In vivo and In vitro of Arctiin Schistosomicidal Activity

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Abstract

Human schistosomiasis, caused by trematode worms of the genus Schistosoma, is one of the most significant neglected tropical diseases, affecting more than 200 million individuals worldwide and praziquantel is the only available drug to treat this neglected disease. Arctiin is a lignan obtained from Arctium lappa (Asteraceae) with anti-inflammatory and antiproliferative activities. Our purpose was to investigate the in vitro and in vivo schistosomicidal activities of arctiin in mice infected with S. mansoni. Arctiin (200 and 100 μM) caused mortality, tegumental alterations, and reduction of motor activity of adult worms of S. mansoni in culture. Oral administration of a single dose of arctiin (25 mg/kg) on day 45 of infection did not reduce worm burden or cause any alteration in the analyzed parameters when compared to infected untreated mice. On the other hand, intraperitoneal treatment with arctiin (50 mg/kg) was able to reduce the hepatic granuloma volume by 20% in comparison to infected untreated mice. In addition, after intraperitoneal administration of arctiin in mice it was shown by HPLC analysis that arctiin was present in murine plasma. More studies should be conducted to verify the possible mechanism of action on inflammatory components present in granuloma formation.

Keywords: Schistosoma mansoni, Arctiin; Arctium lappa; Granuloma

Introduction

Schistosomiasis is a debilitating neglected tropical disease caused by trematode worms of the genus Schistosoma that affects hundreds of millions of people worldwide [1]. According to World Health Organization (WHO) [2], schistosomiasis affects more than 240 million people in tropical and sub-tropical areas and more than 700 million are at risk of infection [3,4]. In Brazil, eight million people from endemic regions stretching from the north to the south-east of the country, mainly in the Minas Gerais State, are infected with this chronic debilitating disease [3].

The pathogenesis of this disease depends on the host-parasite interaction [5]. Then, the early stage of schistosomiasis, after skin penetration of cercariae, is characterized by an allergic reaction, which is known as cercarial dermatitis. The second phase is resulting of schistosomula migration through the circulatory system, originating acute symptoms and, after, the development of hepatic granulomas around eggs in the liver [6].

Currently, praziquantel (PZQ) is the only drug available for treatment and control of S. mansoni [7]. However, PZQ has only a limited effect on already developed liver and spleen lesions and there is a considerable concern about the development of PZQ resistance [8,9]. Such facts have encouraged the scientific community to develop novel and inexpensive drugs against schistosomiasis [7-9]. In this regard, a number of studies have been developed with natural products from medicinal plants to identify a leading compound that can be used for the treatment of schistosomiasis [10-12].

In this regard, Arctium lappa (Asteraceae), also known as burdock, is a medicinal plant popularly used as hepatoprotective, antiseptic and anti-inflammatory that has provided biologically activity compounds, mainly lignans, against some parasites, such as Trypanosoma cruzi [13]. Furthermore, it was shown that the fruits extract of A. lappa improved kidney function in mice infected with S. haematobium [14]. Arctiin, a dibenzylbutyrolactone lignan, is the main constituent found in fruits of A. lappa that displays several biological activities, especially anti-inflammatory (Figure 1A) [15,16]. Thus, the aim of this study was to evaluate the in vitro and in vivo schistosomicidal effects of arctiin in mice infected with S. mansoni, which have not yet been described.

Materials and Methods

Drugs

Arctiin was isolated and purified from the crude hydroalcoholic extract of A. lappa fruits according to methods previously described [17,18]. PZQ (Cestox® tablets, 600 mg) was donated from Farmanguinhos/Fiocruz (Rio de Janeiro, Brazil).
Maintenance of the *S. mansoni* life cycle and ethics statement

*Schistosoma mansoni* (BH strain, Belo Horizonte, Brazil) worms were maintained in *Biomphalaria glabrata* snails as intermediate hosts and *Mesocricetus auratus* hamsters as definitive host at the Adolfo Lutz Institute (São Paulo, Brazil), according to standard procedures previously described [3,10]. At 50 days post infection, adult *S. mansoni* specimens were recovered from each hamster by perfusion in RPMI 1640 medium (Invitrogen, São Paulo, Brazil) supplemented with heparin. The Committee for Ethics in Animal Care of Adolfo Lutz Institute (São Paulo, Brazil) authorized all experiments, in accordance with nationally and internationally accepted principles for laboratory animal use and care (CEUA, 11.794/08). The study was conducted in adherence to the institution's guidelines for animal husbandry. All adequate measures were taken to minimize animal pain or discomfort.

