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Molluscicidal activity of *Persea americana* Mill. (Lauraceae) stem bark ethanolic extract against the snail *Biomphalaria glabrata* (Say, 1818): a novel plant-derived molluscicide?

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Abstract: Plant-derived molluscicides have been indicated as biodegradable and low-cost strategies for control of *Biomphalaria* spp., intermediate host for the *Schistosoma*. This study evaluated whether the crude ethanolic extract of the *Persea americana* stem bark has molluscicidal activity against embryos, newly-hatched and adults of *Biomphalaria glabrata*. The extract was obtained, characterized and its toxicity analyzed by snail embryotoxicity test (144 h) and acute toxicity test with newly-hatching and adult snails (96 h). Results showed the presence of flavonoids, anthraquinone heterosides, coumarins and tannins in the crude ethanolic extract, which showed molluscicidal activity against all life cycle stages of *B. glabrata*. The LC_{50} for embryos, newly-hatched and adults were 27.06, 30.60 and 55.55 ppm, respectively. Embryos exposed to the extract at 50 ppm showed hatching inhibition and at 6.2 and 25 ppm had the highest rates of morphological alterations, such as shell malformations and coagulation of the perivitelline substance. Adult snails exposed to the extract at 75 ppm showed a peak of behavioral changes, such as lethargy and shell reclusion, in addition to answers like hemolymph release in most concentrations. Further studies are required, prioritizing toxicity testing on non-target organisms and further elucidation of the active molecules.

Key words: embryotoxicity, flavonoids, intermediate host, *Schistosoma mansoni*, schistosomiasis control.

INTRODUCTION

Schistosomiasis is a neglected parasitic disease, as it affects low-income populations in tropical and subtropical countries between Latin America, Africa and Asia, caused by trematodes of the genus *Schistosoma* (Weiland 1858), wherein the freshwater snails *Biomphalaria* spp. act as the intermediate host (WHO 2017). The schistosomiasis control program focuses on the prevention of risk groups and the treatment of patients, but these strategies remain insufficient in controlling transmission (King & Bertsch 2015). The niclosamide (Bayluscide®), as it is active for adult and embryo snails, and is relatively safe during handling. On the other hand, the niclosamide has high toxicity to other aquatic animals and plants, high cost and difficulty of solubilization in organic solvents and water (WHO 2017).

The toxicity of secondary plant metabolites against the snail *Biomphalaria* spp. have been related to different mechanisms of action (Singab et al. 2006, Faria et al. 2018, Mendes et al. 2018).

Flavonoids induced rupture of cell membranes (Faria et al. 2018) and heart rate reduction (Singab et al. 2006). Tannins and saponins decreased the reproductive capacity and induced behavior changes (lethargy and alimentary capacity) (Mendes et al. 2018). Anthraquinones changed the cellular homeostasis and the Na^+/K^+-ATPase activity (Liu et al. 1997), whereas coumarins act in the coagulation process (Kady et al. 1992). In other snail species, such as *Lymnaea stagnalis* and *Cornu aspersum*, flavonoids compromised synaptic communication (Xu et al. 2010), may act in synergism with coumarins and saponins and cause DNA damage (Silva et al. 2013).

Persea americana Mill. (Lauraceae), popularly known as avocado, is a dicotyledonous plant native to Central America (Mexico, Guatemala and Antilles), easily adaptable in Brazil and several other tropical and subtropical regions (Ramos et al. 2004). This species has economic and therapeutic importance due to its antimicrobial (Cruz et al. 2019), antiviral (Silva-Mares et al. 2018), antiprotozoal (Jiménez-Arellanes et al. 2013), antinociceptive (Deuschle et al. 2018), anti-inflammatory and antioxidant properties (Tremocoldi et al. 2018). Furthermore, the larvicide activity of the *P. americana* extract was also reported against *Aedes aegypti* (Torres et al. 2014). However, to the best of our knowledge, this study is the first to report the toxicity of *P. americana* extracts on a freshwater snail that acts as an intermediate host.

In this sense, the present study aimed to evaluate the molluscicide activity of the crude ethanolic extract from *P. americana* stem bark against different life cycle stages of the freshwater snail *B. glabrata*. Assumingly, studies such as the present one broaden the possibility of using plant extracts to combat different phases of snails that transmit mansonic schistosomiasis, as well as provide scientific subsidies for the development of economically viable and plant-derived molluscicidal substances.

MATERIALS AND METHODS

Plant and extract

The stem bark of *P. americana* was collected of the same specimen in Aparecida de Goiânia, Goiás, Brazil (latitude: 16°48'33.56"S; longitude: 49°19'12.53"W) on August 10, 2010. The exsiccate was deposited in the herbarium from the Federal University of Goiás (UFG) (protocol n° 43394), and the ethanolic crude extract was obtained accordingly Carvalho et al. (2011). Therefore, stem barks were desiccated in an oven with air circulation at 40 °C and ground in a knife mill. Subsequently, the botanical material was macerated in ethanol (PA). At this stage, 1 kg of the powder was homogenized in 3 L of ethanol (PA) under mechanical agitation and kept at rest for 72 h. The supernatant was filtered on a qualitative filter paper (Unifil®), concentrated in a rotary vacuum evaporator at ~ 40 °C and kept in a freezer at -14 °C until further analysis. A total of 81.84 g of the crude ethanolic extract was obtained. The extraction yield was calculated in relation to the initial mass of botanical material (g) used in the extraction, using the following formula: yield (%) = (mass of the extract / mass of the plant material) *x* 100. To prepare the stock solution (1000 ppm), the extract was weighed and solubilized in dimethylsulfoxide (DMSO) at 1% of the solution concentration and diluted in reconstituted water (ISO 1986).

