EFFECT OF *Piper tuberculatum* EXTRACT ON ADULT *Schistosoma mansoni*: *in vitro* AND *in vivo* TESTS

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ABSTRACT

Schistosomiasis is a severely neglected disease with a wide geographical distribution. It affects approximately 210 million people in the world and at least 800 million people live in risk areas. The search for new drugs to treat this parasitosis is significant due to the appearance of strains of the worm that are resistant to the currently available drugs. The retrieval of compounds extracted from plants that act on these parasites has increased scientific investigation of this subject. The present study demonstrates, *in vitro* and *in vivo,* the action of crude extract of *Piper tuberculatum* on adult *Schistosoma mansoni*. The extract was shown to be quite effective in the *in vitro* tests, causing soft tissue alterations and acting on the reproductive system of females and the mortality of the worms, with a greater effect on males. The *in vivo* experiment was performed with infected *Mus musculus* and a decrease in the number of eggs in the first and second oogram stages was found, suggesting action on oviposition.

KEYWORDS: *Schistosoma mansoni; Piper tuberculatum; in vitro; in vivo*

RESUMO

Efeito do Extrato de *Piper tuberculatum* sobre adultos de *Schistosoma mansoni*: testes *in vitro* e *in vivo*

A esquistossomose, doença negligenciada grave e de larga distribuição geográfica, atinge cerca de 210 milhões de pessoas no mundo e ao menos 800 milhões vivem em área de risco. A busca de novos medicamentos para o tratamento desta parasitose é relevante em razão do aparecimento de linhagens do verme resistentes aos fármacos disponíveis. A obtenção de compostos extraídos de plantas com ação sobre parasitos tem incrementado a investigação científica sobre este assunto. O presente trabalho mostra a ação *in vitro* e *in vivo* do extrato bruto de *Piper tuberculatum* sobre adultos de *Schistosoma mansoni*. O extrato mostrou-se bastante eficaz nos ensaios *in vitro*, provocando alterações tegumentares, tendo ação no sistema reprodutor das fêmeas e na mortalidade dos vermes

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com maior ação sobre os machos. Como resultado em experimentos *in vivo*, realizados em *Mus musculus* infectados, observou-se a diminuição do número de ovos de 1º e 2º estágios em oogramas, o que sugere uma ação na postura de ovos.

DESCRITORES: *Schistosoma mansoni, Piper tuberculatum, in vitro, in vivo.*

INTRODUCTION

Schistosomiasis is a neglected disease that is found in 76 countries and affects approximately 210 million people. It is estimated that 800 million people currently live in risk areas. Considering its mortality rates, it is the second most common parasitic disease in the world, second only to malaria (Steinmann et al., 2006). The disease is caused by worms of the *Schistosoma* genus. Treatment involves the use of oxamniquine (only in cases of schistosomiasis caused by *S. mansoni* in Brazil) and praziquantel (for cases caused by *S. mansoni*, *S. japonicum* and *S. haematobium*) (Cunha, 1992; Katz, 2008). The rare use of oxamniquine currently is due to the related side effects and the fact that there are reports of human strains of the parasite that are resistant to the drug (Dias et. al*.*, 1978; Katz, 2008). Praziquantel has been the drug of choice to replace oxamniquine. Strains of the parasite that are resistant to praziquantel have been confirmed in animal experiments in Ourinhos, SP (Bonesso-Sabadini & Dias, 2002) and in human experiments (Fallon & Doenhoff, 1994). Field observations in Egypt and Senegal have persistently shown the elimination of eggs in the feces of individuals treated for the disease (Cioli, 1998).

People worldwide use plants and their extracts to take care of their health. Studies of medicinal plants and their extracts have been increasing as a result of their easy accessibility, availability and low cost (Kaur et al., 2005; Varanda, 2006).

Piper tuberculatum, commonly known as long pepper or arda pepper, is a species representative of the Brazilian *Piperaceae*. This family is primarily tropical. In Brazil, there are more than 500 species, mostly of the genera *Piper* and *Peperomia*. Species of the *Piper* genus have been widely used in folk medicine. The biological activities observed include anti-tumoral properties (Duh et al., 1990), sedative action and anti-venom properties (Araújo-Junior et al., 1997).

