Anthelmintic activity in vitro and in vivo of Baccharis trimera (Less) DC against immature and adult worms of Schistosoma mansoni

Rosimeire Nunes de Oliveira a,⇑, Vera Lúcia Garcia Rehder b, Adriana Silva Santos Oliveira b, Veronica de Lourdes Sierpe Jeraldo c, Arício Xavier Linhares a, Silmara Marques Allegretti a

a Instituto de Biologia, Depto. de Biologia Animal, Universidade Estadual de Campinas, Unicamp, SP, Brazil
b Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas, CPQBA, Unicamp, SP, Brazil
c Instituto de Tecnologia e Pesquisa, Laboratório de Doenças Infecciosas e Parasitárias, Aracaju, SE, Brazil

HIGHLIGHTS

- The lethal effect in vitro occurred in a dose-dependent manner.
- The SEM revealed morphological changes on the tegument of the worms in vitro.
- B. trimera caused reduction worm’s burden of different stages schistosomula, juvenile and adults of S. mansoni in vivo.
- The in vivo treatments significantly reduced the number of hepatic granulomas.

ABSTRACT

Although its efficiency against all Schistosoma species, praziquantel (PZQ) shows low efficacy against schistosomula and juvenile stages. The potential for development of resistance to PZQ has justified the search for new alternative chemotherapies. In this scenario, studies to new formulations, more comprehensive and without adverse effects, are being conducted. One viable and promising treatment is the study of medicinal plants as a new approach to the experimental treatment for Schistosomiasis. Amongst all the variety of the medicinal species studied, we can highlight Baccharis trimera (Less) DC, known as “Carqueja-amarga”. This paper not only describes the effect of crude dichloromethane extract (DE) and aqueous fraction (AF) obtained from B. trimera, in vitro but also is the first one that investigates the in vivo efficacy of B. trimera against schistosomula, juvenile and adult worms of Schistosoma mansoni BH strain. In the experiment, mice were treated with DE, AF and PZQ (40 and 200 mg/kg) over the period of larval development (3 and 30 post-infection; pi), and adult worms (60 days post-infection; pi). The in vitro results show that the DE and AF effects are dose-dependents, being the 130 μg/mL the most effective one in a shorter period of incubation. The exposure of the in vitro samples over adult parasites were able to inhibit 100% of the oviposition in females. Likewise caused the mortality of the parasites with morphological alterations on the tegument, on the suckers, oral and acetabulum, in both males and females after 6–72 h of exposure. Additionally, the in vivo treatments against juvenile and adult infection were more effective compared to the control group untreated. Administrations of AF and DE in day 30 pi (juvenile worms) show female worm total burden reductions of 75% and 68% respectively. At the same period of infection reductions of respectively 98% and 97% egg/g in the faeces were seen. In relation to...
1. Introduction

Amongst all the parasitosis, the schistosomiasis figures as the second most neglected tropical diseases, being overcome only by malaria. The disease registers high rates of morbidity and mortality especially in tropical and subtropical countries, more specifically in underprivileged communities where there is no access to drinkable water or adequate sanitation structure (Kamel et al., 2011; Steinmann et al., 2006).

Nowadays, the schistosomiasis control is primordially based on chemotherapy. In Brazil, the treatment program of schistosomiasis control is carried out using praziquantel (PZQ) under standard doses of 50 mg/kg for adults and 60 mg/kg for children. However, in preventive chemotherapy programs (e.g., in Africa) the dose used is 40 mg/kg with cure rate of 80–90% (Katz and Coelho, 2008). Although it is efficient against all human schistosomes, praziquantel shows low efficacy against schistosomula and its juveniles stages, allowing lower cure rates in areas with high endemicity (Abath et al., 2000; Doenhoff et al., 2008). Moreover, repetitive use of the drug has brought resistance and tolerance by Schistosoma S. mansoni in different areas and by different strains of the same area, reaffirming an urge to develop new treatment alternatives (Doenhoff et al., 2008; Fallon et al., 1995; Parise-Filho and Silveira, 2001; Pica-Mattoccia and Cioli, 2004).

One viable and promising treatment is the study of medicinal plants as a new approach to the experimental treatment for schistosomiasis (Allegretti et al., 2012; Moraes, 2012; Ndamba et al., 1994). The research on medicinal plants is encouraged by the World Health Organization (WHO, 2002), considering that certain traditional knowledge on curative plants could add up to the development of new pharmaceutical products as well as to the combat against diseases that affects the populations of underdeveloped countries.

