Braz J Vet Pathol, 2021, 14(3), 165 – 172
DOI: 10.24070/bjvp.1983-0246.v14i3p165-172

BJVP
Brazilian Journal of Veterinary Pathology
ISSN 1983-0246

Original Full Paper

The Licochalcone A induces tegumental damages in *Schistosoma mansoni* and impairs its oviposition *in vitro*

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Submitted June, 3rd 2020, Accepted June, 22th 2021

Abstract

In this study, Licochalcone A (LicoA) was investigated in in vitro and in vivo assays. The survival of worms in culture, the pattern of oviposition, the count of intact tubers and the integrity of the coat were adopted in the *in vitro* tests. After the animals were perfused, the number of worms recovered, their location and the oogram study were the parameters analyzed to signal the existence of potential schistosomicidal activity in vivo. We observed reduction on the survival, integument integrity and reproduction of adult worms *in vitro*. Murine models did not show a significant difference in the parasitological parameters analyzed that indicate activity against the worms with an oral single dose of 25 mg/ kg of LicoA or two intraperitoneal injection of 50 mg/ kg LicoA. Nevertheless, it is too early to completely exclude the schistosomicide activity of LicoA, considering that the used dosage form could not provide a regular absorption of the drug.

Key words: Licochalcone A. Schistosomiasis. *Schistosoma mansoni*.

Introduction

Blood fluke parasites of the genus Schistosoma cause a debilitating, chronic and neglected tropical disease called schistosomiasis (12, 24). *Schistosoma mansoni* causes intestinal schistosomiasis form. Depending on the intensity of the infection, it might result in severe complications and lifelong disability (4, 12, 24). Thus, schistosomiasis poses a significant health, social and financial burden to individuals and governments (4, 12, 24). Praziquantel (PZQ) is the only available drug for the treatment of schistosomiasis (4, 12, 24) and is also used for mass-administration to at-risk population as no vaccine is currently available (12, 24). However PZQ has several disadvantages, including inability to prevent reinfection, effectiveness only against adult worms and limited action on the disease progression (4, 12, 24). Furthermore, the emergence of drug-resistant strains is a concern and might compromise the control of the disease globally. Therefore, there is an urge to develop new drugs for better treatment and control of schistosomiasis (12, 21, 24).

Bioactive natural products are a promising source of lead compounds for developing new drugs against *Schistosoma mansoni* (4, 18, 21). The extract of *Glycyrrhiza inflata* (GI) roots and their isolated compounds have promising schistosomicidal activity (3, 23). In this study, we evaluated *in vitro* and *in vivo* the anti-schistosomal potential of Licochalcone A (Fig. 1), the major purified from GI roots in order to contribute further evidence on the anthelmintic effects described for this compound.

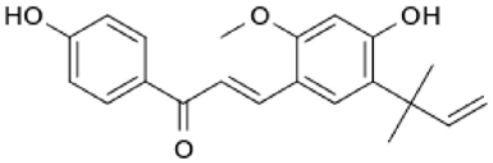

Figure 1. Chemical structure of Licochalcone A.

Material and methods

Isolation and purification of Licochalcone A

Licochalcone A (LicoA) was isolated and purified from the dried extract of *Glycyrrhiza inflata* roots (Shanghai Openchem International Co., Ltd, China) according to the methodology described by Fontes et al. (9). The dried extract of G. inflata (10 g) was chromatographed over silica gel (60H, 100–200 mesh ASTM; Merck, Darmstadt, Germany) under vacuum-liquid chromatography system (glass columns with 5–10 cm i.d.) using dichloromethane (DCM)–methanol (MeOH) mixtures in increasing proportions to afford eight fractions. The resulting fraction 3 (DCM : MeOH 7 : 3 v/v; 1,7 g) was submitted to flash column chromatography (450 × 25 mm glass column, 5 ml/ min) over silica gel (230–400 mesh, Merck) using DCM– MeOH (95 : 5 v/v) as mobile, furnishing 0.5 g of LicoA. The chemical structure of LicoA was established by 1 H- and 13C-NMR data analysis (Bruker ARX 300 spectrometer; Bruker, Ettlingen, Germany) and by comparison of the data with literature. Purity of LicoA was estimated to be higher than 95% by both 13C NMR and HPLC analysis using different solvent systems.

