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Preparation and characterization of a synthetic curcumin analog inclusion complex and preliminary evaluation of *in vitro* antileishmanial activity

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ABSTRACT

The aim of this work was to prepare and characterize inclusion complexes between a synthetic curcumin analog (dibenzalacetone, DBA) and beta-cyclodextrin (β-CD); and to evaluate their *in vitro* antileishmanial activity. DBA was synthetized and characterized by spectroscopic methods and the inclusion complexes were obtained by kneading and lyophilization (LIO) in 1:1 and 1:2 stoichiometries. Phase solubility and dissolution assays showed a 40-fold increase in the aqueous solubility of DBA and its complete dissolution from LIO 1:1 formulation after 120 min respectively. Solid-state characterization by differential scanning calorimetry and near infrared spectroscopy demonstrated the inclusion of DBA in the β-CD cavity at the molar ratios tested, with LIO 1:1 formulation being the most stable. Using nuclear magnetic resonance experiments, the protons inside the cavity of β-CD were the most affected after the inclusion of DBA molecule. The cellular viability of THP-1 macrophage cells treated with plain DBA, β-CD and DBA/CD inclusion complexes showed that the plain DBA and DBA/CD at 1:2 stoichiometry presented toxicity, while β-CD alone and DBA/CD at 1:1 stoichiometry showed no toxicity up to 640 μg mL^{-1}. The *in vitro* assay with free-living promastigotes demonstrated that plain DBA and β-CD had IC_{50} of < 10 and > 320 μg mL^{-1} respectively, while only inclusion complexes with 1:1 stoichiometry showed antiproliferative activity with IC_{50} = 51.3 μg mL^{-1}. Using the amastigote intracellular forms, there was also a difference between the plain and β-CD complexed DBA with complexes of 1:1 and 1:2 stoichiometry presenting EC_{50} = 66.3 μg mL^{-1} and 58.9 μg mL^{-1} respectively. The study concluded that DBA/CD at 1:1 molar ratio has the potential to decrease the intrinsic toxicity of plain DBA towards Leishmania host cells, which may be a therapeutic advantage in the application of these compounds.

1. Introduction

Leishmaniasis is a neglected disease transmitted by phlebotomine sandflies. Depending on the species of the parasite, it can cause cutaneous lesions or visceral symptoms, often leading to death. The countries most affected by the disease are Brazil, India, Ethiopia, Kenya, South Sudan, Somalia, and Sudan, representing more than 90% of new cases. Many factors have been linked to the spread of the disease, including the high incidence of adverse effects of first-choice drugs (pentavalent antimonials) and high toxicity related to second-choice drugs (amphotericin B and pentamidine). The emergence of strains resistant to available treatments may also influence the increased incidence of the disease. In addition, problems related to drug administration, with daily injections, contribute to poor adherence to treatment regimen. Other factors, such as the absence of public policies that favor the registration and purchase of drugs to treat leishmaniasis, may lead to an insufficient stock of drugs to treat patients in endemic areas (Oliveira et al., 2011; WHO, 2020).

Presently, 1 billion people are at risk and there are 20,000–30,000 deaths due to visceral leishmaniasis registered per year (DNDi, 2020) and the treatment for this disease is still a challenge due to drug toxicity and several side effects. While the use of combination therapies has become a common approach to overcome the resistance acquired by parasites to certain drugs, new strategies to fight against this disease are required to enhance therapeutic effectiveness, improve the quality of life of the affected population and, if possible, lower costs (Chappuis et al., 2007). Drug delivery and nanomedicine approaches, and the discovery of new molecules (from natural or synthetic sources) are of utmost importance for safely combatting leishmaniasis (Gutiérrez et al., 2016; Vijayakumar and Das, 2018).

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There are reports in the literature that curcumin (Das et al., 2008; Shahiduzzaman and Daugschies, 2011) and its derivatives (Changtam et al., 2010; Chauhan et al., 2018) have antiparasitic activity. Active curcuminoid analogs exhibited antileishmanial activity against promastigotes of *L. major* and amastigotes of *L. mexicana* (Changtam et al., 2010). Also, an antiproliferative effect of dibenzalacetone (DBA), an analog of curcumin, was reported on the intracellular amastigotes of *L. donovani* (Chauhan et al., 2018). Thus, in this work, the physicochemical properties and antileishmanial activity of an inclusion complex between a curcuminoid compound (dibenzalacetone) and β-cyclodextrin (β-CD) were evaluated. The hypothesis was that the use of β-CD could improve the aqueous solubility and hence, the activity of the tested molecule. Dibenzalacetone (DBA), a curcumin derivative, was synthesized and inclusion complexes were prepared and characterized for subsequent testing in free-living promastigotes and intracellular amastigotes of *Leishmania* sp. Since DBA is a molecule easily and inexpensively obtained, the results for its use in cases of leishmaniasis may be extremely relevant.

2. Methodology

2.1. Molecular modeling

The structure of DBA was constructed, and optimization was performed with density functional theory (DFT) in vacuum, using B3LYP method and 6-31G basis set, at the Gaussian 09W software. The purpose of the minimization of the molecule was to analyze possible interactions with the cavity of cyclodextrin, based on its polarity and shape.

