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Comparative toxicity of *Euphorbia milii* latex and synthetic molluscicides to *Biomphalaria glabrata* embryos

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ABSTRACT

Plant molluscicides have been regarded as possible alternatives to the costly and environmentally hazardous molluscicides currently available. This study was undertaken to compare the developmental toxicity of a plant molluscicide (*Euphorbia milii* latex, LAT) with that of three synthetic molluscicidal compounds. *Biomphalaria glabrata* egg masses (0–15 h after spawning) were exposed to molluscicides for 96 h and thereafter examined up to the 14th day after spawning. Embryo deaths, abnormal embryo development (malformations) and the day of hatching were recorded. Although exhibiting a weak ovicidal effect, LAT markedly impaired the development of snail embryos at concentrations $\geqslant 1000\ \mu g\ L^{-1}$ and produced anomalies ($EC_{50} = 2040\ \mu g\ L^{-1}$) such as abnormal shells, hydropic embryos, cephalic and non-specific malformations. Embryolethal potencies of molluscicides were as follows: triphenyltin hydroxide (TPTH; $LC_{50} = 0.30\ \mu g\ L^{-1}$) > niclosamide (NCL; $LC_{50} = 70\ \mu g\ L^{-1}$) > copper sulphate ($CuSO_4$; $LC_{50} = 2190\ \mu g\ L^{-1}$) $\ggg$ LAT ($LC_{50} = 34\ 030\ \mu g\ L^{-1}$). A few malformations were recorded in embryos exposed to concentrations of TPTH within the range of lethal concentrations, while almost no anomalies were noted among those treated with NCL or $CuSO_4$. A hatching delay (hatching on day 10 after spawning or later) was observed among LAT-exposed embryos. The effects of NCL, TPTH and CuSO4 on hatching were to some extent masked by their marked embryolethality. The no-observed effect concentrations (NOEC) for embryotoxicity were as follows: TPTH, $0.1\ \mu g\ L^{-1}$; NCL, $25.0\ \mu g\ L^{-1}$; $CuSO_4$, $500.0\ \mu g\ L^{-1}$ and LAT, $500.0\ \mu g\ L^{-1}$. Results from this study suggest that, although LAT was not acutely embryolethal after a short-term exposure, it markedly disrupted snail development. The marked embryotoxicity of *E. milii* possibly contributes to its effectiveness as a molluscicide.

1. Introduction

Controlling populations of intermediate-host snails through the use of molluscicides, combined with chemotherapy of infected individuals, rapidly reduces schistosomiasis transmission in endemic areas (Perrett and Whitfield, 1996). Niclosamide (NCL) is currently the only molluscicide recommended by WHO for snail control programmes (World Health Organization, 1993, 2006). Large-scale use of NCL, however, is costly and cannot be afforded by most developing countries where the disease is endemic. Additionally, since NCL at effective molluscicidal concentrations also kills fish, its use becomes a problem where fishing is an important economic activity and or a source of food for the population. In such circumstances, locally available, cheaper and less hazardous plant-derived molluscicides have been regarded as possible alternatives to NCL. *Euphorbia milii* (Crown-of-Thorns, *Euphorbiaceae*) latex possesses several interesting properties for a plant molluscicide (Vasconcellos and Schall, 1986). Although highly toxic to target snails, *E. milii* crude latex is comparatively less harmful to fish and other non-target aquatic species than NCL (Oliveira-Filho and Paumgartten, 2000). Moreover, *E. milii* is easily grown in endemic areas and pilot field studies suggest that its use in the control of intermediate hosts of *Schistosoma mansoni* is feasible (Mendes et al., 1997). A disadvantage of using *E. milii* latex for schistosomiasis control, however, is its weak ovicidal activity (Schall et al., 1998). Molluscicides lacking capacity to kill snail eggs have to be re-applied at short time intervals in order to eradicate snails from infested water bodies (World Health Organization, 1965; Duncan and Sturrock, 1987).