In vitro assays

Adult schistosomes were washed in RPMI 1640 medium supplemented with 200 mg/mL streptomycin, 200 UI/mL penicillin (Invitrogen), and 25 mM HEPES (Sigma-Aldrich). Adult worm pairs (male and female) were incubated in a 24-well culture plate (Techno Plastic Products, TPP, St. Louis, MO, USA), containing the same medium supplemented with 10% heat-inactivated calf serum (Gibco BRL) at 37°C in a 5% CO2 atmosphere. The LicoA was evaluated at concentrations of 5, 10, 25, 50 and 100 μM. Samples were added to the culture from a 4000 mg/mL stock solution in RPMI 1640 containing dimethyl sulfoxide (DMSO- Synth). The final volume in each well was 2 mL. The control worms were assayed in RPMI 1640 medium and RPMI 1640 with 0.5% DMSO as negative control groups and Prazinquantel (Farmaguinhos, Rio de Janeiro, BRA) – PZQ (10 μM) as a positive control group. All experiments were performed in triplicate and were repeated at least two times. Parasites were maintained for 120 h and monitored every 24 h using a light microscope to evaluate their general condition: motor activity, mortality rate and tegumental alterations (14, 16).

- *Assessment of the reproductive fitness of adult worms*

Parasites were continually monitored, and the eggs counted daily for five days, using an inverted microscope as previously described (15) after exposure LicoA. To observe whether the effect on oviposition was reversible, the medium containing LicoA (1.25, 2.5 and 5 μM) was removed after 24 h of drug exposure and the worms were carefully rinsed three times with RPMI to prevent separation of the worm pairs. The worms were also incubated in a drug-free medium and monitored on a daily basis for five days (120 h). The assays were repeated three times independently.

- *Confocal laser scanning microscopy*

Tegumental alteration and quantification of the number of tubercles were performed for LicoA (10, 25, 50 and 100 μM) using a confocal laser scanning microscope. After 120 hours or following the schistosome death, the parasites were fixed in Formalin-Aceto-Alcohol solution (FAA) and analyzed using a confocal microscope (Laser Scanning Microscopy, LSM 510 META, Zeiss) at 488 nm (excitation) and 505 nm (emission) (16). At least three areas of the tegument of each parasite were assessed. The number of tubercles was counted in 20.000 μm2 of area calculated with the Zeiss LSM Image Browser software.

- *Cytotoxicity Assay*

Vero cells (African green monkey kidney fibroblast) were obtained from the American Type Culture Collection (ATCC CCL-81; Manassas, VA). Cytotoxicity was determined as previously described (14) using different concentrations of LicoA (25, 50, 100 and 200 μM). The statistical tests were performed using GraphPad Prism 5® software. Significant differences were determined by one-way analysis of variance (ANOVA) and applying Tukey's test for multiple comparisons with a level of significance set at $p < 0.05$.

In vivo assays

Parasite culture and maintenance: Swiss Albino female mice, ~56 days old and weighing between 35-50 g was used in this study. The mice were subcutaneously infected with approximately 100 cercaria of LE strain per animal (Belo Horizonte - MG). Mice were maintained with ad libitum access to food and water during the experiment. Following 60 days of infection, animals were euthanized by deepening anesthesia (ketamine+xylazine) and perfused using a technique described by Pellegrino and Siqueira (20).

The first test *in vivo* was performed to evaluate the effect of LicoA administrated by gavage on adult worm. Mice were divided into two groups: a control untreated group (n = 5) and LicoA 25mg/ kg group (n = 20). Treatment initiated 45 days post-infection. The worms recovered from the mice's perfusion were counted and grouped according to the mesenteric or hepatic location. The results were compared to those obtained in the untreated control group.

