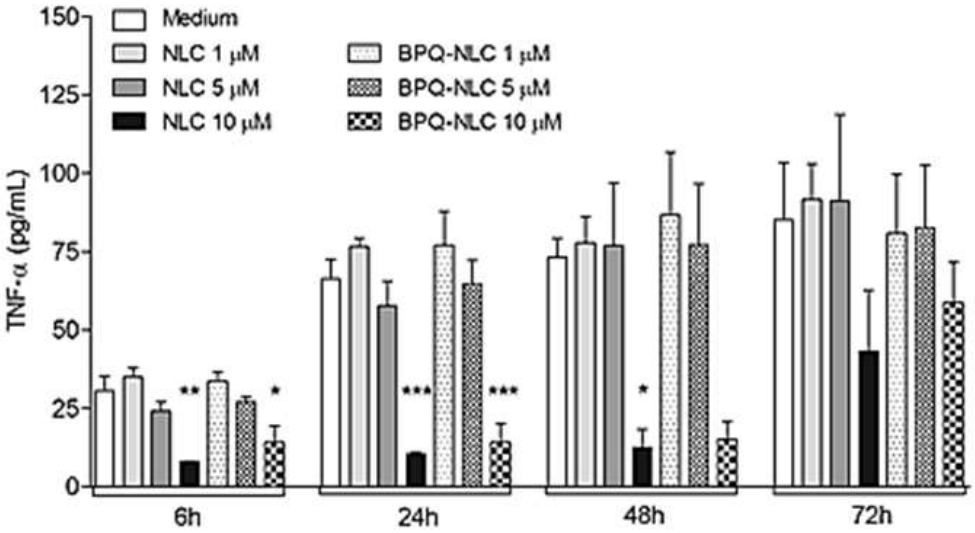


Figure 5. Levels of TNF-α after treatment with NLC or BPQ-NLC. The levels of TNF-α were determined by ELISA assay on supernatants from cultures of J774A.1 macrophages treated with NLC or BPQ-NLC at 1, 5, or 10 μM after 6, 24, 48 and 72 h of incubation. Results presented as means with SEM; *p ≤ 0.05; **p ≤ 0.01; and ***p ≤ 0.001 compared with Medium at the respective period of incubation.

3.8. *In vivo* leishmanicidal activity

For decades, viceral leishmaniasis has been treated with injectable pentavalent antimonials, and only miltefosine has been approved for administration by oral route [46]. Nevertheless, this drug substance never became affordable and available for patients who most need the treatment [46]. Besides, the development of resistance has increasing concerns about the effectiveness of miltefosine drug therapy [47]. Hence, the aim of this work was to evaluate whether BPQ-NLC presents *in vivo* activity against *L. infantum*.

Fig. 6 shows the leishmanicidal activity of BPQ-NLC V1 (0.3 mg/kg/day) and miltefosine (2.0 mg/kg/day), in the spleen and liver. V1 parasite burden reduction in spleen was 94.4% ± 6.0% ($p > 0.05$; $\alpha = 0.05$), while in the liver 83.4% ± 18,2% ($p < 0.05$; $\alpha = 0.05$). For miltefosine it was 92.8% ± 7.9% and 87.2% ± 14.2% in the spleen and in the liver, respectively. For both treatments the parasite burden was reduced when compared to the control (untreated group). In addition, the statistical analysis showed that there is no difference between blank-NLC and control group, for both spleen and liver. The ANOVA p value for the liver and the spleen were <0.05 ($\alpha = 0.05$) and >0.05, respectively. The statistical significance for the spleen could not be achieved due to the high variability of the control. Despite the non-statistical significance for the spleen results, the box plot shows the difference between control and the BPQ-NLC treated group.