Phytochemical screening

The phytochemical screening investigated the presence of the main classes of secondary metabolites, such as flavonoids, anthraquinone heterosides, coumarins, tannins, alkaloids, cardioactive heterosides and saponins in the crude ethanolic extract of the *P. americana*

stem bark. The characterization reactions were performed using the methodologies described and adapted from Matos & Matos (1989), Costa (2001), Matos (2009), Brazilian Pharmacopoeia (Brazil 2010) and Reginatto (2017).

Thin Layer chromatography (TLC)

The presence of quercetin and rutin flavonoids in the crude ethanolic extract of *P. americana* stem bark was analyzed by Thin Layer Chromatography (TLC). The extract (1 g) was diluted in methanol PA (10 mL), kept in an ultrasound bath (Unique®) for 30 minutes at room temperature (25 ± 1 °C), and filtered on qualitative filter paper (Unifil®). Quercetin and rutin standards (0.0025 g) (Sigma-Aldrich®) were diluted separately in methanol PA (10 mL), homogenized in an ultrasonic bath (25 ± 1 ° C) for five minutes and filtered on qualitative filter paper (Unifil®). As a mobile phase, 50 mL of a solution composed of ethyl acetate, acetone, acetic acid and ultrapure water (60:20:10:10 v/v/v/v) were used (Tietbohl et al. 2010). For the detection of constituents, chromatographic plates prepared with silica G/UV_{254} (Macherey-Nagel®) were observed in ultraviolet light (UV) at 254 nm and 365 nm and revealed by sulfuric vanillin nebulization followed by heating (Wagner & Bladt 1996). The retention factors (Rf) of spots obtained at the end of the chromatographic run were calculated.

Total flavonoid content

The total flavonoid concentration, expressed as rutin equivalents, in the crude ethanolic extract of *P. americana* stem bark was performed according to the adapted method of Rolim et al. (2005). The *P. americana* extract (0.2 g) was dissolved in 10 mL of the solution containing methanol PA and 0.02 M acetic acid (99:1 v/v). This solution was kept in an ultrasound bath (Unique®) for 10 minutes (25 ± 1 °C) and filtered on qualitative filter paper (Unifil®). The solution containing methanol PA and 0.02 M acetic acid (99:1 v/v) was used as a blank solution in spectrophotometric readings. Samples containing 100 µL of the stock solution of the extract in sufficient quantity for 2 mL of the blank solution were prepared. For the rutin standard analytical curve, a solution containing 0.01 g rutin (Sigma-Aldrich®) in a 100 mL volumetric flask was prepared and the volume was completed with the blank solution. Seven different concentrations of rutin (5, 10, 15, 20, 25, 30 and 35 µg/mL) were prepared by diluting the stock solution. All steps were performed in triplicate and absorbances were measured at 364 nm using a digital spectrophotometer (PerkinElmer®). Results of concentration and total flavonoid content expressed as rutin equivalents were reported in µg/mL and %, respectively.

Snails

B. glabrata snails (BH lineage; SISGEN number: A6172FD) with shell diameter between 8 and 12 mm (between 8 and 12 weeks old) were obtained and kept in the malacology sector of the Institute of Pathology and Public Health from the Federal University of Goiás, such as recommended by Organization for Economic Cooperation and Development guideline n° 243 (OECD 2016). Adult snails were placed in tanks (32 x 22 x 22 cm) containing 15 L of dechlorinated water and under controlled conditions of temperature (25 ± 1 °C), photoperiod (12:12 h light/dark) and pH (7.0 ± 1), being fed three times a week *ad libitum* with fresh leaves of *Lactuca sativa* lettuce previously washed with dechlorinated water. The ovigerous masses from the adult snails were obtained and transferred to plastic vessels (30 x 25 x 7 cm) until hatching. Newly-hatched snails were fed twice a week *ad libitum* with dehydrated lettuce.

Bioassays

The molluscicidal activity of the crude ethanolic extract of the *P. americana* stem bark against different stages of the *B. glabrata* (embryos, newly-hatched and adults) was assessed following OECD guideline n° 243 (OECD 2016) and Melo et al. (2019). Three control groups were used: negative control [NC = reconstituted water (ISO 1986)]; solvent control (1% DMSO diluted in reconstituted water) and positive control [PC = niclosamide (Sigma-Aldrich®, CAS # 50-65-7)] at 0.08 ppm to embryos and 0.10 ppm to newly-hatched and adults snails.

Snail embryotoxicity test (SET)

Snail egg-clutches containing 14.00 ± 3.62 embryos in the blastula stage (egg-clutches with approximately 12 hours of oviposition - same day) were placed in 12-wells cell culture plates (1 egg-clutch *per* well containing 5 mL of final solution). Embryos were exposed to crude ethanolic extract of *P. americana* stem bark (6.2, 12.5, 25 and 50 ppm) during 144 h under a photoperiod of 12:12 h light/dark, the temperature of 28 ± 1 °C in triplicate design. During the exposure period (24, 48, 72, 96, 120 and 144 h), the frequency of viable and unviable embryos, morphological alterations and development stages was analyzed using a microscope (Leica DM 750) associated with the camera HD Leica ICC50 and LAS EZ software. The mortality rate was determined by the following formula: mortality rate (%) = (number of inviable embryos / total number of embryos) *x* 100 (OECD 2016). Regarding the morphological alterations of embryos, the possible presence of hydropic alterations, malformations in the tentacles, shell, muffle and eyes, nonspecific malformations (dysmorphic embryos), and coagulation of the perivitelline substance was analyzed (Melo et al. 2019).

The early developmental of *B. glabrata* was classified into five stages: blastulae, gastrulae, trochophore, veliger and hypo-stage (Melo et al. 2019). At the end of the exposure period (144 h), hatching rate (%) was determined by the following formula: hatching rate (%) = (number of hatched snails / total number of embryos) *x* 100 and hatched snails were euthanized by hypothermia (OECD 2016).

