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In vitro and in vivo evaluation of cnicin from blessed thistle (*Centaurea benedicta*) and its inclusion complexes with cyclodextrins against *Schistosoma mansoni*

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Abstract

Schistosomiasis, caused by a blood fluke of the genus *Schistosoma*, afflicts over 230 million people worldwide. Treatment of the disease relies on just one drug, praziquantel. Cnicin (Cn) is the sesquiterpene lactone found in blessed thistle (*Centaurea benedicta*) that showed antiparasitic activities but has not been evaluated against *Schistosoma*. However, cnicin has poor water solubility, which may limit its antiparasitic activities. To overcome these restrictions, inclusion complexes with cyclodextrins may be used. In this work, we evaluated the in vitro and in vivo antischistosomal activities of cnicin and its complexes with β-cyclodextrin (βCD) and 2-hydroxypropyl-β-cyclodextrin (HPβCD) against *Schistosoma mansoni*. Cnicin were isolated from *C. benedicta* by chromatographic fractionation. Complexes formed by cnicin and βCD (Cn/βCD), as well as by cnicin and HPβCD (Cn/HPβCD), were prepared by coprecipitation and characterized. In vitro schistosomicidal assays were used to evaluate the effects of cnicin and its complexes on adult schistosomes, while the in vivo antischistosomal assays were evaluated by oral and intraperitoneal routes. Results showed that cnicin caused mortality and tegumental alterations in adult schistosomes in vitro, also showing in vivo efficacy after intraperitoneal administration. The oral treatment with cnicin or Cn/βCD showed no significant worm reductions in a mouse model of schistosomiasis. In contrast, Cn/HPβCD complex, when orally or intraperitoneally administered to *S. mansoni*-infected mice, decreased the total worm load, and markedly reduced the number of eggs, showing high in vivo antischistosomal effectiveness. Permeability studies, using Nile red, indicated that HPβCD complex may reach the tegument of adult schistosomes in vivo. These results demonstrated the antischistosomal potential of cnicin in preparations with HPβCD.

Keywords Cnicin · Schistosomicidal · Cyclodextrins · *Schistosoma mansoni* · Blessed thistle

Introduction

Schistosomiasis, caused by trematode blood fluke flatworms of the genus *Schistosoma*, afflicts over 230 million people worldwide, being the second most important human parasitic disease in terms of public health (Lago et al. 2018; Vale et al. 2017). Currently, schistosomiasis treatment is obtained with a single drug, praziquantel (PZQ). Although it is safe, PZQ exhibits lack of activity against juvenile worms, limited effects on liver and spleen lesions, and its use over the last decades as a single antischistosomal drug may contribute to emerging PZQ-resistance development (Vale et al. 2017). Therefore, the lack of any other effective and safe schistosomicidal compound has raised the urgent need for new antischistosomal drugs that could either complement or replace PZQ chemotherapy (de Santiago

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et al. 2014). As a result, the search for novel anthelmintic compounds, especially from natural sources, has been increased (Lago et al. 2018; de Moraes and Geary 2020).

Centaurea benedicta (Asteraceae, synonymy *Cnicus benedictus*), known as blessed thistle, is a plant used in traditional world medicine as tonic for increasing appetite and gastrointestinal secretion, showing inhibitory effects against inflammation (Szabó et al. 2009; Ghiasy-Oskoee et al. 2018). In addition, cnicin (Cn) is the main germacranolide sesquiterpene lactone found in *C. benedicta* (Ghiasy-Oskoee et al. 2018) that showed anti-inflammatory (Erel et al. 2011), antimicrobial (Barrero et al. 2000), and antitumor (Sen et al. 2017; Saroglou et al. 2005) effects. Recent studies reported that cnicin possess a potent antiparasitic activity against *Trypanosoma brucei* (Kurita et al. 2016) and *Leishmania major* (Chibli et al. 2018). However, despite its biological potential, cnicin has not been evaluated against *Schistosoma*.

Although may be a promising lead natural compound against several diseases, cnicin has some chemical characteristics that may limit its pharmacology use, such as poor water solubility (Erel et al. 2011). To overcome these restrictions, technological alternatives may be developed to improve its biopharmaceutical properties, such as the use of inclusion complexes with cyclodextrins (CDs) (Mangolim et al. 2014).

CDs are cyclic oligosaccharides consisting of six to eight glucopyranose units, linked by an α-1,4-glycosidic bond (Suárez et al. 2014; Lanna et al. 2016). The most common CDs are α, β, and γ CDs, which are composed of six, seven, and eight glucopyranose units, respectively. The size of CDs' cavity allows the complexation of guest molecules or moieties, and, therefore, they can form inclusion complexes stabilized by non-covalent interactions with a wide variety of compounds which can improve the technological aspects of the drugs (Mangolim et al. 2014; Lanna et al. 2016). Besides the natural CDs, 2-hydroxypropyl-β-cyclodextrin (HPβCD) is a hydroxy alkyl derivative that shows a higher water solubility, satisfactory inclusion ability, and less toxicological potential (Gould and Scott 2005). Moreover, HPβCD does not demonstrate any nephrotoxicity via the parenteral route even at high dosages (Irie and Uekama 1997). In addition, HPβCD was the first approved cyclodextrin derivative by Food and Drug Administration (FDA) (Brewster and Loftsson 2007), showing large application in pharmaceuticals, food, and agriculture (de Venturini et al. 2008). Nonetheless, to the best of our knowledge, no previous study regarding cnicin complexes with βCD or HPβCD has been reported.

Thus, in the present study, we isolated cnicin from *C. benedicta* and evaluated its in vitro and in vivo antischistosomal properties. In addition, we prepared and characterized the inclusion complexes of cnicin with βCD and HPβCD and assessed their antischistosomal activities in vitro and in vivo by using oral and intraperitoneal routes in mice infected with *S. mansoni*.

Materials and methods

Materials

βCD (MW = 1134.98 g/mol, with purity ≥ 97%) was purchased from Sigma-Aldrich (St. Louis, MO, EUA), while HPβCD (MW = 1460 g/mol, DS = 5–8, with purity ≥ 95%) was purchased from Cerestar Company (Hammond, IN, EUA). The other reagents used in the experiments were also all reagent grade (≥ 95%) and were used without any treatment. Nile red was purchased from Sigma-Aldrich (St. Louis, MO, EUA).

Isolation of cnicin from *C. benedicta*

This study was developed in line with the Brazilian Federal Law number 13.123/2015 on Access to Genetic Heritage, registered under number AE32DB3. Aerial parts of *C. benedicta* L. (Asteraceae) were collected at the Faculty of Pharmacy's Medicinal Herb Garden, 21°46′38.7″S 43°22′00.5″W, Juiz de Fora city, MG, Brazil, on August 16 in 2017. The plant material was authenticated by Dr. Luiz Menini Neto (Botanic Department, Federal University of Juiz de Fora, Juiz de Fora, MG, Brazil), and a voucher specimen of *C. benedicta* (CESJ 71393) was stored at the Herbarium of Botanic Department at Federal University of Juiz de Fora, MG, Brazil.