2.2. Synthesis and characterization of DBA

0.5 mL of acetone and 1 g of NaOH were added to a 1:1 water:-ethanol mixture. Then, the obtained solution was poured in 0.9 mL of benzaldehyde and stirred with a glass stick for 30 min. Yellow crystals of dibenzalacetone were obtained, filtered under vacuum, washed and recrystallized with ethanol. The final product was filtered again to be characterized by Fourier transform infrared spectroscopy, using a Bruker Alpha II Fourier Transform Infrared Spectrometer and by ^{1}H, ^{13}C and HMQC nuclear magnetic resonance, recorded in a Bruker Ultra-Shield 300 MHz spectrometer using DMSO-$_{d6}$ or $CDCl_3$ as solvent.

2.3. Phase solubility studies and inclusion kinetics

Phase solubility studies were carried out using the protocol adapted from (Higuchi and Connors, 1965). Excess amounts of DBA (6 mM) were added to solutions containing increasing amounts of β-CD (0–12 mM) in triplicate and stirred for 48 h at 37 °C. Samples were centrifuged (14,000 rpm/20 min), filtered and the absorbances were recorded at 230 nm.

The association constant (K_a) was determined using the following equation (Higuchi and Connors, 1965), where S_0 is the intrinsic solubility of DBA:

$$Ka = \frac{slope}{S_0(1 - slope)}$$

The standard Gibbs free energy (ΔG°) was calculated using the equation described in sequence (Patel and Patel, 2010; Yadav et al., 2009), where S_0 and S_s are the solubility of DBA in absence and in presence of β-CD respectively:

$$\Delta G^0 = -2.303RTlog\left(\frac{S_0}{S_S}\right)$$

The inclusion kinetics experiment was run at 37 °C and was used to estimate the time to reach the complexation equilibrium. DBA and β-CD, at 1:1 and 1:2 molar ratios, were placed in ultrapure water and left shaking for 300 min (5 h) in triplicate. A control with plain DBA was performed. At specific times, aliquots were withdrawn and the absorbances were monitored at λ_{max} = 230 nm. The absorbances were normalized to compare the different conditions, as follows:

Normalized Absorbance = Absorbance of DBA at time t/Absorbance of DBA at time zero

The inclusion kinetics plots were analyzed using a linear regression of the data: absorbance *vs* time, ln absorbance *vs* time and 1/absorbance *vs* time, to obtain the order and the kinetic constant of complexation (Moraes et al., 2007a; Rodrigues et al., 2011).

2.4. Preparation of the inclusion complexes

Inclusion complexes were produced in 1:1 and 1:2 stoichiometry using the kneading (KN) and lyophilization (LIO) methods. Briefly, in the KN method DBA and β-CD were weighed and placed in a mortar with a small amount of absolute ethanol. The paste was homogenized for 30 min with a pestle and then kept in a desiccator to dry. In the LIO method the compounds were weighed, and DBA was dissolved in absolute ethanol, while β-CD was dissolved in ultrapure water. The solutions were mixed and rotaevaporated. After rehydration the sample was freeze dried. Physical mixtures (PM) were prepared with the simple contact of the compounds.

2.5. Characterization of the inclusion complexes

2.5.1. Differential scanning calorimetry

Calorimetric assays were performed in a DSC Q200 – TA Instruments. 3–5 mg samples of plain DBA, plain β-CD, DBA/CD inclusion complexes at 1:1 and 1:2 molar ratios, prepared by KN and LIO, as well as PM used as a control, were weighed and placed in aluminum pans with a hole. Scans were performed from 30 to 300 °C with a heating rate of 10 °C/min. The atmosphere was N_2 with a flow rate of 50 mL/min.

2.5.2. Near infrared spectroscopy

The procedure was performed in a NIR-ATR ABB - Model TLA 2000. The same samples prepared for DSC were mounted in a glass blade and the experiment was set for a resolution of 8 cm^{-1}, 75 scans min^{-1}, range from 4,000 to 10,000 cm^{-1}, using the software GRAMS AI 7.0.0.

2.5.3. ^{1}H nuclear magnetic resonance

The objective of the experiment was to observe if there are any changes in the chemical shifts of the protons of plain DBA or β-CD, after the complexation.

^{1}H NMR spectra of the samples: plain DBA, plain β-CD and DBA/CD inclusion complex, were recorded at 20 °C in DMSO-$_{d6}$ solution using a Bruker Ultra-Shield 300 MHz spectrometer. Chemical shifts (δ) were reported in parts per million (ppm) relative to the residual solvent peak.

2.6. Dissolution test

Samples of plain DBA and DBA/CD inclusion complexes at 1:1 and 1:2 molar ratios were placed in erlenmeyers flasks and shaken (200 rpm) for 120 min at 37 °C in triplicate. Aliquots of 2 mL were withdrawn each 5 min and after filtration the absorbances were read at 230 nm. The volume of the samples was replaced with ultrapure water after each estimation (Haroun and El-Halawany, 2010).

