Parasitology

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Antiparasitic effects of ethanolic extracts of *Piper arboreum* and *Jatropha gossypiifolia* leaves on cercariae and adult worms of *Schistosoma mansoni*

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Research Article

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Abstract

New treatment strategies for schistosomiasis should be evaluated, since resistant strains to the only available drug, Praziquantel, have already been described. Thus, we demonstrated antiparasitic effects of ethanolic extracts of *Jatropha gossypiifolia* and *Piper arboreum* on cercariae and adult worms of *Schistosoma mansoni*. The bioassays were performed at 0–10 000 $\mu g\ mL^{-1}$ concentration for 0–72 h. Adult worms were stained with carmine to assess external and internal damage. The chemical screening was performed using high-performance liquid chromatography. *P. arboreum* displayed the best cercaricidal effect, with a 100% reduction in viability in just 60 min. The extract of *J. gossypiifolia* was more effective against adult worms, with 100% viability reduction of male and female worms after 12 and 24 h, respectively. *P. arboreum* and *J. gossypiifolia* were equally effective in inhibiting the oviposition of *S. mansoni* (93% reduction) and causing damage to internal and external structures in adult worms. Flavonoids were identified in both the extracts and phenolic compounds and amides only in *P. arboreum*. Thus, for the first time, it was proven that ethanolic extracts of *P. arboreum* and *J. gossypiifolia* leaves are biologically active against cercariae and adult worms of *S. mansoni in vitro*.

Introduction

Schistosomiasis is a parasitic disease caused by helminths of the genus *Schistosoma*. Currently, an estimated 200 million people in tropical and subtropical regions are infected, with up to 700 million people living in endemic areas at risk of infection and 200 000 deaths occurring annually (Steinmann *et al*., 2006; WHO, 2018; Verjee, 2019). Hence, there is an urgent need to develop new treatment strategies to treat such a large number of infected individuals. The only drug indicated for the regular treatment of schistosomiasis is Praziquantel (PZQ), which has been used since the 1970s (Cioli *et al*., 2014; Neves *et al*., 2015; Bergquist *et al*., 2017). It was the first drug to demonstrate good activity when administered orally or intramuscularly, against all *Schistosoma* species that infect humans (Cioli *et al*., 1995, 2014; Melman *et al*., 2009). However, PZQ has low efficacy against immature forms of the parasite (Pica-Mattocci and Cioli, 2004; Oliveira *et al*., 2014) and several reports, mainly of resistance of *S. mansoni* strains to the drug have already been confirmed (Fallon *et al*., 1995; Ismail *et al*., 1996; Picquet *et al*., 1998; Crellen *et al*., 2016). In addition to the treatment of infected individuals, blocking contact of people with infectious larvae (cercariae), which are shed on a large scale in freshwater bodies by schistosome vector snails (e.g. *Biomphalaria*, *Bulinus*, *Oncomelania*) (Steinmann *et al*., 2006), could help to avoid infection. However, to date, no chemical compounds have been approved or are currently available for this purpose.

Secondary metabolites (such as crude extracts and essential oils) are present in different parts of plant species; these natural products can be used as prototypes for the development of new antiparasitic drugs (Kaiser *et al*., 2003; Silva Filho *et al*., 2009; Newman and Cragg, 2012; Castro *et al*., 2013). Plant extracts are, in general, cheaper and easy to obtain (Kaur *et al*., 2005; Varanda, 2006). In Brazil, there is great vegetal diversity, corresponding to 30% of the world flora, which represents an ideal ground for the search of novel natural compounds for new treatments (Nodari and Guerra, 2001). *Jatropha gossypiifolia* and *Piper arboreum* are plants commonly used in Brazilian folk medicine. *J. gossypiifolia* is popularly known as 'peão roxo' and belongs to the Euphorbiaceae family (Abreu *et al*., 2003). The main biological activities experimentally demonstrated for *J.* gossypiifolia are microbicidal (Nair *et al*., 2007), larvicidal (Rahuman *et al*., 2008), molluscicidal (Pereira-Filho *et al*., 2014) and anti-*Leishmania*

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activities (Martins *et al.*, 2018). *P. arboreum* belongs to the Piperaceae family and is popularly known as 'pimenta de macaco' (Kato and Furlan, 2007; Andrade *et al.*, 2009; Regasini *et al.*, 2009; Ferreira *et al.*, 2010; Paes-Gonçalves *et al.*, 2012). Previous studies have already demonstrated some important biological activities of this plant species, such as antifungal (Silva Costa *et al.*, 2002), trypanosomicidal (Figueiredo *et al.*, 2014), leishmanicidal (Figueiredo *et al.*, 2014) and microbicidal activities (Regasini *et al.*, 2008).

Despite the biological potential displayed against several organisms, antiparasitic activities of the extracts of *J. gossypiifolia* and *P. arboreum* leaves on *S. mansoni* have not yet been evaluated. Thus, we aimed to evaluate the cercaricidal and schistosomicidal effects of ethanolic extracts of *J. gossypiifolia* and *P. arboreum* leaves *in vitro* and to describe preliminary classes of the main chemical compounds present in these extracts.

Material and methods

Collection of plants and preparation of ethanolic extracts

The leaves used for the production of crude extracts were collected in the morning at two collection points – *J. gossypiifolia* in the municipality of São Bento, State of Maranhão, Brazil (S02° 42′06.4″ and W044°49′55.0″) and *P. arboreum* in the municipality of São Luís, Maranhão, Brazil (S02°33′38.5″ and W044°14′19.6″).

The plants were identified at the Rosa Mochel Herbarium of the State University of Maranhão (UEMA), with specimen voucher number '4929' for *P. arboreum* and '4464' for *J. gossypiifolia*. The plant material was collected, cleaned, dried and powdered. The powder was macerated with a 70% hydroethanolic solution, a traditional extraction used in Brazilian folk medicine (Rodrigues *et al.*, 2020) and mixed every 12 h for a total of 48 h at a ratio of 1:5 (w/v). The extract was first filtered five times and then concentrated under reduced pressure to obtain a dry extract (Neiva *et al.*, 2014). The final yield was 16% of the weight of the crushed dry leaves for *P. arboreum* and 48% for *J. gossypiifolia*.

Parasite

Biomphalaria glabrata snails infected with the LE (Luís Evangelista) strain of *S. mansoni* were exposed to light and heat for 4 h (Brasil, 2008) to obtain cercariae for performing experimental infections and bioassays. Subsequently, the cercariae were washed, concentrated, counted and a total of 100 cercariae added to 500 μL of saline (0.9% g NaCl/100 mL dechlorinated water) were percutaneously inoculated in male Swiss mice (3–4 months old, weighing ~40 g) ($n = 12$), to obtain adult worms (Pellegrino and Macedo, 1955). The care of and experiments with mice were approved by the Ethics Committee for the Use of Animals (CEUA) of UEMA, under approval number 03/2018.

