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Major Article

Development of a natural molluscicide prototype kit (MoluSchall) for the control of schistosomiasis mansoni transmission

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Abstract

Introduction: In Brazil, *Biomphalaria glabrata*, *B. tenagophila,* and *B. straminea* are intermediate hosts of *Schistosoma mansoni*, the etiological agent of schistosomiasis mansoni. Molluscicide use is recommended by the WHO for controlling the transmission of this parasite. *Euphorbia milii* latex has shown promising results as an alternative molluscicide. Thus, a natural molluscicide prototype kit based on freeze-dried *E. milii* latex was developed and evaluated against *Biomphalaria* spp. **Methods**: *E. milii* latex was collected, processed, and lyophilized. Two diluents were defined for freeze-dried latex rehydration, and a prototype kit, called MoluSchall, was produced. A stability test was conducted using prototype kits stored at different temperatures, and a toxicity assay was performed using *Danio rerio.* Additionally, MoluSchall was tested against *B. glabrata* under semi-natural conditions according to defined conditions in the laboratory. **Results**: MoluSchall was lethal to three Brazilian snail species while exhibiting low toxicity to *D. rerio.* Regardless of storage temperature, MoluSchall was stable for 24 months and was effective against *B. glabrata* under semi-natural conditions, with the same LD_{100} as observed under laboratory conditions. **Conclusions**: MoluSchall is a natural, effective, and inexpensive molluscicide with lower environmental toxicity than existing molluscicides. Its production offers a possible alternative strategy for controlling *S. mansoni* transmission.

Keywords: *Euphorbia milii*. MoluSchall. Natural molluscicide. Prototype kit. Schistosomiasis control.

INTRODUCTION

Human schistosomiasis is a parasitic disease caused by trematode flukes of the genus *Schistosoma*. More than 240 million people worldwide have schistosomiasis, and almost 700 million are at risk of infection[1]. Of the six *Schistosoma* spp. that cause human infections, only *Schistosoma mansoni* (Sambon, 1907) maintains a life cycle in the American continent. Brazil is considered an important endemic country, with approximately 1.5 million infected individuals[2].

Freshwater snails of the genus *Biomphalaria* (Preston, 1910) are the intermediate hosts of *S. mansoni.* In Brazil, the species *Biomphalaria glabrata* (Say, 1818)*, Biomphalaria tenagophila* (Orbigny, 1835), and *Biomphalaria straminea* (Dunker, 1848) are naturally infected and are essential for the transmission of *S. mansoni*[3].

It is necessary to carry out integrated measures to implement and sustain successful control programs. These integrated

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measures should include the use of chemotherapy in association with the use of molluscicides for snail control, as well as educational interventions and improved sanitation systems, including a regular supply of treated water[4].

For the past few decades, the WHO has recommended the application of the synthetic molluscicide, niclosamide, which is the 2-amino ethanol salt of 2′,5-dichloro-4′-nitro salicylanilide or Bayluscide®, in natural sites where snails are present. Since a report published in 1953 by the WHO, the use of molluscicides in schistosomiasis control has been recommended along with other control measures[5]. In 1961, another report concluded that the application of molluscicides in the fight against intermediate hosts was the single most efficient measure for controlling schistosomiasis[6]. Recently, the WHO published an operational manual to facilitate the reintroduction of practices and protocols for the field use of molluscicides in schistosomiasis control programs, reaffirming their importance in these programs[4].

In Brazil, since the 1970s, the synthetic molluscicide Bayluscide® has been used in national schistosomiasis control programs[7]. However, after 1986, its use progressively decreased and starting in 2002, the Brazilian Ministry of Health did not register the use of Bayluscide® in the country[7]. According to Coelho and Caldeira[7], this decrease was probably associated with the lack of specificity to *Biomphalaria* and to more stringent regulations regarding the use of toxic substances in aquatic ecosystems.

In this context, natural molluscicides are a promising alternative to synthetic chemicals since they are locally available, cheaper, and less hazardous to the aquatic environment[8]. Several authors have demonstrated the molluscicidal effects of some plants against snail species[9,10,11]. The latex of *Euphorbia milii* var. *hislopii* (N.E.Br.) Ursch & Leandri 1955 (Syn. *Euphorbia splendens* var. *hislopii* N.E.Br.), also known as the "Crown of Christ", showed the lowest lethal dose mentioned in the literature[9].