To evaluate the effect *in vivo* of LicoA in juvenile worms, the drug was intraperitoneally administered twice (50 mg/ kg) at an interval of 15 days. The mice were divided into three groups: (I) infected control and untreated group (n = 15); (II) treated group - a single oral dose of PZQ 200mg/ kg (n = 15) according to Shaw and Erasmus (22) (III) treated with LicoA intraperitoneally with two doses of 50 mg/ kg. Treatment was initiated 30 days post-infection. LicoA suspensions were prepared by emulsifying the powder in 10% DMSO in PBS. The

amount of parasites recovered and their location (whether hepatic or mesenteric) obtained in each group were then compared.

The oogram studies were performed with a portion of about one cm of the ileocecal junction of the animal's intestine in each group. The eggs were observed fresh and a minimum of 50 viable eggs counted and classified based on their stage of development, according to Jurberg et al. (10). The oogram was considered altered in the absence of one or more immature stages (19).

The results were presented as arithmetic mean ± standard error, median and / or percentage. Kruskal-Wallis test was used for *in vivo* assay and values of $p < 0.05$ were considered statistically significant. Statistical evaluation of the results was performed using the GraphPad Prism 5®.

Ethics

These studies were approved by the Ethical Committee of the Federal University of Juiz de Fora, Juiz de Fora, Minas Gerais, Brazil under report number 026/2012. The assays were performed according to the Brazilian guideline for care and use of animals for scientific and educational purposes (DBCA/2013), the guide for the care and use of laboratory animals (2011) and the ARRIVE guidelines.

Results

The motor disorders and soft tissue reflect the general state of the parasites in the culture medium (18) and one in the initial test *in vitro* has been proposed. The worms used were intact and intense motor activity to be recovered from the mice. At the end of 24 hours incubation, the drug could cause the death of all worms grown at a minimum concentration of 25µM, as described in Table 1. PZQ (10 µM), the positive control, also caused lethal injury in the tegument of the worms exposed to this drug for 24h.

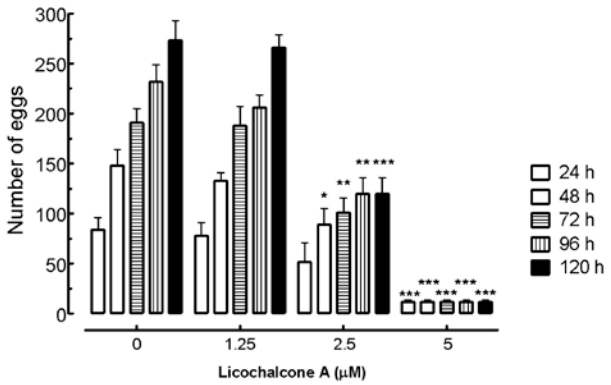

Figure 2. *In vitro* effect of the Licochalcone A on *Schistosoma mansoni oviposition*. Quantitative reduction of eggs occurred from 2.5 µM and becomes quite evident from 5 µM. Worms were grown in RPMI medium at sub lethal concentrations of Licochalcone A and the egg production monitored and scored using inverted microscope at certain time ponits. Values are means ± SD (bars) of ten worm couples compared with untreated groups. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

In the negative control group eggs were found in several stages at the end of the incubation period. Under sub-lethal concentrations of the Licochalcone A (Fig. 2), it was possible to observe certain interference level on oviposition profile of the worms, particularly at 5 µM wherein the reduction of the number of eggs became clear and remained non-rising with time of incubation as observed for the control group.

