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Diethyldithiocarbamate loaded in beeswax-copaiba oil nanoparticles obtained by solventless double emulsion technique promote promastigote death *in vitro*

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ABSTRACT

Leishmaniasis is considered a neglected tropical disease that represents a Public Health problem due to its high incidence. In the search of new alternatives for Leishmaniasis treatment diethyldithiocarbamate (DETC) has shown an excellent leishmanicidal activity and the incorporation into drug carrier systems, such as solid lipid nanoparticles (SLNs), is very promising. In the present work DETC loaded in beeswax nanoparticles containing copaiba oil were obtained by the double emulsion/melt technique. The nanoparticles were characterized and leishmanicidal activity against *L. amazonensis* promastigotes forms and cytotoxicity in murine macrophages were evaluated. SLNs presented size below 200 nm, spherical morphology, negative charge surface, high encapsulation efficiency, above 80%, and excellent stability. Moreover, Fourier transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC) analyses were performed to evaluate the chemical structure and possible interactions between DETC and SLNs. SLNs provided a protection for DETC, decreasing its cytotoxic effects in macrophages, which led to an improvement in the selectivity against the parasites, which almost doubled from free DETC (11.4) to DETC incorporated in SLNs (18.2). These results demonstrated that SLNs had a direct effect on *L. amazonensis* promastigotes without affect the viability of macrophage cell, can be a promising alternative therapy for the cutaneous treatment of *L. amazonensis*

1. Introduction

American Cutaneous Leishmaniasis (ACL) is caused by protozoa of the genus Leishmania and is recognized by the World Health Organization (WHO) as a neglected tropical disease. This disease presents manifestations ranging from the formation of the single ulcer to disseminated forms and can affect the mucous [1,2]. It consists of a Public Health problem, due to its high incidence, wide distribution and great complexity, with the possibility of the appearance of destructive, disfiguring and even incapacitating lesions for infected individuals [3,4]. Despite advances in parasite studies, as well as knowledge of the disease, therapies have now been based on the use of pentavalent antimonials such as sodium stibogluconate (Pentostam®) and antimoniate N-methyl-glucamine (Glucantime®), besides the use of amphotericin B or pentamidines, however they present difficulties of administration, high cost and important side effects [5–7].

In search of new alternatives for Leishmaniasis treatment the Diethyldithiocarbamate (DETC) has shown an excellent leishmanicidal activity, being promising as new antileishmanicidal drug [8–10]. DETC a hydrophilic compound, is a member of the dithiocarbamate family and a potent metal-chelating agent with a dithiocarboxy functional group conjugated to an aliphatic secondary amino group [11,12]. According to Celes et al. (2016), the use of DETC-based bacterial cellulose bio-curatives, a copper chelating agent that targets SOD1, significantly reduces *Leishmania brasiliensis* infection *in vitro* and *in vivo*, due to the increased superoxide levels [8].

The development of new drug-delivery systems to treat leishmaniasis, such as solid lipid nanoparticles (SLNs), represent an excellent alternative for leishmaniasis treatment [7,13]. SLNs have been reported as an efficient drug delivery system due to various advantages such as

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feasibility of carrying lipophilic, as well as hydrophilic drugs, biocompatibility, adapted to different administration routes, drug protection, increase of permeability through cell membranes and bioavailability of encapsulated drug, decrease of the side effects, low cost and ease of scale-up and manufacturing [14–22].

SLNs present a lipid matrix, being suitable system for the encapsulation of hydrophobic drugs. For the encapsulation hydrophilic drugs, SLNs with hydrophilic cores can be prepared by the double emulsion technique. Double emulsion is an excellent alternative for the encapsulation of hydrophilic and hydrophobic compounds/drugs in a same system [21,23]. In addition, when this technique is combined with the melt dispersion technique, the use of an organic solvent, which can present toxicological problems, is avoided [24]. Peres et al. (2016) incorporated both hydrophilic (Sulphorhodamine 101) and hydrophobic (Coumarin-6) molecule in SLNs (stearic acid) by double emulsion/melt dispersion technique. In this study, the authors showed a high encapsulation efficiency, above 60% and 95% for hydrophilic and hydrophobic compounds, respectively.

Herein SLNs were obtained from beeswax. Beeswax has a broad range of applications in the food, cosmetology and pharmaceutical fields [25,26]. Due to its antimicrobial and humectant properties the beeswax can be a potential lipid for the development of SLNs for cutaneous application. [24,25,27,28]. The liquid selected to compose the hydrophobic part of SLNs was copaiba oil, widely used in cosmetics due to its emollient properties [29]. Therefore, the aim of this work was to prepare and characterize novel SLNs from beeswax containing DETC and evaluate their *in vitro* cytotoxicity in murine macrophages, as well as the leishmanicidal activity against *L. amazonensis* promastigotes.

2. Materials and methods

2.1. Materials

White beeswax (GM CERAS), Crodamol GTCC and copaiba oil (Alpha Química) were used as lipid matrix. As surfactants, soy lecithin (Alfa Aesar) and polyoxyethylene-20-sorbitan monooleate (Tween 80, Vetec). The drug sodium diethyldithiocarbamate trihydrate (DETC) was purchased from Vetec. Distilled water was used throughout the experiments.

2.2. Preparation of beeswax nanoparticles containing DETC (DETC-Beeswax)

Beeswax SLNs containing DETC were prepared by double emulsion/melt technique as proposed by [30]. In the first step, a water in oil emulsion was prepared by the emulsification of 0.3 ml of aqueous phase, containing 30 mg mL^{-1} of DETC, in a lipid phase, Crodamol (400 mg) and beeswax (450 mg), melted at 60 °C in the presence of the surfactant lecithin (45 mg). The sonication was carried out at 45% amplitude for 15 s (Fischer Scientific, Ultrasonic Dismembrator Model 500). The sonication was used a 1/2″ (13 mm) probe with replaceable tip. In sequence, this first emulsion was added to 9 mL of the aqueous solution with the second surfactant Tween 80 (90 mg) and kept under magnetic stirring (300 rpm) for 10 min at 60 °C. The second emulsion was sonicated at 60% amplitude for 60 s, forming a double emulsion (water/oil/water). To promote the rapid lipid solidification, the double emulsion was added to 35 mL of water at 2 °C under magnetic stirring (400 rpm) for 5 min. The pH of nanoparticles was maintained close to 7.

