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Synthesis, characterization and cytotoxic evaluation of inclusion complexes between Riparin A and β-cyclodextrin

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ABSTRACT

This study performed a physicochemical characterization of the inclusion complex generated between Riparin A and β-cyclodextrin (Rip A/β-CD) and compared the cytotoxic potential of the incorporated Rip A upon *Artemia salina* larvae. Samples were analyzed by phase solubility diagram, dissolution profile, differential scanning calorimetry, X-ray diffraction, infrared spectroscopy, proton nuclear magnetic resonance, scanning electron microscopy and artemicidal action. Riparin A/β-cyclodextrin complexes presented increased water solubility, A_L type solubility diagram and Kst constant of 373 L/mol. Thermal analysis demonstrated reduction of the melt peak of complexed Rip A at 116.2 °C. Infrared spectroscopy confirmed generation of inclusion complexes, ^{1}H NMR pointed out the interaction with H-3 of β-CD cavities, alterations in the crystalline natures of Rip A when incorporated within β-CD were observed and inclusion complexes presented higher cytotoxic on *A. salina* nauplii, with CL_{50} value of 117.2 (84.9–161.8) μg/mL. So, Rip A was incorporated into β-CDs with high efficiency and water solubility of Rip A was improved. Such solubility was corroborated by cytotoxic evaluation and these outcomes support the improvement of biological properties for complexes between Riparin A/β-cyclodextrin.

1. Introduction

Riparins are natural isolated for the first time from *Aniba riparia* (Ness) Mez fruits. Thenceforth, new potentially active molecules derived from them revealed as promising options for novel drugs. Riparin A (Rip-A) – N-(2-phenylethyl)benzamide – a synthetic alkamide, is the fundamental structural of all riparins (Fig. 1) [1,2].

Recently, we have shown biological *in vitro* properties attributed to the Riparin A, such as antioxidant activity against TBARS, cytotoxic action on HCT-116 colon carcinoma cells and leishmanicidal potential upon *Leishmania amazonensis* promatigostas. Additionally, it exhibits anti-inflammatory properties in carrageenan-induced paw edemas and reduces levels of inflammatory mediators as myeloperoxidase, tumor necrosis factor-α and interleukin-1β [3–5].

Cyclodextrins (CDs) have been used to develop new drugs with new therapeutic systems and produce optimized pharmaceutical formulations since they often increase pharmacological activity and improve physical and chemical properties of drugs. They are macrocyclic oligosaccharides generated by the enzyme glycosyltransferase (CGTase) action on the starch. CDs work as pharmaceutical excipients of natural sources constituted by six (α-CD), seven (β-CD) or eight monomers (γ-CD) of α-D-glucopyranose connected by α-1,4 bonds. However, there are semisynthetic CDs

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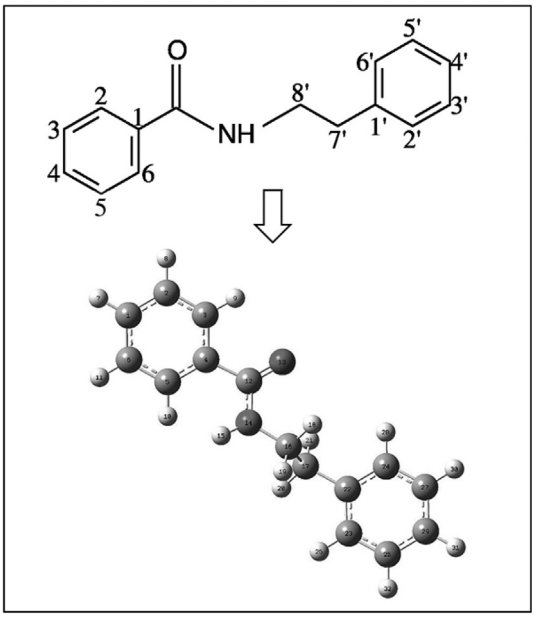


Fig. 1. Molecular structure of Riparin A.

based on substituted derivatives of natural cyclodextrins, such as hydroxypropyl betacyclodextrin [6–8].

Cyclodextrins have a variable concave molecular aspect, hydrophilic external surface with hydroxyl groups and a central lipophilic structure. Thus, they are capable of harboring hydrophobic molecules, improve their solubility, as well as increase their bioavailability. Such inclusion complexes have low oral toxicity even in high doses and superior drug inclusion capacity [9–11]. So, the present study performed a physicochemical characterization of the inclusion complex generated between Riparin A and β-cyclodextrin (Rip A/β-CD) and compared the cytotoxic potential of the incorporated Rip A upon *Artemia salina* larvae.