In the laboratory, ovicidal effects are usually evaluated by scoring embryo deaths after short-term exposures to candidate molluscicidal compounds. As a rule, other endpoints of developmental

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toxicity such as teratogenicity and hatching delay are not examined (Sukumaran et al., 2004; Adenusi and Odaibo, 2009; Rapado et al., 2010). Although being alive, malformed embryos seldom successfully hatch and, even if they do, hatching delay itself may impair post-hatching growth and survival thereby contributing to the reduction of snail population in an infested body of water. The three outcomes of a substance-induced embryotoxicity, therefore, apparently contribute to reducing the viability of molluscicide-exposed egg masses.

The present study was undertaken to evaluate comprehensively the developmental toxicity of *E. milii* latex and three synthetic molluscicides (niclosamide, triphenyltin hydroxide and copper sulphate) to the freshwater snail, *Biomphalaria glabrata*, one of the intermediate hosts of *S. mansoni* in Brazil.

2. Materials and methods

2.1. Plant material

The latex of Crown-of-Thorns (*E. milii*, Euphorbiaceae, syn *E. splendens*) was obtained from plants grown in experimental gardens located within FIOCRUZ campus, Rio de Janeiro, Brazil. Plant identification was made by a botanist (Dr. Ivete Maria da Silva), and a voucher specimen (reference number R202859) was deposited in the herbarium of the National Museum of the Federal University of Rio de Janeiro (UFRJ), Rio de Janeiro, RJ, Brazil. After a longitudinal cut in the plant stem, the latex was collected into glass tubes that were sealed, wrapped in aluminum foil, and transported to the laboratory for lyophilization. Samples of lyophilized latex were stored in tightly closed glass vials that were kept protected from light in the refrigerator (4–6 °C) until further use. Under these conditions of storage, molluscicidal activity of *E. milii* latex is stable for at least 18 months (Schall et al., 1992).

2.2. Test solutions

Niclosamide ethanolamine salt (Bayluscide®, Bayer AG Leverkusen, Germany), copper sulphate ($CuSO_4$, Merck, Brazil), triphenyltin hydroxide (TPTH, 97% pure, Agrevo, Brazil) and *E. milii* lyophilized latex solutions were prepared in synthetic soft assay water with hardness of 40 ± 1 mg L^{-1} as $CaCO_3$, pH 7.0 ± 0.2, as recommended by Brazilian Association for Technical Standardization (ABNT, 2004). TPTH was first diluted in ethanol (stock solution 1 mg mL^{-1}) and subsequently in the assay water. An ultrasonic dispersion apparatus was used for 20 min to facilitate complete dissolution of latex and TPTH in soft water. All test solutions were freshly prepared at room temperature (21 ± 2 °C).

2.3. Snails

B. glabrata (Say, 1818, Mollusca, Gastropoda, Planorbidae) snails used in this study came from a colony maintained at the Department of Malacology of the Oswaldo Cruz Institute (FIOCRUZ), Rio de Janeiro, Brazil. All adult snails were kept in aerated glass aquaria where they were fed *ad libitum* with fresh lettuce leaves.

2.4. Testing for developmental toxicity

Snail embryos were exposed within the egg masses to different concentrations of plant (latex) or synthetic molluscicides for 96 h, from day 1 up to day 4 after spawning, and observed until day 14. Tests were carried out with egg masses laid on small pieces of cellophane sheet that had been left floating on the aquaria water. Within 15 h of spawning, when embryos were still in the blastula stage of development (Camey and Verdonk, 1968; Kawano et al., 1992), pieces of cellophane sheet with adhered egg masses were carefully transferred to Petri dishes where they were further exposed to testing substances (static exposure) dissolved in the assay water. The number of egg masses and the total number of eggs exposed to each concentration of test substances are indicated in Tables 1–5. The 96 h exposure period ended when molluscicide-containing assay water was replaced with control soft water. Control (unexposed) egg masses were similarly treated except that they were not exposed to any molluscicidal substance. Except for the time needed for examination of embryo development and viability, Petri dishes containing egg masses were kept within climatic chambers under controlled photoperiod (12 h light/12 h dark), illumination (provided by fluorescent lamps) and environmental temperature (25 ± 1.0 °C). All egg masses were examined daily under a stereomicroscope up to the 14th day after spawning (spawning = day 1). Mortality, anomalies in embryo development (presence of malformations) and the day of hatching were recorded. Embryos were considered as dead whenever disintegrating embryonic forms were noted within the egg and or, at advanced developmental stages, no heart movements and no motility of the embryo were detected. Embryos with anatomic anomalies were identified and classified as a malformed embryo according to scheme adapted from Geilenkirchen (1966). Malformed embryos were included in one of four categories defined as follows: hydropic malformation, embryo partly or totally swollen to a considerable degree; shell malformation, embryo with an abnormally shaped shell; head malformation, embryo with any anomalies in the cephalic region such as monophthalmia, anophthalmia, cyclopia, eye re-duplication and others; and non-specific malformation, in this group were included all clearly dysmorphic embryos with anomalies which are not classifiable as hydropic, shell or head malformation.