2.3. Preparation of beeswax nanoparticles containing DETC and copaiba oil (DTECBeeswax-OC)

Beeswax SLNs containing DETC and copaiba oil were prepared in a similar way as the DETC-Beeswax SLNs, with the only difference that 100 mg of Crodamol were replaced by 100 mg of copaiba oil.

Table 1
Particle size (Dp), polydispersity index (PdI), zeta potential of SLNs and encapsulation efficiency (EE) of DETC.

	Dp(nm)	PdI	Zeta potential (mV)	EE (%)
Beeswax NPs	192 ± 2	0.169	−43 ± 3	–
Beeswax-OC NPs	187 ± 6	0.141	−42 ± 4	–
DETC-Beeswax NPs	191 ± 4	0.193	−45 ± 2	86 ± 5
DETC-Beeswax-OC NPs	186 ± 4	0.171	−42 ± 2	87 ± 6

2.4. Characterization

The morphology of the beeswax nanoparticles containing DETC and copaiba oil was evaluated using transmission electron microscopy (model JEM 2100 F, 80Kv). The samples were prepared by dropping the diluted dispersion (0.5% solids content) onto the copper grid (300 mesh) with a Formvar/carbon film and dried at room temperature overnight. In sequence, the copper grid was coated with carbon. The intensity average particle diameter and polydispersity index were determined by dynamic light scattering (Nanosizer, Malvern Instruments, U.K). The surface charge of the nanoparticles was investigated by zeta potential measurements (Zetasizer, Malvern Instruments, U.K.). For nanosizer and zetasizer analyses the nanoparticles were washed and resuspended in phosphate-buffered saline (PBS) solution (pH 7.2). All samples were analyzed five times at 25 °C. Results are shown as mean ± standard deviation (mean ± SD). The stability of DETC-OC loaded in beeswax nanoparticles was evaluated for 120 days (2 °C) and the physicochemical characteristics (average particle size, polydispersity index and zeta potential) were evaluated. The colloidal stability of nanoparticles was also evaluated under thermal stress. In this study, 5.0 *g* of total sample (aqueous emulsion containing DETC-Beeswax-OC nanoparticles at pH 7) submitted to higher temperatures without stirring or shaking. The temperature was increased from 40°C to 80°C by steps of 5 °C every 30 min. Physico-chemical characteristics were evaluated before and after thermal stress. Macroscopic analyses were performed in DETC loaded in beeswax nanoparticles, to observe any sign of macroscopic instability (creaming, sedimentation, flocculation or coalescence) before and after thermal stress and for a long period of time (120 days). All samples were analyzed in triplicate (n = 3). For the encapsulation efficiency (EE%), beeswax nanoparticles were centrifuged using Amicon® Ultra centrifugal filter (Millipore, 100 kDa) at 13.400 rpm for 30 min. After centrifugation, the supernatant and the retained in the Amicon® were quantified by UV–vis spectrophotometry (U-1900, Hitachi). The DETC concentration was measured at 281 nm (region in the ultraviolet spectrum) using a calibration curve with different concentrations of DETC (0.5–18 μg mL^{-1}) dissolved in distilled water (pH 7). The coefficient of determination (R^2) exceeded 0.999, with excellent linearity. The EE% was calculated from Eq 1:

$$EE\ (\%) = {C_1 - C_2}/{C_1} \times 100 \quad (1)$$

where C_1 is the mass of DETC loaded to the nanoparticles and C_2 is the free DETC mass. The centrifuged volume was of 500 μL with 2.16% of solid content. The centrifugal force was of = 12.100 x g (rcf). The measures were taken in triplicate (n = 3) at room temperature and the results are shown as mean results followed by standard deviation (mean ± SD).

Fourier transform infrared spectroscopy (FTIR, IRPrestige-21, Shimadzu) analyses were performed with lyophilized samples and prepared in KBr pellets. FTIR spectra were recorded over 4000–400 cm^{-1} using a resolution of 4 cm^{-1} and 32 scans. The differential scanning calorimetry (DSC - 4000 Perkin Elmer) analyses were performed under inert atmosphere (N_2, 20 mL.min^{-1}), over a temperature range from -10 to 120 °C, and heated at a linear heating rate of