The leaf rinsed extract of *C. benedicta* L. was obtained by immersing the fresh leaves (1000 g) in dichloromethane:ethanol (9:1 v/v) for 30 s at room temperature, and the solvent was removed under vacuum at 40 °C, affording 15 g of the rinsed leaves extract (CB). The crude extract CB (15 g) was chromatographed over silica gel (40–63 μm), under vacuum liquid chromatograph system (VLC, glass columns with 5–10 cm i.d.) using chloroform:methanol mixtures in increasing proportions as eluent (chloroform 100%, chloroform:MeOH 98:2 v/v, chloroform:MeOH 95:5 v/v, and chloroform:MeOH 90:10 v/v), furnishing ten fractions. After solvent evaporation, a white powder was obtained in fraction VIII (chloroform:MeOH 95:5 v/v), yielding 3.198 g of an isolated compound. Chemical structure of this compound was established as cnicin by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) (Bruker 500 Advance spectrometer, Bruker Corporation, Dresden, Germany) analysis and comparison with literature (Chain et al. 2014).

HPLC-DAD analysis of cnicin

Cnicin was analyzed using high performance liquid chromatography (HPLC) (Waters Corporation, Milford, MA, USA) equipped with (diode array detection) DAD (Waters 2998), binary HPLC pump (Waters 1525), and an autosampler (Waters 2707). The analytical column used was a SunFire C_{18} column (5 μm particle size, 4.6 mm × 250 mm) with a SunFire C_{18} precolumn (5 μm particle size, 4.6 mm × 20 mm),

both from Waters Corporation (Milford, MA, USA). The mobile phase was a mixture of ultrapure water acidified with 0.5% of phosphoric acid (A) and methanol (B). The gradient method was as follows: 40–100% (B) in 0–60 min, with the flow rate of 1 mL/min. Cnicin was diluted in methanol (HPLC grade) to reach a final concentration of 2 mg/mL. Solution was filtered through 0.45 μm membrane filters and degassed before usage, injecting a volume of 15 μL.

Preparation of cnicin inclusion complexes with βCD and HPβCD

The inclusion complexes of cnicin with βCD (Cn/βCD) and HPβCD (Cn/HPβCD) were prepared by coprecipitation, followed by a freeze-drying method (Lanna et al. 2016; De Miranda et al. 2019). Briefly, cnicin was dissolved in ethanol, and βCD was dissolved in ultrapure water at 1:1 mol ratio. The ethanol solution of cnicin was poured into the aqueous solution of βCD, and the suspension formed was subjected to stirring for 24 h, and the excess of ethanol was removed under vacuum and heating at 50 °C during 20 min. Then, they were subjected to the freeze-drying process to achieve the solid inclusion complex. The same process was applied to HPβCD to yield Cn/HPβCD (Bittencourt et al. 2019).

Characterization of cnicin inclusion complexes with βCD and HPβCD

Fourier Transform Infra-Red (FTIR) spectra of cnicin, βCD, HPβCD, Cn/βCD, Cn/HPβCD, and their respective physical mixtures (PMs), at a molar ratio of 1:1, were recorded between 4000 and 400 cm^{-1} using a Perkin Elmer Spectrum Two™ FTIR spectrometer (PerkinElmer, Boston, MA, USA) and KBr pellets. The spectra were recorded as the average of 16 scans with a spectral resolution of 2 cm^{-1}. Perkin Elmer Spectrum ES 192 software (version 10.03.08.0133) was used for the analysis of the spectra (Ribeiro et al. 2008; Moreira et al. 2018).

Isothermal titration calorimetry (ITC) was performed in duplicate using a Microcal VP-ITC Microcalorimeter (Malvern Panalytical Ltd., Malvern, UK) (Aberkane et al. 2010). Solutions were prepared by dissolution of cnicin and βCD or HPβCD in dimethyl sulfoxide (DMSO):water mixture (9:1, v/v). This solvent mixture was used due to the incredibly low water solubility of cnicin. Experiments consisted of 51 successive injections of Cn solution (30 mmol L^{-1}) into the reaction cell charged with 1.5 mL of βCD solution or HPβCD solution (2.0 mmol L^{-1}) at intervals of 540 s. A blank experiment was performed by injection of Cn solution into the solvent. The concentration correction and integration of the heat flow peaks as well as the calculation of the binding constant (K_b), stoichiometry (N), and enthalpy of reaction ($\Delta_r H^o$) were performed using the Microcal Origin 6.0 software. The Gibbs free energy ($\Delta_r G^o$) and entropy ($T\Delta_r S^o$) of interaction were calculated by the classical thermodynamic equations also provided by the same software (De Miranda et al. 2019).

Studies about the hydrodynamic diameter (D_h) of hydrophobic precipitates were performed by the titration of cnicin, Cn/βCD or Cn/HPβCD DMSO solutions in water. For this purpose, dynamic light scattering (DLS) experiments were performed in a Malvern Zetasizer Nano ZS 90 particle analyzer (Malvern Panalytical Ltd., Malvern, UK) using square polyethylene cells. Concerning the low solubilities of cnicin and their inclusion complexes, stock solution samples were prepared by initial dissolution of 1.0 mg of cnicin or an equimolar amount of Cn/βCD or Cn/HPβCD in 0.5 mL of DMSO. Subsequently, 25 injections of 20 μL of these DMSO solutions were gradually titrated in a greater volume of ultrapure water (2 mL), and the hydrophobic precipitates were spontaneously formed by simple mixture. Samples were submitted to a monochromatic light (4 mW He–Ne laser at 633 nm), and the scattered light intensity was measured at an angle of 90°. The D_h were determined by the average of five independent measurements, each of them obtained as the mean of 30 counts. Zeta potential (ZP) measurements were also determined in the Malvern Zetasizer Nano ZS 90 by means of the laser Doppler microelectrophoresis technique, at a scattering angle of 173°, using a glass cuvette into which the measuring cell (Dip Cell) was immersed. ZP values were calculated as the average of 10 independent measurements, each of them obtained as the mean of 30 counts with the same samples used in DLS experiments.

Parasites

The Belo Horizonte (BH) strain of *S. mansoni* was used in all experiments. This BH strain was maintained by passage through *Biomphalaria glabrata*, as the intermediate host, and Swiss female mice (Anilab, São Paulo, Brazil) as definitive host as previously described (de Moraes et al. 2014). Both mice and snails were kept under environmentally controlled conditions (temperature, 25 °C; humidity, 50%), with unrestricted access to rodent food and water.

