Carvacrol loaded nanostructured lipid carriers as a promising parenteral formulation for leishmaniasis treatment


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ABSTRACT

Leishmaniasis are a group of neglected infectious diseases caused by protozoa of the genus Leishmania with distinct presentations. The available leishmaniasis treatment options are either expensive and/or cause adverse effects and some are ineffective for resistant Leishmania strains. Therefore, molecules derived from natural products as the monoterpene carvacrol, have attracted interest as promising anti-leishmania agents. However, the therapeutic use of carvacrol is limited due to its low aqueous solubility, rapid oxidation and volatilization. Thus, the development of nanostructured lipid carriers (NLCs) was proposed in the present study as a promising nanotechnology strategy to overcome these limitations and enable the use of carvacrol in leishmaniasis therapy. Carvacrol NLCs were obtained using a warm microemulsion method, and evaluated regarding the influence of lipid matrix and components concentration on the NLCs formation. NLCs were characterized by DSC and XRD as well. In addition, the in vitro carvacrol release from NLCs, the in vitro cytotoxicity and leishmanicidal activity assays, and the in vivo pharmacokinetics evaluation of free and encapsulated carvacrol were performed. NLCs containing carvacrol were obtained successfully using a warm microemulsion dilution method. The NLCs formulation with the lowest particle size (98.42 ± 0.80 nm), narrowest size distribution (suitable for intravenous administration), and the highest encapsulation efficiency was produced by using beeswax as solid lipid (HLB = 9) and 5% of lipids and surfactant. The in vitro release of carvacrol from NLCs was fitted to the Korsmeyer and Peppas, and Weibull models, demonstrating that the release mechanism is probably the Fickian diffusion type. Moreover, carvacrol encapsulation in NLCs provided a lower cytotoxicity in comparison to free carvacrol (p < 0.05), increasing its in vitro leishmanicidal efficacy in the amastigote form. Finally, carvacrol encapsulation in NLCs decreased the in vivo pharmacokinetics of free carvacrol (p < 0.05), favoring a higher distribution of carvacrol in the target tissues. Thus, it is possible to conclude that the developed NLCs are a promising delivery system for leishmaniasis treatment.

1. Introduction

Neglected Tropical Diseases (NTDs) comprises several infectious diseases predominant in the poorest regions of the world. These diseases are prevalent in tropical and sub-tropical areas, due to insufficient sanitary conditions. It is estimated that currently there are approximately 20 NTDs prevalent in 149 countries, affecting one billion people worldwide. Among NTDs, it is estimated that annually around 700,000 to 1 million new cases of leishmaniasis occur and 20,000 to 30,000 of deaths worldwide (de Souza et al., 2018; World Health Organization (WHO), 2018).

Leishmaniasis is caused by protozoa of the genus Leishmania in the...
family Trypanosomatidae. This disease presents in two main forms, cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL) (Tiuman et al., 2011). According to the World Health Organization, 25 countries were considered high burden for leishmaniasis in 2015, which means they present over 100 cases of VL and over 2500 of CL. Among these countries, Brazil is the only country to present high burden for both forms of leishmaniasis (de Souza et al., 2018; Organization, 2016). In Brazil, there were 3200 registered cases of VL and 12,690 cases of CL, with the Northeast region most affected by VL with 1523 (47.59%) cases and North region most affected by CL with 5075 (39.99%) registered cases (Ministério da saúde, 2017; Saúde, 2017).

Thus, from an epidemiological point of view leishmaniasis can be considered a public health issue and a major concern for the health surveillance agencies. Since the control of insect vector is difficult and no effective vaccine exists, chemotherapy is the main means of dealing with this disease. In the past 70 years, pentavalent antimonials - sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantime®) - , amphotericin B, pentamidine, paromomycin and miltefosine have been used as gold standard treatment of leishmaniasis (Pham et al., 2014, 2013; Tiuman et al., 2011).

However, conventional leishmaniasis treatment options may cause some undesirable adverse effects such as gastrointestinal disorders, kidney and liver toxicity (Pham et al., 2013). In addition, some of these drugs are expensive, Leishmania species from different strains have shown different susceptibility to them, and resistance of some isolates and/or strains to some drugs have been reported, especially, with antimonials and miltefosine. Thus, there is an urgent need for new molecules to treat Leishmania infections (de Medeiros et al., 2011; Pastor et al., 2015).

Besides the synthesis of drugs, the research of natural products may provide new promising molecules. Nowadays, medicinal plants and/or several of its derivative compounds are employed in the treatment of diseases. As examples, paclitaxel and vinca alkaloids are used in cancer treatment, while artemisinin is a potent molecule against malaria (Polonio and Efferth, 2008).

Carvacrol (CRV) is a phenolic monoterpene that presents various pharmacological activities such as analgesic (de Sousa, 2011), anti-inflammatoty (Silva et al., 2012), antimicrobial (Belda-Galbis et al., 2014), antitumoral (Arunasree, 2010), antioxidant (Beena et al., 2013) and anti-leishmania (Monzote et al., 2014). It is believed that because of its hydrophobic nature, carvacrol is able to be preferentially incorporated in the cell membrane, which renders them permeable to protons and ions as well as affecting vital enzymatic function of the microorganisms (de Medeiros et al., 2011). Furthermore, Silva et al. (2017) observed that the presence of the hydroxyl group associated with the benzene ring in the carvacrol molecule plays an important role for anti-leishmania activity.

De Melo et al. (2013) reported the anti-leishmania activity of carvacrol, which demonstrated lethal inhibition concentration to 50 percent of the Leishmania chagasi (IC50) of 2.3 μg mL⁻¹. In L. amazonensis strains, Pastor et al. (2015) and Monzote et al., 2014 observed for carvacrol IC50 of 15.3 ± 4.6 μg/mL⁻¹. Moreover, in vivo studies in mice demonstrated a reduction in lesion size, caused by cutaneous leishmaniasis, after intraleusmal administration of 30 mg/kg of carvacrol on a day for 15 days in comparison to control group (Monzote et al., 2014).