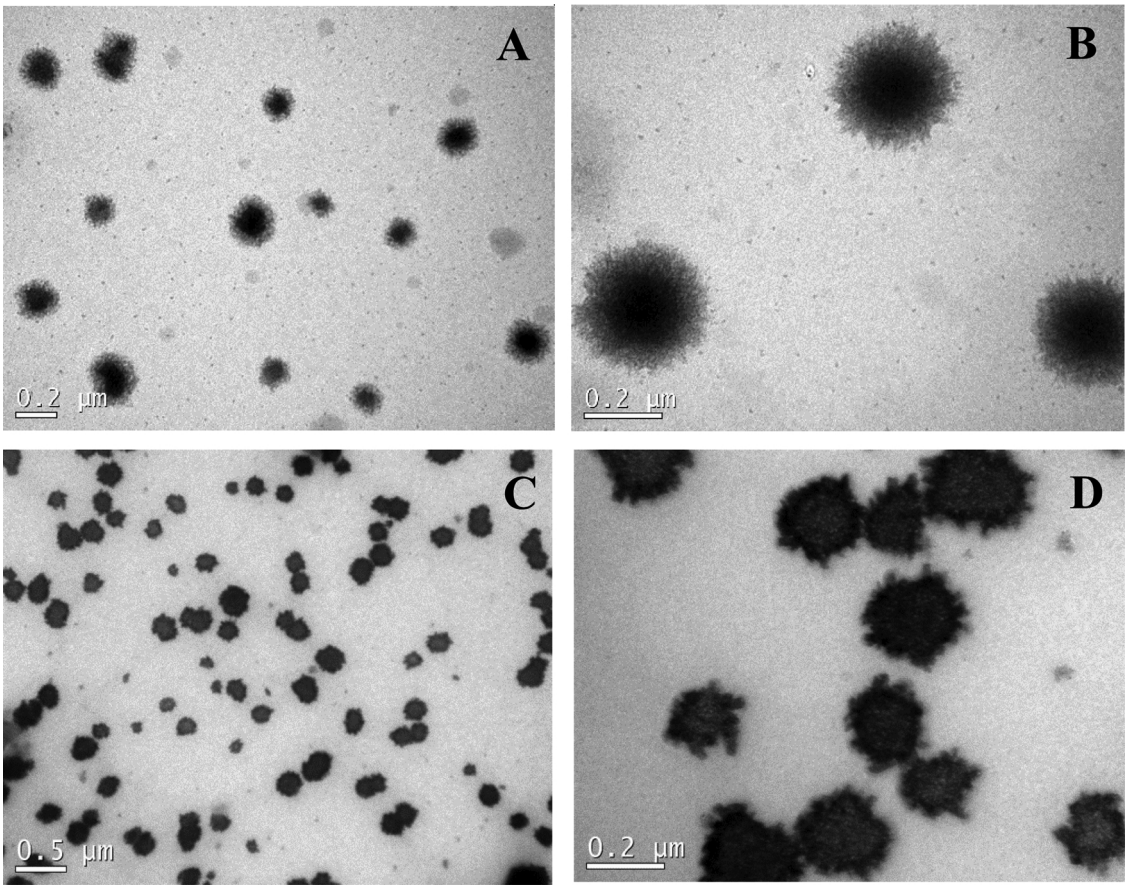

Fig. 1. TEM images of Beeswax-copaiba oil nanoparticles (A–B) and DETC loaded in Beeswax-copaiba oil nanoparticles (C–D).

Table 2
Stability of beeswax SLNs containing DETC with and without copaiba oil under thermal stress.

	Dp (nm)	PDI	Zeta potential (mV)	pH
	After thermal stress (40 °C)			
DETC-Beeswax NPs	191 ± 5	0.189	−45 ± 4	**7.1**
DETC-Beeswax-OC NPs	185 ± 3	0.170	−41 ± 2	**7.0**
	After thermal stress (80 °C)			
DETC-Beeswax NPs	192 ± 4	0.191	−44 ± 2	**7.1**
DETC-Beeswax-OC NPs	188 ± 5	0.172	−42 ± 3	**7.2**

10 °C/min.

2.5. In vitro studies

2.5.1. L. amazonensis maintenance Amazonensis maintenance

Promastigote forms of *L. (L.) amazonensis* (MHOM/BR/1989/166MJO) were maintained in culture medium 199 (M199) (GIBCO, Invitrogen, New York, USA) supplemented with 10% fetal bovine serum (GIBCO - Invitrogen, New York, USA), 1 M HEPES buffer, 1% human urine, 1% L-glutamine, streptomycin, penicillin (GIBCO, Invitrogen) and 10% sodium bicarbonate. The culture was maintained in an incubator at 25 °C in a 25 cm^2 culture flask for five days (stationary growth phase). All promastigote forms were used in the stationary growth phase.

2.5.2. Experimental animals and ethics committee

BALB/c mice weighing 25–30 g aged 6–12 weeks were obtained from Carlos Chagas Institute /Fiocruz-PR, Curitiba, Brazil. The mice were maintained under sterile conditions and used according to the protocol approved by the Ethics Committee for the Use of Animals of the State University of Londrina (nº 24299.2017.66).

2.5.3. Peritoneal macrophage viability analysis

The cytotoxic effect of free DETC and DETC-Beeswax-OC nanoparticles on peritoneal macrophages was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described by Tomiotto-Pellissier et al. [31]. Briefly, macrophages (5×10^4 cells/mL) were recovered from the peritoneal cavity of BALB/c mice with cold PBS supplemented with 3% of FBS and then cultured in 96-well plates with 200 µL of RPMI 1640 medium (10% FBS) for 2 h (37 °C, 5% CO_2). After this time, the wells were washed with PBS to remove the non-adherent cells. Adherent cells were incubated with 0.1, 1 and 10 µM of free DETC and DETC-Beeswax-OC nanoparticles and cultured for 24 h under the same conditions. All assays were performed at equivalent free DETC concentrations. Thereafter the supernatants were discarded and each well washed 3 times with PBS to remove potential interferents from the treatments; and MTT (5 mg/mL) was added for 3 h. After this time, the supernatant was removed and 100 µL of DMSO (dimethyldisulfoxide) was added to solubilize the formazan crystals. The plates were read using a spectrophotometer (Thermo Scientific, Multiskan GO) at 550 nm.

2.5.4. In vitro leishmanicidal activity of free DETC and DETC loaded in beeswax-copaiba oil nanoparticles against L. amazonensis promastigote forms Amazonensis promastigote forms

The antipromastigote activity was performed according to Gonçalves et al. [32]. Promastigote forms of *L. amazonensis* (10^6 cells/mL) were treated with different concentrations of free DETC and DETC-Beeswax-OC nanoparticles (0.1, 1 and 10 µM). The parasites were counted in a Neubauer chamber after 24 h of treatment. Untreated