2. Material and methods

2.1. Obtention of Riparin A

Riparin A was obtained according to Nunes et al. [4] using the Schoten-Bauman reaction, through the mixture of 0.41 mL acyl chloride and 0.89 mL 2-phenylethylamine with triethylamine (as base), followed by magnetic stirring and purification by column chromatography with yield of 84%. The confirmation of synthesis was characterized using usual spectrometric methods (UV, IR, 1H and 13C NMR) [12].

2.2. Proton nuclear magnetic resonance (^{1}H NMR)

The spectrum of Rip A was obtained by nuclear magnetic resonance spectrometer of ^{1}H operating at 500 MHz (Bruker Avance III) with samples solubilized in deuterated dimethylsulfoxide (DMSO-d_6) at room temperature.

2.3. Physical mixture of Rip A and β-CD

Physical mixture (PM) was obtained by homogenization in porcelain mortar for 30 min with pestle in molar ratio of 1:1. Subsequently, the products were separated in a mesh of 250 μm and stored in a desiccator until analysis [13].

2.4. Preparation of the inclusion complex Rip A/β-CD by spray-drying

Equimolar amounts of Rip A and β-CD (ISP Technologies, São Paulo, Brazil, Batch: B701118) were solubilized at room temperature in ethanol and distilled water, respectively. The solutions were homogenized with magnetic stirrer for 30 min and were injected into the bench spray-drying apparatus (Buchi B-290) with an inlet pressure of 0.9 Bar, inlet temperature of 95 °C and sample flow of 3 mL/min. This technique ensured the obtainment of complex with 62% of yield. Complexes were stored in a desiccator until its analysis [14].

2.5. Characterization of the inclusion complex of Rip A/β-CD

2.5.1. Phase solubility diagram

Solubility studies were performed according to Higuchi and Connors [15]. Riparin A was added in tubes with aqueous solutions of β-CD (0, 2, 4, 6, 8 and 10 mM). The samples were kept in constant agitation for 72 h at temperature of 50 ± 2 °C. Then, they were filtered in 0.22 μm membranes and analyzed by spectrophotometer UV–Vis (Shimadzu® UV – 1800) at 225 nm. It was used a mixture of distilled water with ethanol (1:9) as background medium. Experiments were performed in triplicate for each concentration of β-CD. The stability constant (Kst) was calculated by the following equation, where S_0 is the intrinsic solubility of Rip A [14,16,17]: $Kst = Slope\ [S_0 \times (1-Slope)^{-1}]$.

Subsequently, Rip A, PM and Rip A/β-CD complexes were submitted to the standardization analysis.

2.5.2. Dissolution study

Dissolution investigations were conducted in the dissolutor Ethink Technology Model 299 at 50 rpm and 37 ± 0.5 °C. Samples were added in an equivalent way to 10 mg of Rip A. The reaction media with distilled water (1000 mL) was used to evaluate the variation of aqueous solubility. Analysis was performed in triplicate by UV/VIS spectrophotometry (Shimadzu® UV – 1800) after filtration at 225 nm with intervals of 5, 10, 15, 20, 30, 45 and 60 min. The dissolution profile was determined based on the dissolution efficiency at 60 min [18,19].

2.5.3. Differential scanning calorimetry (DSC)

Samples were put inside closed pots and heated between 50 °C and 400 °C at 10 °C/min in nitrogen atmosphere (DSC-2920 - TA Instruments) to obtain the DSC curves.

2.5.4. X-ray diffraction (XRD)

The diffractograms were obtained by X-ray diffractometer (Minifle x® model, λ = 1.5418 Å) with angle of 2θ ranging from 3° to 120° at 2 °C/min.

2.5.5. Infrared spectroscopy (IR)

The infrared vibration spectra were acquired with solid samples of Rip A, β-CD, PM and Rip-A/β-CD in KBr pellets (Perkin Elmer Frontier 1420) and scans ranging from 400 to 4000/cm. Spectra were smoothed and their baselines were automatically corrected using the spectrophotometer software.

2.5.6. Proton nuclear magnetic resonance (^{1}H NMR)

The spectra of the samples were obtained by hydrogen nuclear magnetic resonance spectrometer at 500 MHz (Bruker Avance III). Samples were solubilized in deuterated dimethylsulfoxide (DMSO-d_6) at room temperature (25 °C).

2.5.7. Scanning electron microscopy (SEM)

The morphological characteristics of the samples were analyzed in the electron microscope Shimadzu SSX-550 Superscan to visualize the surfaces of Rip A, β-CD, PM and Rip-A/β-CD. The particles were fixed in metallic support under vacuum with subsequent metallization by a thin gold film. Microphotographs were acquired in different magnifications by acceleration voltages from 8 to 15 kV.