2.5. Statistical analysis

To determine embryolethality, hatching delay and teratogenicity of molluscicides, lethal, inhibitory and effective (50%) concentrations were calculated by use of the Trimmed Spearman Karber method (Hamilton et al., 1977). To establish the no-observed effect concentrations (NOECs) for hatching delay and malformations, differences were evaluated by one-way ANOVA followed by Dunnett's multiple comparison test.

3. Results

The effects of *E. milii* latex, niclosamide, $CuSO_4$ and TPTH on the development of *B. glabrata* embryos are shown in Tables 1–4.

3.1. Embryotoxic effects of niclosamide (NCL)

As shown in Table 1, NCL induced a concentration-dependent increase in embryo deaths. Embryo-mortality during the first 96-h (exposure period) increased from 1% at the lowest concentration tested (25 μg L^{-1}) to 28.5% at 50 μg L^{-1}, 49.8% at 75 μg L^{-1}, 82.6% at 100 μg L^{-1} and 100% at 250 μg L^{-1}. At 50 and 75 μg L^{-1} most deaths occurred between 24 and 96-h of exposure while at the two highest concentrations (100 and 250 μg L^{-1}), most embryos died during the first 24 h thereby indicating that latency to death becomes shorter as NCL concentration increases. Very few snail embryos died after the end of the 96-h exposure period and almost all surviving embryos seemed to have been unharmed by NCL. The hatching of NCL-exposed egg masses was not delayed as compared to the hatching of unexposed egg masses and only a few cases of aberrant embryonic development (shell and cephalic malformations) were noted among the exposed embryos (Table 1). There-

Table 1
Effects of niclosamide (NCL) on the embryonic development of *Biomphalaria glabrata* snails. Egg-masses within 15 h of spawning were exposed to NCL dissolved in the assay water for 96 h and observed until 14 d post-spawning.