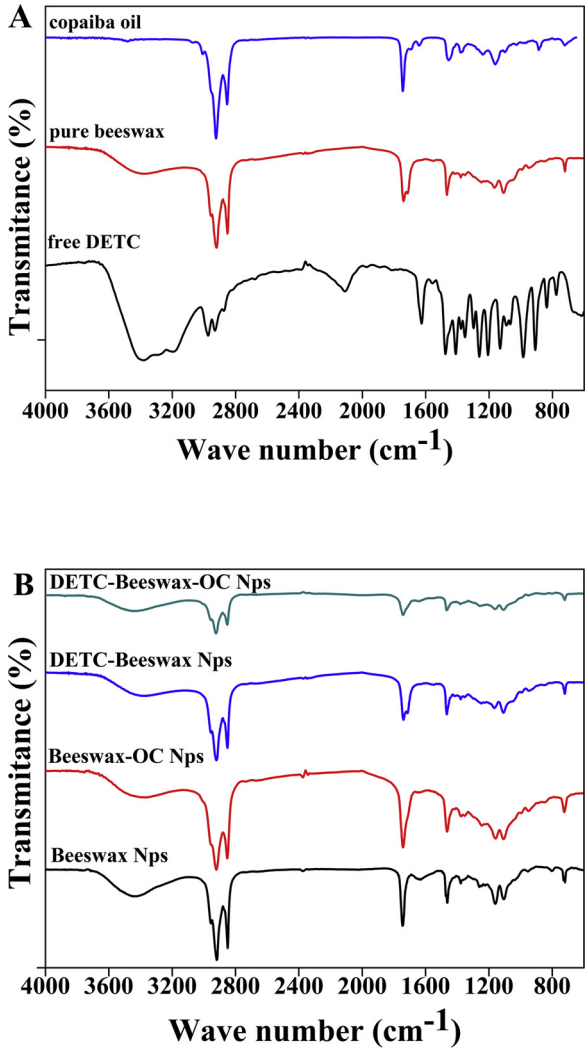

Fig. 2. FTIR spectra of copaiba oil, beeswax, DETC, physical mixture (DETC and beeswax) (A) and SLNS with and without DETC and copaiba oil (B).

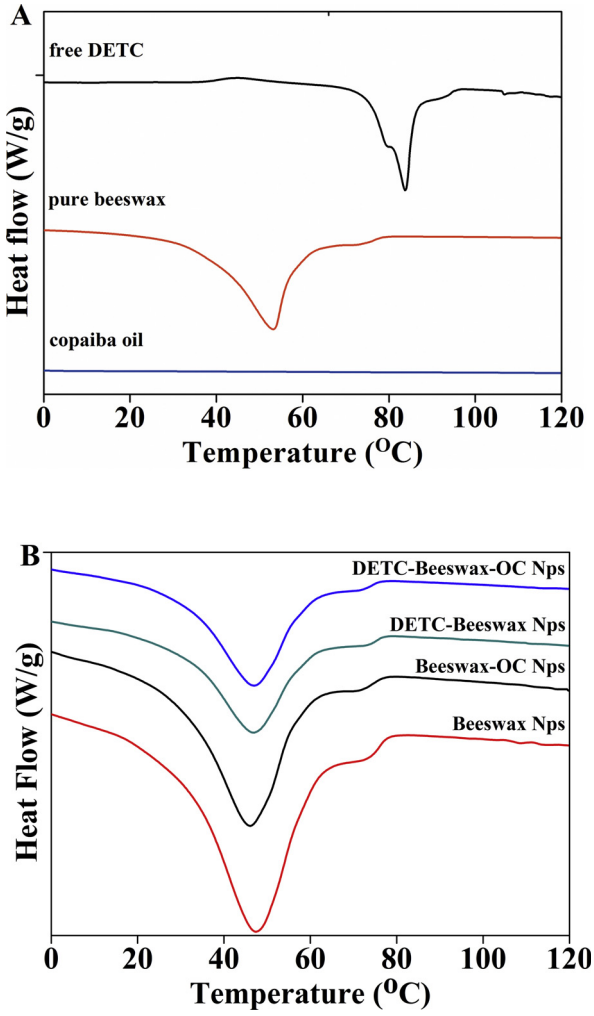

Fig. 3. DSC thermograms of free DETC, pure beeswax, copaiba oil (A) and lipid nanoparticles (B).

promastigotes and those treated with Beeswax-OC nanoparticles were used as controls.

2.5.5. Statistical analysis

The data were from three independent experiments and were expressed as mean ± standard deviation (SD). Significant differences between treatments were determined by ANOVA, followed by the Tukey test for multiple comparisons. $p \leq 0.05$ was considered statistically significant. Cytotoxic (CC_{50}) and leishmanicidal (IC_{50}) activities are the concentrations of compounds able to reduce 50% of murine peritoneal macrophages viability and inhibit 50% of parasites growth, respectively and were calculated by non-linear curve fitting. From CC_{50} and IC_{50} was obtained the selectivity index (SI), as shown by equation 2.

$$SI = {}^{CC_{50}}\!/_{IC_{50}} \quad (2)$$

Statistical analyzes were performed using the software GraphPad Prism 5.03 (San Diego, USA).

3. Results and discussion

DETC was encapsulated in beeswax nanoparticles with and without copaiba oil obtained by the double emulsion technique using pharmaceutically acceptable (non-toxic) lipids and surfactants. When the focus is the biomedical application the study of the physico-chemical properties (size, morphology and zeta potential) of nanoparticles is extremely important, since these can have biological implications, as in the cellular uptake, biological processes and biocompatibility [33–36].

Therefore, the particle size, polydispersity index, zeta potential and EE were determined, as shown in Table 1. Beeswax nanoparticles presented submicrometric sizes (close to 200 nm) with narrow distribution, negative zeta potential and high DETC encapsulation efficiency. The incorporation of DETC and copaiba oil had no major effect on the physico-chemical properties of beeswax nanoparticles. The total solid was of 2.16%.

The morphology of DETC-beeswax-OC nanoparticles was evaluated by TEM. In Fig. 1, it can be verified that nanoparticles present a semispherical morphology and submicrometric size, in agreement with average particle sizes determined by dynamic light scattering analyses (Table 1). In addition, a slight crystallization can be seen in nanoparticles. The appearance of crystals on the surface of the nanoparticles can be related to the crystalline characteristic of lipid. The crystals can also appear when the heated lipid is cooled rapidly, as well as, the presence of the surfactant can force the crystallization of the lipid [37].