Acute toxicity to newly-hatched snails

The newly-hatched snails were obtained from egg-clutches containing embryos in the blastula stage kept in a BOD incubator (temperature 27 ± 1 °C and 70% humidity) for 144 h until hatching. Snails were randomly selected after 96 h of hatching. The experiments were performed in 6-wells cell culture plates (10 snails *per* well containing 10 mL of final solution). Newly-hatched snails were exposed to crude ethanolic extract of the *P. americana* stem bark (12.5, 25.0, 37.5 and 50.0 ppm) during 96 h. The experiments were conducted under a photoperiod of 12:12 h light/dark and temperature 25 ± 1 °C in a triplicate design. Snails were not fed during the exposure period (OECD 2016) and those with immobility, shell depigmentation, hemolymph release, absence of heartbeat and/or visceral mass exposure were considered dead (Melo et al. 2019). At the end of the experiment, the mortality rate (%) was determined by the following formula: mortality rate (%) = (number of dead snails / total number of snails) *x* 100 and the surviving newly-hatched snails were euthanized by hypothermia (OECD 2016).

Acute toxicity to adult snails

Adult snails with shell diameter between 8 and 12 mm were obtained randomly from the maintenance aquarium and exposed to crude ethanolic extract of the *P. americana* stem bark (25, 50, 75 and 100 ppm) during 96 h. The

experiments were conducted in a glass container (6 x 8 cm) containing 180 mL of the final solution (10 snail *per* tank) under controlled photoperiod (12:12 h light/dark) and temperature (25 ± 1 °C) in a triplicate design. The dead snails were removed from the experimental tanks daily. At the end of the experiment, the mortality rate (%) was determined by the following formula: mortality rate (%) = (number of dead snails / total number of snails) *x* 100 and surviving snails were euthanized by hypothermia (OECD 2016).

After 96 h of exposure, the behavioral alterations were analyzed in a real-time during 1 min for each snail, totalizing 10 min per replicate. The following behavioral alterations were recorded: lethargy (after stimulation of the cephalopedious mass with tweezers, the snails did not respond) and shell reclusion (Nolan et al. 1953, Melo et al. 2019). The frequency (%) of each behavioral alteration was determined according to Melo et al. (2019). In addition to behavioral alterations, the frequency (%) of snails with hemolymph release (Mendes et al. 1993, Melo et al. 2019) and mucus secretion (Musman 2010, Melo et al. 2019) was recorded.

Statistical analysis

For the determination of the analytical curve of the rutin pattern in the total flavonoid assay in the crude ethanolic extract of *P. americana* stem bark, the linear regression was used. Lethal concentrations (LC_{10}, LC_{50} and LC_{90}) and their confidence intervals were obtained by regression analysis using the Probit method using Statistica version 7.1 software (Statsoft 2007). To verify the existence of a pattern dependent on the concentrations tested in the mortality of *B. glabrata* exposed to the extract, the linear regression test was used. One-way analysis of variance (ANOVA) was used to verify differences in mortality rates of *B. glabrata* exposed to extract and differences in hatching rates, each embryo morphological alteration and behavioral alteration of adult snails. In cases where ANOVA showed significant difference ($p < 0.05$) Tukey's multiple comparisons test was used. All these tests were conducted using the Vegan package (Oksanen et al. 2017) in the R software (R Core Team 2019). Results were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

Phytochemical characterization

The crude ethanolic extract of the bark of the *P. americana* stem showed a extraction yield of 8.184%. To know the secondary metabolite present in crude ethanolic extract of the *P. americana* stem bark, we pioneered conducted its phytochemical characterization. The presence of phenolic compounds, such as flavonoids, anthraquinone heterosides, coumarins and tannins was detected in the extract. On the other hand, alkaloids, cardioactive heterosides, and saponins were not detected (see Supplementary Material - Table SI). Phenolic compounds were also found in the ethanolic extract of leaves (Neto et al. 2014) and seed of this species (Silva et al. 2016). Similar secondary metabolites have been described in other species belonging to the same family of *P. americana* (Lauraceae), as in the ethanolic, hexanoic and ethyl acetate extracts of stem bark and *Litsea angulata* leaves, except for the presence of alkaloids and terpenoids (Kuspradini et al. 2019). In addition, different flavonoids were detected in the extracts and fractions obtained from *Ocotea notata* leaves (Pereira et al. 2019).

Thin Layer Chromatography (TLC) results showed the presence of quercetin flavonoid in *P. americana* extract (Rf = 0.99 cm), while no rutin-like stain pattern (Rf = 0.68 cm) was observed (Figure 1). The equation of the line and the correlation coefficient of the rutin pattern