**In vitro assays**

Adult worms were washed in RPMI 1640 medium (Gibco) supplemented with 200 µg/mL of streptomycin, 200 UI/mL of penicillin (Invitrogen), and 25 mM of Hepes. Adult worms 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. For the *in vitro* test with *S. mansoni*, arctiin was evaluated at concentrations of 50, 100 and 200 µM, according to procedures previously described [11,12]. Samples were added to the culture from a 4000 µg/mL stock solution in RPMI 1640 containing dimethyl sulfoxide (DMSO). 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 PZQ (5 µM) as positive control group. All experiments were performed in triplicate and were repeated at least twice. Parasites were maintained for 48 h and monitored every 24 h using a light microscope in order to evaluate their general condition: motor activity, mortality rate and tegumental alterations [11,12].

**In vivo assays**

**Animals:** Female Swiss mice (4-7 weeks), weighing approximately 20 g, were obtained and maintained in Schistosomiasis Laboratory of the René Rachou Research Center, Fiocruz/MG. Mice 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 randomly divided into four experimental groups among treated and untreated groups were used analysis of variance (ANOVA). The results were presented in mean/standard deviation and percentage.

**Intraperitoneal** *in vivo* treatment with Arctiin: Sixty female mice were randomly divided into four experimental groups (fifty animals each). The first group received two intraperitoneal doses (interval of two weeks) of arctiin (50 mg/kg), which was injected in a DMSO 10% saline solution. Control group received two intraperitoneal doses (interval of two weeks) of DMSO 10% saline solution, while the other infected group received no treatment. The positive control group was treated with PZQ (200 mg/kg, p.o.). All treatments were made on day 20 post infection, and animals were sacrificed after 60 days post infection.

**Parasitological evaluation of the recovered worms:** On the day 60 after infection, animals were euthanized under deepening anesthesia (xylazine at 10 mg/kg, i.p. and ketamine at 115 mg/kg, i.p.), and then both hepatic and mesenteric vessels were perfused to recover the worms, which were then sexed and counted according adapted to Pellegrino e Siqueira [18,19]. Recovered worms of treated mice were compared to untreated animals.

**Liver weight:** After euthanasia and perfusion, the liver from each mouse submitted to the double-dose regimen arctiin (50 mg/kg weight) as well as those belonging to the control groups-PZQ, DMSO and untreated-were removed and weighted.

**Qualitative and quantitative analysis of oogram:** Parts of the small intestine were used to estimate the tissue egg load and to study the oogram pattern (egg developmental stages); about 1 cm of the ileocecal intestine was removed and prepared for oogram examination. The slides were viewed under microscope (Olympus BX41) in order to verify and quantify the presence of eggs. The eggs were classified as immature, mature, or dead according to Jurberg et al. [20].

**Histopathological studies and granuloma analysis:** Specimens of the liver were fixed in phosphate buffered formalin solution, and stained with various agents, and then examined by light microscopy (Olympus BX41) to assess granuloma diameter and deposition of collagen. Morphometric analysis was carried out by means of the Image Pro-Plus 4.5 software (Media Cybernetics). The total area of granulomas was measured and the results were expressed in square microns (µm²). They were selected randomly and evaluated at 10 granulomas per slide (5 in each group).

**Identification of arctiin in the murine plasma by HPLC**

Determination of arctiin in the murine plasma, after intraperitoneal administration, was made according to methods previously described [17,18]. HPLC analysis was performed using an Alliance 2695 (Waters, USA) with ultraviolet (UV) detector model 2489. The column employed was X-Bridge C18 column, 4.6 × 150 mm, 5 um (Waters, USA) operating at 35°C, a gradient system phosphoric acid solution using 0.1% methanol and the following gradient: 1-5 min (50:50), 5-10 min (60:40). The flow of the mobile phase was maintained at 1 ml/min and injection volume of 10 µl for the standard sample and 20 µl for samples containing mouse plasma, collected after one h after intraperitoneal treatment with arctiin (50 mg/kg). Empower software 3 (Waters, USA) was used for processing the samples, integration of peaks and evaluating the obtained chromatograms. Detection was performed simultaneously at 254 and 280 nm.