2.7. Anti-leishmaniasis assays

2.7.1. Assessment of cell viability in differentiated THP-1 monocytic cells

THP-1 cell line derived from an acute monocytic leukemia patient, were grown in RPMI-1640 medium supplemented with 10% inactivated

fetal bovine serum (iFBS), 100 U/mL penicillin and 100 mg/mL streptomycin and incubated at 37 °C in a humidified atmosphere of 5% CO_2. A cell suspension at 3 × 10^5 cell mL^{-1} in RPMI medium containing 50 ng mL^{-1} of PMA [phorbol 12-myristate 13-acetate (Sigma-Aldrich, Spain)] to promote differentiation into macrophages was prepared and cells were seeded (200 µL) into 96-well plates and incubated at 37 °C, 5% CO_2 for 48 h to allow differentiation. Cells were washed and exposed to the test formulations of plain DBA, plain β-CD and DBA/CD inclusion complexes at 1:1 and 1:2 molar ratios, previously prepared and diluted in RPMI medium at concentrations of 5, 10, 20, 40, 80 and 160 µg mL^{-1} in respect to DBA content. Plates were incubated at 37 °C, 5% CO_2 for 24 h. After washing with PBS, a 0.5 mg mL^{-1} solution of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added in each well (50 µL) and incubated for 3 to 4 h (37 °C, 5% CO_2). At that time, DMSO (200 µL/well) was added to each well to solubilize the formazan-MTT crystals and plates were shaken for 15 min. The plates were read on a microplate reader at 570 nm.

2.7.2. Assessment of antileishmanial activity against promastigote cultures

A *Leishmania major* (MHOM/SA/85/JISH118 strain, kindly provided by Dr. Simon Croft from London School of Hygiene and Tropical Medicine, UK) promastigotes suspension at 2 × 10^6 promastigote mL^{-1} in SCN medium was prepared. 100 µL of promastigotes were seeded into the middle 60 wells of the 96-well plates. Stock solutions of DBA, plain β-CD and DBA/CD inclusion complexes at 1:1 and 1:2 molar ratios were prepared and serially diluted in SCN medium. 100 µL of the diluted solutions at 10, 20, 40, 80, 160 and 320 µg mL^{-1} were added to each well and incubated.

2.7.3. Antileishmanial screening against intracellular cultures

After seeding and differentiation of the THP-1 cells in a 96-well microplate, the infection of the transformed THP-1 cells with *Leishmania infantum* (MHOM/MA/67/ITMAP263 strain, isolated in Morocco, was a kind gift from Prof. Ana Tomás from i3S, Universidade do Porto, Portugal) promastigotes was performed with 2.5 × 10^5 parasites/well. The plates were incubated at 37 °C, 5% CO_2 for 24 h to allow the parasites to infect the differentiated THP-1 cells. Then the infected macrophages were treated with the test formulations serially diluted in RPMI with 2% FBS and the plates were incubated at 37 °C, 5% CO_2 for 24 h. The parasite-rescue-transformation-assay (Jain et al., 2012) was performed with controlled lysis of *L. infantum* amastigotes-infected macrophages. Plates were incubated at 26 °C for 72 h for transformation of rescued amastigotes to promastigotes. MTT assay was done for the quantitative analysis of transformed promastigote.

3. Results and discussion

3.1. Theoretical

DBA is a symmetrical and totally conjugated molecule. Besides the carbon to oxygen polar bond, due to the electronegativity difference, the molecule has a large apolar structure, thus overcoming any possible polar interaction in the central ketone portion.

The optimization of the molecule (Fig. 1) shows that the ketone group has remarkably more tendency to attract electrons (red) than the aromatic rings (green). Although all the electronic density is spread along the axis toward the aromatic rings. This characteristic turns the molecule almost insoluble in water. The cavity of the cyclodextrins could accommodate the rings of the molecule in a 1:1 or 1:2 DBA/CD molar ratios, thus indicating that a stable inclusion compound could be formed and an increase in the water solubility can be reached.

3.2. Synthesis and characterization of DBA

The pale-yellow powder of DBA, obtained in the synthesis, was characterized by FTIR and NMR (Supplementary material S1 and S2 respectively).

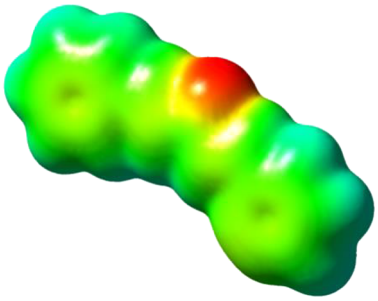


Fig. 1. Energy minimization of the DBA molecule using the software Gaussian 09W.

The first band observed in FTIR was in the range of 3000–3100 cm^{-1}, indicating the presence of a sp^2 carbon connected to hydrogen. An intense peak at 1649 cm^{-1} indicates the presence of the conjugated carbonyl and at 1589 cm^{-1} is the signal of the double bond C═C. At 1494 cm^{-1} is the C—C stretching vibrations of the aromatic ring and the C—H bending vibration appeared at 760 and 596 cm^{-1}, as described in the literature (Periasamy et al., 2014).