Nevertheless, carvacrol exhibits low aqueous solubility, due to its lipophilic nature, rapid oxidation and volatilization, limiting its therapeutic application (Santos et al., 2015). The encapsulation of hydrophobic compounds, such as carvacrol, in colloidal carriers is a possible strategy to overcome these limitations (Bruni et al., 2017; De Almeida et al., 2017). Furthermore, nanoparticles ranging in size from 50 to 500 nm especially, have the advantage of being easily internalized by macrophages that are the main phagocytic cells that hosts Leishmania parasites. Internalization of nanoparticles by macrophages may increase intracellular drug concentration, specifically in the parasitic vacuole, where Leishmania reside (Bruni et al., 2017). This mechanism might favor the increase of therapeutic efficacy by release of active compound directly in macrophage-rich organs such as bone marrow, liver and spleen (de Souza et al., 2018).

Nano-sized carriers such as lipid nanoparticles have been reported to improve the efficacy of the known anti-leishmania and anti-malaria agents, amphotericin B and oxazolin, respectively (Lopes et al., 2012). Nanostructured Lipid Carriers (NLCs) consist in nanometric particles obtained from mixtures between solid and liquid lipids dispersed in a surfactant solution, remaining solids at room and corporal temperature (Müller et al., 2007). Due to its nanoparticulate and matrix nature, NLCs have some advantages such as the controlled release of substances, due to the solid state of the lipid matrix, the improvement of drug stability, low toxicity and biocompatibility, being promising release systems for parenteral or non-parenteral administration of molecules for leishmaniasis treatment (Guterres et al., 2007; Mehnert and Mäder, 2012; Müller et al., 2000).

Thus, the aim of this work was to encapsulate CRV in a pharmaceutically acceptable formulation of NLCs for parenteral administration. This study assesses the influence of manufacturing parameters, such as the choice of solid lipid, surfactant and lipid concentration, as well as the stability during storage time. In addition, the in vitro carvacrol release from NLCs, the in vitro cytotoxicity and leishmanicidal activity assays, and the in vivo pharmacokinetics evaluation of free carvacrol and encapsulated were accessed as well.

2. Material

Stearic acid (SA) was purchased from Dinâmica® and beeswax (BW) from GM Ceras (São Paulo, SP, Brazil). Carvacrol (≥ 99%), p-cresol (≥99.5%) and Kolliphor188® were acquired by Sigma Aldrich® (St. Louis, MO, USA). Acetonitrile (ACN) and acetic acid HPLC grade were obtained from Fisher Scientific® (Fairlawn, NJ). Drug-free Wistar rat plasma with sodium heparin was purchased from Innovative Research, Inc., (Novi, MI). All the other solvents were acquired from Fisher Scientific®.

3. Methods

3.1. Preparation of NLCs by warm microemulsion method

NLCs were prepared by warm microemulsion oil in water (O/W) adapted from Souza et al. (2011). Firstly, the oily phase containing a mixture of the solid lipid (SA or BW) and carvacrol as liquid lipid in 7:3 ratio was heated up to 10 °C over the solid lipid melting point. The aqueous phase composed of the surfactant Kolliphor 188® (known as poloxamer 188) was dispersed in ultrapure water and heated up to approximately to the same temperature of the oily phase. Then, the aqueous phase was poured into the oily phase and submitted to an ultrasound (Newtown, EUA) Vibracell® 130 W model at 50% of amplitude for 10 min. Finally, the coarse emulsion was immediately dispersed in cold water at 2 to 4 °C (placed in an iced bath) in 1:10 ratio followed by stirring at 3400 rpm for 3 min in Ultra-turrax IKA® (Staufen, Germany) T25 model.

For DSC and XRD analysis the resultant nanodispersion was freeze-dried by Freeze Dryer LS3000 TERRONI® for 48 h.

3.2. Factorial design

For the evaluation of the lipid matrix influence on the hydrodynamic radius, polydispersity index (PdI) and zeta potential of the free and carvacrol loaded NLCs, a 2³ factorial design was performed. The variables analyzed were the hydrophilic-lipophilic balance (HLB) required by the solid lipids (9 for BW and 15 for SA) and the lipids (solid + liquid lipids) concentration in the coarse emulsion (2 and 5% w/v). In this case, the surfactant concentration was kept constant at 1%
in all obtained formulations.

A second factorial design $2^3$ was performed aiming the evaluation of the lipids (2 and 5% w/v) and surfactant concentration (1 and 5% w/v) on the hydrodynamic radius, polydispersity index (Pdi), zeta potential, encapsulation efficiency (EE%) of the free and carvacrol loaded NLCs. In this case, the solid lipid used was the BW.

The NLCs formulations were developed following these factorial designs and the obtained results were submitted to STATISTICA® 10.0 (StatSoft Inc®, Tulsa- US) software. The analysis of variance (ANOVA) of the influence of variables and their interactions were performed, being $p < 0.05$ values considered statistically significant.

3.3. HPLC quantification method of carvacrol

All analyses were performed on YL9100 HPLC System (Anyang-si, Korea) equipped with a PDA detector (Model YL9160) operating at 274 nm, a quaternary pump (Model YL9110), a vacuum degasser (Model YL9101), an autosampler (Model YL9150) and a column oven (Model YL9130) at the temperature of 35 °C. A Agilent® Zorbax C18 column (150 × 4.6 mm, 5 µm packing, Santa Clara, EUA) was used. The mobile phase consisted of acetonitrile: water acidified with acetic acid 1% (60:40) at a flow rate of 1 mL min$^{-1}$. The methodology was validated to ensure the reliability of results. All measurements were performed in triplicate.

3.4. Characterization of NLCs

3.4.1. Particle size, polydispersity index and zeta potential

The particle size, polydispersity index (Pdi) and Zeta potential of the prepared NLCs were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments®, UK) with a fixed detector at an angle of 173°, and a 35 mw He-Ne laser with wavelength of 633 nm, at 25 °C. Whenever needed, the samples were diluted with ultrapure water to an appropriate concentration, prior to measurement in order to prevent multiscattering effects. Zeta potential was obtained by the conversion of electrophoretic mobility according to the method of Helmholtz-von Smoluchowski. All measurements were carried out in triplicate.