The colloidal stability of Beeswax SLNs containing DETC was evaluated under thermal stress condition and confirmed based on particle size, polydispersity index, zeta potential and pH results. As can be seen in Table 2, the stability of lipid nanoparticles was confirmed by absence of modification of their physico-chemical properties before and after thermal stress [38,39]. In addition, it is important to emphasize that it

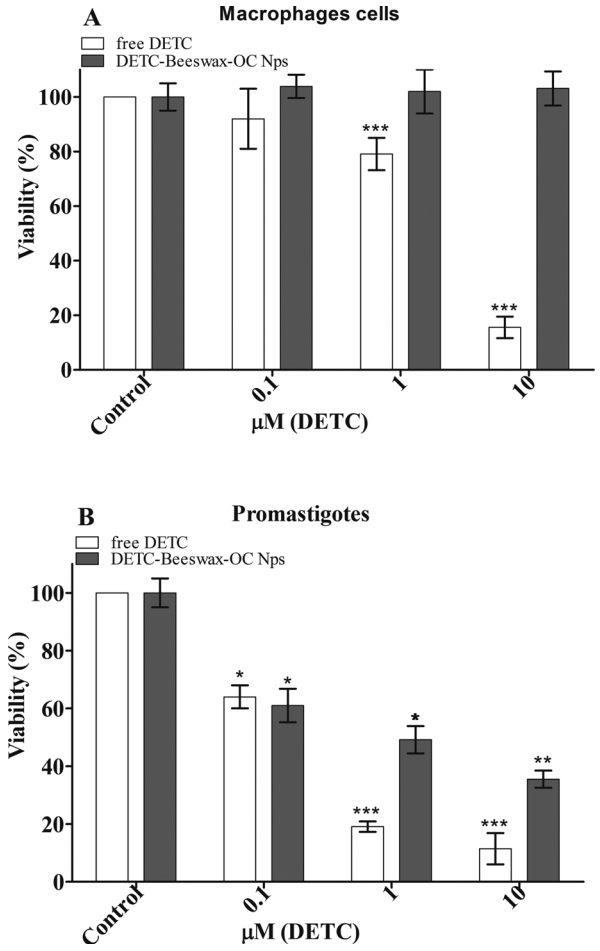

Fig. 4. Cytotoxicity and leishmanicidal activity of free DETC and DETC-Beeswax-OC nanoparticles. (A) Peritoneal macrophages treated for 24 h with free DETC and DETC-Beeswax-OC nanoparticles and cell viability was determined by MTT test. (B) Promastigote forms of *L. amazonensis* were treated (24 h) with free DETC and DETCBeeswax-OC nanoparticles and the viability was assessed by counting in a Neubauer chamber. Values represent the mean ± SD of three independent experiments performed in duplicate. Significantly different compared to the control group. * ($p \leq 0.05$), ** ($p \leq .01$) and *** ($p \leq .001$).

was not observed any sign of macroscopic instability in the beeswax SLNs containing DETC dispersion.

The FTIR spectral analysis was performed to verify the possible physico-chemical interaction between the drug (DETC) and SLNs. As can be seen from Fig. 2A, free DETC presents characteristic peaks at 837, 909, 985 cm^{-1} (C—S), 1070, 2104 (C═S) and 1423 cm^{-1} N-CS_2) [11,40,41]. Copaiba oil presents a characteristic peak at 1740 cm^{-1} (C═O) and a broad medium band between 3100 and 2700 cm^{-1}, attributed to carboxylic acids [42,43]. In the FTIR spectra of beeswax, it was observed peaks at 2922, 2848 cm^{-1} (stretching vibrations of C—H groups), 1736 cm^{-1} (stretching vibrations of the carbonyls of esters), 1467 cm^{-1} (hydrocarbon vibrations) and peaks located at 1300 to 728 cm^{-1} related to the ester group [44,45]. When DETC was encapsulated in SLNs, the characteristic peaks of beeswax and copaiba oil were much more pronounced due to their higher concentration (Fig. 2B).

To characterize further the incorporation of DETC and copaiba oil in the SLNs, samples were analyzed by DSC. DSC thermograms of DETC and pure beeswax showed melting peaks at, respectively, 83 °C and 58 °C (Fig. 3A). The melting peak of copaiba oil was not identified in the evaluated temperature range as it is liquid at temperatures above 0 °C. As can be seen in Fig. 3B, the SLNs presented a melting peak at 45 °C. This reduction can be related with Crodamol and copaiba oil being molecularly dispersed in the SLNs matrix [46–48]. The melting peak of DTEC was not observed at 83 °C. It could imply in a good dispersion of the drug in SLNs matrix. However, due to its low concentration the absence of the melting peak should be regarded with precaution.

In order to evaluate if free DETC and DETC-Beeswax-OC nanoparticles exert cytotoxic effect in peritoneal macrophages, a MTT assay was conducted as shown in Fig. 4A. The results showed that treatments with 1 and 10 µM free DETC presented reduction in the viability of murine peritoneal macrophages; in contrast the treatment with DETC-Beeswax-OC nanoparticles did not affect the cell viability. These results demonstrate that the incorporation of DETC in beeswax nanoparticles protects healthy cells, decreasing the toxic effect of DETC.

As expected, the treatment with free DETC showed antipromastigote effect in all tested concentrations (0.1–10 µM), evidenced more with 1 and 10 µM when compared to DETCBeeswax-OC nanoparticles (Fig. 4B). However, the treatment with DETC-OC-beeswax nanoparticles was also able to eliminate promastigote forms, but without altering the viability of macrophage cells. These results indicate that SLNs provided a protection for the drug, decreasing its cytotoxic effects on macrophages, which led to an improvement in the SI (11.4–18.2) of DETC when incorporated in SLNs against the parasites [49]. Additionally, we observed that the IC_{50} was estimated in 0.23 and 0.55 µM for free DETC and DETC-OC-beeswax nanoparticles, respectively. The CC_{50} was estimated in 2.7 and > 10 µM for free DETC and DETC-Beeswax-OC nanoparticles, respectively. Moreover, it is noteworthy that the Beeswax-OC nanoparticles did not present any cytotoxic on the macrophage cells and parasites.