Amongst all the variety of medicinal species studied, we can highlight Baccharis trimera (Less) DC known as “Carqueja-amarga”, highly used in traditional medicine as a treatment or prevention against hepatic diseases, rheumatism and morbid obesity (Borella et al., 2006; Dickel et al., 2007; Fukuda et al., 2006; Torres et al., 2000; Verdi et al., 2005). Furthermore, other biological effects were already evidenced by Simões-Pires et al. (2005), who could demonstrate the antioxidant and anti-inflammatory activity of the aqueous extract. Gambinini et al. (1991) have already evidenced the reduction of the acidity and also the stress-induced and ethanol-induced gastric wounds in laboratory mice, as well as the relaxation of the smooth bowel musculature. Oliveira et al. (2005) evaluated the antidiabetic activity of the ethanolic crude extract as well the aqueous and butanolic fractions which were obtained from the aerial parts of B. trimera. Pádua et al. (2010) have described the antioxidant activity of the hydroalcoholic crude extract, either in vitro or in vivo tests.

Recently, the promising antischistosomal activity of B. trimera has been evidenced by in vitro tests using essential oils, hydroalcoholic crude extract and hexanic fraction on S. mansoni adult worms (Allegretti et al., 2012; de Oliveira et al., 2012; Oliveira et al., 2013). In the study it was observed that these samples caused significant mortality of schistosome showing morphological changes in the tegument analyzed by scanning electron microscopy (SEM) in a 24-h incubation. The same analysis evidenced the inhibition of the oviposition by females when the worms were exposed to sub-lethal concentrations.

Due to the high prevalence, wide distribution of schistosomiasis, limitation of drugs for the treatment and control of this helminthiasis, it is necessary to study alternative therapeutics. In this scenario, it is necessary to conduct studies to new and more comprehensive formulations without adverse effects. This paper describes the effect in vitro and in vivo of crude dichloromethane extract (DE) and aqueous fraction (AF) obtained from B. trimera against immature and adult worms of Schistosoma mansoni BI strain for the first time.

2. Materials and methods

2.1. Plant material

Leaves of B. trimera, cultivated at CPQBA 1, a variety developed by mass breeding at the Chemical, Biological and Agricultural Pluridisciplinary Research Center (CPQBA) of the State University of Campinas (Unicamp, Campinas, São Paulo, Brazil), were collected in an experimental field. The plant was identified by Prof. Dr. Grady L. Webster (University of California, Davis, USA). Its exsiccata is preserved at the herbarium of CPQBA under the number 1286 (de Oliveira et al., 2012).

2.2. Extraction protocols or preparation of B. trimera extracts

In order to prepare B. trimera dichloromethane extract, the fresh aerial part residues of the plant were used right after the essential oil extraction. An amount of 100 g of dried plant was extracted and so crushed in a (Polytron®) dispenser together with 800 mL of dichloromethane solvent (Sigma–Aldrich®) for five minutes. The extract was filtered using a funnel with porous plate and was dried under vacuum using a rota-evaporator. To obtain the aqueous fraction (AF) 4.0 g of dichloromethane extract (ED) was used. Initially, the ED was solubilized with 120 mL of acetone and 80 mL of distilled water using an ultrasonic bath. The extraction was proceeded in a separatory funnel. The fraction containing water and acetone was dried by the rota-evaporator vacuum until complete the removal of the solvents.

2.3. Testing toxicity of the prepared extracts

A mammalian cell line, vero cells, obtained from Piracicaba Dental School of the State University of Campinas – UNICAMP, was used for evaluating toxicity of the extracts. Stock and experimental cultures were grown in a medium containing 5 mL RPMI-1640 (Gibco BRL, Life Technologies) supplemented with 5% fetal bovine serum. Experimental cultures were also supplemented with penicillin: streptomycin (1000 μg/mL; 1000 U/mL, 1 mL/L). The cells (100 μL cells/well, inoculation density from 3 to 6 x 10<sup>4</sup> cells/mL) in 96-well plates were exposed to different concentrations of dichloromethane extract and aqueous fraction (0.25–250 μg/mL, 100 μL/well) in DMSO/RPMI 1640/FBS 5% at 37°C and 5% of CO<sub>2</sub>, for 48 h. Final DMSO concentration did not affect cell viability. Before and 48 h after sample addition, cells...
were fixed with 50% trichloroacetic acid. The cell proliferation was determined by spectrophotometric quantification (540 nm) of cellular protein content using sulforhodamine B assay, by measuring absorbance at the beginning of the incubation (time zero, T0) and 48 h post-incubation for compound-free (T1) as well as treated (T) cells. The concentration-response curve for vero cells, TGI (concentration that produces total growth inhibition or cytostatic effect) was determined through non-linear regression analysis using software ORIGIN 8.0 (Denny et al., 2008; Shoemaker, 2006).