^{1}H NMR assignments of DBA was recorded in DMSO-$_{d6}$ and also in $CDCl_3$ solvent for unambiguous confirmation of the structure (Supplementary material S2 – a, b and c) and are in accordance with the literature (Chauhan et al., 2018). One doublet in δ_H 7.79 ppm and another in δ_H 7.37, with a coupling constant of 15 Hz, indicates the presence of the *trans* double bond connection, the main link obtained in this reaction. ^{13}C NMR chemical shifts were described in Supplementary material S2 - d and e, with seven carbons. Cross peaks obtained by HMQC NMR demonstrated that proton Ha is linked to C2, Hd-meta is linked to C6, Hd-para is linked to C7 and Hc is linked to C5 (Supplementary material S2 - f).

The mechanism of reaction involves the reaction of one molecule of acetone with two molecules of benzaldehyde. Two aldolic condensations occur in the sequence (Fig. 2).

3.3. Phase solubility and inclusion kinetics

The phase solubility diagram for DBA at increasing concentration of β-CD is presented in Fig. 3. An A_L type profile was clearly observed, and the solubility increased approximately 40-fold with the concentration of β-CD. This could be attributed to the formation of soluble DBA/CD inclusion complexes at 1:1 stoichiometry (Periasamy et al., 2014).

The association constant (K_a) was determined as 1158 L mol^{-1} which indicates the formation of a very stable complex at the temperature tested. Reports in the literature described that K_a values between 200 and 5000 L mol^{-1} are considered more suitable to improve the solubility and stability of molecules with low aqueous solubility

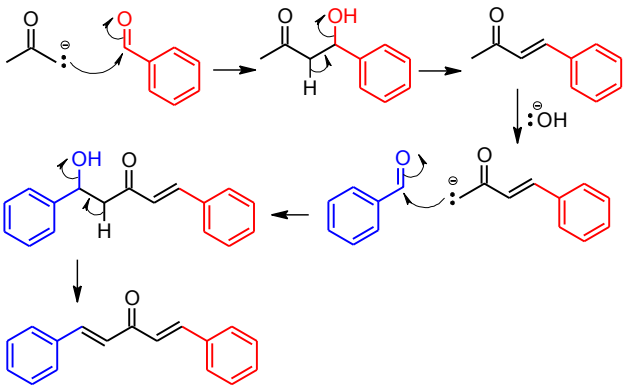


Fig. 2. Proposed mechanism of reaction for the synthesis of DBA.

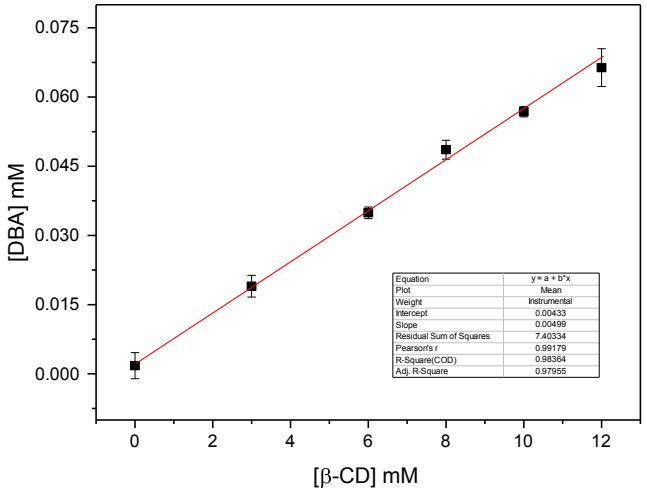


Fig. 3. Phase solubility diagram obtained for DBA in the presence of β-CD at 37 °C.

(Patel and Patel, 2007).

The ΔG° is a thermodynamic parameter that can be related to the transfer of molecules from the aqueous solution toward the cavity of β-CD and gives information about the spontaneity of the process at constant pressure and temperature. Values of ΔG° obtained for DBA were all negative and increased as the concentration of β-CD was raised (Table 1). This was indicative that the process was spontaneous and that the environment inside the cavity of β-CD was more favorable for DBA molecules than water, as the concentration of β-CD increased.

For the inclusion kinetics analysis, when included in the cyclodextrin cavity, the guest molecule undergoes changes in its spectroscopic characteristics, like the intensity and the maximum absorption peak. In solution, there is a balance between the associated and dissociated form of the molecule to be complexed, according to criteria of polarity and steric effects (Chaves et al., 2010; Grillo et al., 2008; Rodrigues et al., 2011) that can be monitored by UV–Vis.

In this work, after approximately 30 min, the tested solutions reached equilibrium in both stoichiometries (data not shown), thus being a relatively short time for preparing the inclusion complexes. No changes were observed in the λ_{max}, only in its intensity.

After analysis of the kinetic profile, a second-order kinetics was determined, with R^2 = 0.99392 and 0.99119 for stoichiometries 1:1 and 1:2 respectively. Thus, when doubling the concentration of the guest molecule, the tendency is for the complexation rate to increase by 2^2. The kinetic constants calculated were k = 0.18557 and 0.25942 h^{-1} for molar ratios 1:1 and 1:2 respectively. These values are in accordance with values of the literature for molecules considered hydrophobic such as bupivacaine (Moraes et al., 2007b) and praziquantel (Chaves et al., 2010; Rodrigues et al., 2011). The results suggest that, in both molar ratios, there was a tendency for the DBA molecule to interact with the cavity of β-CD and the equilibrium was quickly achieved.