3.4.2. Encapsulation efficiency (EE%)

The encapsulation efficiency of carvacrol in the NLCs was determined by the indirect method described by Ribeiro et al. (2016). The NLCs containing carvacrol were centrifuged in a Eppendorf 5804R model (Hamburg, DE) centrifuge using Vivaspin 500 Sartorius (Model YL9101), an autosampler (Model YL9150) and a column oven (Model YL9130) at the temperature of 35 °C. A Agilent® Zorbax C18 column (150 × 4.6 mm, 5 µm packing, Santa Clara, EUA) was used. The mobile phase consisted of acetonitrile: water acidified with acetic acid 1% (60:40) at a flow rate of 1 mL min$^{-1}$. The methodology was validated to ensure the reliability of results. All measurements were performed in triplicate.

\[
EE\% = \frac{(CRV \text{ used in NLC preparation}) - (CRV \text{ filtered})}{(CRV \text{ used in NLC preparation})} \times 100
\]

To perform the next characterizations, in vitro release experiments, in vitro cytotoxicity, in vitro leishmanicidal activity and in vitro pharmacokinetics a CRV-NLCs formulation with 5% (w/v) of carvacrol, 5% (w/v) of surfactants and 1500 µg/mL of carvacrol was chosen.

3.4.3. Differential scanning calorimetry

DSC curves were obtained by DSC NETZSCH DSC 200 F3 (Selb, Germany). The solid lipid beeswax, poloxamer 188, and freeze-dried carvacrol loaded NLCs (3 to 5 mg) were placed in aluminum crucibles and analyzed in the temperature range of 25 to 300 °C, heating rate of 10 °C min$^{-1}$, and nitrogen dynamic atmosphere (50 mL min$^{-1}$).

3.4.4. X-ray diffraction

XRD analysis was performed in a Bruker (Billerica, USA) model D8 Advance; Radiation: Cu Kα (λ = 1594 Å). The solid lipid beeswax, poloxamer 188, and freeze-dried carvacrol loaded NLCs were analyzed in the range of 2 to 40° (2θ), speed of 1° min$^{-1}$, 40 kV and 40 mA.

3.4.5. Transmission electron microscopy (TEM and cryo-tem)

CRV-NLCs were deposited on ultrathin lacey copper grids and negatively stained with 2% (w/v) uranyl acetate and observed through a transmission electron microscope JEOL (Akishima, JP) JEM-1400Plus model at 120 kV. CRV-NLCs were also prepared for Cryo-TEM by quench-freezing thin liquid films in liquefied ethane. The vitrified specimen was transferred to the microscope for imaging on a liquid-nitrogen-cooled Gatan 626 cryoholder. The temperature of the sample was kept below −180 °C throughout the examination by using a transmission electron microscope JEOL (Akishima, JP) JEM-1400Plus model at 120 kV. A low-dose procedure was used to reduce radiation damage in the areas of interest.

3.4.6. In vitro release experiments

The in vitro release experiments were performed according the dialysis bag method reported by Fanguero et al. (2016) adapted. Firstly, cellulose acetate 10.000 MWCO membranes Fisher Scientific® (Whaltam, EUA) were prepared as the supplier recommended. The release medium was composed of a mixture of phosphate buffer saline (pH 7.4) and ethanol in 7:3 ratio (v/v). A carvacrol solution (CRV-free) was prepared using the release medium. Afterwards, 2 mL of the carvacrol loaded NLCs (CRV-NLCs) that was selected in the previous experiments, and CRV-free were put in the dialysis bags and placed into a container containing 10 mL of the release medium. The system was kept at 37.0 ± 0.5 °C under magnetic stirring at approximately 30 rpm for 24 h. 300 µL of the release medium was collected after 0.25, 0.5, 1, 2, 3, 4, 5, 6, 8, 12 e 24 h of the experiment has started, being replaced by new release medium in order to maintain the sink conditions. The experiment was performed in sextuplicate.

The collected samples were quantified by a HPLC-UV quantification method previously validated. Carvacrol release data were analyzed by release kinetic models, including, zero order, first order, Korsmeyer & Peppas and Weibull models by the KinetDS Copyright (C) 2010 Aleksander Mendyk software (Sizilio et al., 2018).

3.4.7. In vitro cytotoxicity in differentiated human monocyte cell line

Human monocyte cells-line THP-1 (ATCC) were cultivated in RPMI 1640 medium with 1-glutamine and 25 mM of HEPES at 37 °C and 5% of CO$_2$. For the differentiation to adherent macrophages, the culture was adjusted to a density of 5 × 10$^8$ cells/mL and 100 ng/mL of phorbol myristate acetate (PMA) were added. Then, 5 × 10$^5$ cells were seeded in each well of a 96 well plate and were incubated at 37 °C and 5% of CO$_2$ for 48 h. After the differentiation, the cells were washed and were incubated in RPMI medium, PMA free. After 24 h, differentiated THP-1 cells were incubated with different concentrations of carvacrol, CRV-NLCs (24.9–150 µg/mL) and a standard drug miltefosine (3–48.9 µg/mL) in RPMI medium at 37 °C and 5% of CO$_2$ for 48 h. Cells without treatment were used as negative control.

After the incubation time, the cells were exposed to 10 µL of MT (Biotium kit) at 37 °C for 4 h. MT is converted, from the viable cells, to formazan, and therefore, 200 µL of dimethyl sulfoxide (DMSO) were added in each well in order to solubilize formazan. The absorbance was measured using a microplate reader at 570 nm and used to calculate the cell viability (%), according to the Eq. (2):

\[
\text{Cell viability} = \frac{\text{Abs (treatment)} - \text{Abs (DMSO)}}{\text{Abs (negative control)} - \text{Abs (DMSO)}} \times 100
\]
analyzed through the Student’s t-test, considering \( p < 0.05 \) as statistically significant.