2.4. Parasites, intermediate and definitive hosts

The life cycle of *S. mansoni* (BH strain from Belo Horizonte, MG, Brazil) is maintained in * Biomphalaria glabrata*, which is the intermediate host, at the Department of Animal Biology, IB, UNICAMP. As definitive host, Swiss/SPF female mice, weighing 20 g and 4 weeks of age, were previously infected by means of exposure to a suspension containing 70 cercariae using the tail immersion technique as described by Oliver and Stirewalt (1952). The experiments were approved by the Ethics Commission for the Use of Animals (CEUA/UNICAMP, protocol no 2170-1), as they were in accordance with the ethical principles of animal experimentation adopted by CEUA.

2.5. Assessment of the anti- Schistosomal effects of the prepared extracts in vitro

Eight weeks following infection, adult *S. mansoni* worms were retrieved by perfusion of the hepatic portal system and mesenteric veins. These were washed in RPMI-1640 (Nutricell®) medium supplemented with 0.05 g/L of streptomycin, 10,000 U/ml of penicillin, 0.3 g/L of l-glutamine, 2.0 g/L of D-glucose, 2.0 g/L of NaHCO3 and 5.958 g/L of Hepes. After being washed, a couple of worms was transferred to each well of a 24-well culture plate (TPP) containing the same medium. Sequentially the plates were incubated in a CO2 greenhouse at 5% and 37 °C (Xiao et al., 2007). *S. mansoni* worm pairs were subjected to treatment with serial concentrations (24, 48, 91 and 130 ng/mL of both extracts). The final volume in each well was 2 mL. The positive control group was treated with a co-ion (5 and 10 ng/mL) of praziquantel, whereas the negative control group was kept in RPMI-1640 medium with the addition of 0.01% of Tween-80 with PBS 2% (the highest concentration of solvent used). The parasites were kept under the same conditions. All the experiments were performed in five replicates. Treated worms were monitored for their motility, mortality rate and oviposition at intervals of 2, 4, 6, 24, 48 and 72 h using an inverted optical microscope DM-500 (Leica®). Furthermore, alterations in the tegument were observed (de Oliveira et al., 2012).

2.6. Scanning electron microscopy examination of the tegument of *S. mansoni*

To observe changes in the tegument of adult parasites, schistosomes were exposed to treatments in vitro with 130 µg/mL of the DE and aqueous AF of *B. trimera* monitored for 24 h and analyzed by scanning electron microscopy (SEM). Briefly, worms were incubated at 37 °C in a CO2 atmosphere (5%) for 24 h. After incubation, the parasites were washed with sodium cacodylate buffer (0.1 M), fixed in 2.5% glutaraldehyde (pH 7.4) (Merk®) during 24 h and then fixed in 1% osmium tetroxide for 1 h. Specimens were dehydrated in increasing concentrations of ethanol (50%, 70%, 80%, 90%, 95% and 100%) for 30 min each, dried in a critical point dryer, mounted on stubs, metalized with gold particles using Sputter Coater and, finally, analyzed and photographed using an electron microscope (Jeol-JSM-820).

2.7. Assessment of the antischistosomal effects of the prepared extracts in vivo

Thirty-day-old Balb/C albino females (18–20 g) were individually infected with 70 *S. mansoni* cercariae through tail immersion, for a period of 2 h, exposed to light and to a temperature of 28 °C approximately (Oliver and Stirewalt, 1952). In the present study, a total of 150 *S. mansoni*-infected mice were used. The samples were weighed on an analytical balance and, after that process, were solubilized in a 2% autoclaved “Phosphate Buffered Saline” (PBS) with 0.01% Tween 80 Polysorbate (Merck®). All groups were treated with a single oral dose through esophageal pipeline. Then concentrations (40 and 200 mg/kg) were calculated according to the in vitro toxicity test results done beforehand in vero cells and also according to the acute toxicity test as described by Dias et al. (2009). The body weight of the animal was also taken into consideration.

2.8. Animal groups

The mice for the experiment were allocated into four groups (I–IV), with 30 animals each, at the time of the experiment. Group (I) was treated after 03 days (lung stage) infection; the group (II) was treated after 30 days (juveniles worms), with a single dose (200 mg/kg); the group (III) was treated after 60 days (adults worms) infection with 40 and 200 mg/kg of DE and AF and same doses for PQ (control pharmacological). Finally, the group (IV) as negative control infected and untreated. All animals were given 0.3 ml of “Phosphate Buffered Saline” PBS on the respective days of treatment. The treated groups are shown in Table 2.