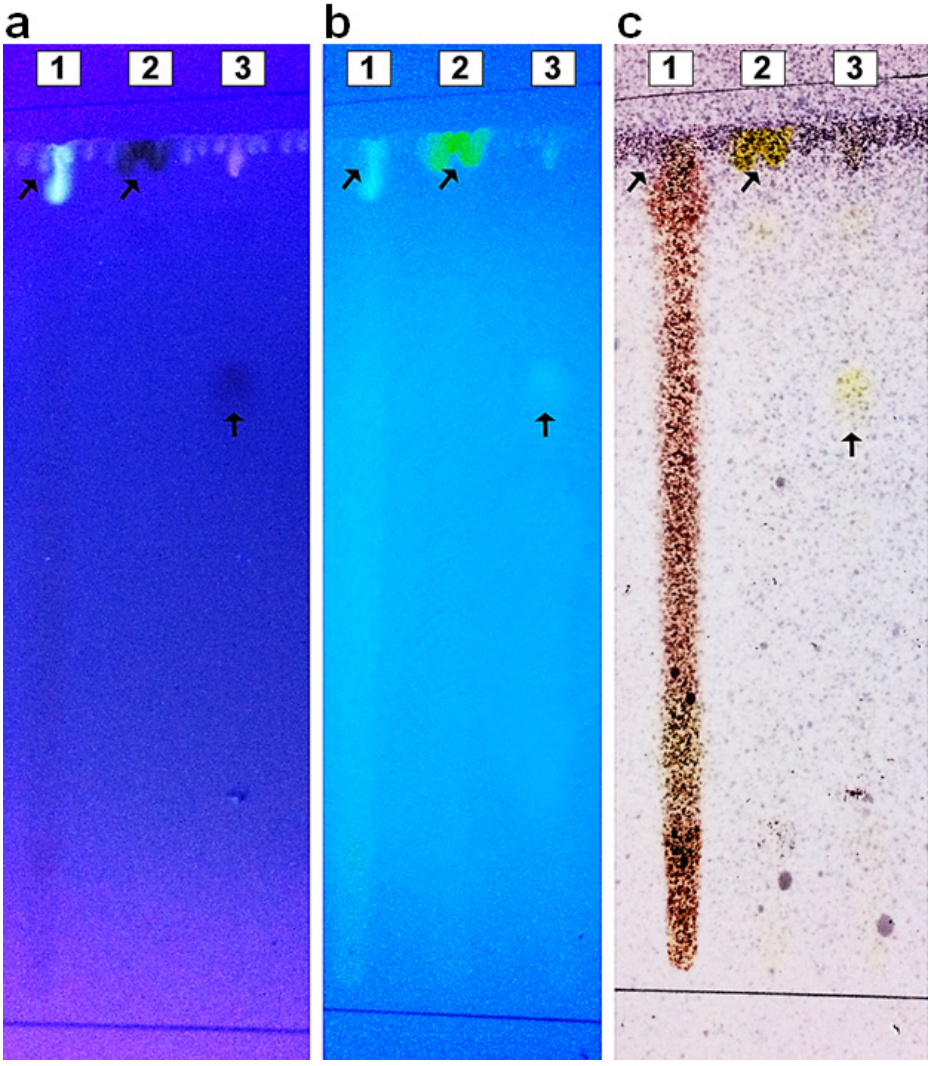

Figure 1. Thin Layer Chromatography (TLC) of the ethanolic crude extract of the *Persea americana* stem bark. (a) Ultraviolet observation at 365 nm. (b) Ultraviolet observation at 254 nm. (c) Observation after nebulization of sulfuric vanillin and heating. (1) *Persea americana*; (2) quercetin; (3) rutin. Mobile phase: ethyl acetate, acetone, acetic acid and ultrapure water (60:20:10:10 v/v/v/v).

analytical curve were: y = 0.0226x + 0.0756; R^2 = 0.9957. The concentration and content of total flavonoids expressed as rutin present in the extracted sample were 160.52 µg/mL and 0.802%, respectively. However, the total flavonoid contents may vary according to the species and the anatomical part of the plant, the collection period, the climate, the type of solvent used in the extraction and the storage form of the extract (Nascimento et al. 2011). The identification of the presence of phenolic compounds in the crude ethanolic extract may contribute to the elucidation of the biological properties of the *P. americana* stem bark.

Several plants biological activities are attributed to flavonoids, a fact that is related to their ability to cause damage to cell membranes and enzyme modulation (Panche et al. 2016). The presence of flavonoids and saponins in the extracts from *Bauhinia variegata* leaves and *Mimusops elengi* bark was considered responsible for the molluscicidal activity in the snail *Radix rufescens*, intermediate host of *Fasciola* sp. (Singh et al. 2012). Besides, Xu et al. (2010) showed the action of the flavonoid quercetin on cysteine-string protein alpha (CSPα), which affected synaptic transmission in *Lymnaea stagnalis*.

Embryotoxicity

B. glabrata embryos exposed to crude ethanolic extract of *P. americana* stem bark for 144 h showed increased mortality ($F_{6,56}$ = 88.38; p < 0.001; Figure 2a) in a concentration-dependent pattern (R^2 adj = 0.87; p < 0.05). After exposure to extract at 25 and 50 ppm, embryos showed higher mortality rate (54.00 ± 3.24% and 100%, respectively) when compared to negative control, solvent control and low concentrations of the extract (6.2 and 12.5 ppm) (p < 0.05; Figure 2a), indicating that the extract at 50 ppm induced similar mortality to positive control (niclosamide). The $LC_{50\text{-}144h}$ of the crude ethanolic extract of *P. americana* stem bark to *B. glabrata* embryos was 27.06 ppm (24.95 - 29.16) (Table I).

The surviving snails completed embryonic development to the last stage (hypo-stage), but the crude ethanolic extract reduced their hatching rate ($F_{6,56}$ = 27.60; p < 0.001; Figure 3). Results showed significant reduction in hatching rate of snails exposed to 25 ppm (1.00 ± 0.33%) and 50 ppm (0 ± 0%) when compared to the negative control group (94.00 ± 2.28%), solvent control (53.00 ± 4.46%) and other extract concentrations (p < 0.05). Snails exposed to niclosamide (positive control) did not hatch (Figure 3). Embryotoxic effects induced by molluscicidal compounds are influenced by molecular characteristics of substances, such as solubility, polarity, weight and size, which determine the transposition capacity of the gelatinous membrane coating the snail embryos (Miyasato et al. 2012, Duarte et al. 2015, Melo et al. 2019). *P. americana* extract showed high solubility in the egg-clutches, evidenced by their color change, especially in the exposure to higher concentrations (Figure 4), indicating the interaction and bioaccumulation of the extract in the egg-clutches.

For the development of new molluscicidal compounds, it is necessary to characterize the toxic effects on the embryos, since the toxicity of molluscicides is dependent on the development stage. In this sense, some compounds may have activity on adult snails, but be inactive on embryos, such as the activity of barbatic acid in *B. glabrata* (Martins et al. 2017). The mortality of embryos exposed to toxic substances may also be related to the inhibition of biological development processes or interference with the hatching mechanism (Adenusi & Odaibo 2009). These responses are interesting in terms of the reduced reproductive capacity of snails, with implications for decreasing population density and distribution of disease-transmitting snails (Kristoff et al. 2011).