The infected animals were kept in ventilated racks and food and water were provided *ad libitum*. After 60 days of infection, the mice were euthanized with an anaesthetic overdose (150 mg kg^{-1} ketamine hydrochloride, Vetanarcol® and 120 mg kg^{-1} xylazine hydrochloride, Rompum®). Next, the circulating blood was perfused with sterile saline solution to recover adult worms (Pellegrino and Siqueira, 1956), which were separated into males, females and couples. The adult worms were immediately placed in RPMI 1640 medium, supplemented with 100 μg mL^{-1} of penicillin, 100 μg mL^{-1} of streptomycin, 10% fetal bovine serum and washed twice (Soares *et al.*, 2017).

Toxicity assay against cercariae

Toxicity tests were performed according to Rodrigues *et al.* (2020), with some modifications. Briefly, 24-well culture plates (Costar, Corning, NY, USA) were used with 10 active cercariae per well. Each well was filled with incomplete RPMI culture medium containing RPMI-1640 (Sigma-Aldrich) supplemented with 10 mM HEPES, 11 mM sodium bicarbonate, 100 U mL^{-1} penicillin, 100 mg mL^{-1} streptomycin, 2 mM L-glutamine, 23 mM L-asparagine and 0.1 mM pyruvic acid. Subsequently, the ethanolic extracts of *P. arboreum* and *J. gossypiifolia* leaves were added at final concentrations of 250, 500, 1000, 5000 and 10 000 μg mL^{-1} (test groups) in a final volume of 500 μL (RPMI). For the negative control (C-), 10 cercariae were added to 500 μL of incomplete RPMI medium. The plates with the cercariae were kept in an oven at 30°C and 5% CO_2. The assessment of cercaricidal activity was based on the visualization and counting of larvae with preserved mobility at 15, 30, 60, 90, 120, 150 and 180 min after adding the extract, using a stereomicroscope. The tests were performed in triplicate (biological replicates) with three replications (technical replicates).

Toxicity assay against adult worms

Two worms (male or female) were distributed into wells in 24-well culture plates containing 2 mL of RPMI complete medium and incubated in an oven (under 5% CO_2 at 37°C). After 1 h of incubation, extracts of *P. arboreum* and *J. gossypiifolia* were added at concentrations of 50, 150 and 250 μg mL^{-1} (test groups). As a negative control group (C-), the worms were incubated only in complete RPMI medium. The positive control group was composed of two worms incubated with 10 μg mL^{-1} of PZQ (Farmanguinhos, Rio de Janeiro, Brazil). An inverted microscope (Olympus CK40) was used to assess the worms' survival at 3, 6, 12, 18, 24, 48 and 72 h of culture. The treatment was considered effective when the worms did not show any movement after 2 min of observation (for analysis time). All procedures were performed according to Rodrigues *et al.* (2020), with some modifications. The tests were performed in triplicate (biological replicates) with three replications (technical replicates).

Egg-laying assay

For this assay, one worm couple was added per well along with 50 μg mL^{-1} of extract for 30 h (test groups). In the positive control group, a sub-lethal dose of PZQ (0.5 μg mL^{-1}) was used, as described by Mendonça *et al.* (2016). In the negative control group (C-), only complete RPMI medium was used. An inverted microscope was used to count the eggs in each well containing viable couples. Worm couples that eventually died after 30 h were not considered for this analysis. The tests were performed in triplicate (biological replicates) with two repetitions (technical replicates) employing a culture protocol as described by Soares *et al.* (2017) and Rodrigues *et al.* (2020).

Assessment of morphological changes in adult worms

The parasites were carefully removed from the wells and fixed in AFA (70% ethanol, 3% formaldehyde and 2% glacial acetic acid) (Torres *et al.*, 2011). The fixed worms were stained in hydrochloric carmine for 30 min and the excess dye was removed with 70% ethanol. Next, the worms underwent gradual dehydration in an alcoholic series (70%, 90% and absolute alcohol, 3 min at each stage) and clarification in methyl salicylate with Canada Balsam (1:2) for preparing permanent slides (Neves *et al.*, 2007). The worms were subjected to morphological analysis

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according to Neves *et al.* (1998) using an optical microscope (ZEISS, Axio Imager).

Preliminary phytochemical screening

High-Performance Liquid Chromatography (HPLC) was used to identify the presence of phytocompounds in ethanolic leaf extracts of *J. gossypiifolia* and *P. arboreum*. For screening with HPLC-PAD (HPLC coupled with Photodiode Array Detection), 1 mg aliquot of each extract was solubilized in ACN (acetonitrile): H_2O (95:5) and subjected to a clean-up process using Sep-pak cartridge RP18 (previously activated with methanol [MeOH]) and filtered through a 0.22 μm membrane. The extracts were injected individually in a Shimadzu analytical liquid chromatography system, model LC-20 A. A CBM-20 A controller with UV-PAD detector was used in gradient mode using 5–95% of B mixed in A (B, ACN; A, H_2O) for 45 min at a flow of 1.0 mL min^{-1}. A LUNA 5μm HPLC C18 (250 × 4.6 mm^2) column was used. All solvents used were HPLC grade.

Statistical analysis

All data were initially analyzed for normality (Kolmogorov–Smirnov test). To compare the averages of parametric data between the groups, the one-way ANOVA test was used, followed by Tukey test (Mishra *et al.*, 2019). Viability rates between groups were comparatively analyzed using the log-rank test (Hazra and Gogtay, 2017). Values of $P < 0.05$ were considered significant. To perform these tests, the GraphPad Prism version 8 software was used.

Results

In the cercariae assay, at the concentrations of 10 000 and 5,000 μg mL^{-1} of *J. gossypiifolia* extract, all the cercariae were killed after 120 and 150 min, respectively. At lower concentrations (1000–250 μg mL^{-1}), the larvae showed a 100% mortality rate only after 180 min. These mortality rates were statistically different when compared to the control group. However, there were no differences found between viability rates of the different concentrations of the extract (Fig. 1A).

For *P. arboreum* extract, it was observed that after exposure to 10 000 μg mL^{-1} for 60 min, all the cercariae were dead. At 5000 and 1000 μg mL^{-1} concentrations, the cercariae viability was totally reduced in 120 and 150 min, respectively. Moreover, at the lowest concentrations (500 and 250 μg mL^{-1}), there was a 100% reduction in viability only after 180 min. These mortality rates were statistically different when compared to the control group. However, the viability rates for different concentrations of the extract were not different from each other (Fig. 1B).