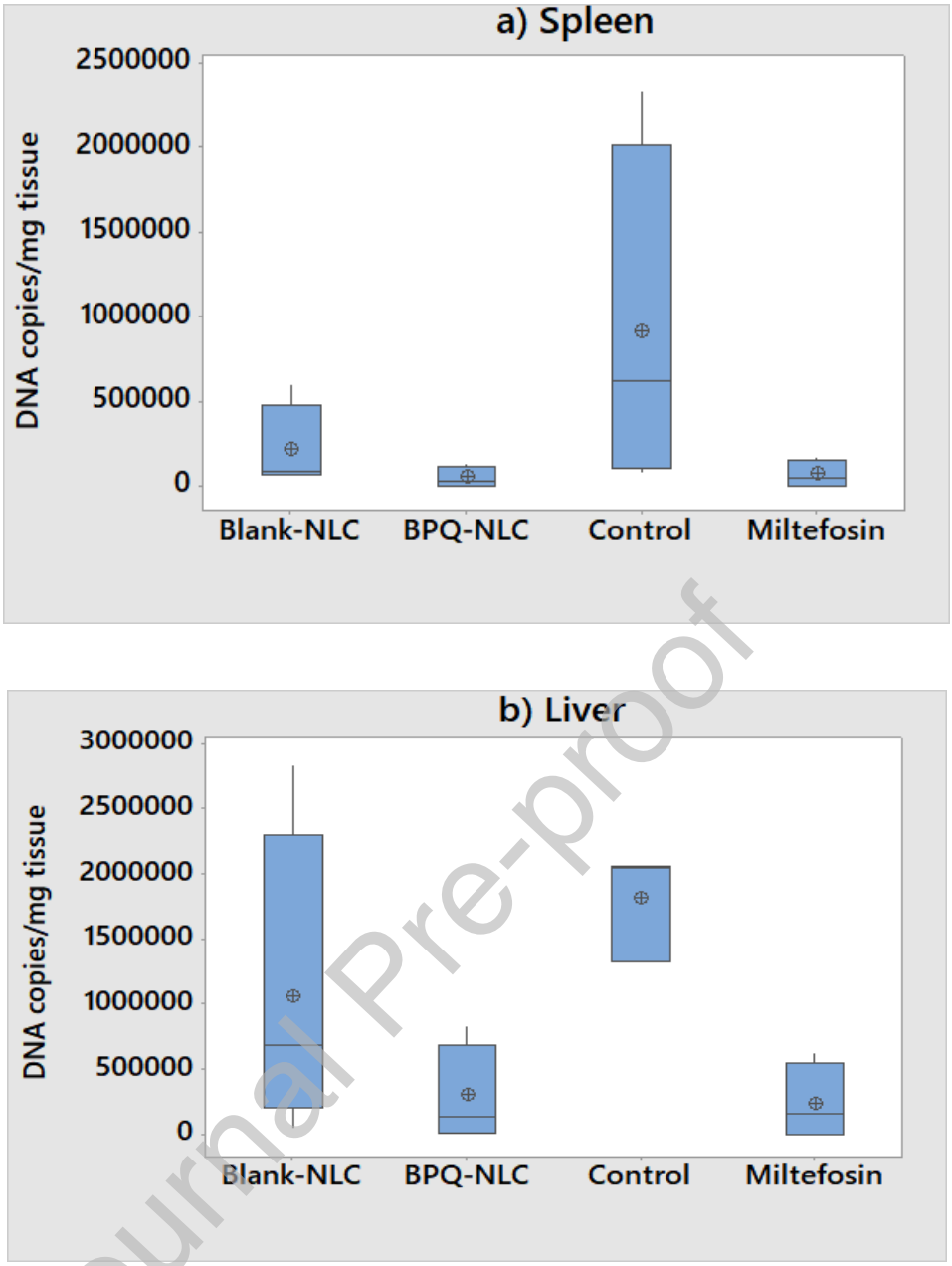


Figure 6. Leishmanicidal activity after oral administration (10 consecutive days) of BPQ-NLC (0.3 mg/kg/day), Miltefosine (2.0 mg/kg/day), blank-NLC and control (untreated group) in a) spleen and b) liver (n = 5/group, young male Golden hamsters <110 g).

A liposome formulation containing BPQ was tested in *L. infantum*-infected hamsters [43]. Initially, three routes of administration were evaluated: sub-cutaneous, intramuscular and intravenous, at 0.4 mg/kg/day (1.2 mol/kg/day), for 10 consecutive days. Sub-cutaneous treatment showed the best efficacy, reducing the parasite burden in the spleen and liver by 98 and 96%, respectively. For authors, the effectiveness of the formulation could be due to a depot effect, which could sustain BPQ release from possible intact nanoparticles. Besides, they also discussed the possibility of drugs reaching the lymphatic system when using injectable liposomes. In this case, the drug could enter the lymphatics by reaching lymph nodes in the site of the lesion, which is a gateway to pathogen dissemination [43,48].

Herein, the expected drug release mechanism involves the digestion of the lipids and the presence of bio-surfactants, both contributing to BPQ solubilization and absorption. The lymphatic system is also associated with oral lipid formulations, but in this case the hypothesis is based on drugs entering the lymphatics by chylomicron pathway. It states that after digestion of lipid formulations, drug and lipids could be packing in the enterocytes during chylomicron synthesis [49]. In the case of BPQ, its high lipophilicity justifies the possibility, but other factors such as particle size or chain length of triglycerides may impact the phenomenon [49].