2.6. Artemicidal cytotoxic assay

Larvae of *Artemia salina* Leach were treated with Rip A and Rip A/β-CD complex according to Meyer et al. [20]. Briefly, *A. salina* eggs were hatched in a synthetic saline (NaCl 36 g/L) at 25 °C with continuous aeration. Following 48 h of hatching, nauplii were collected (n = 10 nauplii/sample/concentration) and samples (Rip A, β-CD and Rip-A/β-CD complex) were added at the concentrations of 50 50, 100, 250, 500 and 1000 μg/mL. Potassium dichromate ($K_2Cr_2O_7$) 0.1% was used as positive control. After 24 h exposure, death nauplii were quantified to obtain CL_{50} (average lethal concentration) values. Experiments were performed in triplicate.

2.7. Statistical analysis

All results were presented as mean ± standard error of mean (SEM). Data were evaluated by analysis of variance (ANOVA) followed by Student-Neuman-Keuls. Differences were considered significant when $p < 0.05$. CL_{50} values and their 95% confidence intervals were obtained by nonlinear regression (GraphPad program, Intuitive Software for Science, San Diego, CA).

3. Results and discussion

3.1. Phase solubility diagram

Complexation is an interaction between a host molecule, such as β-CD, and its guest molecule, as Rip A. Recently, investigations have been conducted to acquire inclusion complexes of active substances, such as neurotransmitters (dopamine), hormones (epinephrine) and the neuroactive amino acid tyramine, and these inclusions improved stability and optimized some pharmaceutical parameters [21,22].

The method of Higuchi and Connors [15] is widely cited to carry out phase solubility diagrams and analysis of inclusion complexes and was used based on the increase of Rip A solubility as function of increasing concentrations of β-CD. Fig. 2A details the UV–Vis absorption spectrum of Rip A in a concentration-dependent way and Rip A solubility diagram. Rip-A revealed absorption at approximately 225 nm in water:ethanol (1:9). In this wavelength, no spectral reading of β-CD absorption was detected, indicating that cyclodextrin does not interfere in the spectrophotometric analysis of Rip A [21,23].

Considerations about the UV–Vis scan of Rip A allowed to obtain a calibration curve: $y = 0.1664x + 0.0008$ ($r^2 = 0.9992$) (Fig. 2B). A linear increase between aqueous solubility of Rip A and β-CD concentrations was found, suggesting inclusion complexes' production in the molar ratio of 1:1 (Rip A:β-CD). This stoichiometric ratio is present in most complexes obtained with β-CD, in which the guest molecule usually has a molecular weight between 100 and 400 Da, and Rip A, with 225.29 Da, has fit parameters. The stability constant (Kst) was 373 L/mol [14,22].

The constant Kst indicates the presence of significant interaction between Rip A and β-CD and generation of stable inclusion complexes, since low Kst values indicate poor interaction and big amount of free host molecules. Ideal Kst values range from 100 to 1000 L/mol, and this was verified in the inclusion complexes of Rip A/β-CD. The intrinsic solubility of Rip A (S_0) 1.48 mM and an enhancement of 240.2% of Rip A solubility was found following the addition of β-CD. Solubility diagram allows the classification of complexes in two profiles: A and B. Profile A consists of soluble complexes and profile B represents limited solubility [24,25]. Herein, inclusion complexes of Rip A/β-CD were classified as type A_L.

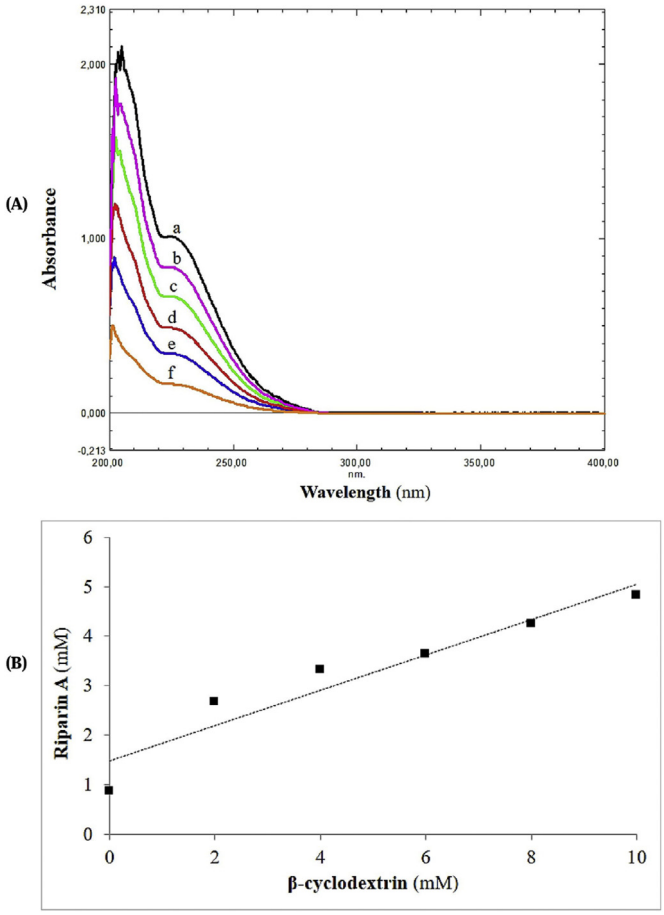


Fig. 2. UV–Vis absorption spectrum of Riparin A (A) and its phase solubility diagram (B) in the presence of β-cyclodextrin.