2.9. Mice perfusion and worm recovery

The group (I) treated after 03 days of infection, was euthanized after 42 days post treatment. The group (II) treated 30 days post infection and group (III) treated 60 days post infection were euthanized after two weeks post treatment by cervical dislocation. The analysis of the group (IV) control, infected but untreated, followed the same scheme. Parasite worms were retrieved through perfusion of the hepatic portal and the mesenteric veins according to Pellegrino and Siqueira (1956). The percentage of worm reduction (WR) was calculated according to Delgado et al. (1992), using the formulation:

\[
\% \text{worm reduction} = \frac{\text{Total worms infected untreated control group} - \text{Total worms treated group}}{\text{Total worms infected untreated control group}} \times 100
\]
3. Determination of the egg counting in treated and control animals

The counting of the eggs eliminated through the faeces was performed on the day of the analysis of the treatments. The faeces were collected before the euthanasia and were examined in sequence, utilizing Kato–Katz quantitative method (Katz et al., 1972). The percentages of the different developmental stages, immature, mature and dead egg (oogram) were examined from the small intestinal wall of infected mice and were computed at day 45 and 60 after infection in accordance with the method described by Hermeto et al. (1994) and Pellegrino et al. (1962).

3.1. Histological methods

Regarding the histological analysis, liver samples (left lobe) were taken from the treated mice and also from the negative control group (infection untreated). The samples were fixed in 10% formaldehyde (Sigma–Aldrich®), dehydrated in increasing concentrations of ethanol, diaphanized in xyloc (Sigma–Aldrich®), and included in paraffin (Merck®). To a better definition of the granulomatous reactions, the histologic sections were colored with Masson trichrome according to Drury and Wallington (1980). To evaluate the sections, the choice was the bright field microscopy followed by image capture and processed by using Leica Image Manager 50. All the granulomas found in 10 histologic section random fields were counted. However, only the diameter was measured; the largest and the smallest ones, from the granulomas presenting one single S. mansoni egg.

3.2. Statistical analysis

The results were analysed throughout statistical and non-parametrical tests, using the SAS 9.1 statistics software (SAS Institute Inc., Cary, NC, EUA). The difference in the mean values was considered significant in a 5% rate.

4. Results

4.1. Effect of dichloromethane extract and aqueous fraction on the survival and the motor activity of S. mansoni in vitro

The survival adult of S. mansoni, which was exposed to the treatment with DE and AF, depended directly on the evaluated concentration and the incubation period. The concentrations 130, 91 and 48 µg/mL were lethal to 100% of males and females in the 6–72 h incubation intervals. However, the individuals exposed to 130 µg/mL of DE showed regurgitation and low contraction over the body. After 4 h and after 6 h of incubation 60% of male worms were dead (Table 1). The concentration – 24 µg/mL – also caused a significant mortality ($p < 0.01$) amongst males and females in 72 h of incubation. The positive control group was treated with PZQ in concentrations of 5 and 10 µg/mL (Table 1) what was lethal to 100% of the worms after 2–4 h of incubation. As expected, all the worms incubated in RPMI-1640 managed to stay alive until the end of the experiment – 72 h. Concerning the motility, a significant reduction in the parasites movements was observed in all concentrations. The percentage of worms that had their motility reduced was directly proportional to the concentration and to the period of incubation, as shown in Table 1.

<table>
<thead>
<tr>
<th>Samples µg/mL</th>
<th>Period of incubation (h)</th>
<th>Couples separated worms (%)</th>
<th>Mortality of the worms (%)</th>
<th>Worms with tegumental alterations (72 h)</th>
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<tr>
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<td>130 µg/mL</td>
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<td>91 µg/mL</td>
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<td>Control</td>
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Incubation period – 72 h; Control – RPMI-1640; I, Insignificant; S, Significant.

4.2. Effect of the dichloromethane extract and the aqueous fraction on the mating of the worms and the oviposition of S. mansoni in vitro

All the tested extracts influenced the process of natural mating, having 100% of the worms been separated, upper to the negative control group, kept in RPMI-1640 medium until 72 h of incubation. Moreover, concentrations that were not 100% lethal to the worms were proven as efficient mating inhibitors, once all of them separated the couples in all samples (Table 1). The egg production in S. mansoni adult females was observed after 2 h of exposure to different concentrations of DE, AF, and PZQ compared to the negative control group. The treatments evidenced the suppression of the elimination of eggs in all concentrations, even the sub-lethal one (24 µg/mL), in 72 h.

4.3. Tegumental changes of S. mansoni adult worms in response to the treatment with B. trimera visualized by scanning electron microscopy (SEM)

Tegument alterations were observed on S. mansoni adult males and females after 24 h incubation in vitro under the concentration...
of 130 μg/mL of DE and AF. The parasites treated with DE presented morphologic alterations (Fig. 1A and B). In male worms the treatment induced tubercles and spine tegumental damage on its dorsal surface, the accession of bubbles surrounding the morphologically altered tubercles, besides the sucker destruction (Fig. 1C and E). The female parasites presented a swollen dorsum (only visible through SEM), tegument scaling, oral sucker alterations and acetabulum alteration (Fig. 1D and F). Concerning the treatment with AF, the male worms presented tuber destruction, spines and sucker alterations (Fig. 2A, C and E). However, female
parasites developed tegument wrinkling and erosion, besides sucker alterations (Fig. 2B, D and F). The control group parasites, RPMI-1640 incubated, did not show any morphological changes (Fig. 3).