### 3.4.8. In vitro promastigote activity

Promastigotes of *L. amazonensis* strain RAT/BA/74/LV78 originally provided by dr. Lynn Soong (UTMB, Texas) were maintained in Schneider medium (Sigma, St. Louis, MO, EUA) supplemented with 10% of heat-inactivated fetal bovine serum and 10 µg/mL gentamycin (Sigma, St. Louis, MO, EUA) at 24 °C. The parasite growth curve was obtained from the daily count of the culture for 7 days. Promastigotes in exponential growth phase (6–8 × 10^6 promastigotes/mL) were used for viability assay. The promastigotes were incubated with different concentrations of carvacrol, CRV-NLCs (6.25–100 µg/mL) and miltefosine (3 – 48.9 µg/mL) (positive control) in Schneider medium at 24 °C for 48 h. As negative control, the promastigotes were kept without any treatment.

After the incubation time, 10 µL of resazurin (TOX-8 *In vitro Toxicology Assay Kit Resazurin Based*, Sigma) were added in each well and the plate was incubated at 24 °C for 2 to 3 h. The absorbance was measured by using a microplate reader at 570 and 600 nm and used to calculate the promastigote viability (%), according to the Eq. (3):

\[
\text{% Viability} = \frac{(\text{Abs 570 nm} \times \text{Abs 600 nm x RO})\text{test}}{(\text{Abs 570 nm} \times \text{Abs 600 nm x RO})\text{control}} \times 100
\]

The inhibitory concentrations to 50% of the promastigotes (IC_{50}) were obtained by nonlinear regression of the sigmoid growth curves using the software Graph Pad Prism 5.0. The results were compared and analyzed through the Student’s t-test, considering \( p < 0.05 \) as statistically significant.

### 3.5. Antiamastigote activity

#### 3.5.1. Maintenance and cultivation of parasites for antiamastigote activity

*Leishmania amazonensis* promastigotes (strain MHOM/BR/77/LTB 0016) were maintained at 26 °C in Schneider* medium (Sigma-Aldrich Corp., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin, 100 U/mL penicillin. Subcultures were performed twice a week until the 5th passage. The parasites were obtained from infected BALB/c mice.

#### 3.5.2. Cytotoxicity to murine peritoneal macrophages

Murine peritoneal macrophages in 96-well plates were treated with carvacrol, lipid formulation at concentrations 7.5 - 960 µg/mL and amphotericin B (0.125 – 20 µg/mL) for 72 h at 37 °C/5%CO₂. After 72 h, resazurin sodium salt (500 µM) (Sigma-Aldrich Corp., St. Louis, MO, USA) was added to the macrophage cultures to a final concentration of 50 µM, and the plates were then incubated at 37 °C/5%CO₂ for additional 4 h. The fluorescence was measured at excitation/emission of 560/590 nm in Spectra Max Gemini XPS (Molecular Devices, Silicon Valley, EUA). The number of viable treated macrophages was expressed as a percentage of the number of viable untreated-control macrophages (taken as 100%). Each point was tested in triplicate with three biological replicates.

#### 3.5.3. In vitro antiamastigote activity assay

Peritoneal macrophages from BALB/c mice were infected with *L. amazonensis* promastigotes (stationary-growth phase) at a 3:1 parasite-to-macrophage ratio, incubated for 4 h in Lab-Tek chambers (Nunc, Roskilde, Denmark), and kept at 37 °C/5%CO₂. After 24 h, the chambers were washed, and the cultures were treated with carvacrol, lipid formulation at concentrations 7.5 - 240 µg/mL and amphotericin B (0.04375 – 1.4 µg/mL) in RPMI medium supplemented with 10% FBS, 1% pyruvate and 1% glutamine for 72 h at 37 °C 5% CO₂. After incubation, the slides were stained, and the infection rate was determined by counting under a light microscope. The infection rate was calculated using the formula: (% of infected macrophages \( \times \) average number of amastigotes per total number of macrophage). The 50% inhibitory concentration (IC_{50}), i.e., the minimum drug concentration that caused a 50% reduction in infection rate in comparison with that in control infection without the compound, was obtained by non-linear regression using GraphPad Prism software. Each point was tested in duplicate with three biological replicates.

#### 3.5.4. Animals

Healthy male cannulated in the jugular vein Wistar rats (weight 300 to 350 g) were obtained from Charles River Laboratories. The rats were fed with standard food pellets and maintained under standard laboratory conditions with a dark/light cycle of 12 h. All rats were fasted overnight before the experiments, but allowed access to water ad libitum. This study was previously approved by the local ethics committee “Institutional Animal Care and Use Committee” da University of Florida (Gainesville, FL) under the protocol number #201710000. The protocol was performed according to the “Principles of Laboratory Animal Care” (publication NIH #85–23, revised 1985).

#### 3.5.5. In vivo pharmacokinetics study

The pharmacokinetic profile of CRV in rat plasma was determined through bolus intravenous administration of 3 mg/kg of free carvacrol prepared in DMSO-saline solution for injection (CRV-S) and encapsulated (CRV-NLCs).

In the day of the experiment, 3 mg/kg of CRV-S and CRV-NLCs were administered in the lateral tail vein of 3 Wistar male rats (for each group) as a bolus. Nine blood samples (300 µL) were withdrawn from the cannula in the jugular vein and stored in EDTA-containing tubes after 0.17, 0.5, 1, 2, 4, 6, 8, 10 and 24 h after dosing. Each of the blood samples were centrifuged at 10.000 rpm for 10 min. Plasma sample analysis was performed using validated HPLC method (paper in communication). The plasma was immediately separated and stored at – 80 °C until analysis. The resulting plasma (100 µL) was vortex-mixed with 900 µL of internal standard (p-cresol) in acetonitrile (3000 ng/mL) for 3 min and centrifuged at 12.000 rpm for 20 min. The supernatant (750 µL) was dried, reconstituted in 100 µL ACN and injected for HPLC analysis.