Type A diagrams are subdivided into i) A_P: positive deviation diagram with more effective solubilization for high concentrations of CDs; ii) A_L: linear diagram with drug solubility increasing linearly with CDs' concentration and iii) A_N: negative deviation diagram in which CDs are proportionally less effective at higher concentrations due to complex interactions between solute-solute and solute-solvent. On the other hand, Type B diagrams can be of two subtypes: i) B_S: diagram with precipitation of insoluble complex with increase of cyclodextrin after initial increase of guest substance solubility and ii) B_I: highly insoluble complexes without the possibility of initial increase in solubility of guest substances [26,27].

3.2. Dissolution study

After 5 min of reaction, dissolution analysis showed free Rip A, PM and Rip A/β-CD complex as 18.4, 26.1 and 67.6% of the dissolution, respectively (Fig. 3). Both free Rip A (61.7%) and PM (62.5%) displayed time-dependent increasing for their percentages of dissolved Rip A until 60 min [18,28].

The establishment of a soluble complex was corroborated by

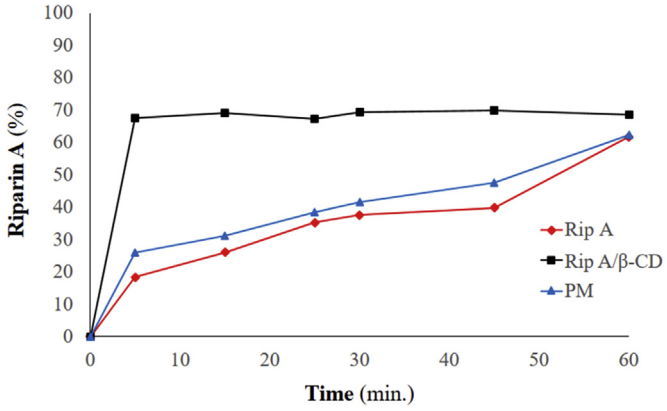


Fig. 3. Dissolution profile of Riparin A (Rip A), PM (physical mixture) and Riparin A/β-cyclodextrin (Rip A/β-CD) inclusion complexes.

dissolution studies, whose outcomes indicated superior solubility of the inclusion complex when compared to free Rip A and PM. It was evident that Rip A/β-CD complexes presented higher concentrations of dissolved Rip A from the first 5 min of experiment than the maximum dissolutions of the other samples, and it reached the maximum dissolution of 68.9% of dissolved Rip-A (Fig. 3). It is likely that the improvement of Rip A water solubility is consequence of interactions between Rip A and β-CD and reduction of Rip A interfacial tension in the dissolution medium. So, these results obtained by the spray-drying method indicated more efficient construction of the inclusion complexes [28,29].

3.3. Differential scanning calorimetry

Fig. 4 illustrates DSC thermograms of the samples whose variations suggest the establishment of inclusion complexes [7]. The DSC curve of β-CD shows a broad endothermic peak at 95.8 °C in comparison to the loss of water, while the Rip A thermogram shows a characteristic, intense and well-defined endothermic peak at 116.2 °C due to the melting point of Rip A and its crystalline nature. A melt peak for Riparin A was partially reduced in the PM thermogram and showed decline in the curve for Rip A/β-CD inclusion complexes.

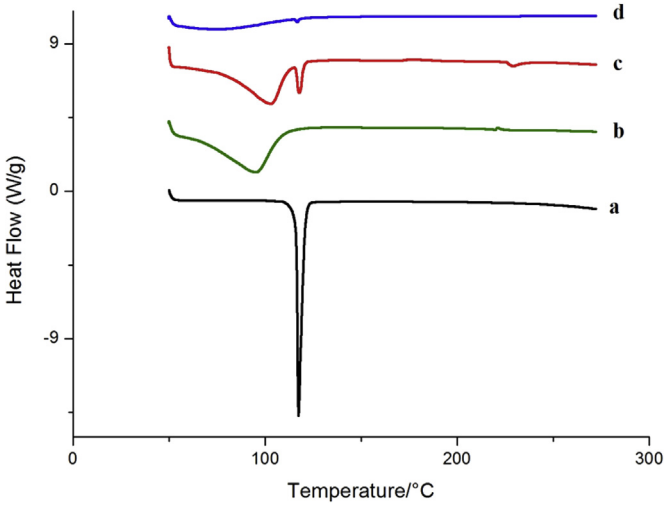


Fig. 4. Differential Scanning Calorimetry (DSC) of Riparin A (a), β-cyclodextrin (b), Physical Mixture (c), and Riparin A/β-cyclodextrin inclusion complexes (d).