4.4. Effect of the prepared extracts on vero cells

It indicates the absence of cytotoxic effects of the studied extracts. Thus, the concentrations in which DE and AF presented the schistosomicidal activity in vitro were not associated with cytotoxic effects on vero cells.

5. Treatment efficacy in vivo

The in vivo treatments performed via oral in a single dose of 200 mg/kg of DE and AF of *B. trimera* significantly reduced the worm burden of the treated mice altering the male and female worm ratio, showing that females were more sensitive. The treatments against juvenile and adult infection were more effective compared to the untreated control group. Concerning the number of eggs excreted in the faeces and different egg developmental stages (oogram), the results showed significant reductions, emphasizing the relationship, due to the reduction in the number of worms, especially the females. The distribution of adult parasites retrieved from the mesenteric veins and the portal system of mice treated with *B. trimera* as well as the control groups – treated with PZQ – and the untreated infected group is summarized on Table 2.

5.1. Response of the immature worms of *S. mansoni* to a single dose of *Baccharis trimera*

The mice treated with a single dose (200 mg/kg) of *B. trimera*, after 03 and 30 days of infection showed significant differences (*p* < 0.01 and *p* < 0.001) regarding the reduction of male, female and total worm burden, especially for the reduction of the excreted eggs in the faeces. The treatments performed against schistosomula showed a moderate reduction of the total worm burden of 47.4%; 42% and 12.2% when the mice received AF, DE and (40 mg/kg) of PZQ, respectively, compared to the untreated infected control group. In contrast, the treatments showed a reduction of the eggs eliminated in the faeces (98%, 97.6% and 69.3% in animals treated with AF, DE and PZQ, respectively).

Regarding the treatments against juvenile worms, a better activity of *B. trimera* was observed in the same parameters as described above. The animals treated with AF, DE and PZQ showed a total reduction of the worm burden of 75%; 68% and 37%, respectively. Promising results were also presented regarding the reduction of eggs excreted in the faeces (98%, 97% and 57% with AF, DE and PZQ treatments, respectively). Comparing the two treatment periods (03 and 30 days PI) in oogram, it was observed that only the variant of the mature eggs, was significantly (*p* < 0.01) different among the tested samples. In Table 2, all results are summarized.

5.2. Response of the adult *S. mansoni* to *B. trimera*

After 60 days of infection, the treatments performed with DE and AF on doses of 40 and 200 mg/kg significantly reduced (*p* < 0.05 and *p* < 0.001) the worm burden of the mice to 52.7% and 66%, 47% and 62% respectively. However the treatments with 40 and 200 mg/kg of PZQ showed a low worm burden of 45% and 49.4% on the adult compared to treatments of *B. trimera* and the untreated infected control group. In relation to the number of eggs excreted in the faeces, the results showed reductions of 62% and 91%; 41% and 76% in response to treatments with 40 and 200 mg/kg of DE and AF, respectively. Nevertheless, the PZQ group showed a reduction of 50% and 63.4% for doses of 40 and 200 mg/kg. Meanwhile, the treatments with DE and AF caused a significant reduction (*p* < 0.001) of immature, mature, and dead eggs retained in the intestinal tissue, when compared to the PZQ group (*p* < 0.01) and with the untreated infected control group, as shown in Table 2.
In recent years, a substantial interest in natural products to the treatment of Neglected Tropical Diseases (NTD’s), including schistosomiasis, has been growing. The interest has been exploited and stimulated as an effort to develop a new medicine as an alternative to this parasitosis treatment (Moraes et al., 2012; Yousif et al., 2007). This study is the first one investigating the in vivo efficacy of B. trimer (Less) DC against schistosomula, juvenile and adult worms of S. mansoni BH strain. Nevertheless, as a first step, in vitro antischistosomal studies were performed on adult worms.

The in vitro assay demonstrates that the DE and AF effects are dose-dependent, being the 130 μg/mL the most effective one in a shorter period of incubation. Furthermore, it was possible to observe that the schistosomes exposed to DE and AF showed slow contractions, motility reduction and paralysis causing, most of the parasites death. According to Noel (2008) that paralysis is associated to important neurotransmitters or neuromodulators such as dopamine, acetylcholine, serotonine, amongst others. In relation to PZQ assays (5 and 10 g/mL), all the worms, males and females, were contracted with no movement whatsoever. Earlier studies by Pica-Mattoccia and Cioli (2004) inform about the PZQ effects on the worms, causing contractions whenever the parasite is exposed to concentrations 0.1 and 1 g/mL. However, it is known that PZQ causes a quick calcium influx followed by contraction, paralysis and tegument destruction. Nevertheless, that process was not completely clarified (Doenhoff et al., 2008).