Regarding the adult worm assay, *P. arboreum* was able to reduce 100% of viability of males and females only after 72 h at the highest concentration (250 μg mL^{-1}), being significantly different from the negative control ($P = 0.003$). At the lowest concentrations (150 and 50 μg mL^{-1}), viable parasites (50% for male and 80% for female worms) were observed even after 72 h (Fig. 2A and B).

Regarding *J. gossypiifolia*, the concentration of 250 μg mL^{-1} was able to kill all male worms after 12 h and all females after 24 h. Even at the lowest concentration (50 μg mL^{-1}), the reduction in viability of the worms (males and females) occurred in less time compared to *P. arboreum* (Fig. 2C and D). All the mortality rates for male and female worms obtained at different concentrations of *J. gossypiifolia* extract were higher than those of the negative control group ($P < 0.02$) (Fig. 2C and D).

In the positive control group (PQZ), there was a 50% reduction in the viability of worms (males and females) in 6 h and after 18 h, all the worms were dead. Treatment with PQZ was more effective

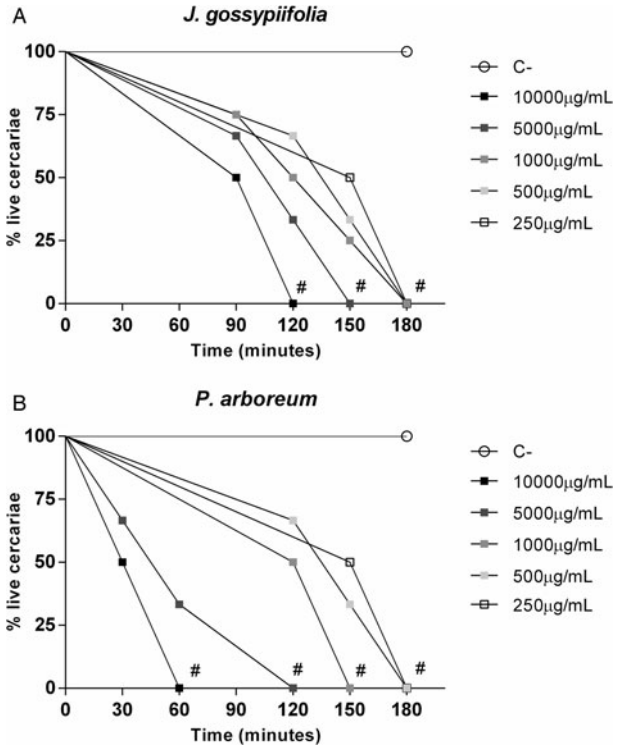

Fig. 1. Survival rate of *Schistosoma mansoni* cercariae exposure to ethanolic extracts of *Jatropha gossypiifolia* (A) and *Piper arboreum* (B) leaves over a time period of 180 min. Ten cercariae per well were used in triplicate at different concentrations (μg mL^{-1}) of the extracts. In the negative control group (C-), only incomplete RPMI was used. # Statistically different from the negative control group. Statistical results were obtained using the log-rank test.

than with all the concentrations of *P. arboreum* extract ($P < 0.05$) (Fig. 2A and B) but was similar to the two highest concentrations (150 and 250 μg mL^{-1}) of *J. gossypiifolia* extract (Fig. 2C and D).

The extracts of *J. gossypiifolia* and *P. arboreum* leaves were also able to reduce the number of eggs laid by the paired females of *S. mansoni* by approximately 93% in the test groups than in the negative control group ($P < 0.0003$) (Fig. 3). Also, no eggs were found in the positive control group ($P < 0.0002$). There was no difference in the number of eggs laid between the test and positive control groups (Fig. 3).

Regarding morphology of the worms, the negative control group showed external and internal morphological integrity (Fig. 4). However, the worms from the positive control group (PZQ) exhibited severe muscle/body contractions, loss of tubercles, damage to the integument and changes in the intestines (Fig. 5).

The worms exposed to the extracts of *J. gossypiifolia* and *P. arboreum* presented atypical body contractions and external microstructural damage. Similar damage of internal structures was observed for all the concentrations. However, an evident worsening of morphological changes was observed at the highest concentration (250 μg mL^{-1}).

Piper arboreum extract induced destruction of tubercles and dilatation of the oesophagus and anterior intestine in male worms. Morphological changes such as destruction of sensorial papillae and tegument layers, as well as the reduction in the vitelline glands, were observed in female worms (Fig. 6).

The extract of *J. gossypiifolia* caused atypical contractions of the body, dilatation of the oesophagus and destruction of tubercles in male worms. In female worms, changes also were observed, such as atypical contractions of the body; destruction of sensorial papillae; dilatation of the uterus, anterior intestine, viteloduct and oviduct; and damage to the eggs present in the ootype (Fig. 7).

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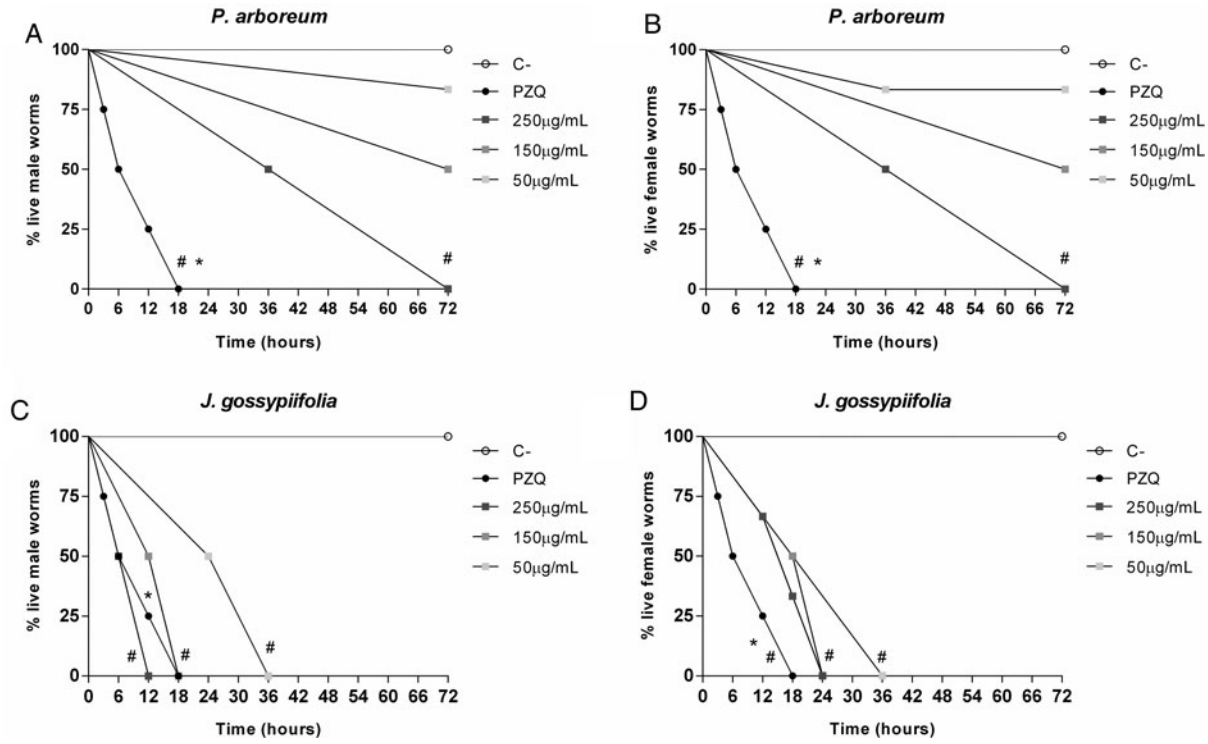