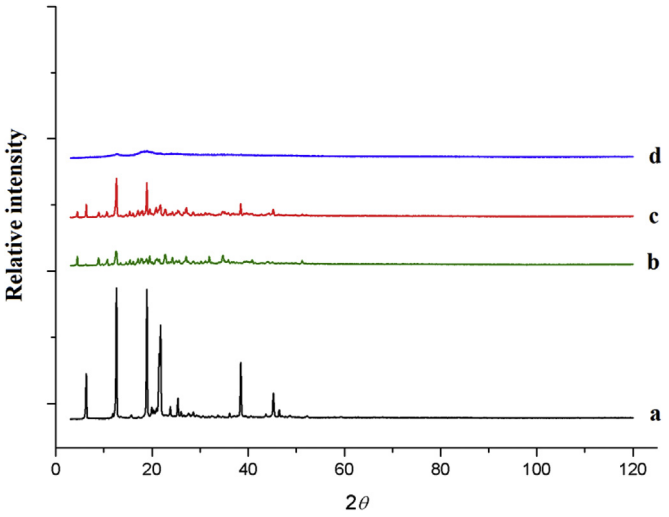


Fig. 5. X-ray diffraction of Riparin A (a), β-cyclodextrin (b), Physical Mixture (c), and Riparin A/β-cyclodextrin inclusion complexes (d).

Thermoanalytical techniques are extra analysis widely used to evaluate inclusion complexes and endorse the presence of complexes. Such methods verify changes related to thermal events as degradation and stability alterations. DSC analysis detects variation in melting, boiling and sublimation points or disappearance of these records due to incorporation of guest molecules into the β-CD cavity [13,30]. So, this technique may also be used to indicate the incorporation of bioactive compounds, such as Rip A, into the CD molecule. We noted that the PM curve corresponds to the association of the β-CD and Rip A curves, and such curves are different from Rip A/β-CD thermogram (Fig. 4), suggesting that PM presented less internalization of Rip A into β-CDs [31].

3.4. X-ray diffraction (XRD)

XRD technique performs structural analysis based on the spreading of X-rays on the samples [32]. The diffraction profile highlighted the Rip-A crystallinity and peak of diffraction angle 2θ at 12.6° followed by secondary peaks at 6.3°, 18.9°, 21.48°, 21.78°, 38.46° and 45.22°. Signals related to typical peaks of Rip A from Rip