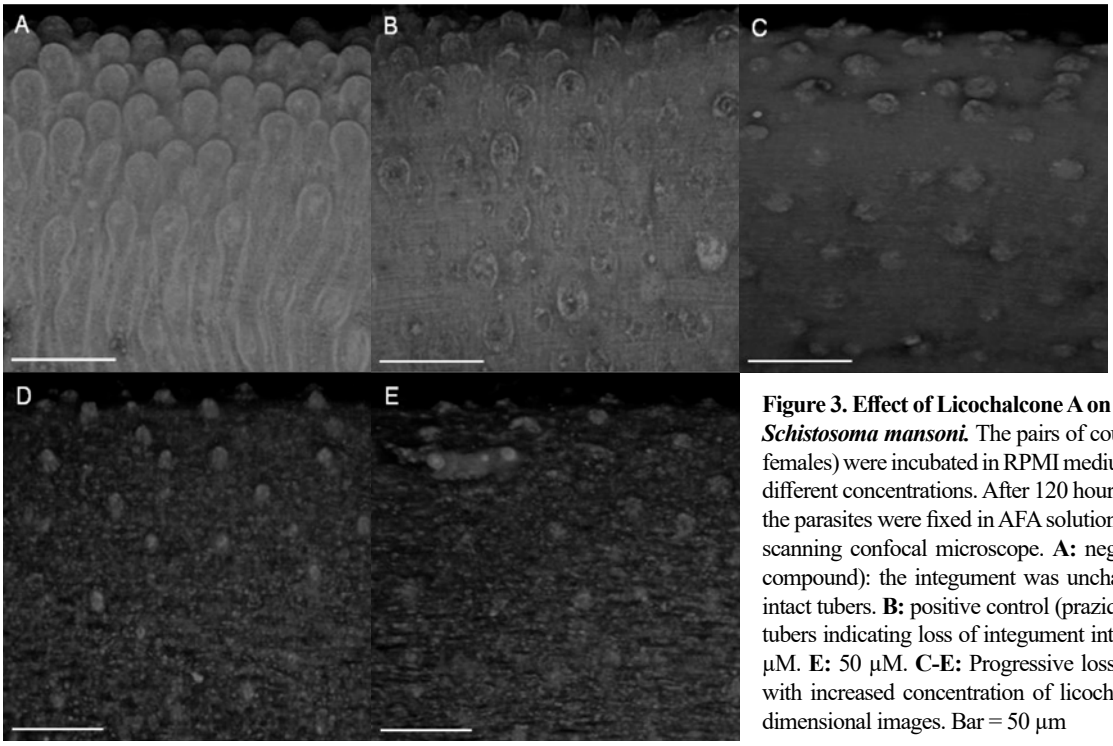

Figure 3. Effect of Licochalcone A on integument of males of *Schistosoma mansoni.* The pairs of coupled worms (males and females) were incubated in RPMI medium containing the drug at different concentrations. After 120 hours or occurrence of death, the parasites were fixed in AFA solution and analyzed with laser scanning confocal microscope. **A:** negative control (no added compound): the integument was unchanged and covered with intact tubers. **B:** positive control (praziquantel 10 uM): ruptured tubers indicating loss of integument integrity. **C:** 10 µM. **D:** 25 µM. **E:** 50 µM. **C-E:** Progressive loss of integument integrity with increased concentration of licochalcone A. **A- E:** Three-dimensional images. Bar = 50 µm

Table 1. *In vitro* effects of LicoA.

Groups	Incubation time (h)	Dead worms (%)[a]	Motor activity reduction (%)[a]		Worms with tegumental alterations (%)[a]	
			Slight	Significant	Slight	Significant
Control [b]	24	0	0	0	0	0
	48	0	0	0	0	0
	72	0	0	0	0	0
	96	0	0	0	0	0
	120	0	0	0	0	0
DMSO 0,5% [c]	24	0	0	0	0	0
	48	0	0	0	0	0
	72	0	0	0	0	0
	96	0	0	0	0	0
	120	0	0	0	0	0
Praziquantel 10 µM	24	100	0	100	0	100
	48	100	0	100	0	100
	72	100	0	100	0	100
	96	100	0	100	0	100
	120	100	0	100	0	100
Licochalcone A 100 µM	24	100	0	100	0	100
	48	100	0	100	0	100
	72	100	0	100	0	100
	96	100	0	100	0	100
	120	100	0	100	0	100
50 µM	24	100	0	100	0	100
	48	100	0	100	0	100
	72	100	0	100	0	100
	96	100	0	100	0	100
	120	100	0	100	0	100
25 µM	24	100	0	100	0	100
	48	100	0	100	0	100
	72	100	0	100	0	100
	96	100	0	100	0	100
	120	100	0	100	0	100
10 µM	24	0	0	0	0	0
	48	0	100	0	0	0
	72	40	20	80	60	40
	96	100	0	100	0	100
	120	100	0	100	0	100
5 µM	24	0	0	0	0	0
	48	0	0	0	0	0
	72	0	30	0	0	0
	96	0	100	0	0	0
	120	0	100	0	0	0