Embryos exposed to the crude ethanolic extract of *P. americana* stem bark showed morphological alterations, such as coagulation of perivitelline substance ($F_{6,56}$ = 7.06; p < 0.0001) and shell malformation ($F_{6,56}$ = 2.92; p = 0.01); however, there were no significant differences for nonspecific malformations ($F_{6,56}$ = 2.06; p = 0.07) (Figure 4; Table II). Embryos exposed to 25 ppm (9.30 ± 1.20%) and 6.2 ppm (5.00 ± 0.87%) of the extract showed a significant increase in the rate of coagulation of perivitelline substance and shell malformation, respectively, compared to the negative control (p < 0.05; Table II). No morphological changes were observed in embryos exposed to 50 ppm extract and niclosamide (positive control), as their toxic effects induced the death of all embryos within the first 24 hours of exposure. Similarly, the exposure of snail embryos to toxic substances that exhibit high solubility in the perivitellin membrane may result in morphological changes during development or cause increased mortality rates, as previously reported for embryos with non-specific alterations (Oliveira-Filho et al. 2010).

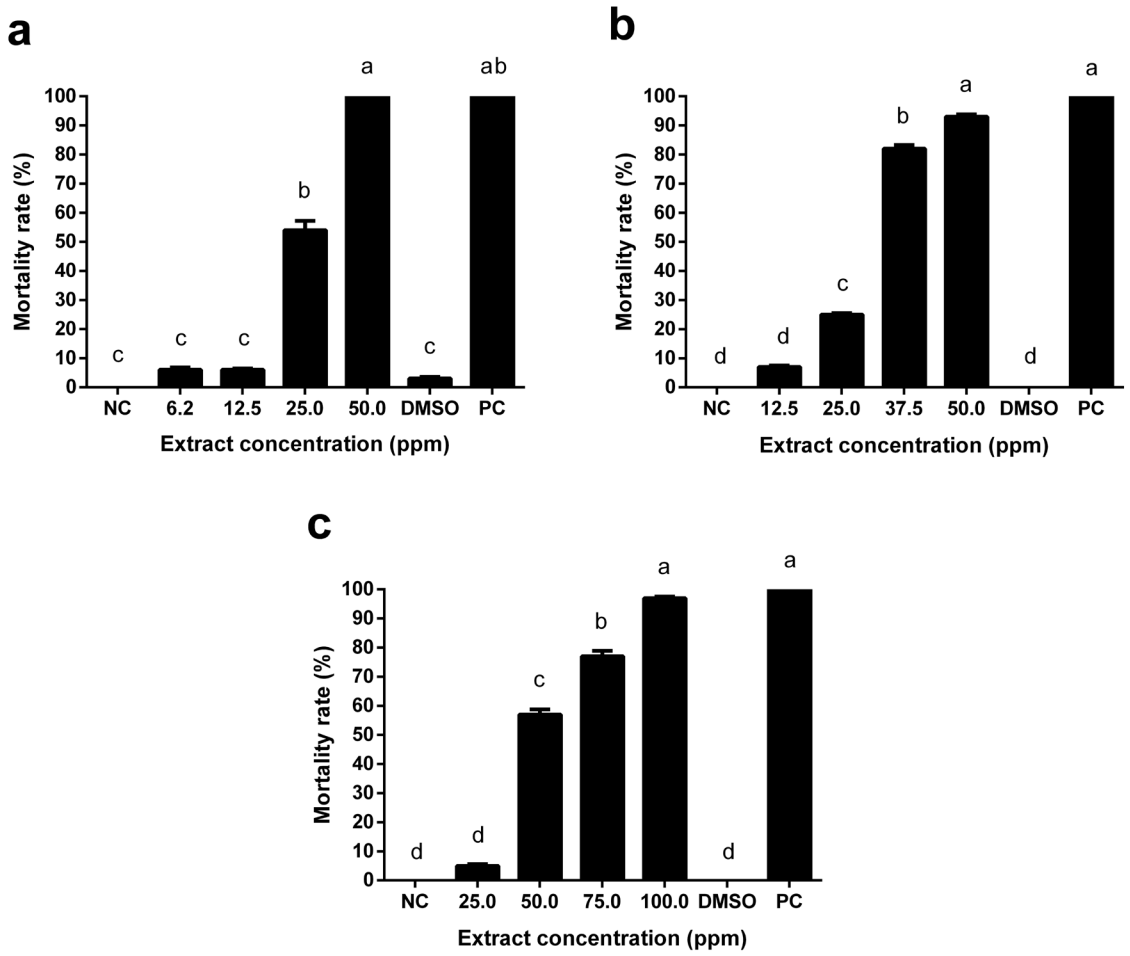

Figure 2. Mortality rate (%) of *Biomphalaria glabrata* embryos (**a**), newly-hatched (**b**) and adults (**c**) exposed to the ethanolic crude extract of the *Persea americana* stem bark. Embryos were exposed for 144 h, while newly-hatched and adults were exposed for 96 h. Results are expressed as mean ± standard deviation. One-way ANOVA followed by Tukey's multiple comparisons test. Different letters indicate differences between groups ($p < 0.05$). (NC) negative control; (PC) positive control (niclosamide); (DMSO) dimethylsulfoxide – solvent control.

Table I. Lethal concentrations [LC10, LC50 and LC90 (ppm)] of the crude ethanolic extract of the *Persea americana* stem bark to embryos, newly-hatched and adult of *Biomphalaria glabrata*. Results represent the mean and confidence interval (95%). (LC50): Mean Lethal Concentration.