The lethal dose defined for *Euphorbia* spp. complies with the WHO recommendations for a plant to be considered a molluscicide[9]. Furthermore, toxicological tests have revealed no toxic effects at the concentrations utilized[12-15].

Here, we present a prototype kit based on *E. milii* latex that is effective against the three species of *Biomphalaria* naturally infected by *S. mansoni* in Brazil.

METHODS

Latex collection

Specimens of *E. milii* var. *hislopii* were cultivated in an area of approximately 1.35 m^2 in the Laboratório Nacional Agropecuário (LANAGRO), Pedro Leopoldo, Minas Gerais, Brazil (19°38'01.9"S, 44°02'42.3"W).

Samples of latex were collected every two months for three years. After longitudinal incisions were made in the stems, specimens were stored in Falcon® tubes (Corning Brasil Ind & Comercio Ltda, São Paulo, SP, Brazil). After collection, the samples were rapidly transported in plastic boxes, at room temperature, to the Instituto René Rachou, Oswaldo Cruz Foundation (IRR/FIOCRUZ), where they were processed.

Snails

All snails used in this study were produced by Moluscário Lobato Paraense, IRR/FIOCRUZ, and included the following: 600 uninfected *B. glabrata* measuring 3 to 6 mm in diameter; 2,430 uninfected *B. glabrata* measuring 10 to 12 mm in diameter; 180 uninfected *B. glabrata* measuring 12 to 17 mm in diameter; and 180 *B. glabrata* measuring 12 to 17 mm in diameter that were infected for 39 days with *S. mansoni* (LE strain). We also used 180 uninfected *B. tenagophila* measuring 10 to 12 mm in diameter and 180 uninfected *B. straminea* measuring 5 to 10 mm in diameter.

E. milii latex preparation and freeze-drying

Crude latex was filtered through surgical gauze and the pH was measured with a pH meter (Hanna Instruments, Woonsocket, RI, USA). The latex was aliquoted in amber vials (5 mL/vial), weighed on an analytical balance (Sartorius AG, Goettingen, GE), and frozen at -80°C for 18 h before being subjected to freeze-drying in an Alpha2-4 LD plus lyophilizer (Martin Christ GmbH, Osterode am Harz, GE) at 0.020 mbar and -55°C for 24 h. At the end of the process, the vials were vacuum sealed, the dry mass was weighed, and the vials were stored in a refrigerator at 2-8°C.

Freeze-dried *E. milii* latex rehydration

In a previous experiment, we observed that water did not fully rehydrate freeze-dried *E. milii* latex. To improve the rehydration, a series of additives were included in the latex preparation, such as 5% trehalose, 5% trehalose + 10% mannitol, 5% trehalose + 10% betaine, 5% and 20% mannitol, 5% and 10% N-carboxymethylcellulose (CMC), 0.5% CMC + 15% mannitol, 5% mannitol + 2% sodium dodecyl sulfate (SDS), 0.16 M N-acetylcysteine (NAC), and 2 mg/mL ethylenediaminetetraacetic acid (EDTA). After the freeze-drying process, the final product was rehydrated with water and the latex dissolution was visually evaluated. After several tests, a formulation was developed consisting of two diluents, denoted in this work as Diluent 1 and Diluent 2. After adding these diluents, it was possible to completely rehydrate the freeze-dried *E. milii* latex.

Freeze-dried *E. milii* latex proof-of-concept

In the proof-of-concept phase, freeze-dried *E. milii* latex was rehydrated with Diluent 1 and Diluent 2 and tested against *B. glabrata*. For this, 90 uninfected *B. glabrata* measuring 10 to 12 mm in diameter were used. The snails were separated into subgroups of 10 snails and placed in triplicate in beakers containing latex diluted to 1 µL/L and 10 µL/L in dechlorinated water. For the control group, snails were separated into subgroups of 10 snails and maintained in dechlorinated water containing Diluent 1 and Diluent 2 under the same conditions. The mortality rates were 80% and 100% at 1 µL/L and 10 µL/L, respectively. There was no mortality in the control group. These results confirmed the molluscicidal activity of the latex after the freeze-drying process.