Non-compartmental analysis of the data was performed in Phoenix 8.0 software (Certara, Princeton, NJ). All pharmacokinetic parameters were calculated for each animal and, the average and standard deviation (SD) of each parameter were determined. The terminal elimination rate constant (\( \lambda_z \)) was estimated from the slope of terminal exponential phase of the logarithmic plasma concentration-time profile using at least 4 data points. Terminal elimination half-life (\( t_{1/2z} \)) was calculated as In(2)/\( \lambda_z \). The area under the concentration-time curve was calculated using the log-linear trapezoidal rule. Clearance (CL) was calculated as the dose divided by the \( \text{AUC}_{0-\infty} \). The volume of distribution \( V_d \) was calculated as the CL divided by \( \lambda_z \). The mean residence time (MRT) was calculated as the area under the first moment curve (\( \text{AUMC}_{0-\infty} \)) divided by \( \text{AUC}_{0-\infty} \).

### 4. Results and discussion

#### 4.1. Influence of the lipid matrix on NLCs formation

Solid lipids play an important role on NLCs matrix composition and, consequently, small differences in its characteristics and properties, may lead to significant changes in NLCs formulations (Mitri et al., 2011; Müller et al., 2002; Yan et al., 2010). Teeranachaideekul et al. (2017) observed that in NLCs preparation, the adequate selection of lipid may lead to significant changes in NLCs formulations (Mitri et al., 2011; Müller et al., 2002; Yan et al., 2010).
required of 9 and 15 respectively were tested in order to select the solid lipid best suited to NLCs formulation. In addition, two pre-emulsion lipid concentration were tested, 2% e 5% (w/v), keeping constant the surfactant concentration in 1%.

Regarding the particle size, we observed that the lower the HLB required by the solid lipid and its concentration (2%) the lower was the particle size (153 ± 2.90 nm). All NLCs formulations presented a narrow particle size distribution with PDI ≤ 0.5, being the CRV-NLCs prepared with BW and 2% of lipids, the formulation with the lowest PDI (0.166 ± 0.04), following the same trend of particle size results (Lakshmi and Kumar, 2010).

Another parameter evaluated was the zeta potential. Zeta potential (ξ) can be used to estimate the nanoparticles charge and/or its electrostatic repulsion. According to DLVO’s theory the system will be considered stable, if the electrostatic repulsion dominates the Van der Walls attractive forces. In general, the aggregation of particles tends to occur less when the system presents high values of ξ (> 30 mV), due to electrostatic repulsion (Han et al., 2008). In Fig. 1, we observed that all

Fig. 1. Surface response charts of experimental design and pareto charts of the standardized effects for CRV-NLCs (a) particle size; (b) polydispersity index; (c) zeta potential in function of the% of lipids and the HLB required by the solid lipid. Color codes stand for the area below the given value. These results were obtained from STATISTICA® 10.0 software and were submitted to ANOVA statistical test, being p<0.05 values considered statistically significant.
Fig. 2. Surface response charts of experimental design and pareto charts of the standardized effects for CRV-NLCs (a) particle size; (b) polydispersity index; (c) zeta potential; (d) encapsulation efficiency in function of the% of lipids and the% of surfactants. Color codes stand for the area below the given value. These results were obtained from STATISTICA® 10.0 software and were submitted to ANOVA statistical test, being $p < 0.05$ values considered statistically significant.
obtained with a heating rate of 10 °C min⁻¹. The DSC curves were
formulated with higher surfactant concentration would present lower
calorimetrically significant effect on particle size (Fig. 2). It is expected that NLCs
for both analyzed variables and its interaction showed statisti-
cally significant effect on particle size (Fig. 2). It is expected that NLCs
lowest particle sizes were found for NLCs with the highest surfactant
and zeta potential were evaluated. In Fig. 2, we observed that all NLCs formu-
the influence of lipid and surfactant concentration on particle size, PDI
value for both analyzed variables and its interaction demonstrated sta-
sion mechanism (Tsai et al., 2012).
As shown in Pareto charts of the standardized effects (Fig. 1), both
studied variables and the interaction between them demonstrated sta-
istically significant effect (p < 0.05) on particle size, PDI and zeta poten-
tial. In view of the presented results, the solid lipid (HLB = 9), since the main stabilization mechanism of these surfactants are due to steric repulsion, besides the electrostatic repul-
sion mechanism (Tsai et al., 2012).
As shown in Pareto charts of the standardized effects (Fig. 1), both
studied variables and the interaction between them demonstrated sta-
istically significant effect (p < 0.05) on particle size, PDI and zeta poten-
tial. In view of the presented results, the solid lipid (HLB = 9) was
selected to prepare CRV-NLCs for enabling the formation of particles
with the lowest size and narrowest size distribution.
Influence of lipid and surfactant concentration on NLCs formation
and encapsulation efficiency of carvacrol
According to Shah and Serajuddin (2012), due to the presence of the
crystallinity through thermal behavior (Galvão et al., 2016).
The stability study of CRV-NLCs formulations stored at room tem-
perature (25 °C) and 4 °C from 24 h until 90 days after the preparation
was performed (shown in the supplementary information). In both storage temperatures the particle size stayed almost constant during the
period. Large fluctuations in particle size within time could indicate the
formation of aggregates, consequently increasing the range of particle
size (Zhang et al., 2018). PdI also kept constant, around 0.2, which is a
value used to indicate a narrow size distribution. Moreover, the zeta
potential of CRV-NLCs formulations varied in the same range, in-
dicating that the particles were kept stable during storage time.
4.2. Stability of NLCs
The evaluation of NLCs stability is essential to confirm that the
structural properties were preserved during the storage time, since
structural changes of NLCs as drug delivery systems may impact its
therapeutic potential (Zhang et al., 2018).
4.3. DSC and XRD characterisations
DSC is an interesting tool to characterize NLCs formulations and its
raw materials, providing information about its physical state and
crystallinity through thermal behavior (Galvão et al., 2016).
In Fig. 3A is shown the DSC curves of the chosen solid lipid, BW (a),
the surfactant P188 (b) and CRV-NLCs. The DSC curve of beeswax
presents an endothermic event in the range of 40 to 70 °C (Tpeak =
64.7°C) characteristic of its melting point. This result was similar to the
event described by our group in previous studies (Dantas et al., 2018;
Galvão et al., 2016). The surfactant used in the CRV-NLCs formulation,
P188 also showed an endothermic event in the interval of 45 to 65 °C
(Tpeak = 56.32 °C) regarding its melting point (Ige et al., 2013).
 According to Shah and Serajuddin (2012) (1), due to the presence of the
copolymer (ethylene oxide) in the structure, P188 may be used as emulsifying
and/or lipid solidification agent, similar to solid PEGs.
The CRV-NLCs exhibited two endothermic peaks in the range of 40 to 70 °C (T_{peak1} = 52.61 °C and T_{peak2} = 63.50). In addition, it was observed that the enthalpy involved in the melting of CRV-NLCs was slightly lowered in comparison to pure bulk BW. This reduction may result from the small particle size of CRV-NLCs and also suggests a disorder of crystalline structure, suggesting that carvacrol is dispersed on lipid matrix (Hu et al., 2006).