In vitro antischistosomal assay

Seven weeks post infection *S. mansoni* were removed from the hepatic portal system and cultured in RPMI 1640 culture medium supplemented with 5% inactivated fetal calf serum (Vitrocell, Campinas, SP, Brazil), 100 U/mL penicillin (Vitrocell, Campinas, SP, Brazil), and 100 μg/mL streptomycin (Vitrocell, Campinas, SP, Brazil) at 37 °C in an atmosphere of 5% CO_2 until usage. For determination of activity against adult schistosomes, cnicin, Cn/βCD, Cn/HPβCD, βCD, and HPβCD were initially tested at the concentration of 50 μM, using DMSO stock solutions (10 mM) diluted in

supplemented RPMI 1640 medium within 24 flat bottom well plates (Tissue Culture plastics, TPP, St. Louis, MO) with a final volume of 2 mL per well (Mafud et al. 2018). Samples were tested in triplicate with two worms of both sexes placed into each well. Wells with the highest concentration of DMSO in medium (0.5%) served as controls. Praziquantel (2 μM) served as positive control. Parasites were kept for 48 h (37 °C, 5% CO_2), and their viability was assessed via microscopic readout (Leica Microsystems, Wetzlar, Germany) (Castro et al. 2015). Cnicin was additionally tested at 25, 12.5, and 6.5 μM as described above, and each experiment was performed at least three times (Silva et al. 2017).

In vivo antischistosomal studies after oral and intraperitoneal treatments

Mice were infected percutaneously with 80 cercariae each (Guerra et al. 2019). At 49 days post infection (adult worm stage, patent infection), groups of five mice were treated orally or intraperitoneally with doses of cnicin, Cn/βCD, Cn/HPβCD, βCD, or HPβCD, which were dissolved in 2% ethanol in water (v/v) (Silva et al. 2017). The doses used of cnicin, βCD, HPβCD, Cn/βCD, and Cn/HPβCD were based on the protocols recommended for the experimental schistosomiasis (Lago et al. 2018). For oral treatment, mice were treated with multiple doses (3 daily doses) of cnicin (100 mg/kg) or its inclusion complexes Cn/βCD and Cn/HPβCD, which were administered with the equivalent dose of 100 mg/kg of cnicin.

Similarly, in the intraperitoneal treatment, mice received multiple doses (3 daily doses) of cnicin (10 mg/kg) and the inclusion complexes of Cn/βCD and Cn/HPβCD, with the same equivalent doses of 10 mg/kg of cnicin. In all protocols, negative group received no treatment, while the control group received only blank βCD or blank HPβCD. At 63 days post infection, animals were euthanized by the CO_2 method and dissected. Surviving schistosomes residing in the mesenteric veins and the liver were counted and sexed as previously described (Silva et al. 2017; de Lima et al. 2018). Activity of the tested samples was determined by comparing the worm reduction in the treated animals relative to the worm burden in the infected but untreated control groups. Finally, the assessment of therapeutic efficacy was also based on the technique of qualitative and quantitative oograms, using a fragment of the intestine (10 mm) of the ascending colon, as well as the Kato-Katz method for quantitative feces examination (de Lima et al. 2018). The difference was considered statistically significant if $P < 0.05$ using the Dunnett's multiple-comparison test (Silva et al. 2017; de Moraes et al. 2014).

Randomization and blinding

For in vivo studies, animals were randomly assigned to the experimental groups, and pharmacological treatments were counterbalanced randomly as well. The animals were euthanized in a random manner inside a group, and all parameters were conducted by different people, done by two different investigators. Therefore, operators of experiments were not the same as the data analysts, to eliminate bias in interpretation (Silva et al. 2017).

In vivo permeability evaluation of HPβCD inclusion complexes in the tegument of *S. mansoni*

The permeability of the HPβCD inclusion complex in adult worms of *S. mansoni* was studied using HPβCD complexed with Nile red (NR), a lipophilic fluorescent pigment (Borgia et al. 2005). Preparation of Nile red in HPβCD was as the same as previously described to cnicin. The in vivo permeability of the HPβCD system was determined in Female Swiss mice (4–7 weeks), weighing approximately 20 g, which were housed under controlled conditions (22 °C; 70% relative humidity; 12/12 h light/dark cycle; standard food and water ad libitum). Each mouse was infected subcutaneously with approximately 80 *S. mansoni* cercariae. On day 45 post infection, 2 h before euthanasia, mice received an intraperitoneal dose of NR/HPβCD (0.6 mg/kg), and the animals were sacrificed. Parasites were collected after perfusion, washed, and examined evaluated by florescence microscopy at 552 nm (excitation) and 578 nm (emission) (Borgia et al. 2005). Images were taken using an inverted fluorescent microscope (Axio Scope, A1 Zeiss) equipped with a monochrome camera. Pictures were recorded setting the camera integration time to 10 ms (Borgia et al. 2005).

In vitro cytotoxicity studies

Cytotoxicity of the compounds was determined in murine peritoneal macrophages using the MTT (Sigma-Aldrich, St. Louis, MO, EUA) assay (Mosmann 1983), according to previous report (de Carvalho et al. 2019) using three independent experiments in duplicate. The values of cytotoxicity concentration to reduce 50% of viable cells (CC_{50}) were obtained using GraFit Version 5 software.

Statistical analysis

For in vivo experimental analysis, a parametric Dunnett's multiple comparison test was applied to compare the vehicle group versus the treated group, where statistical significance was set to $P < 0.05$. In vivo experimental graphics represent data from individual mice and are the combination of two independent experiments. The data and statistical analysis

comply with the recommendations on experimental design and analysis in pharmacology (de Lima et al. 2018; Mengarda et al. 2020).

Results and discussion

Schistosomiasis is a neglected tropical disease with a considerable and serious impact in public health (Lago et al. 2018). Due to the urgent need to identify new drugs, several natural and synthetic compounds, as well as their formulations, have been recently investigated against *S. mansoni* (Lago et al. 2018). Among natural promising substances, cnicin is the main active compound of the blessed thistle (*C. benedicta*) that has been reported to possess a wide range of biological activities, including antileishmanial and trypanocidal properties (Chibli et al. 2018; Kurita et al. 2016). However, to our knowledge, the antischistosomal activities of cnicin have not been yet evaluated against *Schistosoma* sp. Also, despite its pharmacological potential, cnicin has chemical characteristics that may limit its biological profile for the in vivo administration, including its poor water solubility (Erel et al. 2011). Thus, the cnicin inclusion complexes Cn/βCD and Cn/HPβCD were prepared to overcome some of the cnicin drawbacks. Then, in this present study, we evaluated the in vitro and in vivo activities of cnicin and its cyclodextrin complexes against *S. mansoni*.

Isolation and characterization of cnicin

A crude extract of *C. benedicta* was prepared by rinsing the leaves with dichloromethane:ethanol (9:1 v/v), followed by only one step fractionation by vacuum liquid chromatography. The rinsed leaves extract of *C. benedicta* was chosen for fractionation since cnicin and other sesquiterpene lactones in *Centaurea* species are localized mainly at the glandular trichomes of their leaves (Tesevic et al. 2007). After isolation, cnicin (Fig. 1) was chemically identified by ^{1}H- and ^{13}C-NMR data analysis in comparison to literature (Chain et al. 2014), and its purity was estimated to be higher than 95% by HPLC-DAD data analysis (Fig. 1).