A molluscicide prototype kit: MoluSchall

A prototype kit was composed of 10 vials of freeze-dried *E. milii* latex, 10 vials of Diluent 1 (5 mL), and 10 vials of Diluent 2 (5 mL). The molluscicide prototype kit was called MoluSchall in honor of Dra. Virgínia Torres Schall, the researcher who highlighted the molluscicidal activity in her pioneering work[12,16,17].

Lethal Dose (LD_{100}) of MoluSchall against *Biomphalaria* spp.

The latex LD_{100} determination was performed according to the methodology recommended by the WHO and described by Schall et al.[17]. In this experiment, 180 snails of three species, including *B. glabrata, B. tenagophila,* and *B. straminea* were separated into groups A, B, and C, respectively.

Using 150 snails from groups A, B, and C, subgroups of 10 snails were formed. These snails were exposed in triplicate to the molluscicide diluted to 1, 2, 4, 8, and 12 µL/L in dechlorinated water. For controls, 30 snails of each species were separated into subgroups of 10 individuals and kept in triplicate in dechlorinated water containing 12 µL/L of Diluent 1 and 12 µL/L of Diluent 2. The snails were maintained in beakers with 500 mL of the different solutions for 24 h at room temperature. After exposure, the snails were removed from the beakers and washed, and the live snails were returned to the same beakers containing 500 mL of dechlorinated water only. Pieces of lettuce were added to the beakers, and the snails were observed for more than 24 h to assess mortality. Dead and surviving animals were counted after 48 h. Mortality was identified by the loss of muscle contraction and heartbeat, shell discoloration, deterioration of the cephalopodal mass, and hemolymph release via a stereoscopic microscope.

Lethal Dose (LD_{100}) of MoluSchall against infected *B. glabrata*

In addition, molluscicidal activity assays were performed on *B. glabrata* infected with *S. mansoni*. For this experiment, 360 snails were equally separated into an infected and uninfected group. The snails in both groups were separated into subgroups of 10 snails and exposed in triplicate to MoluSchall diluted to 1, 2, 4, 8, and 12 µL/L in dechlorinated water. As controls, 30 snails from each group were separated into subgroups of 10 and kept in triplicate in dechlorinated water containing 12 µL/L of Diluent 1 and 12 µL/L of Diluent 2. Snail mortality was measured as described above in the LD_{100} determination experiment.

Thermal stability of MoluSchall

A thermal stability test was conducted over 24 months using prototype kits stored in a refrigerator (2-8°C), at ambient temperature (22-26°C), and in an incubator (37°C). Every two months, one vial from each prototype kit stored at the different temperatures was randomly chosen, and its physical appearance was visually assessed. Subsequently, the vials were rehydrated with Diluent 1 and Diluent 2 and tested against ten *B. glabrata* snails placed in triplicate in beakers containing MoluSchall diluted to 8 µL/L in dechlorinated water for 24 h at room temperature. As the control group, 10 snails in triplicate were kept in beakers containing 8 µL/L Diluent 1 and Diluent 2 in dechlorinated water under the same conditions. Mortality was assessed as described in the LD_{100} determination experiment.

Toxicity bioassay for MoluSchall

To investigate the toxicity of MoluSchall, *Danio rerio* (Hamilton, 1822) fingerlings that were three days post-fertilization were collected after natural spawning and reared in E2 embryo medium (E2) at 28°C at a density of 60 fingerlings/Petri dish[18,19].

Groups of five *D. rerio* fingerlings were placed in duplicate in culture plates containing MoluSchall diluted to 8, 16, 32, 64, and 128 µL/L in E2 medium. As the control group, 10 *D. rerio* fingerlings were maintained in a culture plate containing only E2 medium. The toxicity of MoluSchall was evaluated every 24 h over 72 h of exposure. At the end of the experiment, mortality was assessed.

Field MoluSchall activity assay

The molluscicidal activity of MoluSchall in semi-natural conditions was also evaluated in three artificial puddles containing 1,000 L of water. For this experiment, 1,800 uninfected *B. glabrata* were used and separated into three groups, puddle A, puddle B, and a control puddle, with each group containing 200 snails that were 3 to 6 mm in diameter, 200 snails that were 7 to 9 mm in diameter, and 200 snails that were 10 to 12 mm in diameter.