a Percentage in relation to 20 worms.
b Medium RPMI.
c Medium RPMI plus DMSO

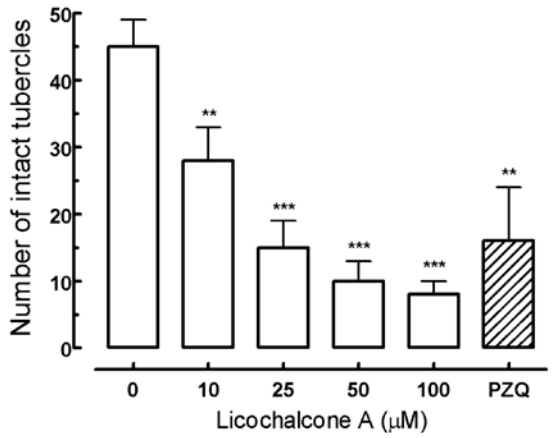

Figure 4. Effect of Licochalcone A on the tubers of *S. mansoni* males. The mated worms (male and female) pairs were incubated in RPMI 1640 medium containing the drug at the indicated concentrations. After 120 hours or death, the parasites were fixed in AFA solution and monitored with confocal microscopy. Indicated are the intact tubers, counted in at least three areas (20.000 μm2) of the dorsal surface of male helminths using the LSM Image Browser (Zeiss) software. Values are average of ten worms. The bars represent the standard deviation of the mean. Significant difference between the negative control and the compounds treated with praziquantel (PZQ 10 μM): * (P <0.05), ** (P <0.01), *** (P <0.001). The number of intact tubers reduced considerably in the presence of certain concentrations of Licochalcone A, especially at 25 μM (Fig. 3D) and 50 μM (Fig. 3E).

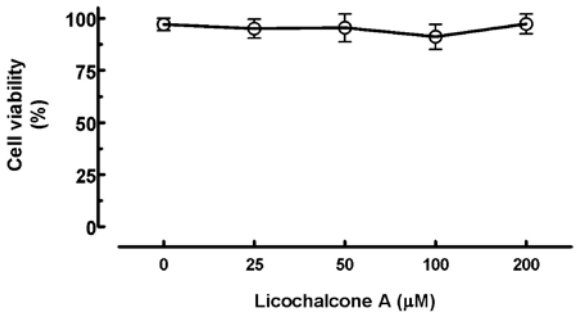

Figure 5. Effect of the Licochalcone A on the viability of mammalian Vero cells. The progressive increase of LicoA did not impair the viability of cells in culture. Cell viability was determined after 48 h of treatment with Licochalcone A by the crystal violet method. Significant differences were determined by one-way analysis of variance (ANOVA) and applying Tukey's test for multiple comparisons.

Confocal microscopy analysis by laser scanning was carried out to get more insights into the surface-tegument morphology changes induced by the LicoA. As shown in Figure 3, LicoA induced extensive damage to the tubers, which became disrupted and atrophied, especially at 25 μM (Fig. 3D) and 50 μM (Fig. 3E) concentrations. The action of LicoA on the tubers of the dorsal region of *S. mansoni* male specimens was quantitatively analyzed and showed in Figure. 4. This aligns with our results in Fig. 3 that already indicated the destruction of these structures. Furthermore, the reduction in the number of tubers was observed in worms exposed to 10 μM of LicoA. This effect was even more pronounced at 100 μM concentration of LicoA.

The LicoA did not exhibit significant cytotoxicity to mammalian cells (Figure 5) 24 h after incubation and demonstrating cell viability even at concentrations higher than that used in the *in vitro* test.