**Statistical analysis:** Statistical analyzes were performed using GraphPad Prism 3.3 software (San Diego, CA, USA). The results with normal distribution were analyzed using: analysis of variance, followed by testing for multiple comparisons Tukey, when comparisons involving more than two variables, the differences were considered significant with p<0.05. To evaluate the homogeneity of infection among treated and untreated groups were used analysis of variance (ANOVA). The results were presented in mean/standard deviation and percentage.
Results

In vitro schistosomicidal activity of arctiin against adult worms of S. mansoni

First, we investigated the in vitro effects of arctiin against adult worms of S. mansoni. As shown (Table 1), arctiin (200 µM) causes 100% mortality in all adult parasites, as well as significant decrease in motor activity and in tegumental alterations. In addition, after 48 h of incubation, arctiin (100 µM), and all worm pairs were dead, also showing tegumental alterations and significant decrease in motor activity (Table 1). However, at 50 µM, arctiin was inactive (Table 1). PZQ (5 µM) also caused 100% mortality with tegumental alterations, whereas no effect was observed in worms in the negative (RPMI 1640 medium) and control (RPMI medium plus 0.5% DMSO) groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Period of Incubation (h)</th>
<th>Dead worms (%)</th>
<th>Motor activity reduction (%)</th>
<th>Worms with tegumental alterations (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Slight</td>
<td>Significant</td>
</tr>
<tr>
<td>Control</td>
<td>24</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DMSO 0.5%</td>
<td>24</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PZQ (5 µM)</td>
<td>24</td>
<td>100</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>100</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Arctiin</td>
<td>200 µM</td>
<td>24</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>100 µM</td>
<td>24</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>50 µM</td>
<td>24</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1: effects of arctiin isolated from A. lappa (AL) against adult worms of S. mansoni. *Percentages relative to the 20 worms investigated.

Natural products are promising sources of new potentially therapeutic compounds, and several lignans, especially dibenzylbutyrolactones, have been reported as promising sources of antiparasitic compounds against neglected parasitic diseases [21]. In this sense, arctiin has also been assessed against some parasites (Figure 1A).

In vivo schistosomicidal activity of arctiin in mice

Then, on the basis of these promising in vitro results, we investigated the therapeutic potential of arctiin on mice. First, the in vivo treatment was performed orally, with a single dose of arctiin (25 mg/kg). However, as shown (Table 2), arctiin did not reduce significantly the total number of worms or any other parameters compared with the corresponding non-treated control (Table 2).

Previous studies on the pharmacokinetic of arctiin, after oral administration, showed that it had a rapid absorption phase followed by a sharp disappearance and rapidly distributed in various organs in rats [22]. So, we hypothesized that oral administration of arctiin could not be effective. Then, in order to change the route of administration, we investigated the availability of arctiin after intraperitoneal administration. Animal plasma samples were collected 1 h after intraperitoneal administration of arctiin (50 mg/kg). As shown by HPLC (Figure 1B), it was possible to verify the presence of arctiin (with Rt approximately in 5 min) in the chromatogram corresponding to the plasma sample collected from mice after 1 h of arctiin intraperitoneal treatment. After results, we supposed that the intraperitoneal treatment with arctiin could be more effective than the oral administration. Then, next, we investigated the in vivo schistosomicidal activity of arctiin administered by intraperitoneal route. Chromatographic analysis of pooled murine plasma samples collected 1 h after arctiin administration was performed.

The chromatographic analysis identified a similar peak (around 5 min) in the standards which is chromatograms A and B and pooled plasma samples collected from treated mice which is chromatograms C (Figure 1B). Pure arctiin diluted in methanol at concentration of 1 mg/mL (chromatogram A) (Figure 1) and plasma of non-treated animal spiked with arctiin at concentration of 1 mg/mL (chromatogram B) (Figure 1) were used as standard.

However, as shown in Table 3, when arctiin was given intraperitoneally (50 mg/kg), it also not produced statistically significant reduction in the analyzed parameters compared with the corresponding infected untreated control group. Thus, these data...
demonstrated that arctiin treatment (at the sued doses) has no activity capable of disrupting infection and eliminate any forms (juvenile worms, adults and eggs) of Schistosoma.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Number of animals</th>
<th>Average of worms</th>
<th>Distribution of worms (%)</th>
<th>Dead worms (%)</th>
<th>Changing of oogram (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treated</td>
<td>Examined</td>
<td>Mesentery</td>
<td>Liver</td>
<td>Lung</td>
</tr>
<tr>
<td>Arctiin</td>
<td>25</td>
<td>20</td>
<td>18</td>
<td>48.4</td>
<td>99.1</td>
</tr>
<tr>
<td>Control untreated</td>
<td>-</td>
<td>-</td>
<td>5</td>
<td>39.6</td>
<td>91.4</td>
</tr>
</tbody>
</table>

Table 2: Preliminary data for the parasitological parameters evaluated. The swiss mice were divided into two groups: untreated control group and the group receiving the test drug orally arctiin a dose of 25 mg/kg body weight. The difference was regarding significates if p<0.05.