Puddles A and B were treated with MoluSchall at 8 µL/L and 12 µL/L, respectively, while the control puddle was not treated. The researchers followed the WHO's safety instructions[4] at all stages of application to prevent direct contact with the skin or eyes.

After 48 h of MoluSchall exposure, the snails were collected and observed, and mortality was assessed. A second experiment was performed on a different day.

Statistical analysis

The results were expressed as percentages of the total number of snails used in the bioassays and subjected to analysis of variance (ANOVA), Tukey's multiple comparisons test, and the Student's t-test (or Mann-Whitney test) using the program GraphPad Prism 4.0.

Ethical considerations

The experimental and breeding procedures used for the *D. rerio* fingerlings were approved by the Ethics Committee for Animal Use of the Federal University of Minas Gerais State (CEUA/UFMG), under protocol no. 9/2012.

RESULTS

Twenty-nine latex samples were collected throughout the study. On average, 60 mL were collected on the appropriate day. The average weight ratio of the crude to the freeze-dried *E. milii* latex showed that 5 mL weighed approximately 1 g post-freeze-drying. However, this equivalency was not constant, as the weight varied from 1.02 to 1.24 g, with a mean of 1.09 g per vial.

The LD_{100} was 8 µL/L against *B. glabrata* and *B. straminea* and 4 µL/L against *B. tenagophila.* There was a statistical difference between the LD_{100} of *B. glabrata* and *B. straminea* when compared to *B. tenagophila* (p = 0.031). No mortality occurred in the control groups, which were exposed to dechlorinated water containing only Diluent 1 and Diluent 2 (**Figure 1**). The snails in all control groups were active, foraging in the beaker, and consuming the lettuce during the recovery period.