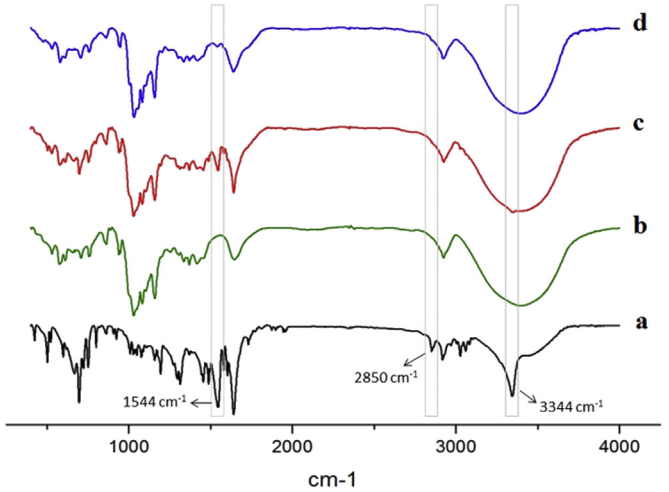
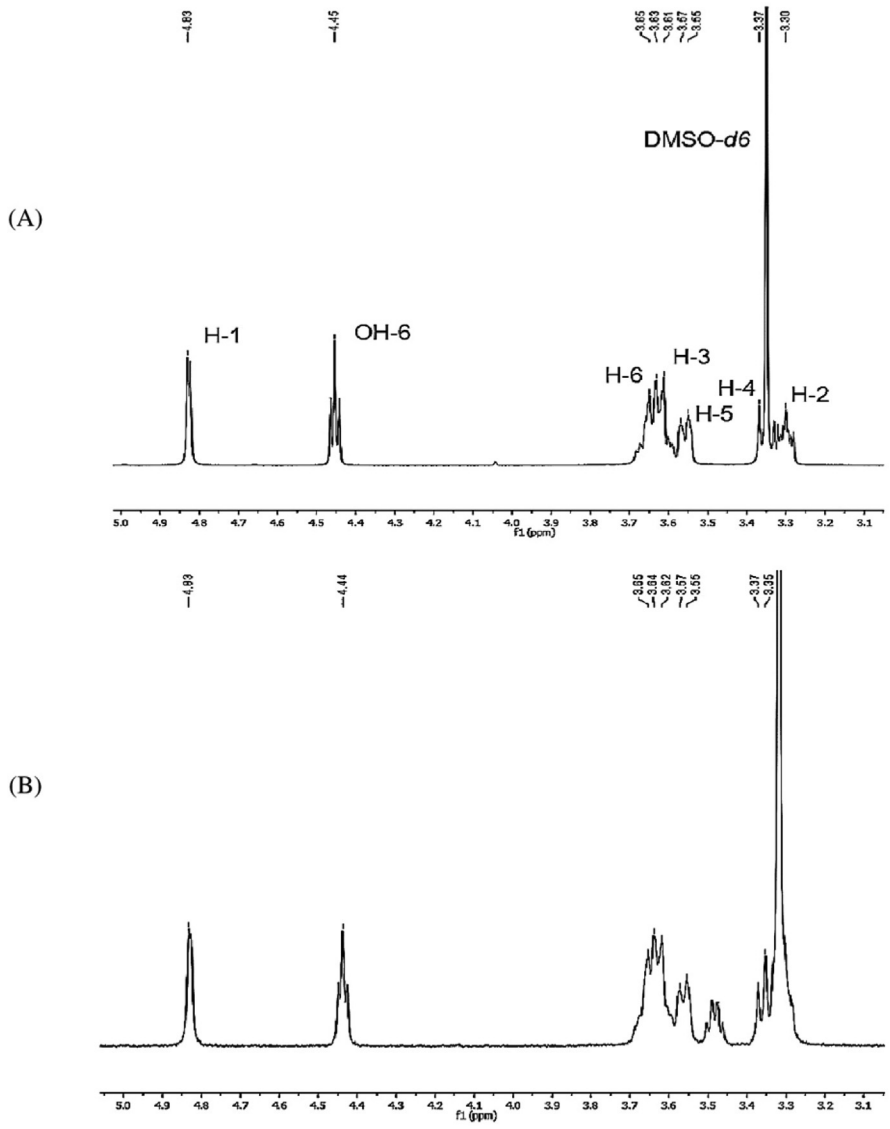


Fig. 6. Infrared spectroscopy of Riparin A (a), β-cyclodextrin (b), Physical Mixture (c), and Riparin A/β-cyclodextrin inclusion complexes (d).



H (δ)	**β-cyclodextrin** (ppm)	**Riparin A/β-cyclodextrin** (ppm)
1	4.83	4.83
2	3.30	3.35
3	3.61	3.62
4	3.37	3.37
5	3.55	3.55
6	3.63	3.64

Fig. 7. Proton nuclear magnetic resonance of β-cyclodextrin (A) and Riparin A/β-cyclodextrin (B) in DMSO-d_6 (500 MHz).

A/β-CD were not observed, which may indicate the efficacious of complexation and crystalline nature modification for an amorphous pattern (Fig. 5). Such finding is not observed in PM diffractograms since Rip A peaks were maintained, though with a fewer intensity, which indicate a less effective complexation in PM samples, as seem in the DSC analysis [33].

3.5. Infrared spectroscopy

NMR and IR spectroscopies are structural elucidation tools extensively used for confirmatory analysis of inclusion complexes. Infrared spectroscopy technique allows generation of characteristic bands by the absorption of infrared radiation at visible light (400–800 nm) from the electromagnetic spectrum and molecular vibration [34,35]. Samples analyzed generated the spectra shown in Fig. 6. Spectrum of Rip A displays symmetric stretching peaks in the region of 3344 cm^{-1} and 1640 cm^{-1} relative to the N–H and C=O bonds of the amide functional group, respectively. The absorption at 2850 cm^{-1} refers to the aliphatic C–H region. The absorption at 3026 cm-1 is related to C–H bonds of aromatic rings. At the 694 and