Table 1
Standard Gibbs free energy (ΔG°) for solubilization of DBA in aqueous solution of β-CD.

[β-CD] mM	ΔG° (KJ mol^{-1})
3	−6.21
6	−7.79
8	−8.64
10	−9.04
12	−9.44

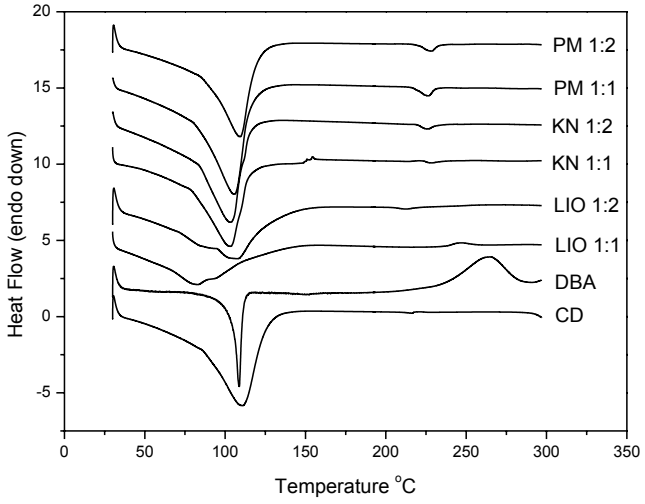


Fig. 4. Thermograms obtained by differential scanning calorimetry of β-CD, DBA, PM and DBA/CD inclusion complexes obtained by LIO and KN at 1:1 and 1:2 stoichiometries.

Table 2
Enthalpy, onset temperature and maximum temperature obtained by DSC.

	Enthalpy (J g^{-1})	Tonset (°C)	Tmax (°C)
β-CD	312.4	77.52	110.83
DBA	50.25	105.22	108.64
	−51.49	243.26	265.41
LIO 1:1	**226.10**	**48.01**	**82.86**
	−2.768	**239.50**	**246.49**
LIO 1:2	242.30	71.16	107.77
KN 1:1	220.6	72.49	102.77
	−0.9476	224.06	228.20
KN 1:2	284.50	72.91	103.23
	2.326	219.91	225.94
PM 1:1	310.50	75.12	105.40
	5.725	217.05	226.06
PM 1:2	290.2	81.30	109.17
	5.150	220.69	228.38

3.4. Characterization of the inclusion complex

3.4.1. Differential scanning calorimetry

DBA presents a sharp endothermic peak at 108 °C related to the melting point of the molecule (Fig. 4 and Table 2). An exothermic transition at 265 °C appears and is probably due to the decomposition of the molecule. On the other hand, β-CD has a unique endo transition at 110 °C related to the loss of water molecules from the cavity (Carvalho et al., 2018; Martins et al., 2020).

It was observed that LIO 1:1 decreases the transition temperature around 100 °C, because the water molecules will leave the cavity before, to accommodate the guest molecule. This is thermodynamic more favorable to occur as the molecule of DBA is less polar than water. The enthalpy observed is in accordance with this statement (Table 2). In LIO 1:2, as the proportion of β-CD is higher, the DBA molecules, when inserted into the cavity, do not displace all the water molecules. However, the exothermic peak observed above 250 °C is completely suppressed in LIO 1:2 and get smaller in LIO 1:1, denoting that, as the molecule is protected by the β-CD, the decomposition does not occur in this temperature. A pronounced decrease was also observed in the enthalpy related to LIO 1:1, more than LIO 1:2, corroborating the discussion that at 1:1 stoichiometry the complexation is preferred.

KN and PM (1:1 and 1:2) have a very similar profile, with no significant decrease in the maximum temperature peak (Tmax), as observed for LIO 1:1. In this case, both KN and PM do not seem to be a good method to prepare the DBA/CD inclusion complexes as it was also

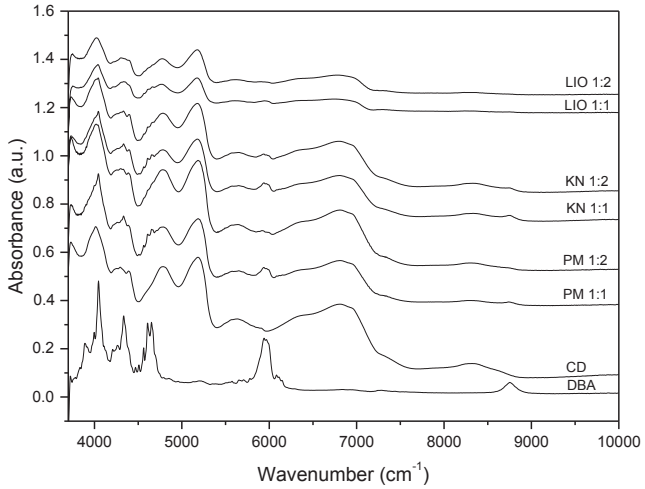


Fig. 5. Near infrared spectra of DBA, β-CD, PM and DBA/CD inclusion complexes obtained by KN and LIO at 1:1 and 1:2 stoichiometries.

confirmed by the NIR experiments.