NCL ($\mu g\ L^{-1}$)	Egg masses	Eggs	Embryos	Days after spawning Exposure						
				1	4	6	8	10	12	14
0	04	58	Dead (%)	0	0	5.2	5.2	6.9	6.9	6.9
			Non-hatched							
			Normal (%)	100	100	63.8	6.9	0	0	0
			Malformed (%)	0	0	0	0	0	0	0
			Hatched							
			Normal (%)	0	0	31.0	87.9	93.1	93.1	93.1
			Malformed (%)	0	0	0	0	0	0	0
25	08	204	Dead (%)	0	1.0	2.0	2.4	2.9	3.4	3.4
			Non-hatched							
			Normal (%)	100	99.0	46.5	14.7	0	0	0
			Malformed (%)	0	0	0.5	0.5	0	0	0
			Hatched							
			Normal (%)	0	0	51.0	82.4	97.1	96.6	96.6
			Malformed (%)	0	0	0	0	0	0	0
50	10	214	Dead (%)	13.6	28.5	29.9	30.4	31.3	31.8	31.8
			Non-hatched							
			Normal	86.4	71.5	51.9	6.5	2.3	7.5	7.5
			Malformed (%)	0	0	0	0	0.5	0	0
			Hatched							
			Normal	0	0	17.3	62.6	65.9	60.7	60.7
			Malformed (%)	0	0	0.9	0.5	0	0	0
75	09	209	Dead (%)	29.5	49.8	50.7	50.7	51.2	51.2	51.2
			Non-hatched							
			Normal (%)	70.3	50.2	49.3	10.5	0	0	0
			Malformed (%)	0	0	0	0	0	0	0
			Hatched							
			Normal (%)	0	0	0	38.8	48.8	48.8	48.8
			Malformed (%)	0	0	0	0	0	0	0
100	07	201	Dead (%)	82.1	82.6	82.6	83.5	83.5	83.5	83.5
			Non-hatched							
			Normal (%)	17.9	17.4	10.4	0	0	0	0
			Malformed (%)	0	0	1.0	0	0	0	0
			Hatched							
			Normal (%)	0	0	6.0	16.5	16.5	16.5	16.5
			Malformed (%)	0	0	0	0	0	0	0
250	12	260	Dead (%)	100	100	100	100	100	100	100
			Non-hatched							
			Normal (%)	0	0	0	0	0	0	0
			Malformed (%)	0	0	0	0	0	0	0
			Hatched							
			Normal (%)	0	0	0	0	0	0	0
			Malformed (%)	0	0	0	0	0	0	0

fore, under the conditions of this study, the no-observed effect concentration (NOEC) for NCL-induced developmental toxicity to *B. glabrata* snails was set at 25 $\mu g\ L^{-1}$.

3.2. Embryotoxic effects of copper sulphate ($CuSO_4$)

Although less potent than NCL, $CuSO_4$ also caused a concentration-dependent increase in embryo-mortality (Table 2). At the lowest tested concentration of $CuSO_4$ (250 $\mu g\ L^{-1}$), embryo-mortality did not differ from that observed in unexposed egg masses. At the second lowest concentration (500 $\mu g\ L^{-1}$), a few deaths occurred during the 96-h exposure period and cumulative mortality rate slightly increased on days 8 and 14 after spawning (Table 2). Among embryos exposed to a concentration of $CuSO_4$ as high as 1000 $\mu g\ L^{-1}$, cumulative mortality rate was 51% on day 10 and did not increase thereafter. At a concentration of $CuSO_4$ as high as 2500 $\mu g\ L^{-1}$, cumulative mortality rates were 82% at the end of the exposure period (96-h) and 83% on day 14 (Table 2). The two highest concentrations of CuSO4 (5000 $\mu g\ L^{-1}$ and 10 000 $\mu g\ L^{-1}$) killed almost all embryos within 24-h of exposure (Table 2). Most surviving embryos seemed to have been unaffected by $CuSO_4$ because very few malformed snails were noted in exposed egg masses and only a small proportion of living exposed embryos (normal and malformed) did not hatch before day 10. Therefore, embryolethality was the most conspicuous outcome of $CuSO_4$–induced impairment of snail embryo development. The study-derived NOEC for $CuSO_4$ induced embryotoxicity in *B. glabrata* was set at 500 $\mu g\ L^{-1}$.

3.3. Embryotoxic effects of triphenyltin hydroxide (TPTH)

TPTH was the most potent ovicidal compound tested in the present assay with *B. glabrata*. During the 96-h exposure period, no TPTH-induced embryo deaths were noted in concentrations ranging from 0.0001 to 0.1 $\mu g\ L^{-1}$. In egg masses exposed to concentrations ranging from 0.001 to 0.1 $\mu g\ L^{-1}$, however, cumulative lethality on post-spawning day 14 was slightly higher than that recorded in unexposed controls (Table 3). At the two highest concentrations (5 and 10 $\mu g\ L^{-1}$), TPTH killed most embryos during the 96-h exposure period such that no surviving snail/

Table 2
Effects of copper sulphate ($CuSO_4$) on the embryonic development of *Biomphalaria glabrata* snails. Egg-masses within 15 h of spawning were exposed to $CuSO_4$ dissolved in the assay water for 96 h and observed until 14 d post-spawning.