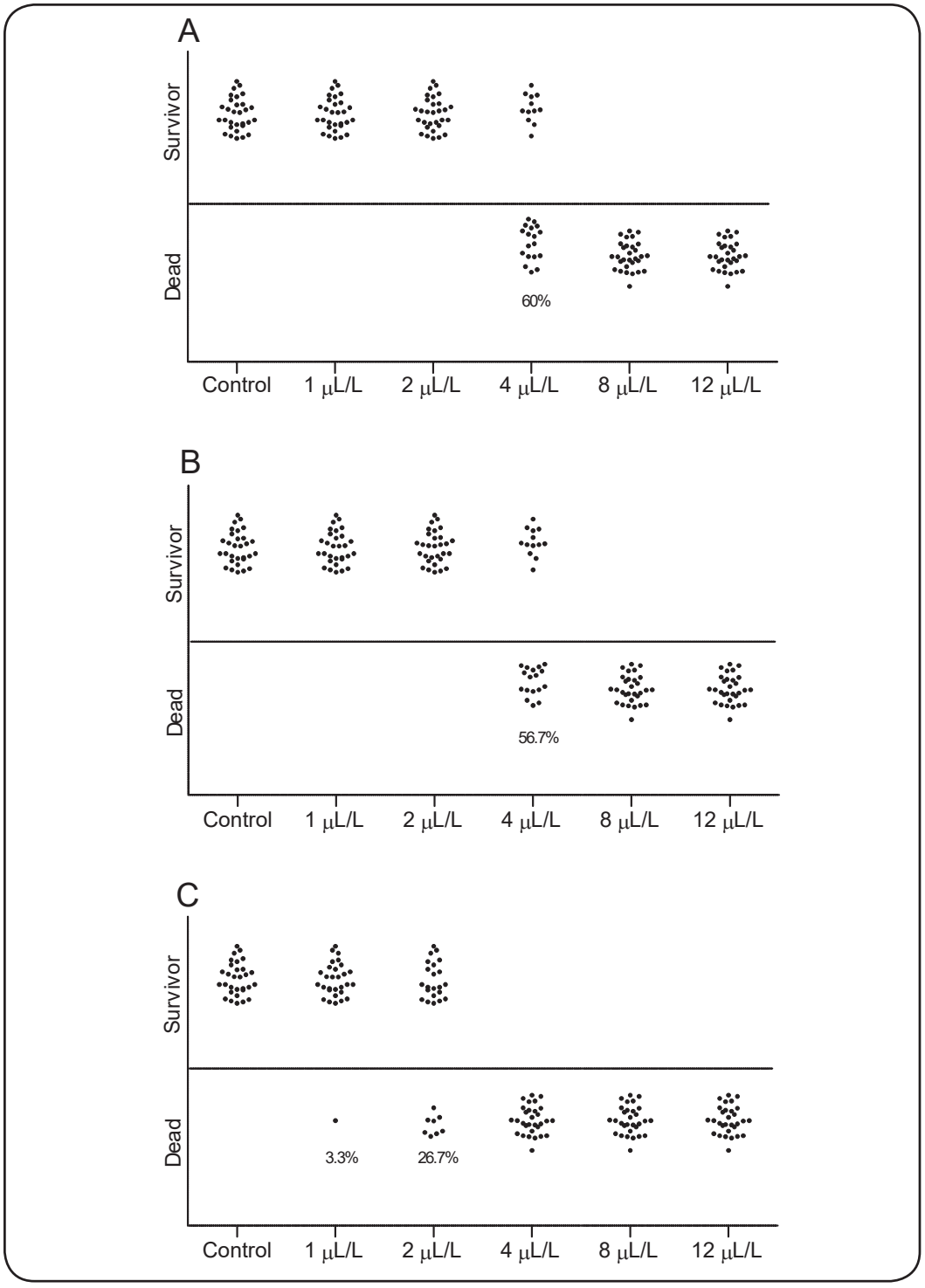

FIGURE 1: The lethal dose (LD_{100}) of MoluSchall against adult *B. glabrata* (A)*, B. straminea* (B), and *B. tenagophila* (C) snails, after 48 h of exposure to MoluSchall. For each dilution, 30 adult snails were placed in three beakers (10 snails/beaker) containing 1, 2, 4, 8, or 12 µL/L of MoluSchall diluted in 500 mL of dechlorinated water. As the control group, 30 *B. glabrata,* 30 *B. straminea,* and 30 *B. tenagophila* snails were distributed in beakers (10 snails/beaker) and exposed to dechlorinated water containing 12 µL Diluent 1 and Diluent 2 under the same conditions. There was a significant difference between the LD_{100} in *B. glabrata* and *B. straminea* when compared to *B. tenagophila*, p = 0.031.

The activity of MoluSchall against *B. glabrata* adults infected with *S. mansoni* is shown in **Figure 2**. When MoluSchall was used at 2 µL/L in dechlorinated water, 63.3% of the infected and 3.3% of the uninfected *B. glabrata* were found dead, and the difference was significant ($p = 0.038$). At 4 µL/L, the mortality rates were 100% and 96.7% for infected and uninfected *B. glabrata*, respectively, with no significant difference ($p > 0.05$). In the infected control group, a mortality rate of 10% was observed, with no significant difference ($p = 0.098$) compared to the uninfected control group exposed to dechlorinated water, with Diluent 1 and Diluent 2.

MoluSchall demonstrated stability for 24 months at the three temperatures tested. Snail mortality was 100% after 48 h of exposure to 8 µL/L molluscicide diluted in dechlorinated water in every activity assay. No mortality occurred in the control group, which was only exposed to dechlorinated water containing Diluent 1 and Diluent 2 (data not shown). MoluSchall diluted to 8 µL/L and 16 µL/L in E2 medium was not lethal to *D. rerio* fingerlings after 72 h of exposure. However, MoluSchall killed 30% of the fingerlings at 32 µL/L and killed 100% of the fingerlings at dilutions of 64 µL/L and 128 µL/L after 72 h of exposure (**Figure 3**).

In the artificial puddles treated with 8 µL/L and 12 µL/L MoluSchall, a mortality rate of 100% was observed for *B. glabrata*, regardless of the snail size, after 48 h of molluscicide exposure (**Figure 4**). In the untreated puddle, the mortality rate was 0.5% and was not significantly different from the expected 0% ($p > 0.05$). As in the laboratory tests, snails were evaluated for mortality by visual inspection and via a stereoscopic microscope using the same criteria.