XRD is a technique that evaluates how the solid lipid is organized, its phase behavior and crystal ordering. NLCs are composed of solid lipids and modifications in crystallinity may affect its physical stability (Yang et al., 2013; Zheng et al., 2013).

Figure 3B shows the XRD profile of BW, P188, and CRV-NLCs. The beeswax presented three main well-defined diffraction peaks in 2θ: 19.40°, 21.53° and 23.96°, characteristic of its crystalline nature, and P188 presented two main peaks in 2θ: 19.23° e 23.33° (Shah and Serajuddin, 2012; Soleimanian et al., 2018). After CRV-NLCs formation, the intensity of main diffraction peaks was reduced in comparison to the raw materials (BW and P188). According to Tamjidi et al. (2013), this reduction can be attributed to a low ordering of the lipid matrix, resulting in a reduction in crystallinity which confirms the results obtained by DSC analysis.

4.4. Transmission electron microscopy (TEM and cryo-TEM)

Fig. 4a shows the TEM images of CRV-NLCs by using negative staining. It was observed that the particles were of mean particle size close to those found in the particle size analysis by DLS (90 to 150 nm). In addition, the particles had darker edges which may be due to the surfactant P188 layer immobilized in the solid lipid.

Cryo-TEM enabled a direct visualization of the morphology of the obtained particles when dispersed in water. This technique allows the formation of images of complex 3D structures with different orientations. CRV-NLCs possess a typical disk-like morphology, presenting low electron density with a circular shape when viewed from the top; or electron-dense bars when edge-on viewed (white arrows) (Fig. 4b). The discoid structure was previously described for NLCs by Esposito et al., 2017.

4.5. In vitro release experiments

The in vitro release profile of carvacrol in solution (CRV-S) and encapsulated (CRV-NLCs) is presented in Fig. 5. Carvacrol was quickly released from CRV-S in comparison to CRV-NLCs, with 100% of cumulative release less than 1 h of starting the experiment. We observed that CRV-NLCs exhibited a biphasic release profile, where a burst release occurred in the first 4 h of experiment (60% of carvacrol), possibly due to the fast diffusion of carvacrol present on the particles surface, followed by an extended release possibly due to diffusion of carvacrol or matrix erosion.

The modeling of active substances release from delivery systems is important for the understanding and the elucidation of the transport mechanisms (Papadopoulou et al., 2006). Therefore, the in vitro release data of carvacrol from NLCs were fitted with Korsmeyer and Peppas and Weibull kinetic models. The general expression of Korsmeyer and Peppas is described by the Eq. (4):

\[ \frac{M_t}{M_\infty} = k t^n \]

where “Mt/M∞” corresponds to the fraction of the active substance released at time t, “k” is the kinetic constant and “n” is the exponent that is related to the diffusion mechanisms involved in the release. Thus, for release systems of sphere type, \( n \leq 0.43 \) corresponds to Fickian diffusion, \( 0.43 < n \leq 0.85 \) corresponds to mixed release mechanism and \( n \geq 0.85 \) to Case II transport (or non Fickian diffusion) (Tsao and Hall, 2017). In the present study the “n” value found was lower than 0.43 (\( n = 0.372 \) e \( r^3 = 0.950 \)), which corresponds to the Fickian diffusion release mechanism, in other words, the release of carvacrol is driven by the concentration gradient and directly related to its solubility in the NLCs matrix and in the release media. A similar release profile was reported by Kumbhar and Pokharkar (2013) (Kumbhar and Pokharkar, 2013) for bicalutamide, a poorly aqueous soluble drug, from NLCs.

The present release profile was also fitted to the Weibull model described by the Eq. (5):

\[ \frac{M_t}{M_\infty} = 1 - \exp(-at^b) \]

where “Mt/M∞” corresponds to the fraction of the active substance released at a time t, and “a” and “b” constants. The “b” value was
correlated to the diffusion mechanisms involved in the release of active substances by Papadopoulou et al. (2006). The authors correlated “b” value obtained by Weibull equation with “n” value obtained by Korsmeyer and Peppas equation, where \( b < 0.75 \) corresponds to Fickian diffusion in the Euclidean space \((0.69 < b < 0.75)\) or fractal \( (b < 0.69)\), and \( b > 0.75 \) corresponds to anomalous transport with more than one release mechanism involved. Therefore, in the present study, the b value found was lower than 0.75 (\( b = 0.69 \) e \( r^2 = 0.9704 \)) which is related to a Fickian diffusion mechanism, confirming the Korsmeyer e Peppas model results.

4.6. In vitro cytotoxicity in differentiated human monocytic cell line

Considering the promising application of CRV-NLCs in the leishmaniasis treatment, cell viability studies are essential in the development of suitable formulations. The Leishmania parasite when in contact with the host, it is phagocytized by macrophages, multiplies and transforms in amastigote forms. Therefore, CRV-NLCs should be capable of being phagocytized by the host cell (macrophage) without causing damage to it. Table 1 shows the mean cytotoxic concentration for 50% (CC50) for THP-1 cells differentiated in macrophages, after 48 h of treatment using CRV-S, CRV-NLCs and miltefosine (conventional anti-leishmania drug). It is possible to observe that CRV-S and CRV-NLCs presented much lower cytotoxicity to THP-1 differentiated cells in comparison to the conventional leishmanicidal drug, miltefosine (MT) (\( p < 0.005 \)). After the encapsulation in NLCs, carvacrol showed lower cytotoxicity in comparison to CRV-S (\( p < 0.05 \)). This result suggests the protective role of NLCs formulations to mammalian cells. Lopes et al. (2012) also reported a decrease in the cytotoxicity of THP-1 differentiated cells after the encapsulation of the drug oryzalin in solid lipid nanoparticles using tripalmitin as solid lipid.