In *in vitro* studies, *Piper* species have shown promise as leishmanicidal and trypanosomicidal drugs, exhibiting low toxicity on the cells and satisfactory activity against the parasites (Ruiz et. al., 2004; Gallego et. al., 2006). Extract from the fruit of *P. tuberculatum* demonstrated a powerful toxic effect against epimastigotes of *Trypanosoma cruzi* (Regasini et al., 2009).

In vitro tests are important because they can directly expose the worm to the effect of the substance to be tested. This step has the function of selecting substances which may have some biological effect so it can later be tested whether the effect is repeated *in vivo*. This type of experiment has previously been used to observe the effect of artemether compound on *S. japonicum* with additional

experiments to be performed at a later stage (Xiao et al., 2003). Recently, *in vitro* studies conducted with piplartine, an amide that can be isolated from several plants of the *Piper* genus, have shown schistosomicidal action in adults (Moraes et al., 2011) and in schistosomula (Moraes et al., 2012). Since the search for new drugs does not always produce correlation between the efficacy of schistosomal compound *in vitro* and *in vivo* tests (Katz, 2008), the importance of the two types of tests in studies seeking an effective way of combating the disease is significant.

Considering the need for new drugs to treat schistosomiasis mansoni and the promising effects of plant extracts on the worm, the aim of the present study was to confirm the *in vitro* and *in vivo* effects of crude *P. tuberculatum* extract on *S. mansoni*.

MATERIALS AND METHODS

S. mansoni strain and its storage in the laboratory: The BH strain of *S. mansoni* was used, maintained by successive passage in sympatric *Biomphalaria glabrata* and Swiss female mice SPF, provided by the *Centro Multidisciplinar de Investigação Biológica* (CEMIB) in the UNICAMP (with approval of the committee of ethics in animal experimentation, protocol number 1249-1). In order to ensure bisexual infection in the mice, with a satisfactory balance between the number of male and female worms, the cercariae were derived from 50 infected mollusks. The cercariae were used to infect the mice and obtain adult worms for the *in vitro* experiments and also to infect the mice in the *in vivo* experiment. This parasite maintenance is performed routinely in the laboratory of Helminthology of the Department of Animal Biology in the Institute of Biology in the Campinas State University (UNICAMP), Brazil.

Preparation of the extracts: After drying (firstly at room temperature and afterwards in a greenhouse at 45 ^{0}C), the plant was mechanically crushed using a slicer to obtain the crude extract. Dichloromethane (2:1) was used to dilute an extract obtained x3 from the crushed plant with methanol and the extracts were maintained at -20 ^{0}C until use. This process was carried out by Dr. Massuo Jorge Kato in the Laboratory of Chemistry of Natural Products in the USP – SP, Brazil.

The extracts used in the experiments were diluted to be administered in liquid form in a single oral application. Dimethyl sulfoxide (DMSO) possesses certain therapeutic properties, including an anti-inflammatory effect (Santos et al., 2003), and was used as the diluent for the *P. tuberculatum* extract.

In vitro test of the *P. tuberculatum* extract: The adult worms were collected from the mesenteric portal system by perfusion (Yolles et. al., 1947) from infected mice previously infected with cercariae derived from 50 *B. glabrata* snails. The worms were washed in RPMI 1640 medium to remove the blood of the host and then studied with a magnifying glass to select the undamaged worms. They were then transferred to 24 well tissue culture plates, each of which contained 2 mL of the

RPMI 1640 medium and serial solutions of *P. tuberculatum* extract in the following concentrations: 25; 50; 100; 250; 500; 750; 1,000 and 2,000 µg/mL. Five couples of the worm were used for each concentration of the extract. The culture plates were maintained in darkness at $37\,^0C$, with 5% CO_2. The worms were examined immediately after exposure to the extract, two hours later and then every 24 hours up to a total of 96 hours (or until the worms died). Eight couples of *S. mansoni,* maintained in the RPMI 1640 medium were used as controls. Damage to the integument, extravasation of the worm's internal contents and movement (internal and external) were assessed in order to confirm the mortality of the worms (Oliveira et. al., 2004).