A BPQ self-nanoemulsifying drug delivery system (SNEDDS) was tested in *L. infantum*-infected BALB/c mice [6]. The BPQ dose was 6.0 mg/kg/day, once daily for 10 consecutive days. The parasite burden reduction in spleen was 94% , and in the liver it showed values of 48 and 56%. Authors attributed the lower activity in the liver to a possible BPQ metabolism by CYP enzymes (2C9, C19 and 3A4) from mouse liver microsomes. They proposed developing SNEDDS with CYP inhibitor fluconazole, besides reducing the amount of long-chain and increasing the amount of short and medium-chain tryglycerides in the formulation, which could diminish liver accumulation of the product to improve BPQ effect.

In our study BPQ parasite reduction in the liver was 83% and close to reference drug miltefosine (87%). Preclinical studies showed that miltefosine is slowly and almost completely absorbed in rats and dogs [50]. Considering its slow elimination and high accumulation [51], no issues related to bioavailability seems to be associated with this drug substance. The lower parasite reduction of both compounds in the liver might be hyphotesized to an early leishmania proliferation in this organ; the spleen, in turn, might serve as a resevoir [45]. Hence, a higher dose could provide a better efficacy result in the liver, but this approach should be accompanied with safety monitoring in clinical studies.

The dose of miltefosine was chosen for two purposes. First, to verify the validity of the animal model; second to estimate an initial dose of BPQ-NLC in one of the species of interest (dogs with visceral leishmaniasis). Therefore, the miltefosine dose was chosen considering that the leishmanicidal activity was statistically equivalent to the BPQ-NLC tested dose. Even being 3 times higher than the recommended dose, using body area equivalence as described by the FDA, there was no total elimination of parasites, as reported by Fortin et al. (2012) [52], and by Hendrickx et al. (2015), which stated that even using 40 mg/kg dose, hamsters failed to eliminate *L. infantum* parasites with a 5-day treatment [53]. Therefore, the dose of 10 mg/kg provided a direct comparison of leishmanicidal activity in a possible future test of BPQ-NLC in dogs. Additionally, it turned out to be a suitable leishmanicidal activity amount to validate the animal model.

In this study, we aimed to develop BPQ-NLC for leishmaniasis oral treatment. Since the product showed equivalent efficacy as reference drug miltefosine, the possibility of combining the two drugs in a therapeutic regimen in the future could be considered. Multidrug therapy may provide therapeutic benefits such as lower doses, higher efficacy and better patient compliance. In addition, reducing dose may help to avoid the selection of drug-resistant lines of the parasite [47].

This is especially interesting considering the raising concerns about miltefosine monotherapy, which encourages the search for therapeutic regimens with better efficacy and safety outcomes [47].

4. Conclusion

In this study we tested the hypothesis whether BPQ-NLC shows *in vivo* activity against *L. infantum* by oral administration. BPQ-NLC showed similar leishmanicidal activity compared to miltefosine, presenting parasite burden reduction higher than 80% in the liver. Therefore, our results supported that this formulation can enable safe and feasible oral therapy for the treatment of leishmaniasis. Thus, this study revealed a promising strategy for treating leishmaniasis, which can expand the limited therapeutic arsenal available for this disease. HPH successfully allowed the BPQ-NLC preparation with suitable particle distribution. Using a statistical approach, it was possible to understand the critical input variables, % lipid phase, and surfactant concentration and their interactions, which influenced the Z-average. The mathematical model derived from the study yield two optimized formulations, V1 (173.9 ± 1.6 nm) and V2 (232.4 ± 1.6 nm), both presented Z-averages close to predicted values, confirming the suitability of the model. BPQ solubility was improved significantly, reaching up 73.0-fold compared with free drug. The dissolution test showed the potential oral administration of the BPQ-NLC by the performance of bio-surfactants and lipolytic enzymes. The assessment of the stability testing data revealed the feasible development of a liquid dosage form, which was prepared using affordable lipids and direct scalable technology.

Declaration of interest

The authors report no declarations of interest.

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CRediT authorship contribution statement

Lis Marie: Conceptualization, Investigation, Validation, Formal analysis, Writing – Original Draft. **Raimar Löbenberg:** Supervision, Methodology. **Eduardo Barbosa:** Writing – Reviewing & Editing. **Gabriel de Araujo:** Methodology. **Paula Sato:** Investigation, Formal analysis, Writing – Reviewing & Editing. **Edite Kanashiro:** Investigation. **Raissa Eliodoro:** Investigation. **Mussya Rocha:** Investigation. **Vera Lúcia:** Methodology. **Nikoletta Fotaki:** Writing – Reviewing & Editing. **Nádia Bou-Chacra:** Project Administration, Funding acquisition, Resources, Writing – Reviewing & Editing.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.