3.4.2. Near infrared spectroscopy

It was observed in the spectra of LIO 1:1 and 1:2 that the characteristic peaks of DBA (~8750 and 6000 cm^{-1}) were suppressed, and the peak for β-CD (~6800 cm^{-1}) decreased (Fig. 5). However, for PM and KN at 1:1 and 1:2 molar ratios, a similar profile was obtained, with the main peaks of DBA and β-CD remaining in the spectra (Jug et al., 2005). So, the results support that PM and KN techniques are not good to incorporate the guest molecule, as already determined by DSC.

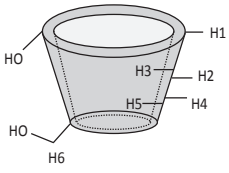
This indicates that the best procedure to prepare the DBA/CD inclusion complexes was LIO and it was the one chosen for the biological tests.

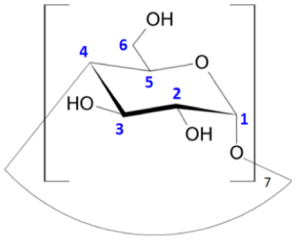
3.4.3. Nuclear magnetic resonance

The experiment gave us an idea of how the inclusion of the DBA molecule occurs inside the cavity of β-CD.

Nine peaks were assigned for the molecule of β-CD (Table 3) and are in agreement with the literature (Marques et al., 1990). The protons

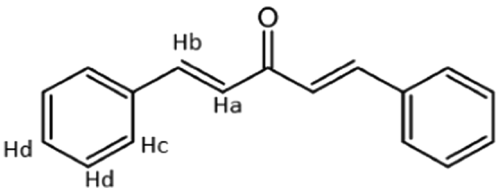
Table 3
^{1}H NMR chemical shifts of β-CD in DMSO-$_{d6}$ before and after the complexation with DBA, at 20 °C.





Hydrogen position	β-CD chemical shift (ppm)	DBA/CD chemical shift (ppm)	Δδ (ppm)
H2	Superimposed with water	3.268	0.062
H4	3.301	3.378	0.077
H5	**3.632**	**3.542**	**0.090**
H3	**3.663**	**3.574**	**0.089**
H6	3.548	3.629	0.081
OH6	4.435	4.468	0.033
H1	4.833	4.819	0.014
OH2	5.695	5.750	0.055
OH3	5.654	5.695	0.041

Table 4
^{1}H NMR chemical shifts of DBA in DMSO-$_{d6}$ before and after the complexation with β-CD, at 20 °C.



Hydrogen position	DBA chemical shift (ppm)	DBA/CD chemical shift (ppm)	Δδ (ppm)
Ha (alfa)	7.371	7.365	−0.006
Hb (beta)	7.791	7.785	−0.006
Hc (Ar orto)	7.824	7.820	−0.004
Hd (Ar meta, para)	7.475	7.469	−0.006

more affected after the inclusion were the H5 and H3, indicating that the molecule of DBA is inserted near them.

On the other hand, the protons of DBA were affected diffusely (Table 4), being the most affected Ha, Hb and the aromatics in meta and para positions. So, probably, as the molecule is symmetric, it is inserted facing the aromatic rings towards the outer face of β-CD and affecting the H6 proton of β-CD. Although the results give an indication that DBA is interacting inside β-CD cavity, further studies using 2D ROESY NMR could help to elucidate the geometry of the inclusion complex formed.

3.5. Dissolution test

The *in vitro* dissolution profiles of DBA before and after complexation with β-CD is shown in Fig. 6. For complexes prepared by kneading at 1:1 and 1:2 molar ratios, the observed profile was similar to those obtained for plain DBA, with very low release. So, for these samples, the complexation using the KN technique did not seem to be an advantage.

On the other hand, the complexes obtained by lyophilization exhibited the fastest dissolution rates, and the tendency to reach a plateau (Wang et al., 2011), demonstrating a zero-order kinetic. The required times for 50% release rate from LIO 1:1 and 1:2 were 40 and 60 min, respectively. This demonstrates that LIO1:1 has a slow release rate than LIO 1:2.

The sample LIO 1:1 reached the maximum cumulative release of the guest molecule after the equilibrium at 120 min, while LIO 1:2 released around 75% of DBA at the same time interval. This indicates that, at

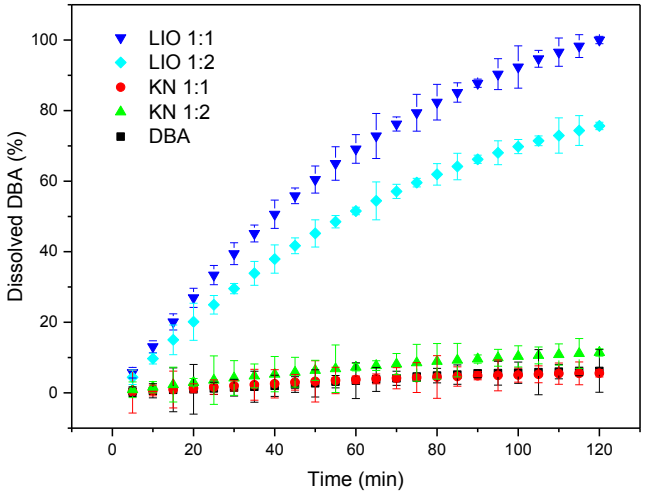


Fig. 6. Dissolution profile of DBA alone and the inclusion complexes obtained by kneading and lyophilization method at 1:1 and 1:2 molar ratio.