$CuSO_4$ ($\mu g\ L^{-1}$)	Egg masses	Eggs	Embryos	Days after spawning Exposure						
				1	4	6	8	10	12	14
0	04	104	Dead (%)	0	1.9	5.2	5.2	6.9	6.9	6.9
			Non-hatched (%)							
			Normal (%)	100	100	63.8	6.9	0	0	0
			Malformed (%)	0	0	0	0	0	0	0
			Hatched (%)							
			Normal (%)	0	0	31.0	87.9	93.1	93.1	93.1
			Malformed (%)	0	0	0	0	0	0	0
250	04	116	Dead (%)	0	1	2.0	2.4	2.9	3.4	3.4
			Non-hatched (%)							
			Normal (%)	100	99.0	46.5	14.7	0	0	0
			Malformed (%)	0	0	0.5	0.5	0	0	0
			Hatched (%)							
			Normal (%)	0	0	51.0	82.4	97.1	96.6	96.6
			Malformed (%)	0	0	0	0	0	0	0
500	08	115	Dead (%)	13.6	28.5	29.9	30.4	31.3	31.8	31.8
			Non-hatched (%)							
			Normal (%)	86.4	71.5	51.9	6.5	2.3	7.5	7.5
			Malformed (%)	0	0	0	0	0.5	0	0
			Hatched (%)							
			Normal (%)	0	0	17.3	62.6	65.9	60.7	60.7
			Malformed (%)	0	0	0.9	0.5	0	0	0
1000	18	206	Dead (%)	29.5	49.8	50.7	50.7	51.2	51.2	51.2
			Non-hatched (%)							
			Normal (%)	70.3	50.2	49.3	10.5	0	0	0
			Malformed (%)	0	0	0	0	0	0	0
			Hatched (%)							
			Normal (%)	0	0	0	38.8	48.8	48.8	48.8
			Malformed (%)	0	0	0	0	0	0	0
2500	11	213	Dead (%)	82.1	82.6	82.6	83.5	83.5	83.5	83.5
			Non-hatched (%)							
			Normal (%)	17.9	17.4	10.4	0	0	0	0
			Malformed (%)	0	0	1.0	0	0	0	0
			Hatched (%)							
			Normal (%)	0	0	6.0	16.5	16.5	16.5	16.5
			Malformed (%)	0	0	0	0	0	0	0
5000	08	202	Dead (%)	100	100	100	100	100	100	100
			Non-hatched (%)							
			Normal (%)	0	0	0	0	0	0	0
			Malformed (%)	0	0	0	0	0	0	0
			Hatched							
			Normal	0	0	0	0	0	0	0
			Malformed (%)	0	0	0	0	0	0	0
10 000	09	209	Dead (%)	100	100	100	100	100	100	100
			Non-hatched (%)							
			Normal (%)	0	0	0	0	0	0	0
			Malformed (%)	0	0	0	0	0	0	0
			Hatched (%)							
			Normal (%)	0	0	0	0	0	0	0
			Malformed (%)	0	0	0	0	0	0	0

embryo from exposed egg masses was found on post-spawning day 6 (Table 3). On post-spawning days 6–10, a slight increase in the occurrence of malformed embryos was noted in egg masses exposed to concentrations of TPTH in the range of 0.001–0.1 $\mu g\ L^{-1}$ (Table 3). No hatching delay was apparent at any of the tested concentrations of TPTH (Table 3). Owing to the potent embryo-lethal effect of TPTH the NOEC was set at a concentration of 0.1 $\mu g\ L^{-1}$.