There was no significant difference between the means ± SED of worm recovery after a single dose of licochalcona A (oral) administered to mice 45 days post-infection and to the untreated control group (37.3 ± 3.57 and 38 ± 3, 22, respectively). In both cases, there was a greater concentration of worms in the mesenteric veins (92.8% and 91.4%, respectively) and none showed changes in the oogram. The mean recovery of worms after a single dose of licochalcona A (oral) administered to mice 45 days post-infection and to the untreated control group corresponded to 37.3 ± 3.57 and 38 ± 3.22, respectively. The effects on worm burden and distribution and egg production of LicoA 50 mg/ Kg intraperitoneal administration juvenile worms are summarized in Table 2.

Discussion

The limited therapeutic arsenal aimed at controlling morbidity and the alarming emergence of strains resistant to the treatment of choice with praziquantel signal the need for studies involving new schistosomicidal agents. Herein, we demonstrated the potential of LicoA, a natural compound with a range of biological activities, including anti-protozoal effects against *Leishmania* spp. and *Plasmodium* spp. (13).

The *S. mansoni* tegument is an interface that plays an important role in protecting the parasite against the host immune system, thus allowing survival and establishment of the parasite in the host (16). Some schistosomicidal drugs, such as praziquantel, damage the parasite's tegument, thereby

Table 2. Effects of two doses of licochalcone A administered (intraperitoneal) to mice at 30 and 45 days post-infection

	Dose (mg/Kg)	Number of animals		Average of worms ± SED	Distribution of worms (%)		Changing of oogram (%)
		Treated	Examined		Mesentery	Liver	
Licochalcone A	50 (two doses)	15	14	17,3±2,42	91,5	8,5	0
Praziquantel	200 (single dose)	15	15	25,0±3,28	88,8	11,2	0
Control untreated	-	-	15	20,3±2,53	92,7	7,3	0

eliciting a strong antigenic response (18). Herein, the effect of LicoA on the survival of schistosomes was initially evaluated *in vitro*. A concentration-dependent effect on tegument integrity was observed. The minimum concentration of LicoA tested (5 μM) caused mild and progressive motor disorders. Increased concentration of LicoA (10 μM) induced later and progressive injuries, resulting in the death of all worms after four days of incubation. At higher concentrations (25-100 μM), LicoA induced significant tegumental damage, reduction of worm's motility and ultimately killed all the worms in culture after 24 hours (Table 1). Similar results were previously described for the Licoflavone B (25-100 μM), a flavonoid, that kills adult schistosomes *in vitro* by causing tegumental alterations and a dramatic reduction in the parasite motility (3). Our results align with a previous work (23) that reported activities of LicoA (~ 10 μM) *in vitro*, likely the excess of free radicals caused by the contact of adult worms with the compound. This effects was caused by an increased the superoxide anion level and decreased the superoxide dismutase activity in *S. mansoni* adult worms in culture (23).

At the microscopic level, LicoA caused a significant injury in the integument of adult male worms. Castro et al. (4) described a similar effect of another flavonoid, the cardamonin, which was able to disintegrate the tubercular surface of the worm in a dose-dependent manner by inhibiting the enzyme ATP diphosphohydrolase (4). These results reinforce the potential of LicoA to damage the worm tegument, thus compromising the tegument, which acts as a protective barrier which controls nutrient absorption and secretion and protects the worm against the host immune system (14, 15, 16). Despite the worm's capacity of renewing the tegument (18), extensive damage as those observed here might prevent tegument repair *in vivo* due to the host immunity system. Thus, the injuries induced by LicoA might be irreversible in the course of the infection *in vivo*.