4. Conclusion

Diethyldithiocarbamate (DETC) is a promising drug for cancer treatment that presented excellent leishmanicidal activity, but also presented cytotoxic effect on peritoneal macrophages. Thus, DETC was successfully encapsulated in beeswax nanoparticles by the double emulsion/melt technique forming SLNS. These SLNs presented a size below 200 nm, high encapsulation efficiency and excellent colloidal stability. Furthermore, DETC encapsulated in beeswax nanoparticles also showed a good leishmanicidal activity against *L. amazonesis* promastigotes. Unlike free DETC, SLNs did not present any cytotoxic effect on peritoneal macrophages, thus presenting a selectivity index almost two times higher than free DETC. Based on these promising results its leishmanicidal activity should be evaluated in animal models.

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References

[1] P. Desjeux, Focus: Leishmaniasis, Nat. Rev. Microbiol. 2 (2004) 692–693, https://doi.org/10.1038/nrmicro981.
[2] V.S. Amato, F.F. Tuon, H.A. Bacha, V.A. Neto, A.C. Nicodemo, Mucosal leishmaniasis. Current scenario and prospects for treatment, Acta Trop. 105 (2008) 1–9, https://doi.org/10.1016/j.actatropica.2007.08.003.
[3] W.M. Monteiro, H.C. Neitzke, M.V.C. Lonardoni, T.G.V. Silveira, M.E.M.C. Ferreira, U. Teodoro, Distribuição geográfi ca e características epidemiológicas da leishmaniose tegumentar americana em áreas de colonização antiga do Estado do Paraná, Sul do Brasil, Cad. Saude Publica 24 (2008) 1291–1303, https://doi.org/10.1590/S0102-311X2008000600010.
[4] B. Gontijo, Leishmaniose tegumentar americana American cutaneous leishmaniasis, Med. Trop. 36 (2003) 71–80, https://doi.org/10.1590/S0104-42302006000600015.
[5] J. Alvar, I.D. Vélez, C. Bern, M. Herrero, P. Desjeux, J. Cano, J. Jannin, M. de Boer, Leishmaniasis worldwide and global estimates of its incidence, PLoS One 7 (2012), https://doi.org/10.1371/journal.pone.0035671.

[6] A.K. Haldar, P. Sen, S. Roy, Use of antimony in the treatment of leishmaniasis: current status and future directions, Mol. Biol. Int. (2011) 1–23, https://doi.org/10.4061/2011/571242 2011.

[7] R. Lopes, C.V. Eleutério, L.M.D. Gonalves, M.E.M. Cruz, A.J. Almeida, Lipid nanoparticles containing oryzalin for the treatment of leishmaniasis, Eur. J. Pharm. Sci. 45 (2012) 442–450, https://doi.org/10.1016/j.ejps.2011.09.017.

[8] F.S. Celes, E. Trovatti, R. Khouri, J. Van Weyenbergh, S.J.L. Ribeiro, V.M. Borges, H.S. Barud, C.I. de Oliveira, DETC-based bacterial cellulose bio-curatives for topical treatment of cutaneous leishmaniasis, Sci. Rep. 6 (2016) 38330, https://doi.org/10.1038/srep38330.

[9] R. Khouri, F. Novais, G. Santana, C.I. de Oliveira, M.A.V. dos Santos, A. Barral, M. Barral-Netto, J. Van Weyenbergh, DETC induces Leishmania parasite killing in human invitro and murine in vivo models: a promising therapeutic alternative in leishmaniasis, PLoS One 5 (2010) 8–9, https://doi.org/10.1371/journal.pone.0014394.

[10] N.B. Barros, V. Migliaccio, V. a Facundo, P. Ciancaglini, R.G. Stábeli, R. Nicolete, I. Silva-Jardim, Liposomal-lupane system as alternative chemotherapy against cutaneous leishmaniasis: Macrophage as target cell, Exp. Parasitol. 135 (2013) 337–343, https://doi.org/10.1016/j.exppara.2013.07.022.

[11] L. Li, Q. Qu, W. Bai, F. Yang, Y. Chen, S. Zhang, Z. Ding, Sodium diethyldithiocarbamate as a corrosion inhibitor of cold rolled steel in 0.5M hydrochloric acid solution, Corros. Sci. 59 (2012) 249–257, https://doi.org/10.1016/j.corsci.2012.03.008.

[12] A.C. Matias, T.M. Manieri, S.S. Cipriano, V.M.O. Carioni, C.S. Nomura, C.M.L. Machado, G. Cerchiaro, Diethyldithiocarbamate induces apoptosis in neuroblastoma cells by raising the intracellular copper level, triggering cytochrome c release and caspase activation, Toxicol. In Vitro 27 (2012) 349–357, https://doi.org/10.1016/j.tiv.2012.08.017.

[13] M. Heidari-Kharaji, T. Taheri, D. Doroud, S. Habibzadeh, A. Badirzadeh, S. Rafati, Enhanced paromomycin efficacy by solid lipid nanoparticle formulation against Leishmania in mice model, Parasite Immunol. 38 (2016) 599–608, https://doi.org/10.1111/pim.12340.

[14] W.N. Omwoyo, B. Ogutu, F. Oloo, H. Swai, L. Kalombo, P. Melariri, G.M. Mahanga, J.W. Gathirwa, Preparation, characterization, and optimization of primaquine-loaded solid lipid nanoparticles, Int. J. Nanomed. 9 (2014) 3865–3874, https://doi.org/10.2147/IJN.S62630.

[15] R.H. Müller, Mader, S. Gohla, Solid lipid nanoparticles (SLN) for controlled drug delivery: a review of the state of the art, Eur. J. Pharm. Biopharm. 50 (2000) 161–177, https://doi.org/10.1016/S0939-6411(00)00087-4.

[16] S.S. Shidhaye, R. Vaidya, S. Sutar, A. Patwardhan, V.J. Kadam, Solid lipid nanoparticles and nanostructured lipid carriers–innovative generations of solid lipid carriers, Curr. Drug Deliv. 5 (2008) 324–331 http://www.ncbi.nlm.nih.gov/pubmed/18855604.