4.7. In vitro leishmanicidal activity

The mean inhibitory concentration for 50% (IC50) of L. amazonensis promastigotes treated with CRV-S, CRV-NLCs and miltefosine is shown in Table 1. Miltefosine was tested as positive control, since this drug is used in the leishmaniasis conventional treatment. Carvacrol in the present study showed an IC50 of 28.1 ± 0.3 µg/mL, being close to the IC50 observed by Silva et al. (2017), 25.4 ± 2.4 µg/mL, for the same Leishmania species. Youssefi et al. (2019) reported a lower IC50 when the carvacrol was tested against other species, Leishmania infantum (9.8 µg/mL). As observed in Table 1, CRV-NLCs presented IC50 close to CRV-S. This is probably due to the presence of CRV in NLCs surface (~60%), which was quickly released according to the in vitro release profile shown previously. Therefore, it is suggested that the actual CRV-NLCs activity would be even higher than CRV-S considering that part of CRV is still confined in NLCs (~40%), and not in direct contact with the parasite. Other studies in the literature have also reported unchanged activity over promastigotes after encapsulation. Moreno et al. (2015) reported that the β-lapachone encapsulated in chitosan-lecithin nanoparticles maintained the same level of activity over L. major promastigotes compared to free β-lapachone. Van de Ven et al. (2011) also observed that the saponin β-aescin encapsulated in PLGA nanoparticles showed the same efficacy against L. infantum promastigotes in comparison to free β-aescin.

4.8. Antiamastigote activity

In Table 2, it is presented the mean inhibitory concentration for 50% (IC50) of L. amazonensis amastigotes treated with CRV-S, CRV-NLCs and amphotericin B (used as positive control). The antileishmanial activity of CRV-S in the amastigote form compared with the promastigote form did not change significantly (IC50 = 28.1 and IC50 = 27.40). Moreover, CRV loaded in NLCs showed higher activity in the amastigote form (IC50 = 19.65) than in promastigote form (IC50 = 28.2). According to Moreno et al., 2015, this may suggest a possible immunomodulatory effect for the system. In addition, CRV-NLCs showed less toxicity to peritoneal macrophages than CRV-S and consequently increasing the selectivity index (SI) of carvacrol.

4.9. In vivo pharmacokinetics study

Fig. 6 shows the in vivo pharmacokinetic profile of carvacrol in solution (CRV-S) and encapsulated (CRV-NLCs) in Wistar rats after I.V. bolus administration (3 mg/kg). We observed that CRV, in the administered dose, suggests that this phenolic monoterpene probably undergo enterohepatic recirculation, since it is possible to observe initially (two first hours) a decrease in plasma concentration followed by an increase in concentration and a decrease again, which is called “zigue zague” profile. In the enterohepatic recirculation, the active substance arrives in the systemic circulation, passing through the liver, being metabolized and its metabolites are excreted with bile, by the gall bladder in the duodenum. In the gut, its metabolites are converted to the original active substance by the gut flora and soon after being re-absorbed by portal circulation. Therefore, some fraction of the active substance present in the gut may be reabsorbed and returns to the liver where the enterohepatic recirculation repeats (Roberts et al., 2002).

Several drugs have been reported to undergo enterohepatic circulation such as warfarin, morphine, erythromycin, doxycycline, ceftriaxone, and others. A recent review identified more than 45 drugs that undergo enterohepatic circulation (Gao et al., 2014). In general, drugs or active substances that undergo enterohepatic circulation are small
nonpolar molecules as carvacrol. Other phenolic compounds have been reported to present pharmacokinetic profile similar to carvacrol, such as, the ginger derivatives, 6-gingerol (6G), 8-gingerol (8G), 10-gingerol (10G) e 6-shogaol (6S) (Mukkavilli et al., 2017).

Plasma concentration-time profile was submitted to non-compartmental analysis (Table 3). CRV presented elimination half-life ($t_{1/2}$) of 51.07 ± 2.80 h, clearance (CL) of 0.057 ± 0.003 L/h and initial plasma concentration ($C_0$) of 0.59 ± 0.075 µg/mL. These results are in agreement with expectations for compounds that undergo enterohepatic circulation since the gut reabsorption tends to promote a long elimination half-life and low clearance value. There are no reports in the literature about the enterohepatic circulation of carvacrol. This is probably because the dose used on the past studies was much lower in comparison to the encapsulated (CRV-NLCs). However, after non-compartmental analysis, we observed that $C_0$, $t_{1/2}$ and mean residence time (MRT) of CRV-NLCs was higher than CRV-S ($p < 0.05$). This increase in $V_{dss}$ probably occurred due to the lipid nature of NLCs that favored a higher distribution of carvacrol in tissues, consequently resulting in a higher $C_0$, $t_{1/2}$ and mean residence time (MRT) than CRV-S. In the literature, drugs and active substances encapsulated in NLCs have been reported to increase the $t_{1/2}$ and MRT (Shi et al., 2016; Singh et al., 2016). According to Tsai et al. (2012), the active compound can be protected from metabolism and excretion when it is carried in NLCs.

However, as the pharmacokinetic profile showed that CRV undergo enterohepatic circulation and consequently providing a long $t_{1/2}$ and higher circulation time, it is expected that after the encapsulation in NLCs the profile did not change much from the CRV-S that explains why the area under the curve total ($AUC_{total}$) did not change significantly after carvacrol encapsulation in NLCs (ns). Although the CRV-NLCs showed similar exposure ($AUC_{total}$) to CRV-S, the higher MRT and $V_{dss}$ of encapsulated carvacrol would favor a higher distribution of carvacrol in the target organs, such as liver and spleen, where Leishmania resides.