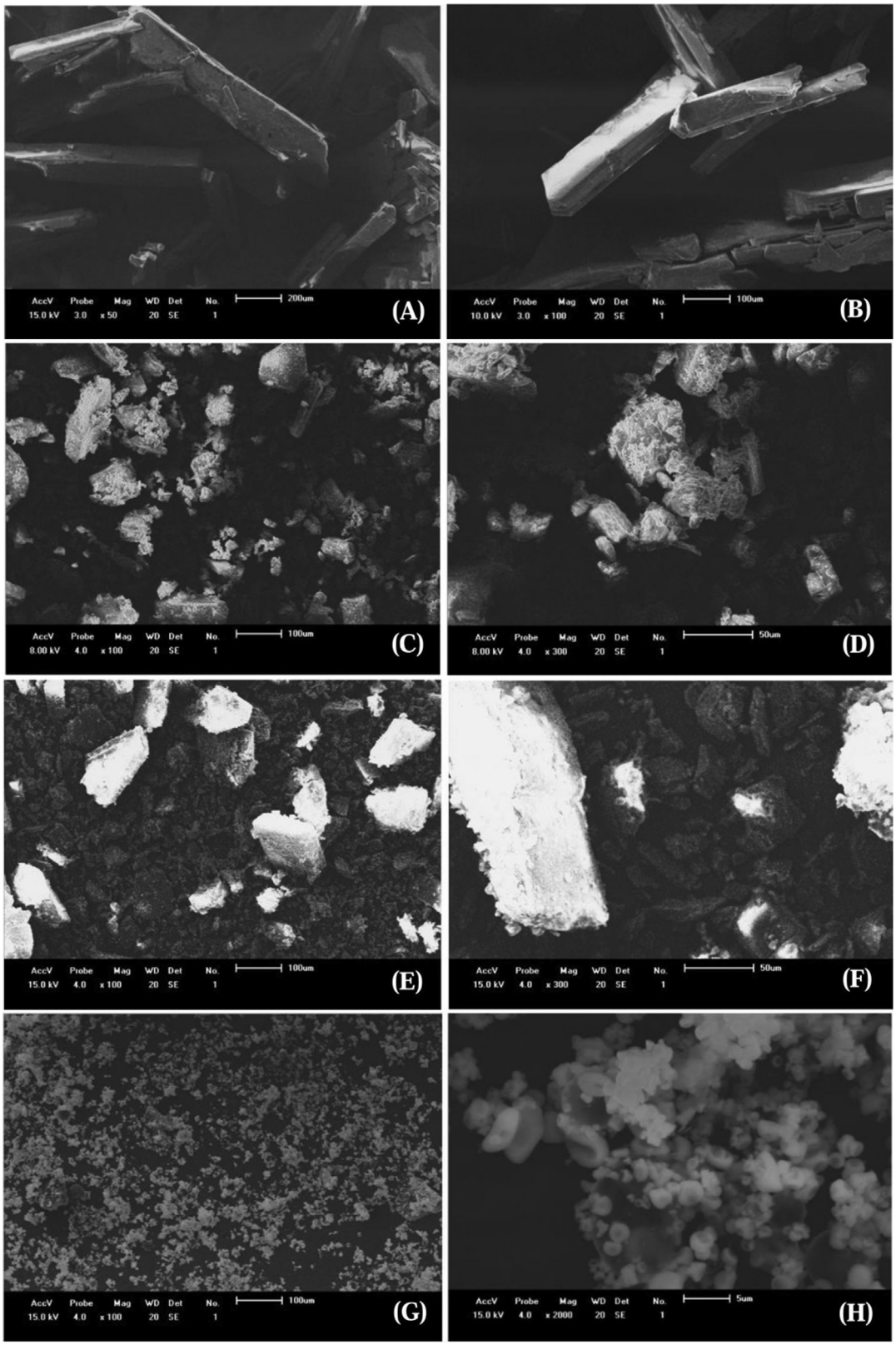


Fig. 8. Photomicrographs of Scanning Electron Microscopy (SEM) of RiparinA (A–B), β-cyclodextrin (C–D), physical mixture (E–F) and Riparin A/β-cyclodextrin complexes (G–H).

750 cm^{-1}, we found the angular deformation outside of the C–H plane of monosubstituted rings and peak at 1544 cm^{-1} of C=C aromatic ring stretching [14].

β-CD showed a large signal at 3400 cm^{-1} corresponding to the O–H groups and absorption bands at 2923 cm^{-1}, 1157 cm^{-1} and 1028 cm^{-1} related to C–H, C–O and C–O–C, respectively. The

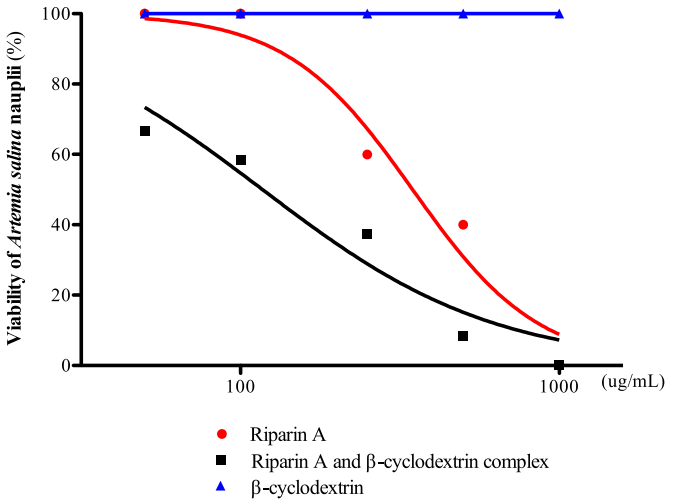


Fig. 9. Curves of concentration-effect of Riparin A, β-cyclodextrin and Riparin A/β-cyclodextrin inclusion complexes on nauplii viability of *Artemia salina* with 48 h-old after 24 h exposure. Results were obtained by nonlinear regression (n = 10 nauplii/sample/concentration).