Parameters	Embryos	Newly-hatched	Adults
LC10	9.67 [6.99 – 12.35]	14.74 [12.28 – 17.21]	22.82 [15.21 – 30.43]
LC50	27.06 [24.95 – 29.16]	30.60 [29.01 – 32.19]	55.55 [51.07 – 60.03]
LC90	44.44 [41.12 – 47.76]	46.45 [44.10 – 48.81]	88.28 [82.33 – 94.22]

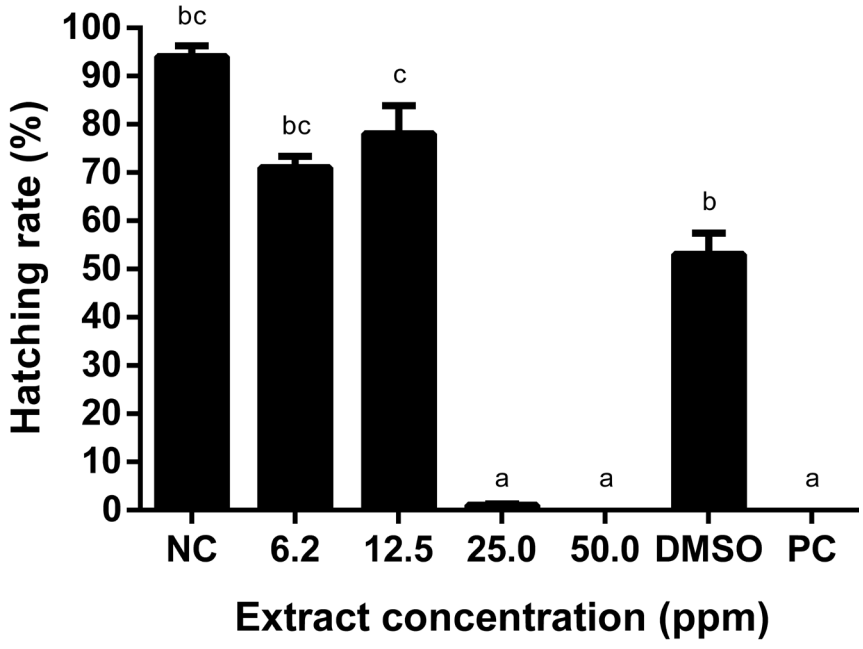

Figure 3. Hatching rate (%) of the *Biomphalaria glabrata* after exposure to the ethanolic crude extract of the *Persea americana* stem bark for 144 h. Results are expressed as mean ± standard deviation. One-way ANOVA followed by Tukey's multiple comparisons test. Different letters indicate differences between groups ($p < 0.05$). (NC) Negative control. (PC) Positive control (niclosamide); (DMSO) Dimethylsulfoxide – solvent control.

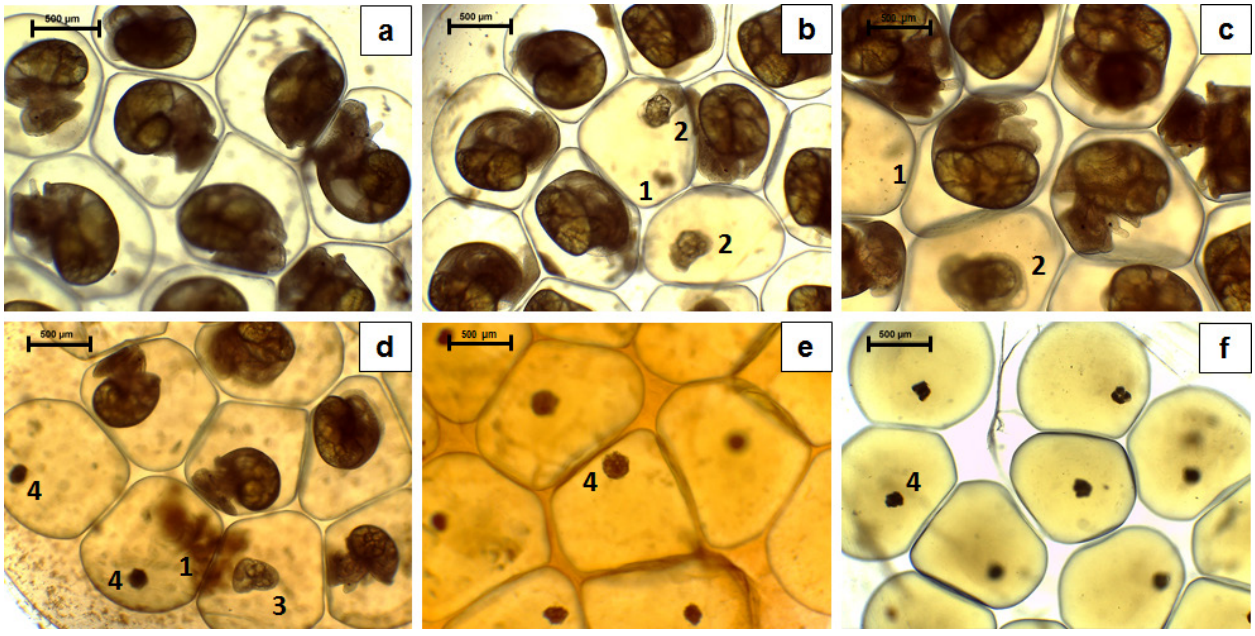

Figure 4. Morphological alterations and mortality of *Biomphalaria glabrata* embryos after exposure to the crude ethanolic extract of the *Persea americana* stem bark for 144 h. (**a**) Negative control (NC = reconstituted water). (**b**) Embryos exposed to 6.2 ppm. (**c**) Embryos exposed at 12.5 ppm. (**d**) Embryos exposed to 25 ppm. (**e**) Embryos exposed at 50 ppm. (**f**) Positive control (PC = niclosamide). (1) Coagulation of perivitelline substance. (2) Shell malformation. (3) Non-specific malformation. (4) Dead embryos. Magnification: 40 *x*. Scale: 500 µm.

Table II. Morphological alterations and mortality frequency (%) of *Biomphalaria glabrata* embryos after exposure to the crude ethanolic extract of the *Persea americana* stem bark for 144 h. Results are expressed as mean ± standard deviation. One-way ANOVA followed by Tukey's multiple comparisons test. Different letters indicate differences between groups (p < 0.05). (NC) Negative control. (PC) Positive control (niclosamide); (DMSO) Dimethylsulfoxide – solvent control.