Whereas that the inverted optical microscopy does not allow to detail the tegumental changes presented in the parasite, a qualitative analysis to evaluate the tegumental damage of specimens after culture in vitro, through the scanning electron microscopy (SEM) was used in this study. According to Sert and Giller (1977), the SEM allows to observe details of the morphology of the tegument of S. mansoni making it possible to interpret its functionality. This technique has been employed by several authors (e.g., El Ridai et al., 2012; de Oliveira et al., 2012; Neves et al., 2011; Oliveira et al., 2013) in order to elucidate the mechanisms of action of drugs/compounds used in the experimental treatment of schistosomiasis.

### Table 2

Effects of B. trimeria efficacy against Schistosomula (03 days PI), juvenile (30 days PI) and adult (60 days PI) S. mansoni BH strain.

<table>
<thead>
<tr>
<th>Infection</th>
<th>Samples</th>
<th>Single dose (mg/kg)</th>
<th>Mean worm burden ± SE (liver and portal-mesenteric)</th>
<th>Total worm burden% reduction</th>
<th>Number of eggs/g faeces % reduction</th>
<th>% Egg developmental stage ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Couples</td>
<td>Total</td>
</tr>
<tr>
<td>Schistosomula infection</td>
<td>Dichloromethane extract</td>
<td>200</td>
<td>4 ± 0.91</td>
<td>1.6 ± 0.21</td>
<td>5.4 ± 0.80</td>
<td>15.7 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>Aqueous fraction</td>
<td>200</td>
<td>2.3 ± 0.49</td>
<td>0.7 ± 0.21</td>
<td>5.7 ± 0.63</td>
<td>14.2 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>Praziquantel</td>
<td>PBS</td>
<td>4.3 ± 0.86</td>
<td>2.8 ± 0.71</td>
<td>8.3 ± 0.78</td>
<td>23.7 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>Control infected</td>
<td></td>
<td>6.7 ± 1.1</td>
<td>2.6 ± 0.61</td>
<td>8.8 ± 0.89</td>
<td>27 ± 2.4</td>
</tr>
<tr>
<td>Juvenile infection</td>
<td>Dichloromethane extract</td>
<td>200</td>
<td>5.4 ± 1.60</td>
<td>1.0 ± 0.27</td>
<td>4.9 ± 1.12</td>
<td>14.9 ± 2.07</td>
</tr>
<tr>
<td></td>
<td>Aqueous fraction</td>
<td>200</td>
<td>3.9 ± 0.86</td>
<td>0.2 ± 0.2</td>
<td>3.9 ± 0.56</td>
<td>11.9 ± 1.40</td>
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<td></td>
<td>Praziquantel</td>
<td>PBS</td>
<td>5.9 ± 1.17</td>
<td>2.5 ± 0.84</td>
<td>10.6 ± 1.11</td>
<td>29.6 ± 2.10</td>
</tr>
<tr>
<td></td>
<td>Control infected</td>
<td></td>
<td>12.9 ± 1.1</td>
<td>9.9 ± 1.20</td>
<td>13.1 ± 1.06</td>
<td>47.6 ± 3.27</td>
</tr>
<tr>
<td>Adult infection</td>
<td>Dichloromethane extract</td>
<td>200</td>
<td>5.6 ± 0.6</td>
<td>3.6 ± 0.7</td>
<td>5.6 ± 0.7</td>
<td>20.1 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>Aqueous fraction</td>
<td>200</td>
<td>4 ± 0.7</td>
<td>1.8 ± 0.1</td>
<td>8.3 ± 1</td>
<td>22.5 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>Control infected</td>
<td></td>
<td>4.5 ± 1.00</td>
<td>2.2 ± 0.59</td>
<td>6.0 ± 0.74</td>
<td>11.5 ± 1.52</td>
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<tr>
<td></td>
<td>Aqueous fraction</td>
<td>200</td>
<td>4.4 ± 0.85</td>
<td>1.8 ± 0.44**</td>
<td>7.1 ± 0.65</td>
<td>12.9 ± 0.73**</td>
</tr>
<tr>
<td></td>
<td>Praziquantel</td>
<td>PBS</td>
<td>3.6 ± 0.70</td>
<td>2.3 ± 0.80</td>
<td>8.3 ± 0.77</td>
<td>23.3 ± 2.08</td>
</tr>
<tr>
<td></td>
<td>Control infected</td>
<td></td>
<td>5.2 ± 1.8</td>
<td>2.9 ± 0.7</td>
<td>7.6 ± 1.6</td>
<td>21.5 ± 3.2</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SEM. Number of animals/group = 10.

The difference was significant "P < 0.01 and ""P < 0.001.

### Table 3

Granuloma diameter (μm) in the liver of the treated and non – treated groups 60 day PI.