DISCUSSION

This study utilized a molluscicide prototype kit, which was developed using freeze-dried *E. milii* latex and appropriate diluents. The important choice of diluent led to the success of this

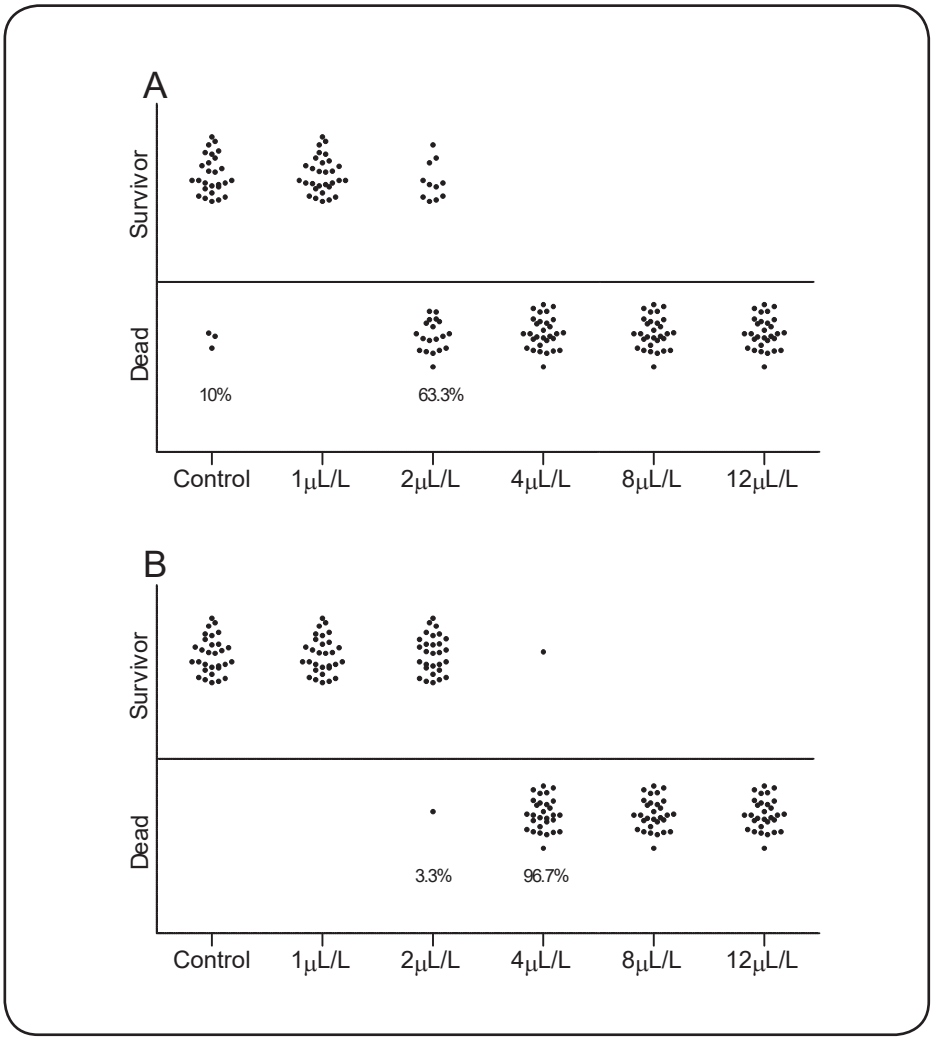

FIGURE 2: The lethal dose (LD_{100}) of MoluSchall against *B. glabrata* infected (A) and uninfected (B) with *S. mansoni*, after 48 h of exposure. The results correspond to the total number of snails (30) placed in the three beakers (10 snails/beaker) containing 1, 2, 4, 8, or 12 µL/L of MoluSchall diluted in dechlorinated water. As the control group, 30 infected and uninfected *B. glabrata* were placed in beakers and exposed to dechlorinated water containing 12 µL Diluent 1 and Diluent 2 under the same conditions. There was a significant difference between infected (A) and uninfected (B) *B. glabrata*; $p = 0.038$.