Characterization of the cnicin inclusion complexes with βCD and HPβCD

FTIR spectra of cnicin, βCD, HPβCD, Cn/βCD, Cn/HPβCD, and their physical mixtures (PMs), as well as their respective attributions are shown in supplementary material (Supplementary Fig. S1). The βCD, HPβCD, and cnicin FTIR spectra are all in accordance with those previously reported in the literature (Egyed 1990; Bratu et al. 2004). The investigation of interactions between Cn with βCD or HPβCD was performed by comparison of FTIR spectrum of free compounds with the correspondent inclusion compound. FTIR spectra of Cn/βCD and Cn/HPβCD (Supplementary Fig. S1) revealed significant modifications in the profile bands if compared with FTIR spectrum of free Cn or cyclodextrins, indicating the establishment of host-guest interactions in solid state. More details about these interactions are available in supplementary material.

In solution, thermodynamic parameters of binding ($\Delta_b G^o$, $\Delta_b H^o$, $T\Delta_b S^o$) and the binding constants between cnicin and cyclodextrins (named $K_{Cn/\beta CD}$ and $K_{Cn/HP\beta CD}$) were determined by ITC experiments, and the results are reported in Supplementary Fig. S2 and Supplementary Table S1. According to the titration curves (Supplementary Fig. S2) for Cn/βCD and Cn/HPβCD systems, the interactions between both formed complexes were considered relatively weak so that the sigmoid pattern was not observed (de Miranda et al. 2019). Also, the found values of $K_{Cn/\beta CD}$ = 123 and $K_{Cn/HP\beta CD}$ 24.5 were similar to others already reported in literature for host-guest interactions in non-aqueous solvent (Moreira et al. 2018). However, although qualitatively the two systems presented similar characteristics, especially exothermic and entropic driven interactions, based on the $\Delta_b H^o$ and $T\Delta_b S^o$ values, their specific mechanisms of interactions must be enough different. For Cn/βCD system, entropy contributes with ≈ 93% for the spontaneity of the process ($\Delta_b G^o$), while enthalpy only with ≈ 7%. In otherwise, for Cn/HPβCD system, entropy contributes with only ≈ 3% for interaction, while enthalpy contributes with ≈ 97%. These differences in the thermodynamic properties are probably caused by the presence of hydroxypropyl groups in the HPβCD, which supposedly allows the HPβCD access more easily specific sites of cnicin, forming more stable local interactions (giving rise a more exothermic process), with less deep inclusion and, consequently, a lower desolvation degree (with lower entropic contribution). For the Cn/βCD system, the more rigid architecture of cyclodextrins may cause a deeper inclusion, with greater desolvation degree, and explain, therefore, the greater entropy values found for this system. Also, the hydrodynamic diameter (D_h), zeta potential (ZP), and electrical conductivity (k) measurements were recorded in order to evaluate the effect of βCD and HPβCD on the colloidal properties of complexes produced in DMSO/water solution (Supplementary Fig. S3). All systems (cnicin, Cn/βCD, and Cn/HPβCD) produced hydrophobic precipitates in DMSO/water mixture with submicrometric size, ranging from 200 to 600 nm. In addition, the negative ZP values, ranging from − 25 to −10 mV for all systems, match with the partial ionization of their hydroxyls. The hydrophobic precipitates of free cnicin showed the more negative ZP values, with slightly lower size of particles. Indeed, lower size of particles is expected at higher ZP values, so that the presence of electrical charges on surface causes repulsion between the particles, making difficult the inelastic collisions and particles growing.

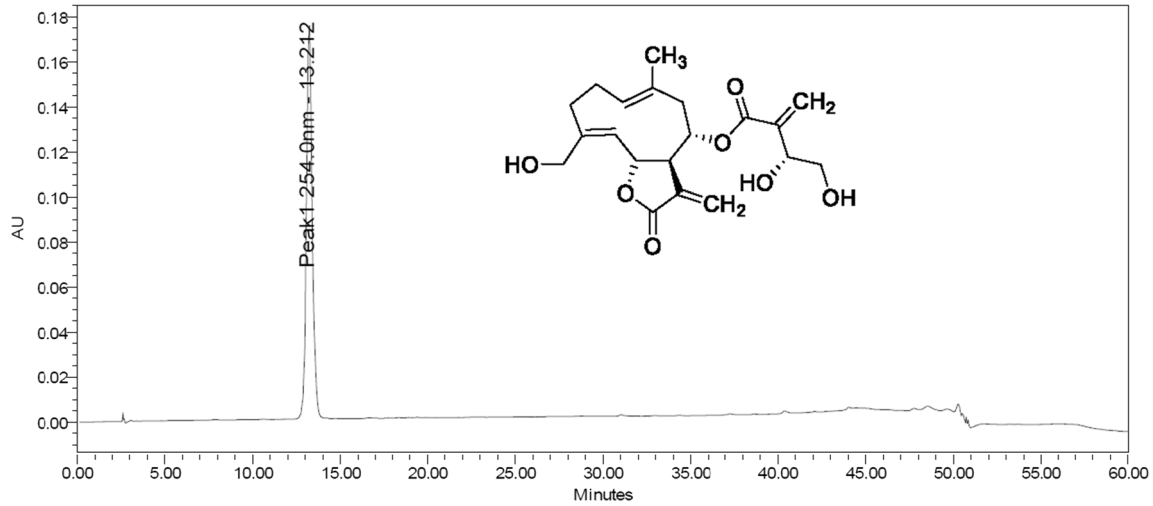

Fig. 1 HPLC chromatogram and chemical structure of cnicin isolated from *C. benedicta* (Asteraceae)

However, less negative ZP values are observed for the hydrophobic precipitates of inclusion compounds. These phenomena can be attributed to the unavailability of hydroxyls of both cnicin and cyclodextrins, due to the local hydrogen bonding formation. This hypothesis is corroborated by measurements of electrical conductivity, where the greater k values are observed for free cnicin over large range of concentration. For Cn/HPβCD, as it was supposed that the hydroxypropyl groups could more easily access the hydroxyls of cnicin, making stable local interactions, a lower dissociation degree is expected, and, consequently, lower electrical conductivity values are observed. Consequently, lower colloidal stability is observed for this system, with greater trend of precipitation. This hypothesis is corroborated by the larger size values and larger standard deviation, especially above [Cn] ≈ 0.4 mM.