<table>
<thead>
<tr>
<th>Sample and doses</th>
<th>Granuloma diameter average (μm) ± SE</th>
<th>Number of granulomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dichloromethane extract 40 mg/kg</td>
<td>263 ± 21.4</td>
<td>61*</td>
</tr>
<tr>
<td>Dichloromethane extract 200 mg/kg</td>
<td>216 ± 5.85**</td>
<td>45**</td>
</tr>
<tr>
<td>Aqueous fraction 40 mg/kg</td>
<td>261 ± 19.4*</td>
<td>50**</td>
</tr>
<tr>
<td>Aqueous fraction 200 mg/kg</td>
<td>253 ± 7.89**</td>
<td>51**</td>
</tr>
<tr>
<td>Praziquantel 40 mg/kg</td>
<td>261 ± 6.29</td>
<td>60*</td>
</tr>
<tr>
<td>Praziquantel 200mg/kg</td>
<td>267.5 ± 19</td>
<td>66</td>
</tr>
<tr>
<td>Control infected untreated</td>
<td>291 ± 5.9</td>
<td>129</td>
</tr>
</tbody>
</table>

The difference was significant "P < 0.01 and ""P < 0.001.

### 6. Discussion

Decreases in granuloma sizes with minimal degenerative changes in the liver tissue were observed in the liver of different treated groups. Considering the histologic analysis of the liver samples, extracted from the animals treated with DE and AF (40 and 200 mg/kg) there was a significant reduction (p < 0.001) in relation to the number of perivascular granulomas formed around the degenerate egg. At the same time, there was a reduction in the size of the granulomatous inflammation, indicating the anti-inflammatory effect of B. trimer. Thus, the hepatic granuloma average diameter was significantly smaller (p < 0.01) in the groups treated with DE and AF, in comparison to the group treated with (40 and 200 mg/kg) of PZQ (Table 3). The histologic observations on the livers from the untreated infected control group, evidenced a chronic granulomatous inflammation characterized by the presence of granulomas altogether with damaged eggs, as well as a large inflammatory infiltration (lymphocytes, macrophages, eosinophils) and fibrous tissue. Fig. 4 shows the effect of the treatment with DE, AF, and PZQ on groups of untreated infected animals.
The main changes induced by treatments with samples ED and AF are related to damage in suckers, oral and acetabular in both male and female schistosomes. Additionally, qualitative analysis showed that samples caused a extensive peeling of the tegument especially in the dorsal region, resulting in the exposure of the antigens of this surface. Furthermore, blebs were visible on the tegument of the parasite, may be accompanied by an increasing of the exposure to antigens on the surface of the worm. Those changes are identified and connected with the host immune response, required to complement the activity of the drug (Brindley et al., 1989; Doenhoff et al., 1987). For this reason, this structure has been investigated in the development of new antischistosomal drugs since the late 40s to the present days.

The in vivo treatments against immature and adult worms of \( S. \) mansoni modify the proportion between male and female parasites and cause the reduction of the density of the eggs eliminated in the faeces and the ones found in the treated animals intestinal tissue, as well as the number of hepatic granulomas. In this study, we also evaluated the effect \textit{in vivo} of praziquantel on schistosomula, juveniles and adults worms. However, the treatments with the usual dose (40 mg/kg) presented a low worm burden reduction corroborating with the study of Utzinger et al. (2003) which shows that the schistosomulas are not susceptible to PZQ. Additionally, it was possible to observe that DE and AF \textit{in vivo} treatments against schistosomula and juveniles worms with a single dose of 200 mg/kg present superior results when compared to the pharmacological control group, treated with PZQ. Thus, considering PZQ as a pure synthetic drug, we can infer that the DE and AF richness of the compounds brings a synergic effect potentializing its schistosomicidal action.

Comparable results were obtained by previous trials \textit{in vitro} and \textit{in vivo} conducted by the other authors; using compounds isolated from species (e.g., Pipilartina), isolated from \textit{Piper tuberculatum}, \textit{Artemether} and 8-Hydroxyquinoline derivatives from \textit{Artemisia annua} and have demonstrated activity against schistosomula and juvenile worms of \( S. \) mansoni. However, the mechanisms of action are not elucidated (Allam et al., 2013; El-Lakkany et al., 2013, Moraes et al. 2012a).

Treatments \textit{in vivo} with \( B. \) trimera showed greater sensitivity on the female worms. It is known that schistosomes, males and females, respond differently to therapy concerning the drug activity. However, the present study corroborates with Mostafa et al. (2011) who observed an increased sensitivity of the female worms \( S. \) mansoni after the treatment with 500 mg/kg of the aqueous extract of ginger (\textit{Zingiber officinale}). Nour et al. (2006) evaluated the efficacy of methanol extract of \textit{Randia Nilotic}, \textit{in vivo}, and observed that the female worms are more susceptible to treatment. Lescano et al. (2004) observed the effect of artemether and reported that females were more sensitive to treatment than male worms. In studies with PZQ, Delgado et al. (1992) observed in their experiments that the \textit{in vivo} concentration of 250 mg/kg, administered 6 weeks after infection, acts preferentially on females of this parasite. In contrast, Drescher et al. (1993), Connert and Andrews (1977), Pellegrino et al. (1977) and Mehlhorn et al. (1981) describe that the subcurative doses of PZQ act in the same proportion on males and females, with no significant differences in the therapeutic sensitivity to either genders.