Morphological alterations (%)	NC	Extract concentration (ppm)				DMSO	PC
		6.2	12.5	25.0	50.0		
Normal	100 ± 0	83.00 ± 1.56	86.50 ± 2.05	35.00 ± 4.59	0 ± 0	94.30 ± 2.05	0 ± 0
Coagulation of the perivitelline substance	0 ± 0 [a]	6.00 ± 0.97 [ab]	5.40 ± 1.36 [ab]	9.30 ± 1.20 [b]	0 ± 0 [a]	0 ± 0 [a]	0 ± 0 [a]
Shell malformation	0 ± 0 [a]	5.00 ± 0.87 [b]	0.70 ± 0.33 [ab]	0 ± 0 [a]	0 ± 0 [a]	1.70 ± 0.67 [ab]	0 ± 0 [a]
Nonspecific malformations	0 ± 0	0 ± 0	1.40 ± 0.44	1.70 ± 0.50	0 ± 0	1.00 ± 0.33	0 ± 0
Dead	0 ± 0	6.00 ± 0.83	6.00 ± 0.50	54.00 ± 3.24	100 ± 0	3.00 ± 0.53	100 ± 0

Acute toxicity to newly-hatched and adult snails

After the exposure period (96 h), newly-hatched snails exposed to crude ethanolic extract from *P. americana* stem shell showed higher mortality rate ($F_{6,35}$ = 294.40; p < 0.001; Figure 2b) in a concentration-dependent pattern (R^2 adj = 0.89; p < 0.05). Newly-hatched exposed to extracts at 37.5 ppm (82.00 ± 1.33%) and 50 ppm (93.00 ± 0.82%) exhibited higher mortality rates compared to negative control, solvent control (0 ± 0%) and other concentrations of the extract (p < 0.05; Figure 2b). The $LC_{50\text{-}96h}$ of the ethanolic crude extract of *P. americana* stem bark to newly-hatched *B. glabrata* was 30.60 ppm (29.01 - 32.19) (Table I). At the end of the experiment, there was no mortality of newly-hatched snails from the negative control and solvent groups, while exposure to niclosamide (positive control) resulted in 100% mortality (Figure 2b). Niclosamide is a molluscicide that causes mortality in the first hours of exposure in newly-hatched embryos and adults and is recommended by the WHO for the control of snails *Biomphalaria* spp. in endemic areas of schistosomiasis (Rocha-Filho et al. 2015). However, the ethanolic crude extract of *P. americana* stem bark at 50 ppm showed similar molluscicidal activity to niclosamide, indicating that this plant-derived product is potential candidate for control of snails.

After the exposure (96 h), adult snails showed increased mortality ($F_{6,35}$ = 122.50; p < 0.001; Figure 2c) in a concentration-dependent pattern (R^2 adj = 0.90; p < 0.05). Adult snails exposed to 50 ppm (57.00 ± 1.75%) and 75 ppm (77.00 ± 1.86%) showed higher mortality rates compared to negative control, control solvent (0 ± 0%) and 25 ppm (5.00 ± 0.55%) (p < 0.05; Figure 2c). The group exposed to 100 ppm showed a similar response to that observed in niclosamide exposure (positive control), which resulted in 100% mortality (p > 0.05). No mortality was observed in the negative control and solvent

groups. The $LC_{50\text{-}96h}$ of the crude ethanolic extract of *P. americana* stem bark in adult *B. glabrata* was 55.55 ppm (51.07 - 60.03) (Table I).

The present study showed that the crude ethanolic extract of *P. americana* stem bark has molluscicidal activity at all stages of the *B. glabrata* life cycle and the early developmental stages (embryos and newly-hatched snails) are more sensitive to the toxic effects of extracts when compared to adults. Because it is crude extract, the MoA of secondary metabolites may be the result of individual activity or synergism between compounds (Silva et al. 2013). Results indicated that the LC_{50} of *P. americana* extract was lower for embryos (27.06 ppm) and newly-hatched (30.60 ppm) compared to adult snails (55.55 ppm), demonstrating that the toxicity of *P. americana* extract to *B. glabrata* is dependent on developmental stage. However, certain compounds have higher toxicity in adult snails than in embryos and may even be inactive at embryonic developmental stages (Rapado et al. 2013). Such differences can be identified in the same phase of the life cycle, which is related to several factors, such as tested concentrations and/or relationship between concentration and biometric data of snails, exposure time, size of molecules, shapes, and release time of compounds (Oliveira-Filho et al. 2019).

Interestingly, the *P. americana* extract LC_{50} in embryos, newly-hatched and adult *B. glabrata* was lower than the values observed for other plant extracts and derivatives, such as *Euphorbia milii* latex ($LC_{50\text{-}96h:}$ 69.44 ppm) (Oliveira-Filho et al. 2010), *Curcuma longa* isolated curcumin ($LC_{50\text{-}24h:}$ 42.29 ppm) (Matos et al. 2019), the crude hydroalcoholic extract of the *Manilkara subsericea* leaf ($LC_{50\text{-}96h:}$ 118.70 ppm) (Faria et al. 2018) and the n-hexane fraction obtained from the methanolic crude extract of *Ficus radicans* leaf ($LC_{50\text{-}24h:}$ 400.00 ppm) (Naressi et al. 2012). These findings reinforce that *P. americana* extract presents more effective molluscicidal activity when compared to other plant species investigated.