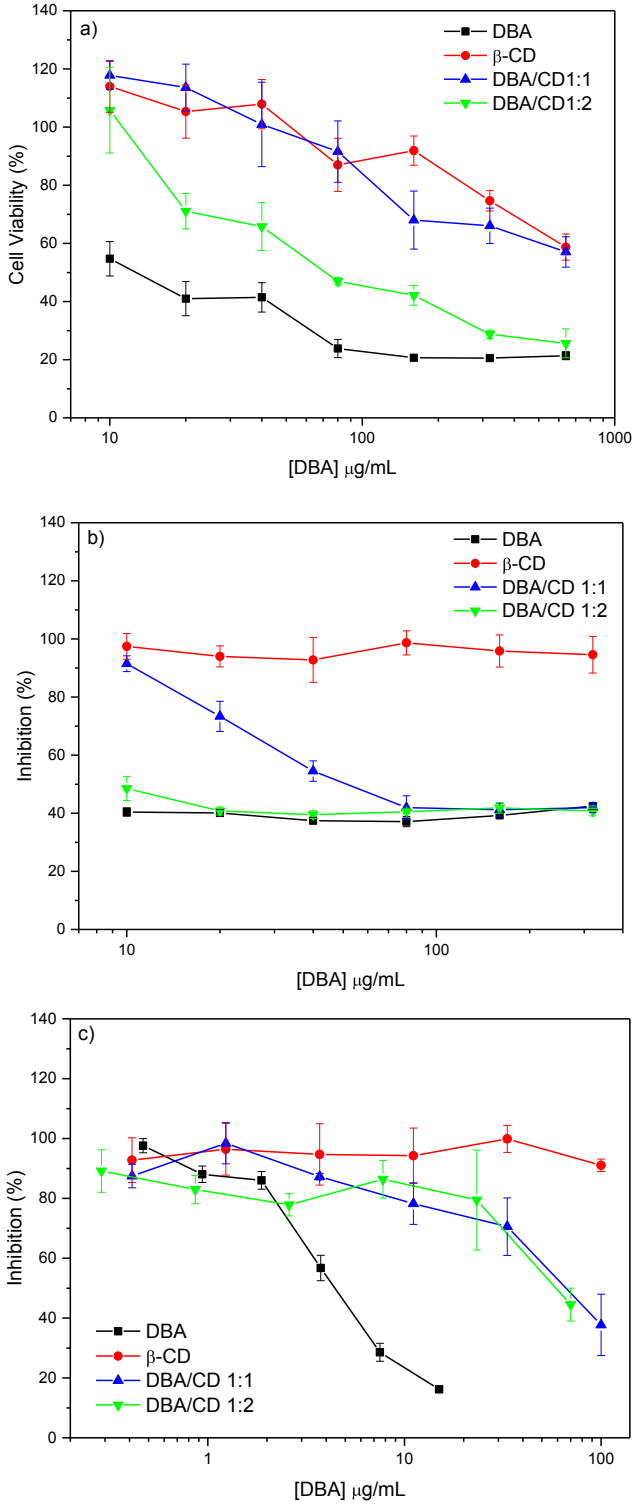


Fig. 7. (a) Cellular viability of THP-1 macrophages. (b) Viability of free-living promastigotes of *L. major*. (c) Viability of intracellular amastigotes of *L. infantum*.

1:1 molar ratio the DBA molecule had a slower release compared to 1:2 molar ratio.

3.6. Biological tests

Using the MTT endpoint, THP-1 macrophage cells were tested in order to verify if the compounds would harm the cells of the host (Fig. 7a). It was observed that DBA alone and DBA/CD at 1:2 molar ratio presented toxicity for these cells, with CC_{50} = 12.7 and 71.51 μg mL^{-1}, respectively, while β-CD alone and DBA/CD at 1:1 molar ratio showed no toxicity up to 640 μg mL^{-1}. So, the complex at 1:1 stoichiometry was not damaging the host cells, in all the concentrations tested.

Tests with free-living promastigotes of *L. major* (Fig. 7b), showed inhibition of the parasite's growth for plain DBA and DBA/CD at 1:2 molar ratio, with IC_{50} < 10 μg mL^{-1}. The effects of curcumin on promastigotes parasites have been demonstrated earlier in reference strains of *L. major*, *L. tropica* and *L. infantum* (Saleheen et al., 2002), with IC_{50} = 4.5, 5.7 and 5.9 μM respectively which are in good agreement with the value obtained for plain DBA. Plain β-CD had IC_{50} > 320 μg mL^{-1} being unable to kill the parasites. DBA/CD at 1:1 molar ratio showed antiproliferative activity, with an IC_{50} = 51.3 μg mL^{-1}.