[17] P. Severino, T. Andreani, A. Jäger, M.V. Chaud, M.H.A. Santana, A.M. Silva, E.B. Souto, Solid lipid nanoparticles for hydrophilic biotech drugs: optimization and cell viability studies (Caco-2 & HEPG-2 cell lines), Eur. J. Med. Chem. 81 (2014) 28–34, https://doi.org/10.1016/j.ejmech.2014.04.084.

[18] R. Kumar, A. Singh, N. Garg, P.F. Siril, Solid lipid nanoparticles for the controlled delivery of poorly water soluble non-steroidal anti-inflammatory drugs, Ultrason. Sonochem. 40 (2017) 686–696, https://doi.org/10.1016/j.ultsonch.2017.08.018.

[19] V.S.S. Gonçalves, A.A. Matias, S. Rodríguez-Rojo, I.D. Nogueira, C.M.M. Duarte, Supercritical fluid precipitation of ketoprofen in novel structured lipid carriers for enhanced mucosal delivery - a comparison with solid lipid particles, Int. J. Pharm. 495 (2015) 302–311, https://doi.org/10.1016/j.ijpharm.2015.08.026.

[20] M. Ghadiri, S. Fatemi, A. Vatanara, D. Doroud, A. Rouholamini, M. Darabi, A. Abbas, Loading hydrophilic drug in solid lipid media as nanoparticles: Statistical modeling of entrapment efficiency and particle size, Int. J. Pharm. 424 (2012) 128–137, https://doi.org/10.1016/j.ijpharm.2011.12.037.

[21] M. Iqbal, N. Zafar, H. Fessi, A. Elaissari, Double emulsion solvent evaporation techniques used for drug encapsulation, Int. J. Pharm. 496 (2015) 173–190, https://doi.org/10.1016/j.ijpharm.2015.10.057.

[22] P. Ganesan, D. Narayanasamy, Lipid nanoparticles: different preparation techniques, characterization, hurdles, and strategies for the production of solid lipid nanoparticles and nanostructured lipid carriers for oral drug delivery, Sustain. Chem. Pharm. 6 (2017) 37–56, https://doi.org/10.1016/j.scp.2017.07.002.

[23] A.J. Almeida, E. Souto, Solid lipid nanoparticles as a drug delivery system for peptides and proteins, Adv. Drug Deliv. Rev. 59 (2007) 478–490, https://doi.org/10.1016/j.addr.2007.04.007.

[24] L. Becker Peres, L. Becker Peres, P.H.H. de Ara??jo, C. Sayer, Solid lipid nanoparticles for encapsulation of hydrophilic drugs by an organic solvent free double emulsion technique, Colloids Surf. B Biointerfaces 140 (2016) 317–323, https://doi.org/10.1016/j.colsurfb.2015.12.033.

[25] S. Kheradmandnia, E. Vasheghani-Farahani, M. Nosrati, F. Atyabi, Preparation and characterization of ketoprofen-loaded solid lipid nanoparticles made from beeswax and carnauba wax, Nanomed. Nanotechnol. Biol. Med. 6 (2010) 753–759, https://doi.org/10.1016/j.nano.2010.06.003.

[26] S.W. Tan, N. Billa, Lipid effects on expulsion rate of amphotericin B from solid lipid nanoparticles, AAPS Pharm. Sci. Tech. 15 (2014) 287–295, https://doi.org/10.1208/s12249-013-0056-9.

[27] H.M. Nguyen, I.C. Hwang, J.W. Park, H.J. Park, Photoprotection for deltamethrin using chitosan-coated beeswax solid lipid nanoparticles, Pest Manag. Sci. 68 (2012) 1062–1068, https://doi.org/10.1002/ps.3268.

[28] A.A. Attama, C.C. Müller-Goymann, Effect of beeswax modification on the lipid matrix and solid lipid nanoparticle crystallinity, Colloids Surf. A Physicochem. Eng. Asp. 315 (2008) 189–195, https://doi.org/10.1016/j.colsurfa.2007.07.035.

[29] P.A. da Rocha Filho, Nanoemulsions as a vehicle for drugs and cosmetics, JSM Nanotechnol. Nanomed. 2 (2014) 2–5.

[30] H. Reithmeier, J. Herrmann, A. Göpferich, Lipid microparticles as a parenteral controlled release device for peptides, J. Control. Release 73 (2001) 339–350, https://doi.org/10.1016/S0168-3659(01)00354-6.

[31] F. Tomiotto-Pellissier, D.R. Alves, M.M. Miranda-Sapla, S.M. de Morais, J.P. Assolini, B.T. da Silva Bortoleti, M.D. Gonçalves, A.H.D. Cataneo, D. Kian, T.B. Madeira, L.M. Yamauchi, S.L. Nixdorf, I.N. Costa, I. Conchon-Costa, W.R. Pavanelli, Caryocar coriaceum extracts exert leishmanicidal effect acting in promastigote forms by apoptosis-like mechanism and intracellular amastigotes by Nrf2/HO-1/ferritin dependent response and iron depletion: Leishmanicidal effect of Caryocar coriaceum leaf ex, Biomed. Pharmacother. 98 (2018) 662–672, https://doi.org/10.1016/j.biopha.2017.12.083.

[32] M.D. Gonçalves, B.T.S. Bortoleti, F. Tomiotto-Pellissier, M.M. Miranda-Sapla, J.P. Assolini, A.C.M. Carloto, P.G.C. Carvalho, E.T. Tudisco, A. Urbano, S.R. Ambrósio, E.Y. Hirooka, A.N.C. Simão, I.N. Costa, W.R. Pavanelli, I. Conchon-Costa, N.S. Arakawa, Dehydroabietic acid isolated from Pinus elliottii exerts in vitro antileishmanial action by pro-oxidant effect, inducing ROS production in promastigote and downregulating Nrf2/ferritin expression in amastigote forms of Leishmania amazonensis, Fitoterapia 128 (2018) 224–232, https://doi.org/10.1016/j.fitote.2018.05.027.