Table 1 In vitro schistosomicidal and cytotoxic activities of cnicin and its inclusion complexes Cn/βCD and Cn/HPβCD

Groups	Concentration (μM)	Incubation period (h)	Motor activity reduction (%)[a]		Dead worms (%)		Cytotoxicity CC_{50} (μM)[d]
			Male	Female	Male	Female	
Control[b]	[b]-	24	0	0	0	0	ND
		48	0	0	0	0	
PZQ	2	24	100	100	100	100	ND
		48	100	100	100	100	
DMSO 0.5%	[c]-	24	0	0	0	0	ND
		48	0	0	0	0	
Cnicin							21.83 ± 0.34
	50	24	100	100	100	100	
		48	100	100	100	100	
	25	24	100	100	100	100	
		48	100	100	100	100	
	12.5	24	30	60	0	30	
		48	60	100	30	60	
	6.25	24	0	10	0	0	
		48	0	100	0	100	
Cn/βCD	50	24	0	0	0	0	19.55 ± 0.03
Cn/HPβCD	50	24	0	0	0	0	29.28 ± 0.89
βCD	50	24	0	0	0	0	> 150
HPβCD	50	24	0	0	0	0	> 150

[a] Percentages relative to the 20 worms investigated

[b] Wells with RPMI 1640

[c] Wells with DMSO 0.5% in medium served as controls

[d] CC_{50} values (50% cytotoxic concentration) on macrophages

ND not determined

Effects of cnicin and its inclusion complexes on the survival of adult worms

According to recent literature (Corrêa et al. 2019), in vitro assays are essential tools to the initial selection of a potential anthelmintic drug. Preliminary in vitro assays against *S. mansoni* showed that cnicin, when incubated for 24 h, caused 100% mortality against adult *S. mansoni* (Table 1). Also, significant contractions and paralysis after incubation with cnicin (50 to 25 μM) were noted. Interestingly, it appeared that adult female worms were more susceptible than male after in vitro incubation with cnicin, especially at low concentrations. The exposure to cnicin (6.5 μM) resulted in 100% mortality of female adult worms after 48 h incubation, whereas no mortality in male worms was observed. Previous studies have shown more susceptibility of female schistosomes to artesunate (Mitsui et al. 2009), *N*-alkylamino-thiosulfuric acids (Penido et al. 1994), and other compounds (Guimarães et al. 2015). In addition, experiments showed a significant and concentration- and time-dependent in vitro schistosomicidal activity for cnicin.

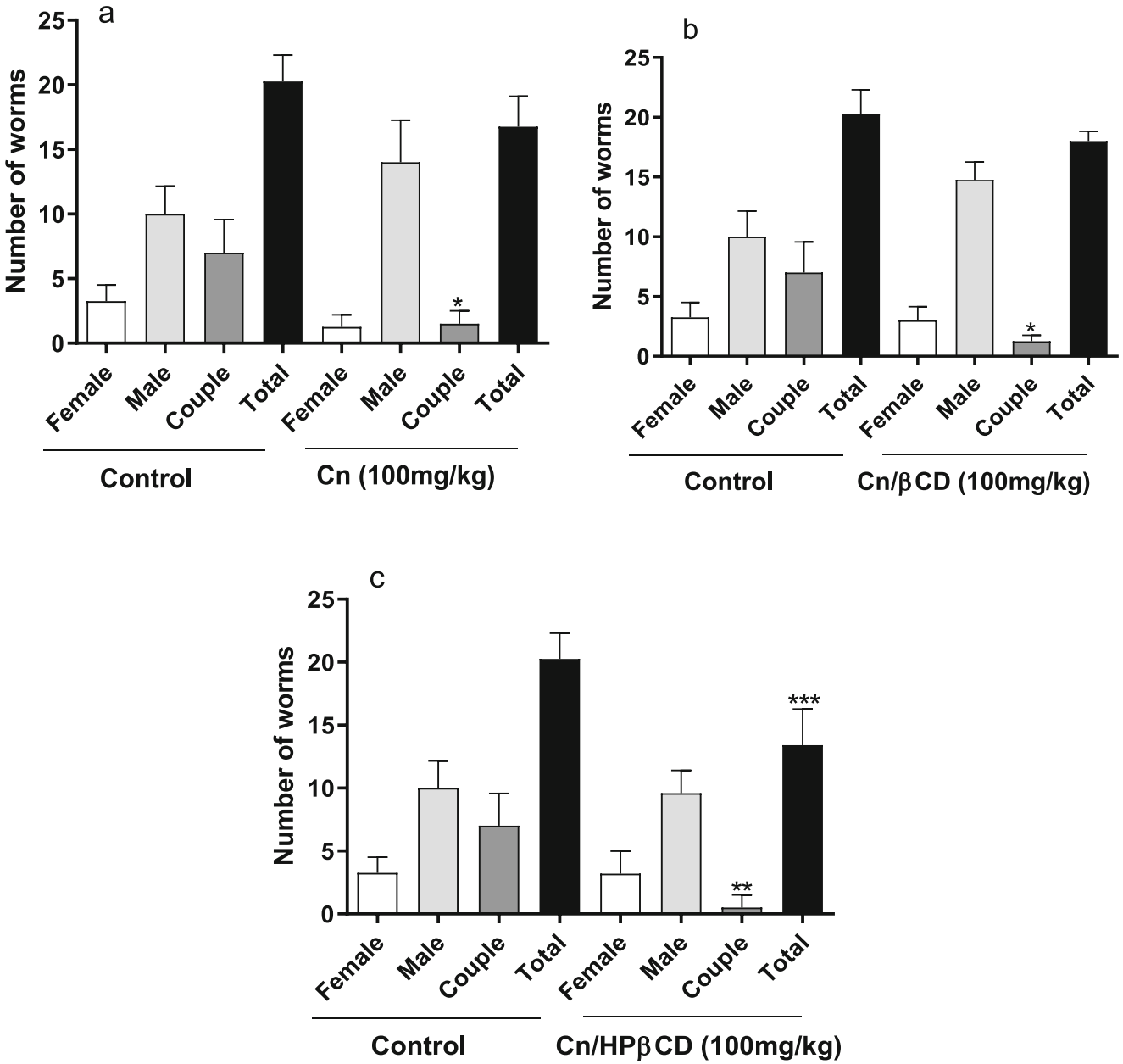

Fig. 2 Effect on worm burden after treatment with multiple oral doses of cnicin (**a**), Cn/βCD (**b**), and Cn/HPβCD (**c**) administered to mice harboring adult *S. mansoni* infection, at the same cnicin nominal doses of 100 mg/kg, p.o., stratified by sex. Bars represent data from individual mice that were infected and treated with samples or infected and untreated (control). HPβCD and βCD alone did not show significant results. *$P < 0.05$, *** $P < 0.001$ compared with untreated groups