Fig. 2. Viability of adult worms (male and female) of *Schistosoma mansoni* after 72 h of exposure to ethanolic extracts of *Piper arboreum* (A and B) and *Jatropha gossypiifolia* (C and D) leaves. Two adult male or female worms were used per well in triplicate at different concentrations ($\mu g\ mL^{-1}$) of the extracts. In the negative control group (C-), only complete RPMI medium was used, whereas, in the positive control group, PZQ ($10\ \mu g\ mL^{-1}$) was used. *Viability of worms in the positive control group was significantly different from all the concentrations of *P. arboreum* and from $50\ \mu g\ mL^{-1}$ concentration of *J. gossypiifolia*. #Statistically different compared to the negative control group. Statistical results obtained by the log-rank test.

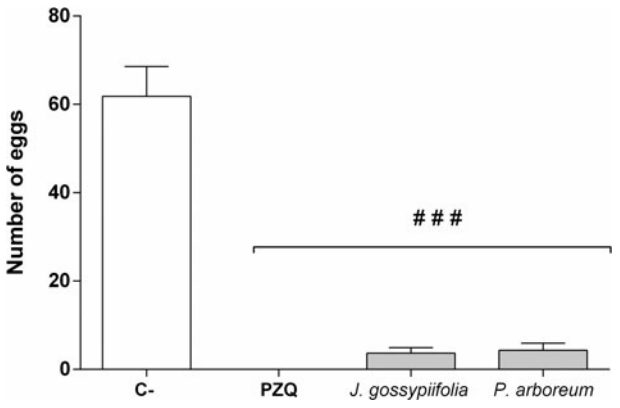

Fig. 3. Number of eggs laid by female *Schistosoma mansoni* worms after 30 h of culture with $50\ \mu g\ mL^{-1}$ of ethanolic extracts of *Jatropha gossypiifolia* and *Piper arboreum* leaves. In the negative control group (C-), only complete RPMI medium was used; in the positive control group, PZQ ($0.5\ \mu g\ mL^{-1}$) was used. One couple of worms per well was used in triplicate. #Statistically significant compared to the negative control. Statistical results obtained using one-way ANOVA test.

A HPLC-PAD analysis of the sample of ethanolic extract of *J. gossypiifolia* leaves was observed in the chromatogram at 330 nm; peaks of medium polarity in the retention times (Rt) in the range of 13.2–22.0 min with absorptions at 269, 348 nm; 267, 347 nm; 269, 337 nm; and 270, 337 nm characteristic of flavonoid compounds were observed (Fig. 8A) (Granados *et al.*, 2015; Cavalcante *et al.*, 2020).

For the ethanolic extract of *P. arboreum* leaves, the HPLC profile demonstrated in the chromatogram at 330 nm was as follows: (i) high polarity peaks were observed at Rt from 1.83 to 2.16 min with absorptions at 276 and 290 nm, respectively, which could be attributed to the presence of amides; these results are consistent with those of Nascimento *et al.* (2015) and Silva Costa (2002); and (ii) medium polarity peaks at Rt of 13.1, 14.2 and 24.4 min with absorption bands at 268, 338, 269, 338 nm, suggestive of flavonoids (Tong *et al.*, 2018) and at 324 nm that may be characteristic of phenolic compounds (Fig. 8B) (Ferreres *et al.*, 2014).

Discussion

The search for new anti-*Schistosoma* drugs has intensified due to reports of resistance of some strains of *S. mansoni* to the currently used drugs (Fallon *et al.*, 1995; Ismail *et al.*, 1996; Picquet *et al.*, 1998; Crellen *et al.*, 2016). Thus, researches with the purpose to identify of biologically active compounds from plant origin against *S. mansoni* are the basis for the development of new therapies (Ndjonka *et al.*, 2013; Seif El-Din *et al.*, 2014).

Although several studies have demonstrated potential effects of plant products against *S. mansoni* (Yousif *et al.*, 2007; Braguine *et al.*, 2010; Caixeta *et al.*, 2011; Koné *et al.*, 2011); to date, there are no reports on the cercaricidal and schistosomicidal activity of ethanolic extracts of *J. gossypiifolia* and *P. arboreum* leaves. Thus, antiparasitic effects against *S. mansoni* demonstrated in *in vitro* tests of this study are described for the first time, which may lead to alternative controls of schistosomiasis.

Regarding the cercaricidal assay, although both the extracts reduced 100% of larval viability, the best effect was observed with *P. arboreum* (when compared to *J. gossypiifolia*), which had the potential to induce the death of all cercariae in 60 min at the highest concentration. The antiparasitic effect of plant extracts on *S. mansoni* cercariae was also verified by Castro *et al.* (2015), who demonstrated mortality of cercariae after 1 h of incubation with the substance 7-epiclusianone (derived from an ethanolic extract of *Garcina brasiliensis*); however, approximately 100% of the cercariae were killed only after 8 h. Al-Sayed *et al.* (2014) tested the extract of *Eucalyptus globules*