Infection of the mice and *in vivo* test of the *P. tuberculatum* extract: Five groups of six mice were assembled. The mice were weighed and separated so that each group had a stipulated mean weight of 19 grams. The *P. tuberculatum* were administered to the mice by gastric tubing as follows: 500 mg/kg (group A); 1,000 mg/kg (group B); 2,000 mg/kg (group C); 4,000 mg/kg (group D). The fifth group (group E) received saline solution with DMSO 2%. Each mouse received 0.3 mL of the solution corresponding to their group. The objective was to establish a dose/response curve that showed the toxicity of the extract and the sensitivity of the mice, observing directly if the animals showed any adverse reactions such as regurgitation, diarrhea, alopecia, itching, loss of appetite or weight loss.

After determining the dose/curve response, a dose was selected to be used in the *in vivo* tests with mice infected with *S. mansoni*. Thirty Swiss female mice (30 days old and approximate weight) were used. The mice were separated randomly into three groups of ten animals: Control Group: infected animals that received no treatment; Piper Group: infected animals that, after 45 days of infection, received 0.3 mL of a solution containing 252 mg/mL of *P. tuberculatum* extract diluted in saline with 2% DMSO; DMSO Group: infected mice that were treated with a saline solution with 2% DMSO after 45 days of infection.

Mice were infected with 100 cercariae by exposing the tail to larvae for a period of 2 hours under light and heat (60W incandescent lamp and temperature of 28 ºC). After this time, the number of penetrating cercariae was confirmed (Magalhães, 1969). The extract was administered 45 days after infection through a gastric tube. Before administering the extract, feces of the rodents were collected to confirm the number of *S. mansoni* eggs using the Kato-Katz method (Katz et al., 1972). A microscopic slide was prepared for each animal and all animals were examined. Sixty days after the infection, the mice were euthanized by cervical displacement in order to recover the worms from the mesenteric-portal system, confirming the presence of the worms in the intra-hepatic branches of the portal vein, the portal vein itself and the mesenteric veins. After the worms were removed, a segment of the ileum of each mouse was used for oogram tests (Cunha & Carvalho, 1966), in order to count and compare the stages of development and the viability of the eggs (Pellegrino & Faria, 1965). At this time, the numbers of granuloma in the liver and eggs in the feces were also confirmed (Zanotti-Magalhães et al., 1993).

In order to confirm the number of granulomas in the liver, a small fragment of the organ was fixed in aqueous Bouin prior to making histological sections, which were 5 μm thick and were stained with Masson's trichrome. The granulomatous reactions around the *S. mansoni* eggs were counted and measured. The granulomas were measured using a light microscope and a computer program for this purpose (IM50/Leica) and the counting of granulomas was performed per microscopic field, the examined area corresponding to 0.9847 mm^2.

RESULTS

In vitro test: *P. tuberculatum* acted effectively on *S. mansoni*, even at low concentrations. Complete mortality of the worms occurred after 72 hours of observation. The death of the worm was confirmed by the lack of motility, the lack of adhesion of the suction cups on the plate, and by contraction of the body and the digestive tract. Males were more sensitive to the treatment than females (Table 1) and died more rapidly. In the statistical analysis perfomed through the multiple comparison test - Duncan's group - in which the mortality of the worms was compared, certain concentrations acted equivalently in terms of the mortality of worms (Table 2). Seventy-two hours after the experiment began, when all of the worms tested with the plant extract had died, 10 worms from the 16 initial worms were still alive in the control group (five males and five females). Figure 1 shows the damage that the treatment with the extract of *P. tuberculatum* caused in females (Figure 1- A, B, C) highlighting the damage to the tegument, the reproductive system, particularly at the vitelline glands and in the development of the eggs when compared with the control group (Figure 1 D). The action of the extract in males can be seen in Figure 2, with the damage mainly to the tegument (Figure 2- A, B, C and D) when compared with the worms from the control group (Figure 2- E and F).