Figure 1: Plasmatic availability assessment arctiin one hour after its intraperitoneal administration. The analysis of pure arctiin sample diluted in methanol (A-1 mg/mL) and diluted in murine plasma untreated (B) showed a peak in the chromatogram measured at 254 nm with retention of 5 min. The animal plasma sample collected after 1 h of treatment with arctiin under the same experimental conditions (C), revealed a peak similar to pure sample, confirming that arctiin is available in plasma following administration.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Number of animals</th>
<th>Average of worms ± SED</th>
<th>Distribution of worms (%)</th>
<th>Average of liver relative weight</th>
<th>Changing of oogram (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treated</td>
<td>Examined</td>
<td>Mesentery</td>
<td>Liver</td>
<td>Liver</td>
</tr>
<tr>
<td>Arctiin</td>
<td>50 (two dose)</td>
<td>15</td>
<td>12</td>
<td>19.83 ± 8.66</td>
<td>90.76</td>
</tr>
<tr>
<td>Praziquantel</td>
<td>200 (single dose)</td>
<td>15</td>
<td>12</td>
<td>20.75 ± 7.37</td>
<td>88.35</td>
</tr>
<tr>
<td>Control untreated</td>
<td>-</td>
<td>15</td>
<td>12</td>
<td>22.67 ± 9.46</td>
<td>93.38</td>
</tr>
<tr>
<td>Control DMSO</td>
<td>(two dose)</td>
<td>15</td>
<td>12</td>
<td>23.08 ± 7.77</td>
<td>95.67</td>
</tr>
</tbody>
</table>

Table 3: Parasitological parameters evaluated. The swiss mice, infected with 50 Schistosoma mansoni cercariaes, were divided into four groups: untreated control group, control group DMSO and the route of intraperitoneally administration, the control group treated with 200 mg
praziquantel/kg body weight orally and the group receiving the test drug, arctiin an intraperitoneally dose of 50 mg/kg body weight, the doses were interleaved for a period of 15 days. The difference was regarding significates if p<0.05.

In addition, oogram analysis (data not shown) revealed that arctiin did not reduce the number of eggs in the intestinal tissue. Also, analysis of the oogram pattern of the arctiin-treated group showed eggs in all development stages, as well as no significant differences in the prevalence of immature or mature eggs in treated experimental groups. Then, it was suggested that arctiin exerted no effect on the development of eggs, like was observe in PZQ treatments in the literature.

On the other hand, histopathological analysis showed a significant anti-inflammatory effect of arctiin. As shown (Figures 2A and 2B), marked inflammation of liver parenchyma was observed in tissue of untreated mice. After hepatic tissue sections were stained with hematoxylin and eosin, it was possible to measure the areas of granulomas. Results showed that arctiin reduced, by approximately 20%, the area of granuloma in comparison with the untreated group (Figure 2).

Discussion

The spread of schistosomiasis endemic areas and increasing infection rate [1-9] support the need of new drug discovery and development [5-9]. Research of natural products has provided remarkable new drugs or drug leads to combat several diseases [14-18].

Several biological activities have been described for Arctiin, a molecule isolated from the Arctium lappa L., such as anti-inflammatory [16] and anti-proliferative [23-26]. In this work, we sought to investigate schistosomicidal potential of this molecule

In vitro effects of arctiin at 50, 100 and 200 μM concentrations on parasite viability, motor activity and tegument damage evaluated on daily basis for 3 days [2,7]. Arctiin (100 and 200 μM) affected vital parasite functions, causing soft tissue injuries and motor activity decrease.

Other substances displaying schistosomicidal activity in vitro have been described. Acetate carvacrol (6.25 μg/mL) affected motility, parasite tegument and oviposition [7]. Similar results were also found for phloroglucinol derivatives (aspidina, flavaspidico acid, methylene-bis-aspidinol and desaspidina) [21], dermaseptina antimicrobial peptide [27], essential oils of Piper cubeba and Tagete serecta [21,28].
Sometimes **in vivo** activity does not reflect **in vitro** efficacy. In addition, appropriate dose for **in vivo** assays from **in vitro** results is a challenging task [30]. Arctin schistosomicidal activity was evaluated using a murine model, initially as a single 25 mg/kg dose regimen and then two doses of 50 mg/kg (15 day time interval).