5. Conclusion

Using a warm microemulsion method CRV-NLCs were optimized with beeswax (HLB = 9) as the solid lipid and the combination of 5% of lipids and surfactant. The selected CRV-NLCs formulation shows particle size, PDI and zeta potential suitable for parenteral administration. High encapsulation efficiency of CRV in NLCs formulation was achieved. Preliminary stability studies showed that CRV-NLCs were stable during the storage time of 90 days. Furthermore, the crystal order of beeswax was disturbed in the inner cores of CRV-NLCs according to DSC and XRD profiles.

The in vitro release of carvacrol from NLCs demonstrated that the release mechanism is probably by Fickian diffusion. Moreover, carvacrol encapsulation in NLCs provided lower cytotoxicity in comparison to free carvacrol and increased its in vivo leishmanicidal efficacy in the amastigote form, improving the selectivity index of CRV. Finally, the in vivo pharmacokinetics of carvacrol after IV bolus administration suggests that this phenolic monoterpene undergoes enterohepatic circulation and therefore shows a long half-life ($t_{1/2}$) and low value of clearance (CL). In addition, $C_0$ mean residence time (MRT) and $V_{dss}$ of encapsulated carvacrol were higher than free carvacrol favoring a higher distribution of carvacrol in the target tissues (liver and spleen). Thus, CRV-NLCs are a promising formulation for leishmaniasis treatment, and may be in future this formulation may serve as a platform for surface modifications for the binding of ligands with affinity for macrophage receptors, aiming to further increase the selectivity of carvacrol for the Leishmania parasite. Moreover, the next step in the development of drug delivery system to treat leishmaniasis would be the in vivo efficacy assays in mice.

Table 2

Mean inhibitory concentration ($IC_{50}$) for 50% of L. amazonensis amastigotes and mean cytotoxic concentration for 50% of peritoneal macrophages ($CC_{50}$) after 72 h of incubation with different concentrations of carvacrol in solution (CRV-S) or encapsulated (CRV-NLCs) and amphotericin B (used as positive control). Results are representative of three independent experiments. Data are expressed as mean ± SD. $n = 3$, ns, no significant statistical difference, *$p < 0.05$, significant statistical difference in comparison to CRV in solution (CRV-S) (Student’s t-test).

<table>
<thead>
<tr>
<th>Samples</th>
<th>L. amazonensis amastigotes(µg/mL)</th>
<th>$CC_{50}$ (µg/mL)</th>
<th>S/I (Peritoneal macrophages)</th>
<th>$CC_{50}$ (µg/mL)/(THP1)</th>
<th>S/I (THP1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRV-S</td>
<td>27.40 ± 2.05</td>
<td>35.27 ± 5.79</td>
<td>1.28</td>
<td>64.1</td>
<td>2.3</td>
</tr>
<tr>
<td>CRV-NLCs</td>
<td>19.65 ± 1.3**</td>
<td>49.05 ± 2.04***</td>
<td>2.50</td>
<td>73.5</td>
<td>3.74</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>0.27 ± 0.05</td>
<td>&gt;20</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

The pharmacokinetic profile of carvacrol in solution (CRV-S) was similar to the encapsulated (CRV-NLCs). However, after non-compartmental analysis, we observed that $C_0$, $t_{1/2}$ and mean residence time (MRT) of CRV-NLCs was higher than CRV-S ($p < 0.05$). Moreover, the volume of distribution ($V_{dss}$) of CRV-NLCs was higher than CRV-S ($p < 0.01$). This increase in $V_{dss}$ probably occurred due to the lipid nature of NLCs that favored a higher distribution of carvacrol in tissues, consequently resulting in a higher $C_0$, $t_{1/2}$ and mean residence time (MRT) than CRV-S. In the literature, drugs and active substances encapsulated in NLCs have been reported to increase the $t_{1/2}$ and MRT (Shi et al., 2016; Singh et al., 2016). According to Tsai et al. (2012), the active compound can be protected from metabolism and excretion when it is carried in NLCs.

Table 3

Pharmacokinetic data obtained from the non-compartmental analysis of in vivo pharmacokinetic profile after intravenous administration of carvacrol in solution (CRV-S) and encapsulated (CRV-NLCs) in Wistar rats. Results are representative of three independent experiments. Data are expressed as mean ± SD. $n = 3$, ns, no significant statistical difference, *$p < 0.05$ and **$p < 0.01$, significant statistical difference in comparison to CRV in solution (CRV-S) (Student’s t-test).

<table>
<thead>
<tr>
<th>Pharmacokinetic parameters</th>
<th>CRV-S</th>
<th>CRV-NLCs</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t/2$ (h)</td>
<td>51.07 ± 2.80</td>
<td>69.33 ± 7.30</td>
<td>0.0150</td>
</tr>
<tr>
<td>$C_0$ (µg/mL)</td>
<td>0.59 ± 0.075</td>
<td>0.89 ± 0.14*</td>
<td>0.027</td>
</tr>
<tr>
<td>CL (L/h)</td>
<td>0.057 ± 0.003</td>
<td>0.048 ± 0.005</td>
<td>ns</td>
</tr>
<tr>
<td>$V_{dss}$ (L)</td>
<td>4.37 ± 0.079</td>
<td>4.83 ± 0.12**</td>
<td>0.0052</td>
</tr>
<tr>
<td>AUC total (µg h/L)</td>
<td>13.62 ± 0.311</td>
<td>13.38 ± 0.57</td>
<td>ns</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>75.29 ± 4.09</td>
<td>99.67 ± 9.99*</td>
<td>0.0166</td>
</tr>
</tbody>
</table>

Fig. 6. In vivo pharmacokinetic profile after intravenous administration in bolus (3 mg/kg) of carvacrol in solution (CRV-S) and encapsulated (CRV-NLCs) in Wistar rats. Results are representative of three independent experiments. Data are expressed as mean ± SD.
Supervision.


Esposito, E., Drechsler, M., Mariani, P., Carducci, F., Servadio, M., Zheng, K., Jia, L., 2014. Drug en-