Table 1.   Number of live male (M) and female (F) *S. mansoni* after *in vitro* exposure with different concentrations of *P. tuberculatum* extract

Concentration (μg/mL)	0 hours M	0 hours F	2 hours M	2 hours F	24 hours M	24 hours F	48 hours M	48 hours F	72 hours M	72 hours F
2,000	5	5	0	0	0	0	0	0	0	0
1,000	5	5	0	0	0	0	0	0	0	0
750	5	5	0	2	0	0	0	0	0	0
500	5	5	1	5	0	0	0	0	0	0
250	5	5	4	5	0	0	0	0	0	0
100	5	5	4	5	0	2	0	1	0	0
50	5	5	5	5	1	4	0	2	0	0
25	5	5	4	5	2	5	0	2	0	0

In vivo test: None of the mice exhibited adverse reactions (regurgitation, diarrhea, alopecia, itching, loss of appetite or weight loss) to the doses administered to determine a dose/response curve to the *P. tuberculatum* extract in healthy animals. They were all alive and healthy after 10 days of observation.

Table 2.   *In vitro* Duncan group test applied with concentrations of *P. tuberculatum* extract to kill the worms of *S. mansoni* (male and female)

Concentration (µg/mL)	Duncan Mean		Duncan Grouping			
D1 (2000)	4.0	A				
D2 (1000)	4.0	A				
D3 (750)	3.8	A	B			
D4 (500)	3.4	A	B	C		
D5 (250)	3.1		B	C	D	
D6 (100)	2.9			C	D	
D7 (50)	2.3				D	
D8 (25)	1.4					E

Concentrations followed by same letter do not differ significantly (α = 0.05)
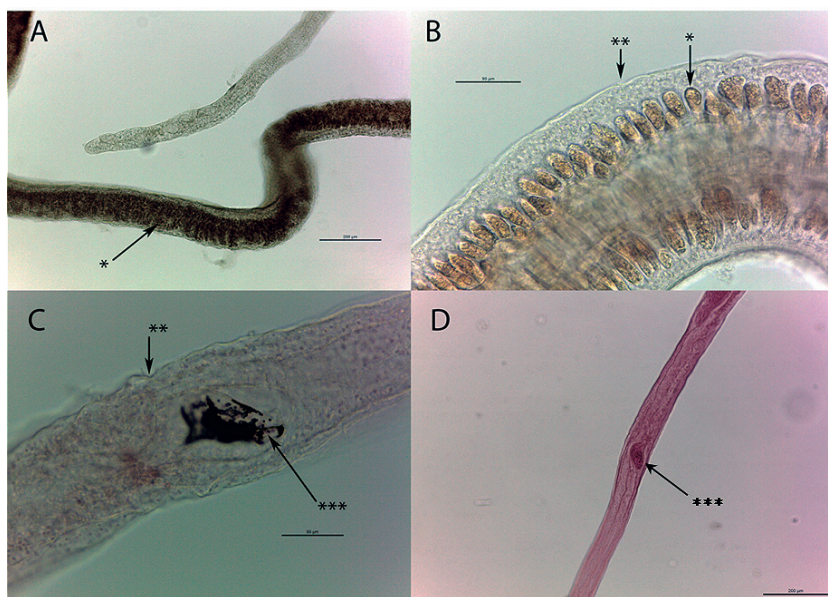
Notice the changes at the vitelline gland (*) at the integument (**) and at the formation of the egg (***) after the treatment. In C, normal aspect of the egg (***)

Figure 1.   Females of *S. mansoni*, *in vitro* test, 24 hours after the application of the extract of *P. tuberculatum*: A (2,000 µg/mL); B (750 µg/mL); C (500 µg/mL) and C (control group, without application of the extract, 72 hours).

There was no significant difference (p=0.12) between the treatment types (Control, Piper and DMSO) in terms of the number of *S. mansoni* eggs eliminated in the feces before and after treatment. In all three treatment types, there were more males than females in the liver (p=0.05). There was also no significant difference in

the number of worms found in the portal vein (p=0.55) or in the mesenteric veins (p=0.88), when comparing the treatment types (Table 3). The three groups showed statistically similar parasite loads.

The size of the area of hepatic granuloma was measured by a photomicroscope (Image Manager 50 software) and no significant difference was found between the size of the granuloma found in the Piper and DMSO treatment groups (p=0.761), although both exhibited significantly smaller granulomas than the control group (p=0.022). No significant difference was found between the mean number of hepatic granulomas in the three treatment types tested (p=0.97). Table 3 displays these results.