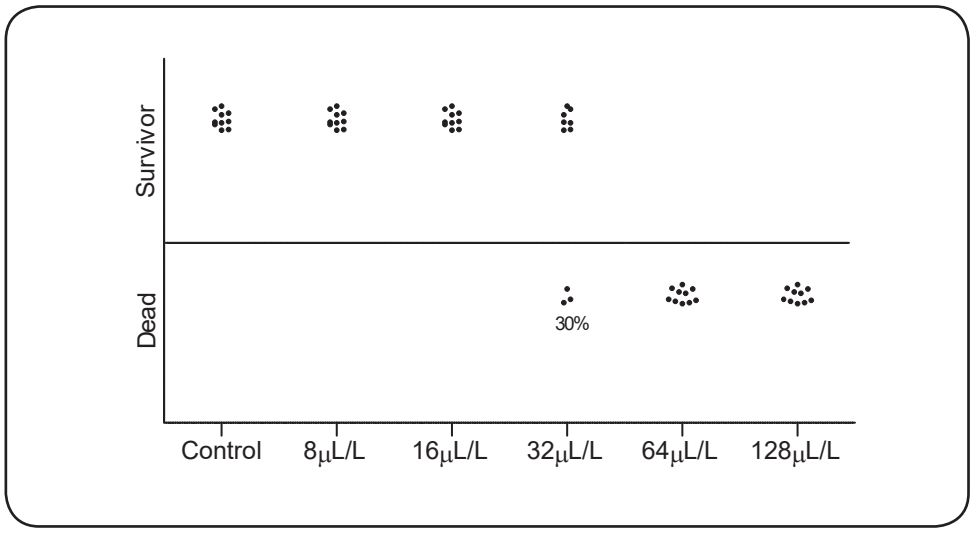

FIGURE 3: Representation of the toxicity assay for different dilutions of MoluSchall against *D. rerio* fingerlings after 72 h of exposure. The results correspond to the number of surviving and dead *D. rerio* fingerlings (10) exposed to each dilution for 72 h.

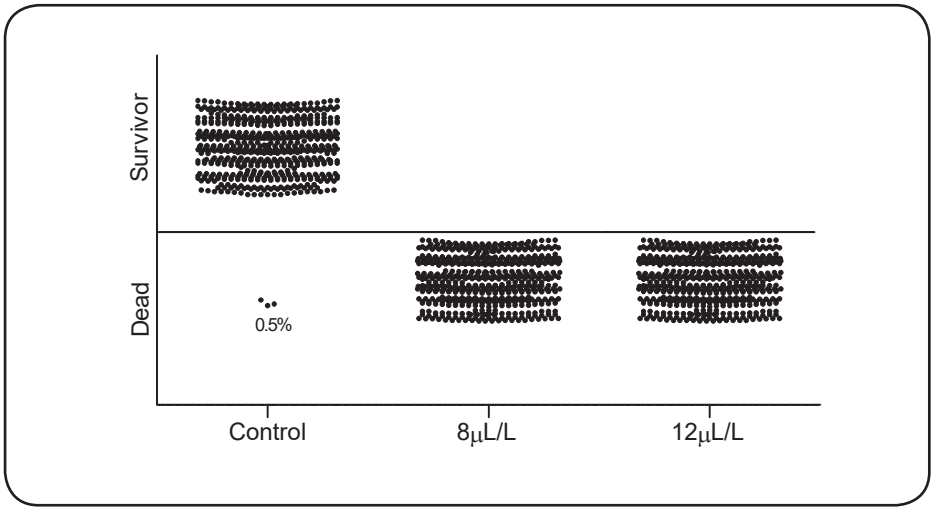

FIGURE 4: Representation of MoluSchall activity against *B. glabrata* under semi-natural conditions. The results correspond to the total number of snails exposed on different days to 8 µL/L and 12 µL/L MoluSchall in water for 48 h. The control group was kept in only water for 48 h in a separate puddle.

prototype kit. Previously, Mendes et al.[20] had reported problems in the field with reconstitution of the lyophilized latex. These authors associated the low mortality rate with low solubility of the *E. milii* latex. We also had difficulty reconstituting lyophilized latex in water alone or when substances other than Diluent 1 or Diluent 2 were used.

Studies involving *E. milii* latex as a potential molluscicide have previously been performed. Vasconcellos and Schall[16] showed that the lethal concentrations that killed 90% of snails (LC_{90}) were lower than 0.5 ppm against *B. glabrata* and *B. tenagophila* that were grown under laboratory conditions and 4.0 ppm against *B. tenagophila* obtained from the natural environment. Later, Schall et al.[17] used freeze-dried *E. splendens* latex and observed an LD_{100} of 0.2 ppm against adult and newly hatched *B. glabrata* and 0.4 ppm against adult *B. tenagophila* and *B. straminea*. In this study, we found LD_{100} values of 4 µL/L (4 ppm, v/v) against *B. tenagophila* and 8 µL/L (8 ppm, v/v) against *B. glabrata* and *B. straminea*, which are higher than the concentrations found by Schall et al.[17]. We believe that this inconsistency can be explained by the different study conditions. Unfortunately, the lethal effects between *E. Splendens* latex and freeze-dried *E milii* latex were not compared in this study.