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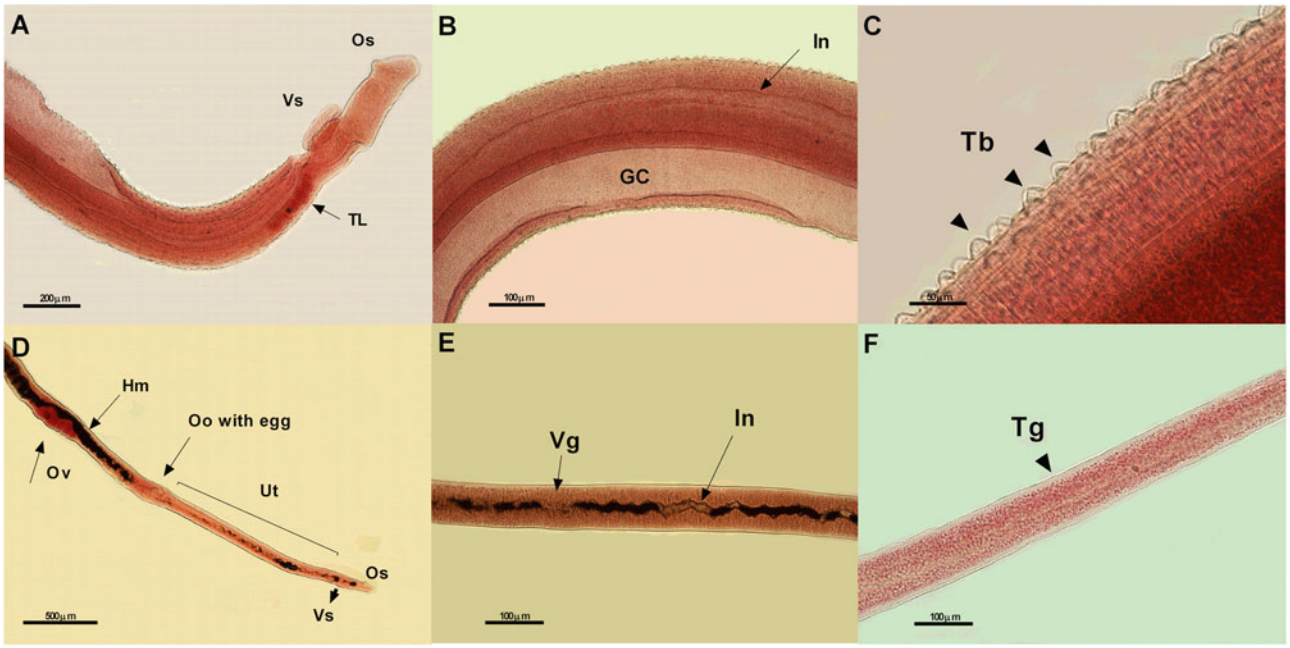

Fig. 4. Photomicrographs of male (A – 10x, B – 20x and C – 40x) and female (D – 5x, E – 20x, and F – 20x) worms of *Schistosoma mansoni* from the negative control group (treated with only complete RPMI) showing normal morphological characteristics. Os, oral sucker; *Vs*, ventral sucker; TL, testicular lobes; In, intestine; GC, ginecophore canal; Tb, tubercles; Hm, hemozoin; Ov, ovary; Oo, ootipus; Ut, uterus; Vg, vitelogenus glands; and Tg, tegument; μm, micrometers.

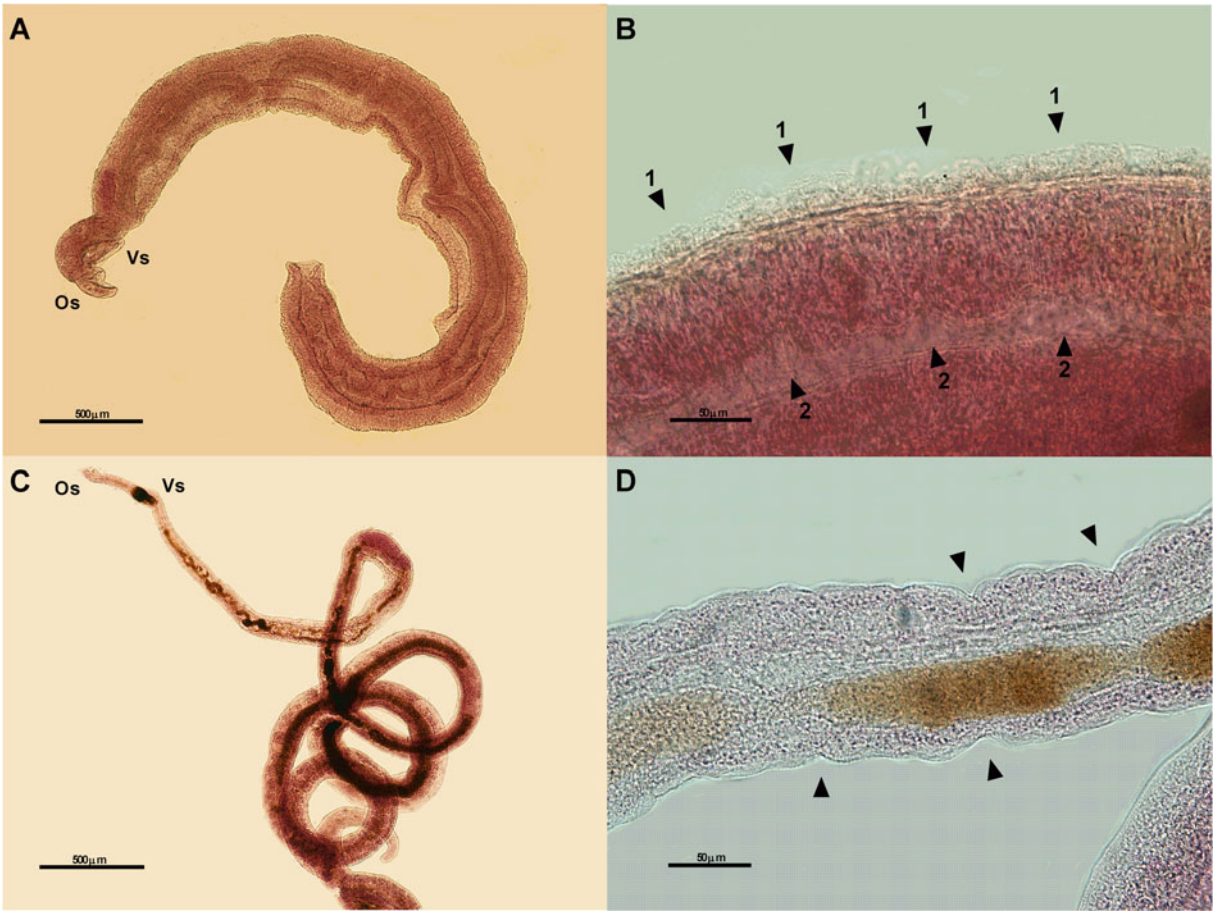

Fig. 5. Photomicrographs of male (A – 5x and B – 40x) and female (C – 5x and D – 40x) worms of *Schistosoma mansoni* from the positive control group (exposed to 10 μg of PZQ) showing some changes: atypical worm body contractions in male (A), destruction of the tubercles (arrows 1) and dilation in the anterior intestine (arrows 2) (B), atypical contractions of the female worm body (C), destruction of the integument (arrows) (D). Os, oral sucker; *Vs*, ventral sucker; μm, micrometers.