For the intracellular assay (Fig. 7c) it was observed that β-CD showed no toxicity for the parasites, with EC_{50} > 100 μg mL^{-1}, while plain DBA destroys the amastigotes with IC_{50} of 4.4 μg mL^{-1}. DBA/CD 1:1 and 1:2 presented EC_{50} of 66.3 and 58.9 μg mL^{-1} respectively.

Plain β-CD did not kill neither the parasites nor the macrophage host cells, being therefore a safe carrier for DBA.

The selectivity index (SI) is considered as the highest exposure to the drug that produces the desired efficacy with no toxicity to that exposure. SI values were determined using the relationship CC_{50} against THP-1/EC_{50} against each parasite strain. It appears from results display in Table 5 that only DBA/CD at 1:1 molar ratio showed potentially high SI for both *Leishmania* strains. This is mainly due to the lack of cytotoxicity demonstrated against the host cells (in this case THP-1).

DBA/CD at 1:1 molar ratio kills the free living or intracellular parasites at higher concentrations than DBA alone, however no toxicity was observed for the host macrophage cell, even in the highest concentrations. Therefore, the adjustment of the dosage, can be made with more security, since higher concentrations can be administered, with no host cell damage.

Plain DBA is toxic for *Leishmania. sp* in concentrations less than 10 μg mL^{-1} both for free living and intracellular parasites, but from around 12 μg mL^{-1} it is also toxic for the host cells, being not safe above this concentration.

DBA/CD at 1:2 molar ratio also fights the free living or intracellular parasites, as well as 1:1. But, in this case, it presents toxicity for the host cells from around 71 μg mL^{-1} and could not be considered as safe.

Differences between 1:1 and 1:2 inclusion complexes may be due to the distinct behavior observed in the dissolution profiles (item 3.5). At 1:1 stoichiometry DBA seemed to be more available for interaction with the infected cells, since it can be more easily released from the cavity of one molecule of β-CD than at 1:2 stoichiometry.

Table 5
Cytotoxicity in THP-1 cells (CC_{50}, μg mL^{-1}), activity against *L. major* promastigotes and *L. infantum* amastigotes (EC_{50}, μg mL^{-1}) and selectivity index values of DBA formulations.

	THP-1	*L. major*		*L. infantum*	
	CC_{50}	EC_{50}	SI[a]	EC_{50}	SI[a]
DBA	13.2	< 10	> 1.3	4.4	3.0
DBA/CD 1:1	> 640	51.3	> 12.48	66.3	> 9.6
DBA/CD 1:2	71.5	< 10	> 1.8	58.9	1.2
β-CD	> 640	> 320	na	> 100	na

[a] Selectivity index (SI) was calculated by the ratio between the CC_{50} (μg mL^{-1}) and EC_{50} (μg mL^{-1}) values of DBA formulations. CC_{50} and EC_{50} values were obtained in experiments against THP-1 cells, *L. major* promastigotes and *L. infantum* amastigote-infected THP-1 cells.

4. Conclusion

The molecule of DBA has a very low tendency to interact with water molecules, being extremely insoluble, as observed by theoretical energy minimization. Synthesis of the molecule was successful performed and well documented, by FTIR and NMR spectra and the mechanism of the synthesis was proposed.

There was a considerable improvement in the aqueous solubility of the guest, after inclusion into β-CD cavity, also improving its dissolution profile. This would improve the bioavailability of the molecule and thus its activity as an antiparasitic medicine.

The curcumin molecule has already been successfully incorporated into cyclodextrin (Chen et al., 2018) and other drug carrier systems such as liposomes (Wei and Lee, 2017) and nanoparticles (Tiwari et al., 2017) to improve its solubility and resolve other limitations of the molecule. The results obtained from these previous efforts corroborates those observed for the curcumin analog compound synthetized in this work and reiterates the potential utility of these drug delivery strategies in enhancing the therapeutic outcomes from leshmaniasis treatment.

Lyophilization at 1:1 molar ratio demonstrated to be the best methodology for preparation of the complex and the *in vitro* tests evidenced that this formulation has activity against *Leishmania* sp. parasites, with no toxicity to the macrophages host cells.

Since SI values of DBA/CD at 1:1 molar ratio were ≥ 10 for both *Leishmania* strains, we may expect that this formulation would be therapeutically safe for leishmaniasis infections.

Thus, the results indicate a therapeutic advantage to use the DBA/CD inclusion complex at 1:1 molar ratio upon leishmaniasis infected cells, because it would be safer than the drug alone. *In vivo* assays should be undertaken to confirm if the bioavailability of the drug will be reached with the same toxicological security.

CRediT authorship contribution statement

Luciana de Matos Alves Pinto: Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing. **Oluwatomide Adeoye:** Validation, Investigation, Writing - review & editing. **Sérgio Scherrer Thomasi:** Conceptualization, Resources, Writing - review & editing. **Ana Paula Francisco:** Methodology, Formal analysis, Writing - review & editing. **Manuela Colla Carvalheiro:** Methodology, Resources, Writing - review & editing. **Helena Cabral-Marques:** Resources, Writing - review & editing, Supervision.

Declaration of Competing Interest

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.

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Appendix A. Supplementary material

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