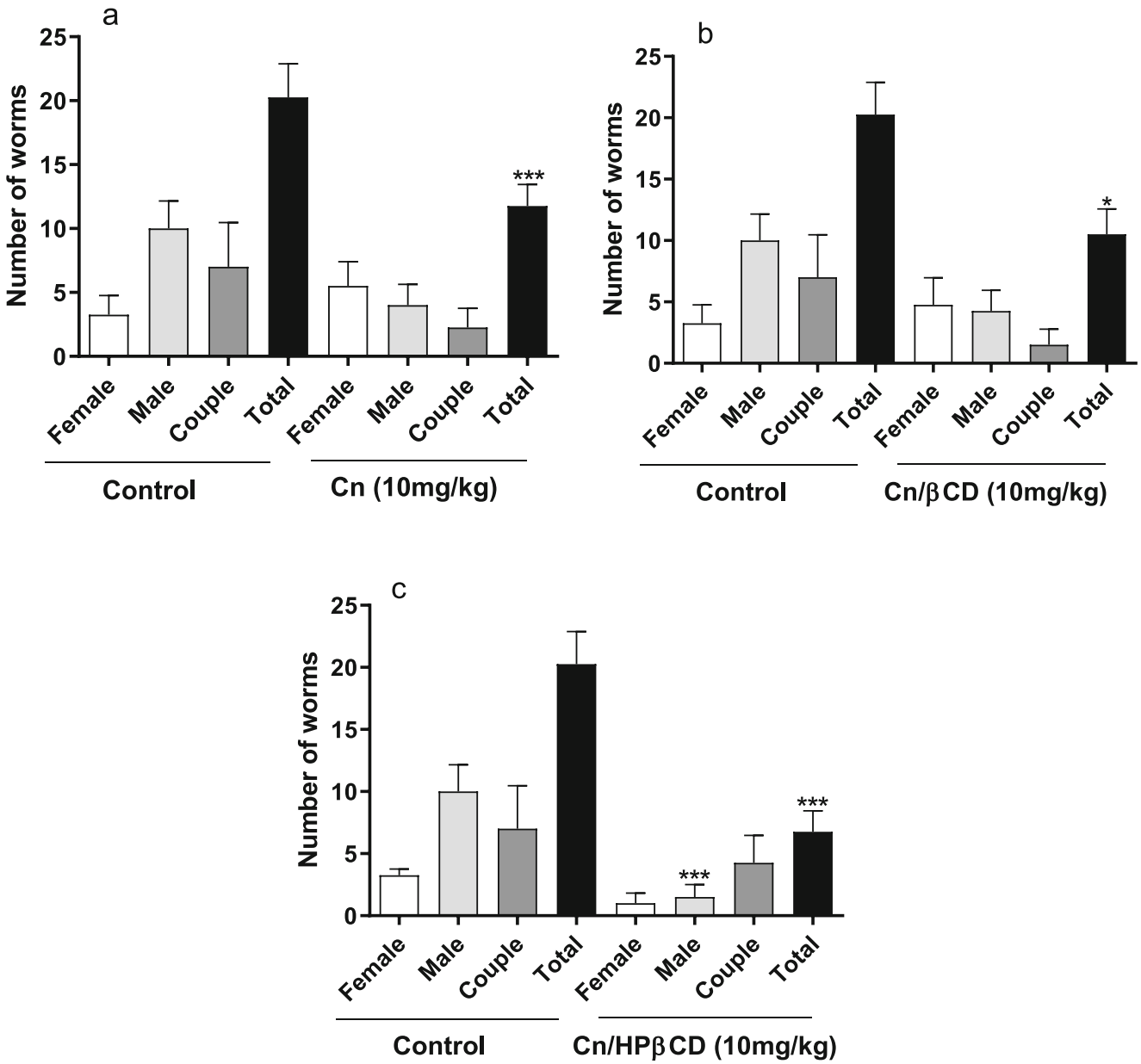

Fig. 3 Effect on worm burden after intraperitoneal treatment with multiple doses of cnicin (**a**), Cn/βCD (**b**), and Cn/HPβCD (**c**) administered to mice harboring adult *S. mansoni* infection at the same cnicin nominal doses of 10 mg/kg, i.p., stratified by sex. Bars represent data from individual mice that were infected and treated with samples or infected and untreated (control). HPβCD and βCD alone did not show significant results. *$P < 0.05$, *** $P < 0.001$ compared with untreated groups

In contrast, βCD, HPβCD, and the cnicin-cyclodextrins hydrophobic precipitates (Cn/βCD and Cn/HPβCD) did not show any activity for schistosomes after 24 h of incubation at the highest tested concentration (50 μM). PZQ (2 μM) caused death in all schistosomes, whereas no effect was observed in worms in the control (RPMI 1640 medium) and vehicle (RPMI medium plus 0.4% DMSO) groups.

Regarding toxicity to mammalian cells, cnicin and the complex Cn/βCD showed similar toxic effects against murine macrophages (CC_{50}: 21.83 and 19.55 μM, respectively) (Table 1), while the inclusion complex of Cn/HPβCD was able to slightly decrease the toxicity (CC_{50}: 29.28 μM) in comparison with free cnicin. In contrast, free cyclodextrins (βCD and HPβCD) showed no significant cytotoxicity to macrophages, as shown in Table 1.

Cnicin was as potent in vitro as most of the antischistosomal natural compounds reported so far (Lago et al. 2018). However, cnicin showed in vitro cytotoxic potential to murine macrophages. The cytotoxicity of cnicin was also reported in a previous work, in which cnicin caused significant damage to a human derived monocyte THP-1 cell line (Bach et al. 2011). In this regard, previous studies have shown the antimyeloma activity

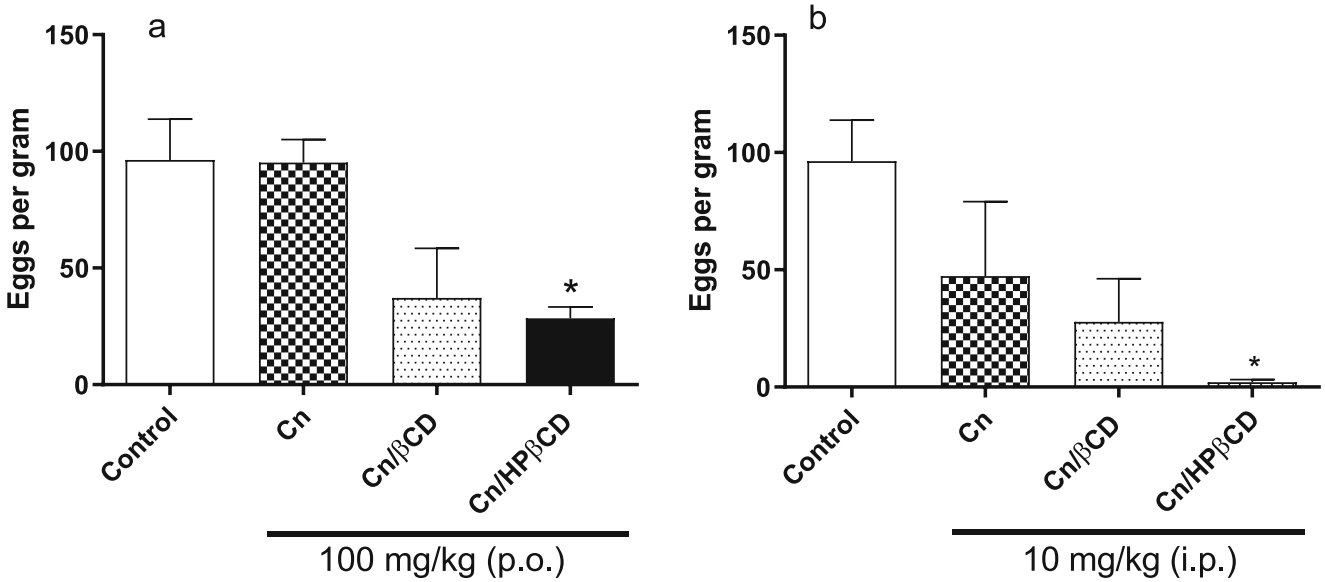

Fig. 4 Effects on egg load of multiple oral (**a**) or intraperitoneal (**b**) doses of Cn, Cn/βCD, and Cn/HPβCD administered to mice harboring adult *S. mansoni* infection at the same cnicin nominal doses of 10 mg/kg, i.p. or 100 mg/kg, p.o. Bars represent data from individual mice that were infected and treated with samples or infected and untreated (control). *$P < 0.05$ compared with untreated groups