[33] Y. Zhang, M. Yang, N.G. Portney, D. Cui, G. Budak, E. Ozbay, M. Ozkan, C.S. Ozkan, Zeta potential: a surface electrical characteristic to probe the interaction of nanoparticles with normal and cancer human breast epithelial cells, Biomed. Microdevices 10 (2008) 321–328, https://doi.org/10.1007/s10544-007-9139-2.

[34] C. He, Y. Hu, L. Yin, C. Tang, C. Yin, Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles, Biomaterials 31 (2010) 3657–3666, https://doi.org/10.1016/j.biomaterials.2010.01.065.

[35] A. Verma, F. Stellacci, Effect of surface properties on nanoparticle-cell interactions, Small 6 (2010) 12–21, https://doi.org/10.1002/smll.200901158.

[36] A. Valério, P.E. Feuser, L.D.S. Bubniak, M.C. Dos Santos-Silva, P.H.H. de Araújo, C. Sayer, *In Vitro* biocompatibility and macrophage uptake assays of poly(urea-urethane) nanoparticles obtained by miniemulsion polymerization, J. Nanosci. Nanotechnol. 17 (2017) 4955–4960, https://doi.org/10.1166/jnn.2017.13434.

[37] C. Dal Pizzol, F.B. Filippin-Monteiro, J.A.S. Restrepo, F. Pittella, A.H. Silva, P.A. de Souza, A.M. de Campos, T.B. Creczynski-Pasa, Influence of surfactant and lipid type on the physicochemical properties and biocompatibility of solid lipid nanoparticles, Int. J. Environ. Res. Public Health 11 (2014) 8581–8596, https://doi.org/10.3390/ijerph110808581.

[38] J. Carpenter, V.K. Saharan, Ultrasonic assisted formation and stability of mustard oil in water nanoemulsion: effect of process parameters and their optimization, Ultrason. Sonochem. 35 (2017) 422–430, https://doi.org/10.1016/j.ultsonch.2016.10.021.

[39] V.C. Gumiero, P.A. da Rocha Filho, Babassu nanoemulsions have physical and chemical stability, J. Dispers. Sci. Technol. 33 (2012) 1569–1573, https://doi.org/10.1080/01932691.2011.625219.

[40] T. Gao, J. Yu, Y. Zhou, X. Jiang, The synthesis of graphene oxide functionalized with dithiocarbamate group and its prominent performance on adsorption of lead ions, J. Taiwan Inst. Chem. Eng. 71 (2017) 426–432, https://doi.org/10.1016/j.jtice.2016.11.033.

[41] P. Yan, M. Ye, S. Sun, X. Xiao, W. Dai, N. Zhang, Removal performances and mechanisms of action towards ethylenediaminetetraacetic acid nickel (II) salt by dithiocarbamate compounds having different carbon chain lengths, J. Clean. Prod. 122 (2016) 308–314, https://doi.org/10.1016/j.jclepro.2016.02.037.

[42] A.C. Pinto, W.F. Braga, C.M. Rezende, F.M.S. Garrido, V.F. Veiga, L. Bergter, M.L. Patitucci, O.A.C. Antunes, Separation of acid diterpenes of Copaifera cearensis huber ex ducke by flash chromatography using potassium hydroxide impregnated silica gel, J. Braz. Chem. Soc. 11 (2000) 355–360, https://doi.org/10.1590/S0103-50532000000400005.

[43] V.R. De Almeida Borges, J.H. Da Silva, S.S. Barbosa, L.E. Nasciutti, L.M. Cabral, V.P. De Sousa, Development and pharmacological evaluation of in vitro nanocarriers composed of lamellar silicates containing copaiba oil-resin for treatment of endometriosis, Mater. Sci. Eng. C. 64 (2016) 310–317, https://doi.org/10.1016/j.msec.2016.03.094.

[44] W. Luo, T. Li, C. Wang, F. Huang, Discovery of Beeswax as binding agent on a 6th-century BC Chinese Turquoise-inlaid Bronze sword, J. Archaeol. Sci. 39 (2012) 1227–1237, https://doi.org/10.1016/j.jas.2011.12.035.

[45] S. Makled, N. Nafee, N. Boraie, Nebulized solid lipid nanoparticles for the potential treatment of pulmonary hypertension via targeted delivery of phosphodiesterase-5-inhibitor, Int. J. Pharm. 517 (2017) 312–321, https://doi.org/10.1016/j.ijpharm.2016.12.026.

[46] M. Yasir, U.V.S. Sara, Solid lipid nanoparticles for nose to brain delivery of haloperidol: in vitro drug release and pharmacokinetics evaluation, Acta Pharm, Sin. B. 4 (2014) 454–463, https://doi.org/10.1016/j.apsb.2014.10.005.

[47] S.V.K. Rompicharla, H. Bhatt, A. Shah, N. Komanduri, D. Vijayasarathy, B. Ghosh, S. Biswas, Formulation optimization, characterization, and evaluation of in vitro cytotoxic potential of curcumin loaded solid lipid nanoparticles for improved anticancer activity, Chem. Phys. Lipids 208 (2017) 10–18, https://doi.org/10.1016/j.chemphyslip.2017.08.009.

[48] N. Tahir, A. Madni, V. Balasubramanian, M. Rehman, A. Correia, P.M. Kashif, E. Mäkilä, J. Salonen, H.A. Santos, Development and optimization of methotrexate-loaded lipid-polymer hybrid nanoparticles for controlled drug delivery applications, Int. J. Pharm. 533 (2017) 156–168, https://doi.org/10.1016/j.ijpharm.2017.09.061.

[49] T.G. Ribeiro, M. a Chávez-Fumagalli, D.G. Valadares, J.R. França, L.B. Rodrigues, M.C. Duarte, P.S. Lage, P.H.R. Andrade, D.P. Lage, L.V. Arruda, D.R. Abánades, L.E. Costa, V.T. Martins, Ca P. Tavares, R.O. Castilho, E.A.F. Coelho, Aa G. Faraco, Novel targeting using nanoparticles: an approach to the development of an effective anti-leishmanial drug-delivery system, Int. J. Nanomed. 9 (2014) 877–890, https://doi.org/10.2147/IJN.S55678.