Behavioral alterations

The crude ethanolic extract of *P. americana* stem bark induced behavioral alterations in adult snails after 96 h of exposure, such as lethargy and shell reclusion ($F_{6,35}$= 7.96; $p < 0.0001$; Figure 5a). In addition to behavioral alterations, snails exposed to crude ethanolic extract showed hemolymph release ($F_{6,35}$ = 122.50; $p < 0.0001$; Figure 5b), which was related to mortality. The snails exposed to 50 ppm (2.00 ± 0.41%) and 100 ppm (3.00 ± 0.52%) exhibited low lethargy and shell reclusion rate. On the other hand, the snails exposed to 75 ppm (23.00 ± 1.86%) exhibited significantly higher lethargy and shell reclusion rate compared to negative control, solvent control (0 ± 0%) and other concentrations of the extract ($p < 0.05$; Figure 5a), showing the possibility of the snails being evaded from the medium containing the substance harmful to them (Pieri & Jurberg 1981) which reaches its peak at 75 ppm. Also, adult snails exposed at 50 ppm (57.00 ± 1.75%) and 75 ppm (77.00 ± 1.86%) showed increased hemolymph release rate compared to negative control, solvent control (0 ± 0%) and 25 ppm of the extract (5.00 ± 0.55%) ($p < 0.05$; Figure 5b), indicating the occurrence of tissue injury and loss of biological functions (Kady et al. 1992, Faria et al. 2018). It is worth mentioning that after exposure to the crude ethanolic extract at 100 ppm (97.00 ± 0.52%), the snails showed increasing hemolymph release rate and behavior changes similar to those exposed to niclosamide ($p > 0.05$; Figure 5b), except for mucus secretion observed in all snails from the positive control. At the end of the experiment, the negative and solvent control groups remained with usual behaviors.

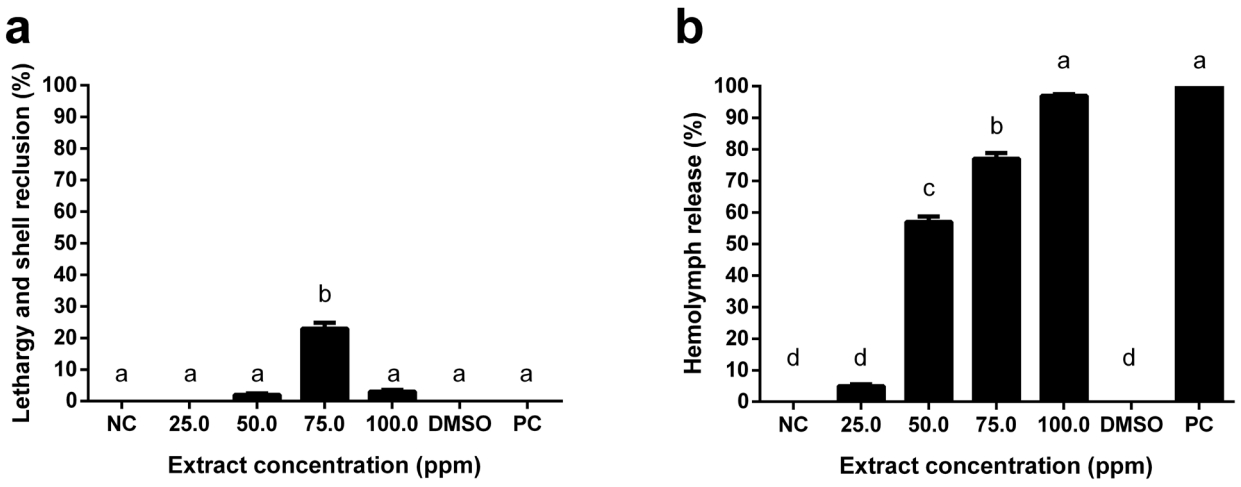

Figure 5. Frequency (%) of lethargy and shell reclusion (a) and hemolymph release (b) of the *Biomphalaria glabrata* adults after exposure to the ethanolic crude extract of the *Persea americana* stem bark for 96 h. Results are expressed as mean ± standard deviation. One-way ANOVA followed by Tukey's multiple comparisons test. Different letters indicate differences between groups ($p < 0.05$). (NC) Negative control. (PC) Positive control (niclosamide); (DMSO) Dimethylsulfoxide – solvent control.

The release of hemolymph into the medium in snails exposed to extract may have been a response to interference in the coagulation process by coumarin (Kady et al. 1992) or flavonoid-mediated cell membrane disruption (Faria et al. 2018). Several saponins can complex phospholipids and cell membrane proteins and, in mollusks, are capable of causing cell lysis (or hemolysis), hemolymph release and death (Mc Cullough et al. 1980, Mendes et al. 2018). Similar behavioral alterations and hemolymph release were reported for *B. glabrata* after 96 h of exposure to Polyhexamethylene biguanide hydrochloride (PHMB) (Melo et al. 2019), indicating systemic toxicity of both compounds. As observed in the present study, in adverse ecophysiological conditions, such as exposure to molluscicidal agents or infection by *S. mansoni*, the intermediate host may present several defensive behaviors to increasing its fitness. In this sense, the snails can "commite suicide" (change the time and nature of your death) and/or exhibit behavioral alterations in order to increase the possibility of predation and consequent death, as well as reduce the rate of capture and accumulation of toxic compounds (Trail 1980).

CONCLUSION

The crude ethanolic extract of *P. americana* stem bark has phenolic compounds, such as flavonoids, anthraquinone heterosides, coumarins, and tannins, and showed molluscicidal activity to all stages of the *B. glabrata* life cycle. The extract inhibited hatching and induced morphological alterations in the embryos, reinforcing its action potential in the early stages of the *S. mansoni* intermediate snail host cycle. In addition, the early developmental stages (embryos and newly-hatched snails) are more sensitive to the toxic effects of extracts when compared to adults. In adult snails, behavioral alterations such as lethargy and shell reclusion were observed, in addition to answers like hemolymph release,

suggesting that the extract caused an imbalance in the homeostasis of snails. Thus, the crude ethanolic extract of *P. americana* stem bark is a potential candidate for molluscicide, contributing to the control of host snails and reducing the transmission of schistosomiasis. Further studies are required, prioritizing extract fractionation, toxicity testing on non-target organisms, and further elucidation of the active molecules.

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SUPPLEMENTARY MATERIAL

Table SI.

How to cite

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