of cnicin, which preferentially killed tumor cells in vitro, displaying CC_{50} values between 3 and 13 μM (Jöhrer et al. 2012). Also, cnicin has cytotoxic effects towards different cancer cell lines, such as human malignant melanoma (SK-MEL) and human ductal carcinoma (BT-549) cells (Sen et al. 2017). In contrast, Cn/HPβCD complexes slightly decreased the in vitro cytotoxic potential of cnicin, suggesting that encapsulation of compounds in HPβCD may be an additional advantage for cells (Teixeira et al. 2015; Szente et al. 2018). Moreover, the inclusion complexes Cn/βCD and Cn/HPβCD did not produce any mortality or motility effects in adult schistosomes after 24-h incubation. These results may be related to the controlled release of cyclodextrin complexes (Barba et al. 2015; Woldum et al. 2008), probably due to the longer time necessary to the dissociation of cnicin from its complexes and the release of free cnicin.

In vivo effects of cnicin and cnicin-loaded cyclodextrins against *S. mansoni*

Cnicin and its cyclodextrin complexes (Cn/βCD and Cn/HPβCD) were first orally in vivo evaluated in chronic murine model of schistosomiasis. The doses used of cnicin, βCD, HPβCD, Cn/βCD, and Cn/HPβCD were based on the protocols recommended for the experimental schistosomiasis (Lago et al. 2018), such as the used for the in vivo antischistosomal evaluation of oxadiazole and derivatives (Sayed et al. 2008). In mice harboring adult *S. mansoni*, after oral treatment with cnicin (100 mg/kg) and Cn/βCD (corresponding to 100 mg/kg of cnicin), no significant total worm reductions were found with both samples (Fig. 2a, b). On the other hand, the oral administration of Cn/HPβCD (Fig. 2c) (corresponding to 100 mg/kg of cnicin) markedly decreased the total worm load by 56.8% ($P < 0.001$) in comparison with infected untreated group. Interestingly, although no difference was observed between male and female worm burden reduction, many adult worm pairs were separated into individual male and female worms following oral administration of cnicin (Fig. 2a), Cn/βCD (Fig. 2b), or Cn/HPβCD (Fig. 2c), indicating that all samples were equally active against both worm sexes.

As observed, the oral administration of Cn/βCD showed no significant reductions in worm burden, while Cn/HPβCD achieved a significant therapeutic efficacy in mice infected with adult *S. mansoni*. Since Cn/HPβCD was more effective in parasitological reduction than the free cnicin, the observed activity can be attributed to the advantages of using HPβCD systems. Previous reports showed that the association of PZQ and HPβCD allowed this complex to change the chemical properties of PZQ, improving its bioavailability and, consequently, the in vivo efficacy (Cugovčan et al. 2017). Additionally, other poor soluble anthelmintic drugs, such as albendazole and mebendazole, increased their efficacy after complexation with cyclodextrins (Buchter et al. 2020; Pacheco et al. 2018). Also, previous antischistosomal study showed that the administration of HPβCD or βCD alone is not orally active against *Schistosoma* (Jesus et al. 2010). Besides, HPβCD is considered safe for parenteral and oral administration (Carneiro et al. 2019; Gould and Scott 2005; Rajewski and Stella 1996).

The antischistosomal efficacy of cnicin and its cyclodextrin complexes was also assessed by intraperitoneal route (Fig. 3). The intraperitoneal treatment with cnicin (10 mg/kg) and Cn/βCD (at the same nominal Cn dose of 10 mg/kg) decreased

the total worm load by 41.9% ($P < 0.001$) and 48.1% ($P < 0.001$), respectively, in comparison with untreated control (Fig. 3a, b). Additionally, the intraperitoneal administration of Cn/HPβCD (at the same nominal cnicin dose of 10 mg/kg) had the best efficacy, showing a significant total worm burden reduction of 66.7% ($P < 0.001$) (Fig. 3c). Similar to oral treatment, which showed no susceptibility between male and female schistosomes, pairs of adult worms were separated following the intraperitoneal administration of cnicin (Fig. 3a) and Cn/βCD (Fig. 3b). In contrast, males were more vulnerable to Cn/HPβCD treatment than females (Fig. 3c).

Moreover, in feces collected from infected mice treated, the number of eggs per gram (OPG) was evaluated. Since *S. mansoni* females are able to produce hundreds of eggs per day, which are closely related to their immunopathogenesis of schistosomiasis (Hiatt et al. 1979; Warren 1982), drugs with potential in decreasing oviposition is of great importance. Then, the efficacy on patent infection was also assessed by egg load after administration of cnicin and its inclusion complexes to mice harboring adult *S. mansoni*. No significant reduction in OPG was found after oral or intraperitoneal administration of cnicin or Cn/βCD (Fig. 4a, b). On the other hand, the oral treatment with Cn/HPβCD (at the same nominal cnicin dose of 100 mg/kg) was able to reduce in 70.5% ($P < 0.05$) the OPG (Fig. 4a), while after intraperitoneal administration of Cn/HPβCD (nominal cnicin dose of 10 mg/kg), the OPG was reduced in 97.9% ($P < 0.05$) in comparison with the infected untreated control group (Fig. 4b). Then, the quantitative feces examination shows a significant reduction in the number of eggs in feces after treatment with Cn/HPβCD. This finding could be attributed to a high reduction in the worm burden due to the treatment with Cn/HPβCD and/or to the inhibition of oviposition by adult helminths. In contrast, no antischistosomal activities of blank HPβCD or βCD (data not shown) were observed in both in vitro and in vivo studies, reinforcing that the experimental antischistosomal activities of HPβCD are related to the delivery of encapsulated cnicin in complexes.