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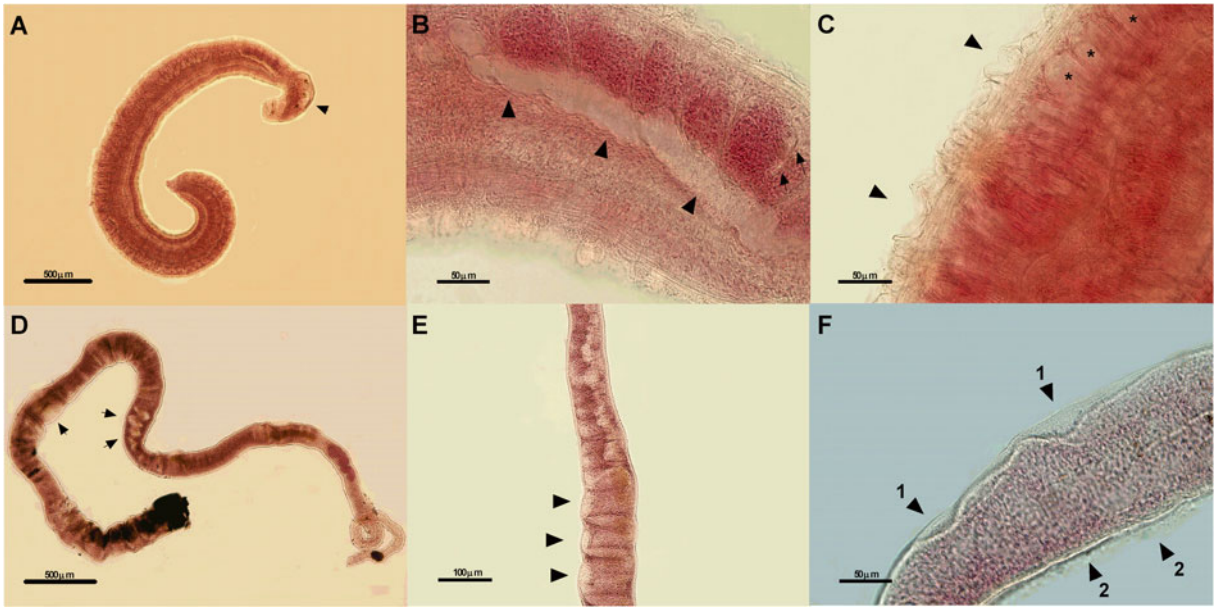

Fig. 6. Photomicrographs of male (A-5x, B-40x, and C-40x) and female (D-5x, E-20x, and F-40x) worms of *Schistosoma mansoni* exposed to 250 μg mL^{-1} of the ethanolic extract of *Piper arboreum* leaves for 72 h. Morphological changes observed are dilation of the oesophagus (arrow) (A), dilation of the intestine (big arrows) and cellular reduction of the testicular lobes (small arrows) (B), destruction of the tubercles (arrows), tegumentary dilation (asterisks) (C), atypical contraction of the female body and reduction of the vitelline glands (arrows) (D), dilation and reduction of the area of vitelline glands (arrows) (E), integument lift (arrows 1) and destruction of the sensorial papillae (arrows 2). μm, micrometers.

leaves at 20 ppm and observed a 100% mortality rate after 2 h of exposure. Thus, based on these studies, the extract of *P. arboreum* leaves presented modest results.

Although the extract of *P. arboreum* leaves exhibited the best cercaricidal effect in this study, the same efficacy was not observed in adult worms. However, we observed that the extract of *J. gossypiifolia* leaves displayed the best performance, with 100% mortality of males and females after 12 and 24 h, at a concentration of 250 μg mL^{-1}.

Previous research using plant extracts in a schistosomicidal assay demonstrated that couples of *S. mansoni* worms subjected to the essential oil of *Dysphania ambrosioides* (L.) at 25 and 12.5 μg mL^{-1} concentrations were killed after 24 and 72 h, respectively (Soares *et al.*, 2017). However, Matos-Rocha *et al.* (2016), using the essential oil of *Mentha x villosa,* demonstrated a mortality rate for all adult male worms of *S. mansoni* only at 500 μg mL^{-1} concentration after 24 h, while the lowest concentrations (5 and 10 μg mL^{-1}) were not able to induce mortality even after 120 h of exposure.

Interestingly, we observed that male worms were more susceptible than females at all concentrations of both the *J. gossypiifolia* and *P. arboreum* leaf extracts. Previous studies have also found that male *S. mansoni* worms were more susceptible than females when exposed to PZQ, ginger extract, diamines, amino alcohols and some essential plant oils (Pica-Mattoccia and Ciolli, 2004; Mostafa *et al.*, 2011; Tonuci *et al.*, 2011; Oliveira *et al.*, 2012; Fernandes *et al.*, 2013). However, these are still controversial data, since Oliveira Penido *et al.* (2008) demonstrated that female worms are more susceptible than males in experiments with amino-alkane-thiosulfuric acid. Despite these reports, the mechanisms involved in the differences in susceptibility between male and female parasites of *S. mansoni* still remain unclear.

Although the viability of adult *S. mansoni* worms was differently affected by both the extracts, a similar efficacy was observed on inhibition in the oviposition assay because at 50 μg mL^{-1} concentration both the extracts were able to reduce the egg-laying capacity of *S. mansoni* worms.

The inhibition of oviposition in female worms of *S. mansoni* was also observed by Sanderson *et al.* (2002), who demonstrated that the extract of ginger ethyl acetate (*Zingiber officinale*) at a concentration of 50 mg mL^{-1} was able to significantly inhibit the cumulative production of eggs in females mated *in vitro*. Godinho *et al.* (2014) also showed that the essential oil of aerial parts of *Tanacetum vulgare* caused complete inhibition of oviposition, but only at 200 μg mL^{-1} after 120 h of incubation. The inhibition of *S. mansoni* oviposition is particularly of great interest since the pathology of schistosomiasis is caused by an inflammatory reaction around the eggs that are retained in the vertebrate host tissue, mainly in the liver and intestines, resulting in the formation of granulomas (Schwartz and Fallon, 2018).

Moreover, we also demonstrated the presence of internal and external morphological changes in adult *S. mansoni* worms, which could constitute one of the main mechanisms of action exerted by the extracts of *P. arboreum* and *J. gossypiifolia*, thereby explaining the mortality rates and inhibition of oviposition observed in this study.