The LD_{100} values determined in the current study do not exceed the 20 mg/L estimated by the WHO[21] as the value that will kill 90% of snails after exposure for 24 h. The LD_{100} values found against infected and uninfected *B. glabrata* with shell diameters of 12 to 17 mm were lower than those with diameters of 10 to 12 mm. Furthermore, a mortality rate difference between infected and uninfected *B. glabrata* was observed at the 2 µL/L dilution ($p = 0.038$). This difference was likely to

be due to lesions in the digestive gland, the hepatopancreas, as a consequence of parasite action[22]. Mello-Silva et al.[22] analyzed glucose content variation in snails exposed to a sub-lethal dose of *E. splendens* latex for 24 h and also determined that infected *B. glabrata* were more susceptible to latex than uninfected snails.

Some snails exposed to higher dilutions for 24 h presented a reduction in muscle contraction and heart rate and were retracted into their shell. This behavior suggests that the substances affected the snails even though no mortality was seen after 24 h of exposure (data not shown). According to Mello-Silva et al.[23], physiological changes occur in snails exposed to low doses of *E. milii* latex, altering the glycogen reserves in the digestive gland and the protein content in the hemolymph of *B. glabrata.* These authors believe that the reduced snail movement could be related to the increase in glycogen content in the cephalopodal mass. McCullough et al.[24] reported that molluscicides probably induce stress on the water balance system, which is thought to be under neurosecretory control in gastropods. Araújo et al.[25] demonstrated histological alterations, such as degeneration, necrosis, and liquid accumulation in the digestive gland and kidney tissues of *Lymnaea columella* (Say, 1817) exposed to *E. splendens* latex.

Another study demonstrated that the exposure of *S. mansoni* cercariae to a low concentration of *E. milii* latex produced effects in adult worms, including a reduction in the parasite burden and the number of eggs released, changes in the male tegument, and alterations in molecular functions[26]. Regarding the stability of the prototype kit, its physical aspects and molluscicidal activity against *B. glabrata* were maintained at all storage temperatures tested for 24 months. These results corroborate the work of Schall et al.[27], which showed effective molluscicidal activity of lyophilized latex for 24 months when stored in a refrigerator.

It was not possible to verify the seasonal variation of the molluscicidal activity of *E. milii* latex, which was a limitation of this study. However, Schall et al.[27] collected *E. milii* latex from different regions of Brazil throughout the year and demonstrated the consistency of its molluscicidal activity against *B. tenagophila*. In contrast, Vasconcellos and Amorin[28] tested *E. splendens* latex against *L. columella* and observed seasonal variations in the molluscicidal activity.

In the current study, only dilutions eight times higher than the LD_{100} defined for molluscicide use were lethal to *D. rerio*, which is a freshwater fish known for its significant sensitivity to chemical substances[18]. These animals can quickly absorb compounds that are added to the water and these compounds accumulate in different tissues, mainly in the central nervous system[29]. Similarly, Oliveira-Filho and Paumgartten[15] observed that freeze-dried *E. milii* latex was approximately four times less toxic than niclosamide to *D. rerio* adults.

The field molluscicidal activity of MoluSchall was the same as that observed in the laboratory. The 8 µL/L dilution of MoluSchall, defined as the LD_{100} against *B. glabrata*, was effective in killing all snails after 48 h of exposure. Therefore, the yield per vial of the MoluSchall kit is 1,250 L of treated water. Thus, the kit can treat up to 12,500 L of water. Although the results are promising, it is necessary to conduct additional MoluSchall field studies under natural conditions, including both lentic and lotic environments, as mentioned by Mendes et al.[20,30].

Considering the results presented, MoluSchall is an excellent candidate for a natural molluscicide and it is useful as an alternative strategy for controlling schistosomiasis transmission.

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Conflict of interest

The authors declare that there is no conflict of interest.

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