In comparison with the oral route, the intraperitoneal treatment with cnicin and its inclusion complexes proved to be more effective. Possible explanations for these differences in efficacy may be related to the low availability of cnicin and its possible metabolization when it is orally administered (Jöhrer et al. 2012). Sesquiterpenes lactones, such as cnicin, are in general metabolized after the first passage through the liver (Lee et al. 2016). This passage may inactivate a considerable amount of cnicin, which may cause a decrease in its in vivo activities after oral administration. In addition, considering that cnicin has low water solubility (Locken and Kelsey 1987), cnicin complexes with HPβCD may allow the improvement of solubility, which may enhance the bioavailability of cnicin. HPβCD possesses hydroxyl and hydroxypropyl groups that increase in more than 30 times the water solubility

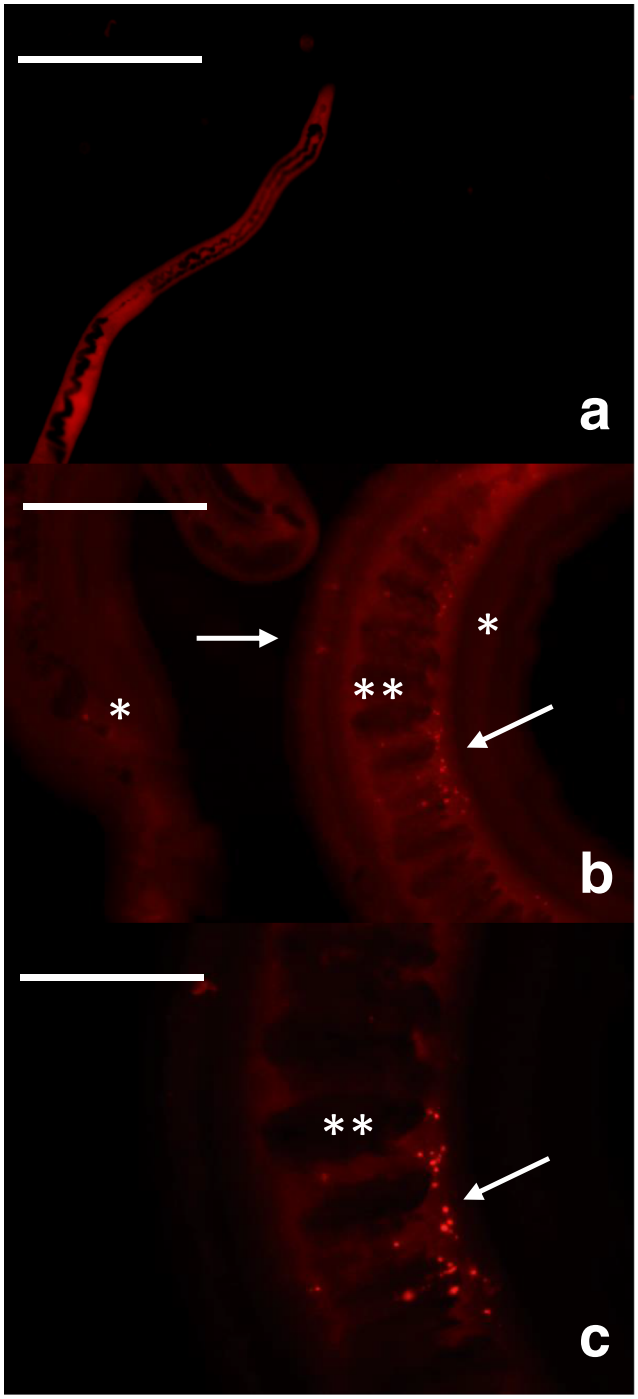

Fig. 5 Representative fluorescence microscopy images of *S. mansoni* male and female adult *S. mansoni* collected from female mice after intraperitoneal application of Nile red-HPβCD. In (**a**) a female incubated only with blank HPβCD (without Nile red), showing no specific areas of fluorescence, only the characteristic autofluorescence of adult worms. (**b**) After treatment with Nile red-HPβCD, the adult worms were able to reveal the presence of the lipophilic probe being incorporated through the tegument, as shown in the image of adult coupled worms highlighted by the arrows. (**c**) Female (**) is housed in the gynecopharyngeal canal of the male (*). In a singular way, females incorporated high amount of Nile red-HPβCD, as shown in the image highlighted by the arrows. Scale bare represents 100 μm

in comparison with βCD (Hedges 1998). Then, we speculated that the higher in vivo antischistosomal activity of Cn/HPβCD could be due to a more water solubility obtained when cnicin is complexed with HPβCD.

In vivo permeability studies with inclusion complexes of Nile red-HPβCD

Furthermore, to visualize the in vivo permeability of the inclusion complexes with HPβCD in the tegument of adult schistosomes, Nile red was used as fluorescent indicator and complexed with HPβCD. In this experiment, parasites were obtained from mice that received the complex Nile red/HPβCD and, after, evaluated by fluorescence microscopy. Representative images of schistosome worms recovered from mice treated with Nile red/HPβCD are shown in Fig. 5. When administered, Nile red/HPβCD was able to reach adult *S. mansoni* in vivo, penetrating the tegument of male and female adult worms (Figs. 5b–c). It was possible to verify the characteristic fluorescence in the adult worms, indicating that the complex Nile red/HPβCD had already reached the parasites. On the other hand, blank HPβCD did not show any fluorescence under the same conditions (Fig. 5a). This result demonstrates that the inclusion complexes with HPβCD reach the desired target, allowing the delivery of compounds to *Schistosoma*.

Conclusions

In this work, cnicin was isolated from blessed thistle (*C. benedicta*) and successfully encapsulated in βCD and HPβCD cyclodextrins. Cnicin presented in vitro effects against adult schistosomes, also showing in vivo antischistosomal efficacy by intraperitoneal route but was no effective in vivo after oral administration. Unlike free cnicin, the complex Cn/HPβCD showed in vivo efficacy against *S. mansoni* after intraperitoneal and oral treatments. The resultant complex obtained from the inclusion of cnicin in HPβCD showed high antischistosomal in vivo effectiveness, enabling the complexed cnicin to be active against *S. mansoni*. Permeability studies indicated that inclusion complex of HPβCD may reach the adult schistosomes in vivo. These results demonstrated the antischistosomal potential of cnicin in preparations with HPβCD.

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Author's contributions Lucas S. Queiroz: investigation, formal analysis, and writing—original draft. Everton Allan Ferreira: investigation and formal analysis. Ana C Mengarda: investigation and formal analysis. Ayla das C. Almeida: investigation, formal analysis, and writing—original draft. Priscila de F. Pinto: investigation and formal analysis. Elaine S. Coimbra: investigation and formal analysis. Josué de Moraes: conceptualization, investigation, formal analysis, writing—original draft, and funding acquisition. Ângelo M. L. Denadai: conceptualization, investigation, formal analysis, writing—original draft, and funding acquisition. Ademar A. da Silva Filho: conceptualization, investigation, formal analysis, writing—original draft, resources, project administration, and funding acquisition.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics statement All experiments were conducted in conformity with the Brazilian Law for Guidelines for Care and Use of Laboratory Animals (Law 11790/2008). The protocol for experimental design was approved by the Comissão de Ética no Uso de Animais (CEUA), Brazil (Protocols ≠ CEUA 031/2017 and ≠ CEUA 007/2018). Animal studies are reported in compliance with the ARRIVE guidelines.**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00436-020-06963-2.

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