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Assessment of toxicity of *Moringa oleifera* flower extract to *Biomphalaria glabrata*, *Schistosoma mansoni* and *Artemia salina*

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HIGHLIGHTS

- *M. oleifera* flower extract delayed the development of *B. glabrata* embryos.
- The extract promoted mortality of adult snails (LC_{50}: 2.37 ± 0.5 mg mL^{-1}).
- Embryos generated by snails exposed to the extract were also affected.
- The extract was active even after exposure to tropical environmental conditions.
- Unrestricted use of extract should be avoided due to its toxicity to *A. salina*.

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ABSTRACT

This study reports the effect of an aqueous extract from *Moringa oleifera* Lam. flowers on *Biomphalaria glabrata* embryos and adults and on *Schistosoma mansoni* adult worms. The extract contains tannins, saponins, flavones, flavonols, xanthones, and trypsin inhibitor activity. The toxicity of the extract on *Artemia salina* larvae was also investigated to determine the safety of its use for schistosomiasis control. After incubation for 24 h, the flower extract significantly ($p < 0.05$) delayed the development of *B. glabrata* embryos and promoted mortality of adult snails (LC_{50}: 2.37 ± 0.5 mg mL^{-1}). Furthermore, treatment with the extract disrupted the development of embryos generated by snails, with most of them remaining in the blastula stage while control embryos were already in the gastrula stage. Flower extract killed *A. salina* larvae with a LC_{50} value (0.2 ± 0.015 mg mL^{-1}) lower than that determined for snails. A small reduction (17%) in molluscicidal activity was detected when flower extract (2.37 mg mL^{-1}) was exposed to tropical environmental conditions (UVI index ranging from 1 to 14, temperature from 25 to 30 °C, and 65% relative humidity). Toxicity to *A. salina* was also reduced (LC_{50} value of 0.28 ± 0.01 mg mL^{-1}). In conclusion, *M. oleifera* flower extract had deleterious effects on *B. glabrata* adults and embryos. However, unrestricted use to control schistosomiasis should be avoided due to the toxicity of this extract on *A. salina*.

1. Introduction

Biomphalaria glabrata (Gastropoda, Planorbidae) is a snail found in streams, lakes, and wetlands in South and Central Americas as well as in Africa. It is an intermediate host of the trematode worm *Schistosoma mansoni*, which causes schistosomiasis (bilharziasis), a chronic disease that affects 240 million people every year (Gryseels et al., 2006; World Health Organization, 2014). Schistosomiasis control focuses on periodic and large-scale preventive chemotherapy using praziquantel, which although effective and inexpensive, causes allergic and hypersensitivity reactions and does not prevent reinfection, which consequently lead to selection of resistant parasites (Aires et al., 2014; World Health Organization, 2014).

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Control of *B. glabrata* comprises an important element in integrated strategies for reducing the spread of schistosomiasis, and the single molluscicide recommended by the World Health Organization is the organic compound niclosamide. This molluscicide possesses low toxicity to mammals and is relatively non-persistent; however, it is toxic to fishes, amphibians, and crustaceans (Salem et al., 2014; World Health Organization, 2014).

Plant compounds with molluscicidal activity, which usually possess a high degree of degradability, are promising alternatives that may expand the range of molluscicides available for *B. glabrata* control, including use with niclosamide in rotation programs (Al-Zanbagi et al., 2000; Schall et al., 2001; Lima et al., 2002; Luna et al., 2005). Lectins, essential oils, flavonoids, saponins, terpenes and tannin from plants have been investigated as candidates for snail control (Diab et al., 2012; Singh et al., 2012; Teixeira et al., 2012; Albuquerque et al., 2014). Furthermore, toxicity to *S. mansoni* associated with tannins, saponins, and flavonoids has been reported (Abozeid et al., 2012; Braguine et al., 2012; Allan et al., 2014).

Assessing the environmental risk of new molluscicides is essential since this can limit their use. An assay using brine shrimp *Artemia salina* (Brachiopoda, Artemiidae) is an efficient method to evaluate environmental contamination, water quality, and toxicity of biocides (Nunes et al., 2006).

Flowers of *Moringa oleifera* Lam. (Moringaceae) contain bioactive compounds including antioxidants, antimicrobial alkaloids, lectin, and trypsin inhibitor (Lizzy et al., 1968; Sánchez-Machado et al., 2006; Santos et al., 2009; Pontual et al., 2014). Aqueous extract from *M. oleifera* flowers containing trypsin inhibitor, β-amyrin, sterol, kaempferol, and quercetin exerted a larvicidal effect against *Aedes aegypti*, and the trypsin inhibitor isolated from it arrested the development of *A. aegypti* larvae (Pontual et al., 2012, 2014). In the present study, aqueous extract from *M. oleifera* flowers was prepared following methods used in previous studies, and was evaluated for molluscicidal activity on *B. glabrata*, and for schistosomicidal and artemicidal activities.