The rigidity of the integument of *S. mansoni* performs essential functions for its survival, such as osmoregulation, protection, synthesis and secretion of metabolites, parasitic defence against the host's immune system and represents an important structure for the action of drugs (Xiao *et al.*, 2002; Faghiri and Skelly, 2009; Yepes *et al.*, 2014; Pereira *et al.*, 2015). Specifically in females, changes in the integument may result in decreased egg formation. Indeed, the main mechanism of action of PZQ is also to target the integument of this parasite, acting on the permeability of its membrane, increasing the calcium influx, thus resulting in abnormal contraction of the parasites, paralysis and death (Novaes *et al.*, 1999; Cioli and Pica-Mattoccia, 2003; Cioli *et al.*, 2014).

Microstructural changes in the integument, similar to those identified in our study, such as bubbles, erosion/flaking and body retraction, were also observed with the use of the antibiotic, doxycycline, especially at higher concentrations (Dias *et al.*, 2019). In the same study, the disappearance, flattening and the collapse of tubercles were also observed. Damage to the tubercles of *S. mansoni* male worms was also caused by a hydroalcoholic extract of *Arctium lappa* fruits at the concentrations of 100 and 200 mg mL^{-1} (Dias *et al.*, 2017).

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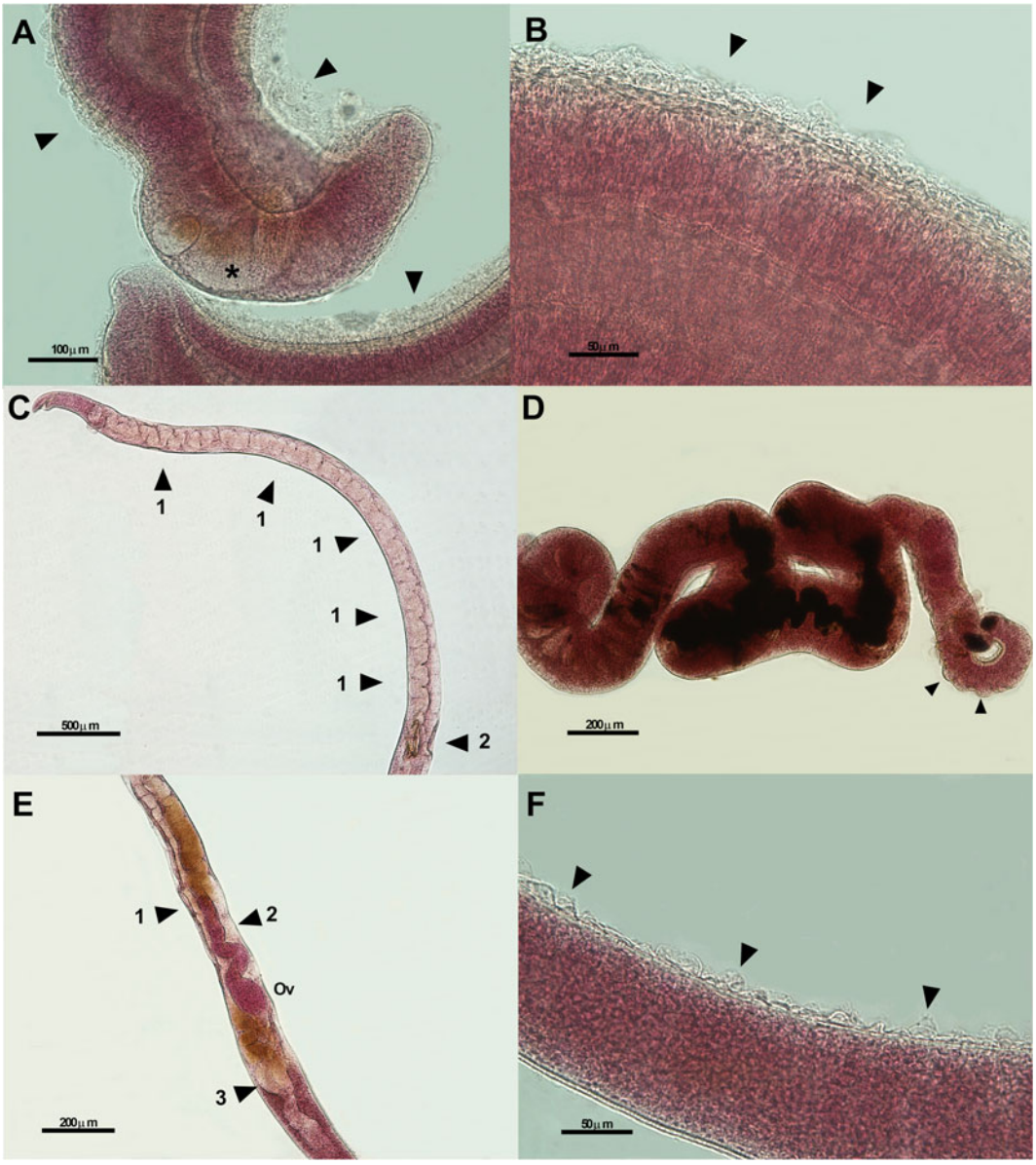

Fig. 7. Photomicrographs of male (A-20x and B-40x) and female (C-5x, D-10x, E-10x, and F-40x) of *Schistosoma mansoni* worms exposed to 250 μg mL^{-1} of the ethanolic extract of *Jatropha gossypiifolia* leaves for 72 h. Morphological changes observed are esophageal dilation (asterisk) and destruction of the tubercles (arrows) (A), destruction of the tubercles (arrows) (B), dilation of the intestinal and uterus areas (arrows 1) and destruction of the egg inside the ootype (arrow 2) (C), atypical contraction of the female body and integument lift (arrows) (D), dilation of the viteloduct (arrow 1), oviduct (arrow 2) and the area of the vitellin glands (arrow 3) (E), destruction of the sensorial papillae (arrows) (F). Ov, ovary; μm, micrometers.

Internal changes in the digestive system were also demonstrated by Oliveira *et al.* (2019), who described esophageal changes (bulb-shaped oesophagus) in female worms exposed to tamoxifen (anti-cancer drug). According to Matos-Rocha *et al.* (2016), internal changes were also verified by transmission electron microscopy; the presence of vacuoles was found in the region of the syncytial matrix and of glycogen granules close to muscle fibres in adult worms of *S. mansoni* treated with the essential oil of *Mentha x villosa* leaves (500 μg mL^{-1}), similar to the tegumentary dilations found in our study. Simões *et al.* (2015) also demonstrated that the crude extract of *P. tuberculatum* is capable of acting internally in female worms, modifying some regions of the vitelline glands. However, the alterations in the female reproductive system are yet less explored; hence, the details mentioned in our study should be considered, as they may provide valuable information on new drug targets.