Oviposition occurs under appropriate conditions at the first day of incubation *in vitro*. The egg production hits the peak between the third and fourth day, reflecting the worm adaptation to the culture microenvironment (10, 13). The reproductive fitness of the adult schistosomes was not influenced by our culture conditions since the worms maintained a growth rate of egg-laying until the end of the incubation period in a drug-free microenvironment (negative control group). LicoA (5 μM) progressively inhibited the oviposition of adult schistosomes (Fig. 2). This inhibition seems to be irreversible since it persisted after removal of LicoA from the medium. We hypothesize that LicoA affects the oviposition by inhibiting the P-glycoprotein (Pgp) present at the worm's digest system (7). Pgp and other ABC multidrug transporters mammalian cells disrupt schistosome egg production ex vivo by potential interaction with SMDR2, a Pgp orthologue from *S. mansoni* (11). Oviposition is required for the transmission life cycle transmission of schistosome and their eggs induce severe immunopathology in the host (7, 8, 10). Therefore, in the absence of a vaccine to prevent the spread of the disease, oviposition-inhibiting therapy has the potential to reduce transmission of schistosomiasis and mitigate the immunopathology caused by the eggs.

LicoA had no significant toxic effect to Vero cells at the evaluated concentrations. Minimal toxicity has been also reported for other mammalian cell lines such as Chang normal human liver cells by Choi et al. (6) and CHO-K1 cells by Souza et al. (23). Taken together, our promising results *in vitro* and the absence of cytotoxicity encouraged us to further investigate LicoA anti-schistosomal effects in animal models.

A subcurative dose of PZQ (200 mg/ Kg) was administered as a positive control. Previous works reported a significant reduction in parasite burden in animals treated with this subcurative dose compared to the untreated group (5, 21). It has been hypothesized that such dose damages the tegument of the adult worm and exposes antigen, enabling the reduction of the parasitic load by a synergistic action with the host immune system (5, 21). However, in this study, PZQ (200 mg/ Kg) did not significantly reduce the worm burden neither alter worm distribution (Table 2). This disagreement might be explained by the administration of PZQ in different time points. Previous studies administered PZQ 50-55 days post-infection when adult worms are already present. Herein, PZQ was administered 30 days post-infection when only schistosomulas (juvenile worms) were present. As PZQ acts only on adult worms (4, 12, 24), it is not a surprise that we did not observe a decrease in worm burden (Table 2).

LicoA failed to reduce the worm burden in both models, adult worms (25 mg/ Kg - one oral dose) and schistosomulas (50 mg/ Kg two intraperitoneal doses), investigated in this work. However, we cannot completely rule out the potential effect of LicoA against *S. mansoni*. It is possible that the doses or route of administration were not ideal to reach the parasite in the host in relevant concentrations, either by the influence of metabolic degradation or by the pharmacokinetics presented by the substance itself in the mice.

Changes in the oogram pattern suggest *S. mansoni* fecundity impairment and is a promising indication of drug efficacy (19). No available drug is able to directly act on mature or immature eggs *in vivo*. Abdul-Ghani et al. (1) observed a complete absence of two immature stages in the oogram after the animals infected with an Egyptian strain of *S. mansoni* were treated with artemether. Although, the treatment of Swiss and BALB/c mice infected with of BH strain of *S. mansoni* with β-lapachone and phytol, respectively did not change the stages of development, these drugs reduced egg production and oviposition kinetics (2, 17). Although LicoA has inhibited reproduction *in vitro*, this was not confirmed *in vivo* since this drug did not modify the egg production rate or oviposition kinetics since all immature stages were visualized in the intestinal wall of the sacrificed animals.

Conclusions

We demonstrated evidence of the *in vitro* LicoA potential. This compound reduced schistosome survival, reproduction and the worm's tegument integrity. However, these effects were not replicable in our preliminary *in vivo* models, likely due to dose regime and route employed in this study that might have resulted in poor absorption/ metabolization. Collectively, our findings provide new directions to explore the promising therapeutic potential of LicoA as a schistosomicidal drug.

Declaration of Competing Interest

None of the authors has conflict of interest with respect to the study.

Acknowledgments

Each named author contributed equally to the design of the study, its implementation, and/or the writing of the manuscript. This work was supported by FAPEMIG (PPM-00296-16) and CAPES, PIBIC/CNPq/UFJF and CNPq for fellowships.

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