spectra of PM and inclusion complexes confirmed incorporation of Rip A into β-CD molecule due to the absence of absorptions related to N–H and C–H aliphatic bonds and evident reduction at 1544 cm^{-1}. This may indicate involvement of the central region of Rip A for the production of inclusion complexes (Fig. 6) [36].

3.6. Proton nuclear magnetic resonance

This technique quantifies number of hydrogen atoms within a molecule exposed to a magnetic field [37–40]. Interaction between Rip A and β-CD can be demonstrated by the spectral variations of the complex spectrum and chemical shifts derived from β-CD changes after the binding with Rip A. This can be represented by variations of internal hydrogens (H-3 and H-5).

Yang et al. [41] demonstrated that chemical shifts of 0.01–0.03 ppm are significant findings for molded inclusion complexes. This variation indicates presence of interactions (between RipA and β-CD), and ratifies the occurrence of complexes. We found that complexes with Rip A generated changes in the δ of H-2, H-3 and H-6, especially for the displacement of H-3 (0.01 ppm). This displacement can demonstrate interaction of Rip A with the β-CD cavity in its larger portion. This may be caused by hydrogen bonds involving the Rip A amide group and β-CD oxygen atoms (Fig. 7) [40].

Results obtained with ^{1}H NMR for Rip A are in accordance with the literature [4]. It was observed a doublet at 7.82 ppm for hydrogens at the C-2 and C-6 positions; a triplet at 7.45 ppm for hydrogens at the C-3, C-4 and C-5 positions; a doublet at 7.24 ppm related to the 2′ and 6′ hydrogens, and a triplet at 7.30 ppm corresponding to hydrogens at the C-3′, C-4′ and C-5'. Moreover, triplet occurred at 2.85 ppm and at 3.47 ppm a multiplet related to the hydrogens at the C-7′ and C-8′ positions. Finally, at 8.56 ppm, we found a triplet related to the NH functional group (data not shown).

3.7. Scanning electron microscopy

Images obtained are able to demonstrate the establishment of inclusion complexes. Herein, SEM demonstrated the morphology of isolated samples (Rip A and β CD), as well as the PM and the Rip A/β-CD complex. Changes in Rip A crystalline structure and globular structure of β-CD were noted (Fig. 8).

β-CD did not demonstrate a morphological similar pattern to the inclusion complexes between Rip-A and β-CD. So, images obtained by SEM demonstrated changes in the morphological pattern when compared to the isolated samples [29]. On the other hand, amorphous materials were seen, a distinctive characteristic for inclusion complexes, confirming results verified by XRD.

3.8. Artemicidal cytotoxic assay

Artemia sp. shrimps are very used in rapid and easy-to-perform tests based on the number of dead specimens after exposure to the tested substance and there is a tendency to use them in toxicological tests for drug discovery [42]. Indeed, this method has many advantages as low cost, quick outcomes, easy handling and dispensation of aseptic techniques, suggesting that *A. salina* assay could replace the MTT method since it requires animal serum [42,43]. The results for nauplii viability after treatment with free and incorporated Rip A are shown in Fig. 9. No deaths were noticed for β-CD (CL_{50} > 1000 μg/mL). On the other hand, free Rip A and Rip A/β-CD complexes revealed CL_{50} values of 346.7 (199.6–602.1) and 117.2 (84.9–161.8) μg/mL, respectively, indicating higher activity of incorporated Rip A when compared with the alone substance ($p < 0.05$). These results may be associated to the better water solubility of the inclusion complex in relation to free Rip A, and support the improvement of biological properties for complexes between Riparin A/β-cyclodextrin.

4. Conclusion

Riparin A was incorporated into β-CDs with high efficiency, physicochemical tools, such as solubility diagram, dissolution profile, differential scanning calorimetry, X-ray diffraction, infrared spectroscopy, proton nuclear magnetic resonance and scanning electron microscopy confirmed inclusion complexes and water solubility of Riparin A was improved. Such solubility was corroborated by cytotoxic evaluation whose outcomes support the improvement of biological properties for complexes between Riparin A/β-cyclodextrin.

Conflict of interest

The authors report no conflict of interest.

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