Table 3.   Mean and standard deviation of the parameters analyzed in the *in vivo* tests, when compared to the groups of mice treated with *P. tuberculatum* extract, as well as the DMSO and Control groups

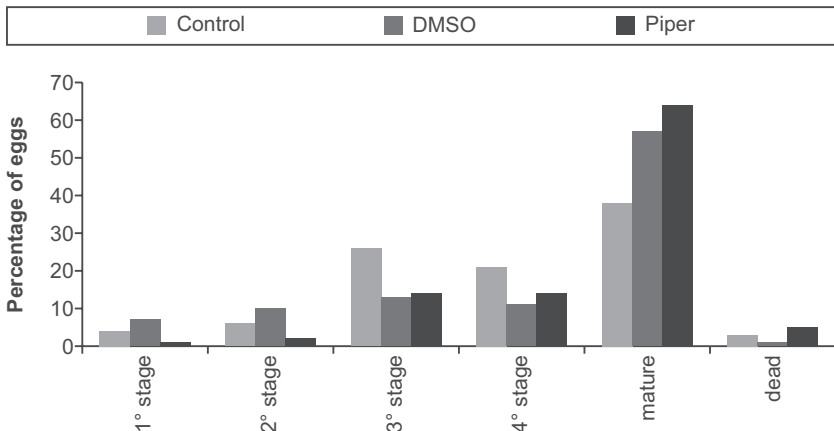|	*P. tuberculatum*	DMSO	Control
Male worms (liver)	2.66 ± 1.73	1.125 ± 0.64	3.875 ± 1.45
Female worms (liver)	2.11 ± 0.92	1.125 ± 0.64	2 ± 0.92
Male worms (Portal vein)	6.33 ± 2.54	5.875 ± 1.64	9 ± 5.92
Female worms (Portal vein)	7.77 ± 3.76	6 ± 2.77	6.87 ± 4.01
Male worms (Mesenteric veins)	14.88 ± 3.37	11.75 ± 3.80	18.25 ± 5.92
Female worms (Mesenteric veins)	16.66 ± 4.24	11.87 ± 4.88	15.87 ± 4.15
Total number of worms	50.44 ± 8.06	37.75 ± 6.69	55.87 ± 16.31
Number of eggs before treatment	2,567.34 ± 640.61	872.01 ± 415.79	2,855.68 ± 1,454.62
Number of eggs after treatment	3,653.52 ± 2,168.28	1,302.28 ± 476.86	4,978.46 ± 3,563.90
Number of hepatic granuloma	6 ± 0.86	6 ± 1.65	5.87 ± 0.99
Area of the hepatic granuloma	121,350.33± 37,672.38	124,197.54 ± 47,407.16	145,955.8± 44,876.11

The number of worms shown takes into account the gender, location and total quantity of the worms, the number of eggs per gram of feces before treatment (45 days of infection) and after treatment (60 days of infection). It also shows the number of hepatic granulomas in 0.9847 μm^2 of tissue (area of the optical microscope objective in which the histological cuts of the liver were examined) and the size of the area (in μm^2) of the hepatic granuloma analyzed.

In the analysis of the number of eggs per stage and per treatment (oogram), a difference was found in the proportion of the number of eggs per stage and per treatment (p=0.02). For eggs in stage 1, the differences were as follows: Piper group *vs.* DMSO group: p=0.03; Piper group *vs.* Control group: p=0.02. For eggs in stage 2, the differences were as follows: Piper group *vs.* DMSO group: p=0.01; Piper group *vs.* Control group: p=0.01. Figure 3 displays these results.
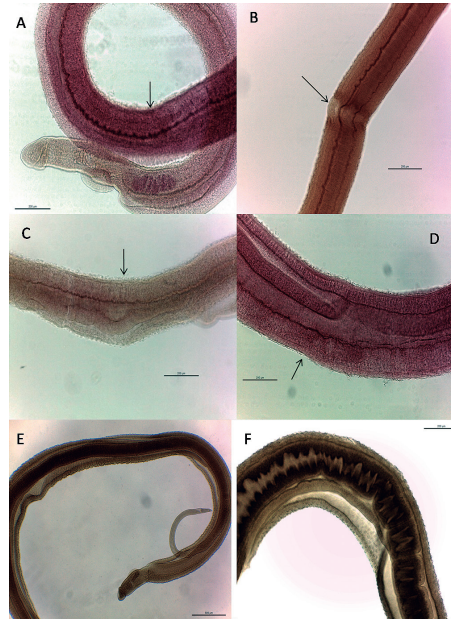
Note the changes in the integument (arrows).