Arctin bioavailability after administration was a main concern, since the literature describes that microorganisms in the intestine convert this compound into arctigenina [31]. To address this issue, plasmas from animals treated with arctin were collected one hour after drug administration, pooled and submitted to chromatographic analysis. Noteworthy, spectral scan showed that analysis using 254 nm wavelengths reduces interference effects from plasma constituents. Chromatographic analysis of pooled plasma from treated animals revealed a sharp peak at 5 min which is the same retention time for the peak found when analysing the standards at same condition.

For the **in vivo** assays, Swiss mice experimentally infected were treated with two intraperitoneal doses of arctin 50 mg/kg. Prasite load, liver weight, oogram and granuloma development were evaluated to assess the drug effectiveness.

Fever, headache, lethargy, inflammation and scarring (collagen deposition and fibrosis) of the liver are the main clinical characteristics of schistosomiasis. The granuloma, resulting from the host inflammatory response against *S. mansoni* eggs trapped in the liver is the main cause of hepatomegaly [25]. Therefore, the reduction of worm number and oviposition can be directly related to a decrease of the granulomatous reaction and liver weight [30]. Fabri et al. [30] demonstrated that treatment with methanolic extract of *M. frigidus* avoided increase of liver and spleen weight during *S. mansoni* infection. Studies with episipoliturna alkali at dosage of 40 mg/kg achieved similar results and the effects with higher doses were not satisfactory [27,32]. Treatment with arctin at concentration of 50 mg/kg had not impact on liver weight, since no statistically significant difference was found when compared with control groups.

Schistosomiasis control is still quite limited. Currently it relies just on sanitation policies and intermediate host (mollusks) control, since vaccine is not available. Thus, chemotherapy remains as the main control strategy. Praziquantel is the only drug available for treatment of all types of schistosomiasis and it is used in mass treatment. The lack of efficacy against immature stage of the parasite is the major limitation [6].

Schistosomes exhibit a biphasic susceptibility to praziquantel. Sensitivity is observed in early-stage migrating larvae. Then, from 2 to 4 weeks post infection, the susceptibility reduces and it is gradually restored afterwards [6,33]. Our results are in line with this finding, since treatment of experimentally infected mice 20 days post-infection with praziquantel 200 mg/kg did not affect the number of parasite recovered. Of special note, treatment with praziquantel changed the distribution of worms in the liver and mesentery. However, this change in the distribution induced by praziquantel, was not able to promote the parasitological cure. Both arctin treatment regimes were also ineffective.

The oogram technique allows assessing *S. mansoni* fecundity and egg development. Qualitative and quantitative oogram evaluation showed that arctin did not affect oviposition kinetics or egg maturation. Similar effects on oogram were described with treatment of infected animals with dexamethasone (50 mg/kg) [34]. In addition, Aires et al. [35] observed eggs in all development phases following administration of β–lapachone, however the percentage of immature eggs was significant lower, indicating a kinetic change in the egg development.

In our study, the majority of the granulomas were in the exudative-productive stage, with prevalence of inflammatory infiltrates and loose and disorganized collagen fibers, characteristics of granuloma acute phase. Qualitative changes of granulomatous infiltrates were not observed on any experimental group, all characterized by a significant number of eosinophils and presence of neutrophils, lymphocytes and macrophages to a lesser extent.

Better disease prognosis depends on fewer granuloma formation and granuloma resolution when they are present. In our experiments, the calculated granuloma areas did not vary between the negative control groups (untreated and DMSO) and praziquantel group. Interestingly, the granuloma size decreased around 20% compared to untreated group. This effect must be related with arctin anti-inflammatory activity previously described [12]. These data suggest the use of arctin as an adjuvant therapy with praziquantel. Combination of these two drugs would achieve a synergic effect, since praziquantel has no effect on egg development and granuloma resolution.

The results presented here indicate that although arctin lacks schistosomicidal activity **in vivo**, this molecule is a potential adjuvant therapeutic for *S. mansoni* infection treatment, since its anti-inflammatory activity is able to reduce infection morbidity. Further studies should be carried out to shed light on the molecular mechanism of action of arctin.

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