According to Harder (2002), the death of adult schistosomes, owing to the conducting treatment with drugs/compounds with anti-\( S. \) mansoni action, is assigned to metabolic disorders as well as mechanical and muscle destruction of worms after exposure to drugs. Furthermore, it is noteworthy that the susceptibility of this parasite is associated with biological factors such as age, sex, host and evaluated strain. Such factors may interfere with susceptibility of infection, fecundity and consequently the production of eggs (Incani et al., 2001; Liang et al., 2010).

Considering the above, the \textit{in vitro} treatments suppressed the oviposition of all the concentrations evaluated and the \textit{in vivo} treatments presented a reduction in the number of eggs eliminated in the faeces after treatments against juvenile and adult worms to both DE and AF treatments besides the demonstration of a significant difference in the percentage of mature eggs kept on the intestinal tissue of the treated animals (Table 2) when compared to the percentages of the control groups, treated with PZQ and infected untreated. Pellegrino et al. (1962) reported that mice infected with \( S. \) mansoni have started eliminating eggs around the 30th day of infection. However treatments performed before and after infection are important at this period. Our results are confirmed by Pra-ta (1957) in a classic theory which says that if the oviposition ceases are reduced within the evaluated drug administration, the oogram must show more mature eggs than immature ones, as a result of anterior oviposition.

The results presented, concerning the reduction of eggs, should be considered extremely significant once the egg is responsible for...
the transmission of the parasite and maintenance of its biological cycle interfering, thus, in the epidemiology of schistosomiasis in endemic areas. Furthermore, according to Gryseels et al. (2006) the pathology of the schistosomiasis is not directly associated to the presence of the adult worm, but to the large number of eggs that are stuck in the host’s tissues and organs, especially the liver, triggering an inflammatory process called granulomas.

Finally, the study of the activity of a drug/compound on the granulomatous reaction that forms around the eggs of S. mansoni is very important once it is responsible for the major pathogenesis of a severe chronic phase of schistosomiasis characterized by fibrosis, especially in the liver tissue (Lichtenberg, 1964). According to Friedman (2008), the process of fibrosis in the granulation tissue of the liver, induced by the release of egg antigens of S. mansoni, depends on the activation of a particular cell type called stellate parasinusoidal cell, known as the major source of fibrillar collagens.

The histopathological analysis of the livers of animals treated with ED and AF after 60 days of infection showed a reduction of the number of granulomas and significant differences between the average diameters, compared to the groups treated with praziquantel and the negative infected and untreated control group. Classic investigations such as done by Doenhoff et al. (1986) report that there is a significant correlation between the numerical decrease in the diameter of the hepatic granulomas and the number of eggs eliminated in the faeces, both mediated by specific cells of the host. This study showed a percentage reduction of 91% and 76% of eggs eliminated in the faeces for the samples DE and AF, respectively, confirming the observations made by Doenhoff et al. (1986). Badawy et al. (1991) report that the reduction in diameter of hepatic granuloma may be related to the reduction of pro collagen type III, responsible for the formation of granulomas.

We believe that the reduction of granulomas and granulomatous infection of the animals treated with ED and AF, is due to the fact that B. trimera presents pharmacological actions such as anti-inflammatory and hepatoprotective activities (Soike and Leng-peschlow, 1987; Gené et al., 1996). Another factor that could promote the reduction of granulomas would be the inhibition of soluble antigens of the parasite eggs (SEA), resulting in the inactivation or the immune response reduction of the host, mediated by T cells (TDH), Th1 and Th2 lymphocytes, what mediates the development of the granulomatous inflammatory response (Wynn and Cheever, 1995).

7. Conclusion

According to the results, the crude dichloromethane extract and its respective aqueous fraction, extracted from B. trimera, caused significant schistosome mortality through tegument, morphological alterations, and, also, oviposition reduction when females were exposed to in vitro assays. However, the treatments in vivo with a single dose against different stages of the schistosomula, juveniles and adults of S. mansoni, caused a reduction in the parasite burden in the treated animals, as well as the reduction of the viable eggs elimination, decreasing the number of hepatic granulomas. Therefore, this study opens up perspectives for future researches on the substance or the compound isolation, making possible the elucidation of the mechanism of action of B. trimera what could be used as a potential new treatment alternative against S. mansoni.

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References