These external and internal morphological changes in the *S. mansoni* male and female adult worms may have been caused by the presence of secondary metabolites present in *J. gossypiifolia* and *P. arboreum* leaves. Chemical screening suggested the presence of flavonoid compounds in the ethanolic extracts of *J. gossypiifolia* and *P. arboreum* and phenolic compounds and amides only in the *P. arboreum* extract.

Among these compounds, flavonoids and amides seem to have an important anthelmintic effect (Silva *et al.*, 2008; Moraes *et al.*, 2011, 2012; Botura *et al.*, 2013). Indeed, amides are one of the main compounds found in plants of the genus *Piper* and recent studies have shown that the main amide isolated from *P. tuberculatum*, named piplartine, was active against adult worms (Moraes *et al.*, 2011) and schistosomules of *S. mansoni* (Moraes *et al.*, 2012). However, future work must be carried out to accurately identify the major compounds of *P. arboreum* and *J. gossypiifolia* that could explain the different effects observed with the use of these extracts on cercariae and adult worms of *S. mansoni*.

It is important to highlight that to continue exploring plant extracts that could be used as therapeutics in humans in the

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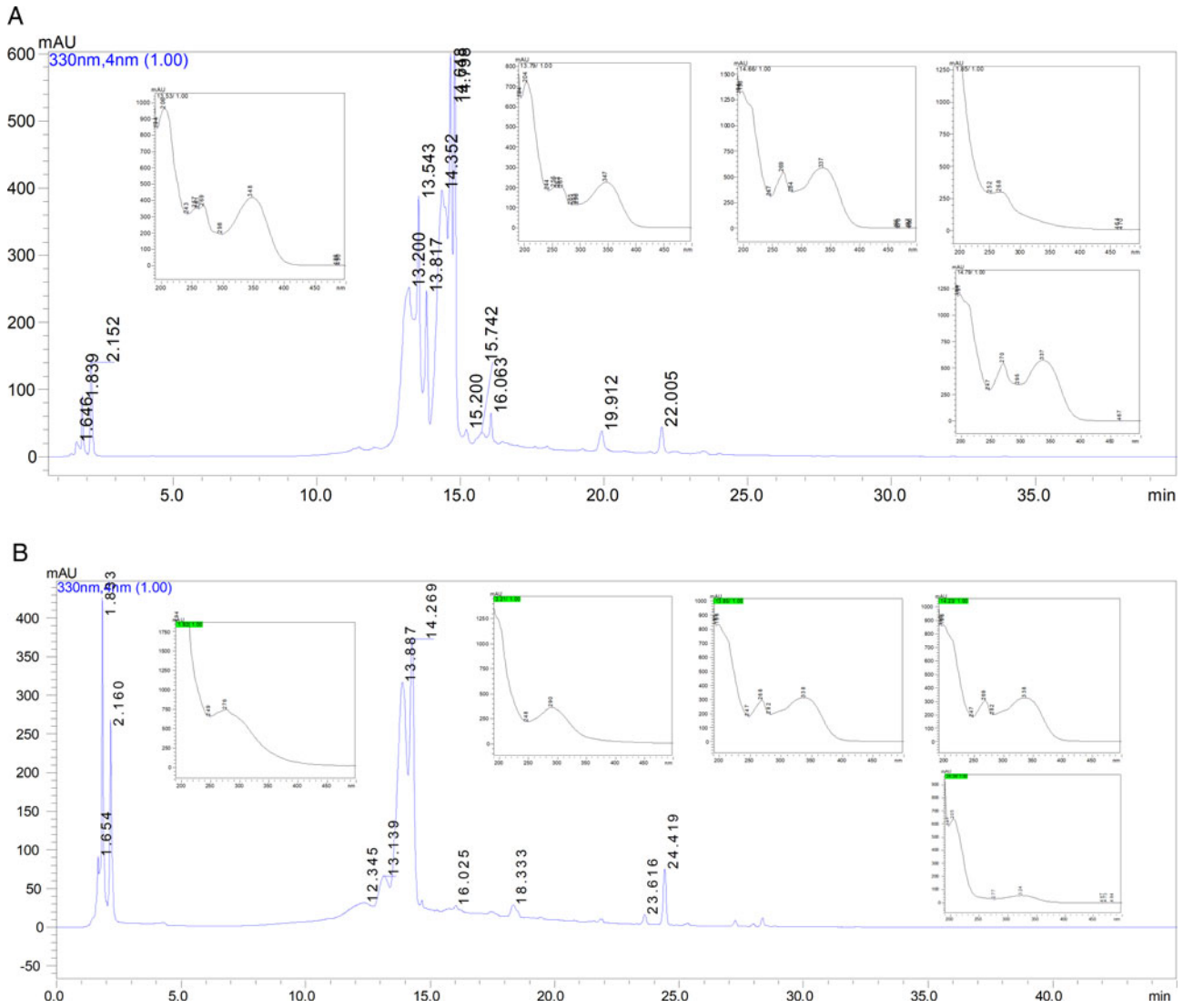

Fig. 8. Representative high-performance liquid chromatography (HPLC) profile and UV spectra of ethanolic extracts of *Jatropha gossypiifolia* (A) and *Piper arboreum* (B) leaves.

future, it is important to perform toxicity tests. Although these tests were not carried out in this study, previous research has already demonstrated reduced toxicity of *P. arboreum* and *J. gossypiifolia* extracts (Nagaharika *et al.*, 2013; Macedo *et al.*, 2019). However, more tests are needed to be carried out to confirm the absence of toxicity of the extracts used in this study in non-target organisms and cells.

In summary, the ethanolic extracts of *J. gossypiifolia* and *P. arboreum* leaves are biologically active against cercariae and worms of *S. mansoni in vitro*. External and internal morphological damage is implicated to be involved in the mortality of adult worms. Moreover, flavonoids, phenolic compounds and amides may be responsible for this process. However, future *in vivo* studies, as well as isolation of the main substances present in these plant extracts, should be carried out to establish the true potential of *J. gossypiifolia* and *P. arboreum* as new therapeutic and prophylactic strategies against schistosomiasis.

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Author contributions.
GSM, and JGMR conceptualized the study. RRSA, JGMR, AT-R, RAN, ICLL, MGSL, RSA, TJASA and GSM performed experiments. RRSA, JGMR, AT-R, RAN, ICLL, MGSL, TJASA, NS-S and GSM analysed the data. GSM supervised the project administration. RRSA, JGMR, RAN, ICLL, MGSL, TJASA, NS-S and GSM drafted the manuscript. All the authors reviewed and approved the manuscript.

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Conflicts of interest. The authors declare there are no competing interests.

Ethical standards. The care of and experiments with mice were approved by the Ethics Committee for the Use of Animals (CEUA) of UEMA, under approval number 03/2018.

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