2. Materials and methods

2.1. M. oleifera flower extract

Flowers (500 g) were collected from *M. oleifera* trees in July 2012 at the campus of the *Universidade Federal de Pernambuco* (Recife City, State of Pernambuco, Northeastern Brazil) with authorization (38690-1) of the *Instituto Chico Mendes de Conservação da Biodiversidade* from the Brazilian Ministry of Environment. A voucher specimen (number 73345) is deposited at the herbarium "Dárdano de Andrade Lima" (*Instituto Agronômico de Pernambuco*, IPA, Recife, Brazil).

The extract was obtained according to the method described by Pontual et al. (2012). Flowers (50 g) were added to distilled water (100 mL) and homogenized in a blender for 10 min at 27 °C. The extract (clear supernatant) was obtained after filtration through cotton gauze (Cremer, Blumenau, Brazil) and centrifugation (9000×g, 15 min, 4 °C). The extract was then lyophilized to dryness for 24 h in a LIOTOP freeze-dryer, model L101 (Liobras, São Carlos, Brazil). After lyophilization, the extract was stored at −20 °C for use in the bioassays.

2.2. Evaluation of compounds in M. oleifera flower extract

Flower extract was investigated for the presence of alkaloids, tannins, saponins, triterpenoids, and steroids according to standard procedures of analysis (Moreira, 1979; Lock-de-Ugaz, 1988; Matos, 1997) using different reagents (Table 1).

Table 1
Reagents used in the phytochemical screening of *M. oleifera* flower extract and reactions observed for positive results.

Secondary metabolite	Reagents	Positive reaction
Alkaloids	Bouchardat reagent	Reddish orange precipitate formation
Tannins/ polyphenols	1% $FeCl_3$ aqueous solution	Color change or precipitate formation
Saponins	80GL: ethanol/distilled water hydroalcoholic solution	Formation of stable foam for more than 30 min
Steroids and triterpenoids	$CHCl_3$, acetic anhydride and concentrated H_2SO_4	Succession of colors from evanescent blue to persistent green

The presence of anthocyanins, anthocyanidins, flavones, flavonols, xanthones, chalcones, and aurones was investigated using the assay described by Matos (1997) which involves adjusting the pH of the sample to 3.0, 8.5, or 11.0. When anthocyanins and anthocyanidins are present in a sample, the colors red, lilac, and purple-blue are observed at pH 3.0, 8.5, and 11.0, respectively. Flavones, flavonols, and xanthones do not alter the color of the sample at pH 3.0 and 8.5, but appear as yellow or reddish orange at pH 11.0. Chalcones and aurones appear red at pH 3.0, have no effect on color at pH 8.5, and change to purple-red at pH 11.

The presence of lectin in flower extract was investigated by determining the hemagglutinating activity in microtiter plates (TPP-Techno Plastic Products) according to the method described by Paiva and Coelho (1992). Hemagglutinating activity was evaluated by mixing a twofold serial dilution of flower extract (50 μL) in 0.15 M NaCl in microtiter plates. Next, 50 μL of a suspension (2.5% v/v) of glutaraldehyde-treated rabbit erythrocytes in 0.15 M NaCl was added to each well and the plate was incubated at 27 °C for 45 min. One hemagglutination unit was defined as the reciprocal value of the highest dilution of sample that promoted full agglutination of erythrocytes.

The presence of trypsin inhibitor activity was evaluated according to the method described by Pontual et al. (2014). Bovine trypsin (5 μL; 0.1 mg mL^{-1} in 0.1 M Tris–HCl pH 8.0 containing 0.02 M $CaCl_2$) was incubated for 5 min at 37 °C with plant extract (20 μL; 0.334 mg) in one well of a 96-well microplate. The volume was then adjusted to 195 μL with Tris–HCl pH 8.0. Next, the substrate *N*-benzoyl-DL-arginyl-ρ-nitroanilide (8 mM) dissolved in dimethyl sulfoxide (DMSO) was added (5 μL) and the mixture was incubated for 30 min at 37 °C. As a control (100% substrate hydrolysis) the reaction was performed by replacing the flower extract with distilled water (20 μL). Substrate hydrolysis was followed by measuring the absorbance at 405 nm using a microplate spectrophotometer (μQuant, MQX200; BioTek Instruments, Inc., VT, USA). Blank reactions were performed under the same conditions, without the substrate or without enzyme. One unit of trypsin inhibitor activity was defined as the amount of inhibitor that decreased the absorbance by 0.01 after 30 min at 37 °C, compared with the control. Specific trypsin inhibitor activity (U mg^{-1}) corresponded to the ratio between the inhibitor activity (U) and protein concentration (mg), which was determined according to the method described by Lowry et al. (1951).

2.3. Bioassays using embryos and adults of B. glabrata

Adult *B. glabrata* snails that were not infected by trematodes were reared in the *Departamento de Biofísica e Radiobiologia*, *Universidade Federal de Pernambuco* (Recife, Brazil). The snails were