Figure 2. Comparative micrographs of male *S. mansoni* after being submitted to *in vitro* tests for groups treated with *P. tuberculatum* at A (50 µg/mL after 24 hours); B (750 µg/mL after 48 hours); C (1,000 µg/mL after 2 hours) and D (100 µg/mL after 24 hours) and the control group, which was alive after 72 hours (E and F).

Figure 3. *In vivo* test - Oogram: mean number of eggs per stage and per treatment, in the control, DMSO and *Piper tuberculatum* groups

DISCUSSION

P. tuberculatum extract acted effectively on the BH strain of S. mansoni in the in vitro tests. The sensitivity of the parasite was greater when the concentration of the extract was greater. The worms died within 72 hours. In the highest concentrations (2,000 and 1,000 µg/mL), death occurred in the first two hours. Studies conducted with praziquantel have reported that the mortality of the worm was also higher with higher doses, and lower doses took longer to damage and destroy adult S. mansoni worms (Xiao et al., 1985).

The integument of the worm has been considered a promising target for schistosomicidal drugs. Alterations in the integument of the parasite are described after the use of drugs such as praziquantel, causing swelling in certain structures, vacuolization, blisters and surface demarcation. Compounds isolated from the extract of the Artemisia annua plant have been shown to be capable of damaging the integument in two strains of S. mansoni (Frezza et al., 2013), demonstrating the potential of tests with plant extracts and their compounds. The in vitro tests conducted in the present study showed that P. tuberculatum extract damaged the integument of the worms. The extract exhibited different forms of S. mansoni mortality depending on the gender. Males were more sensitive than females (Table 1). The fact that the females remain sheltered in the interior of the gynecophoric canal of the males may have protected them from the effect of the extract. However, this hypothesis was not shown to apply in tests with praziquantel and artemether (Shushua et al., 2000), which led to equal mortality rates among males and females. Shaw and Erasmus (1983) described the mechanism of action of praziquantel on S. mansoni. They used electronic scanning microscopy with worms collected from in vivo tests and noted that male worms exhibited damaged integument more rapidly than females.

Based on the difference between the concentration of compounds in different parts of the plant studied (Soberòn et al., 2006), and bearing in mind the potential action demonstrated by P. tuberculatum on S. mansoni, it is possible to suggest further studies using extracts produced separately with leaves, stems and inflorescence.

In the in vivo tests, P. tuberculatum was not toxic to mice, even in high concentrations, and it was not possible to determine the lethal dose for the rodents. Cytotoxicty tests on the extract of P. tuberculatum were described in literature and presented reduction of the cell viability at a concentration of 30 µg/mL (Moraes, 2011).

The 30 mice used in the in vivo experiment eliminated a similar number of eggs in their feces in the three groups tested. The treatment did not affect this parameter and did not affect the distribution of the worms in the portal vein and the mesenteric veins.

It was not possible to detect differences in the number of eggs in the stool, but examining the oogram, which is not a quantitative but a qualitative test, there was the least amount of eggs at the early stages in the treated group, which may suggest a delay in the oviposition.

Dimethylsulfoxide (DMSO), when used as a diluent of the extract, was tested separately in order to compare and isolate its anti-inflammatory properties and to discover if it interfered in the effect of the *P. tuberculatum*. Its action can be seen in the decrease in the area of hepatic granulomas in the two treatment types that contained DMSO.

Similar to the number of eggs in the feces, there was no significant difference in the number of granulomas in the liver when counted in the histological analysis of the three treatment types.

The decreased number of eggs in the 1st and 2nd stages of the animals treated with *P. tuberculatum* extract indicates action on oviposition, which is a significant result considering that only one dose was applied. It is also significant that any alteration in the oviposition of *S. mansoni* must be considered, due to the importance of the role of eggs in the pathogenesis and dissemination of the species. The results obtained by a single dose of *P. tuberculatum* with mice corroborate the alterations observed in the vitelline glands of the females treated with extracts in the *in vitro* experiments.

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