3.4. Embryotoxic effects of Euphorbia milii latex (LAT)

No increase of embryolethality was observed in egg masses exposed to LAT concentrations $\leqslant$500 $\mu g\ L^{-1}$ (Table 4). At higher concentrations (1000–200 000 $\mu g\ L^{-1}$), however, LAT caused a concentration-dependent enhancement of embryo mortality (Table 4). Up to concentrations as high as 25 000 $\mu g\ L^{-1}$, most embryo deaths occurred some days after the end of exposure to LAT. Latency to death, however, became shorter as concentrations of LAT increased and, at the two highest concentrations, most embryos died during the 96 h exposure period (Table 4). LAT also caused a marked increase in the incidence of malformed individuals, particularly in egg masses exposed to concentrations ranging from 1000 to 50 000 $\mu g\ L^{-1}$ (Table 4). In some cases, the abnormal embryonic development resulted into death although it was also associated with a delay of hatching as a number of malformed embryos did not hatch until post-spawning day 14 (Table 4). Among malformed individuals that successfully hatched, some died soon after hatching, while a number of malformed snails were still alive

Table 3
Effects of triphenyltin hydroxide (TPTH) on the embryonic development of *Biomphalaria glabrata* snails. Egg-masses within 15 h of spawning were exposed to TPTH dissolved in the assay water for 96 h and observed until 14 d post-spawning.

TPTH ($\mu g\ L^{-1}$)	Egg masses	Eggs	Embryos	Days after spawning Exposure						
				1	4	6	8	10	12	14
0	04	114	Dead (%)	0	0	0	0	0	1.7	1.7
			Non-hatched (%)							
			Normal (%)	100	100	78.1	35.1	16.7	14.1	10.6
			Malformed (%)	0	0	0	0.9	0.9	0	0
			Hatched (%)							
			Normal (%)	0	0	21.9	64.0	82.4	84.2	87.7
			Malformed (%)	0	0	0	0	0	0	0
0.0001	04	114	Dead (%)	0	0	0.9	0.9	2.6	2.6	2.6
			Non-hatched (%)							
			Normal (%)	100	100	98.2	12.3	2.6	0	0
			Malformed (%)	0	0	0.9	1.7	0	0	0
			Hatched (%)							
			Normal (%)	0	0	0	85.1	94.7	97.4	97.4
			Malformed (%)	0	0	0	0	0	0	0
0.001	05	104	Dead (%)	0	0	0	1.0	2.9	4.8	5.8
			Non-hatched (%)							
			Normal (%)	100	100	85.5	16.3	6.7	1.0	1.0
			Malformed (%)	0	0	4.8	3.8	2.9	1.0	0
			Hatched (%)							
			Normal (%)	0	0	9.6	78.9	87.5	93.2	93.2
			Malformed (%)	0	0	0	0	0	0	0
0.01	08	249	Dead (%)	0	0	2.0	3.2	4.4	4.4	6.8
			Non-hatched (%)							
			Normal (%)	100	100	85.1	40.2	5.6	0	0
			Malformed (%)	0	0	2.0	1.6	1.6	0.4	1.2
			Hatched (%)							
			Normal (%)	0	0	10.8	55.02	87.9	92.4	92.0
			Malformed (%)	0	0	0	0	0.4	1.6	0
0.1	08	229	Dead (%)	0	0	0.4	1.7	1.7	5.7	5.7
			Non-hatched (%)							
			Normal (%)	100	100	95.2	59.4	17.0	8.7	8.7
			Malformed (%)	0	0	3.0	4.4	5.7	1.7	1.7
			Hatched (%)							
			Normal (%)	0	0	1.3	34.5	75.5	83.8	83.8
			Malformed (%)	0	0	0	0	0	0	0
1.0	08	206	Dead (%)	1.4	3.4	5.82	5.82	5.82	–	–
			Non-hatched (%)							
			Normal (%)	98.6	96.6	76.2	76.2	70.4	–	–
			Malformed (%)	0	0	8.7	8.7	9.2	–	–
			Hatched (%)							
			Normal (%)	0	0	5.8	5.8	11.6	–	–
			Malformed (%)	0	0	3.4	3.4	3.4	–	–
5.0	10	269	Dead (%)	0	65.8	100	100	100	100	100
			Non-hatched (%)							
			Normal (%)	100	34.2	0	0	0	0	0
			Malformed (%)	0	0	0	0	0	0	0
			Hatched (%)							
			Normal (%)	0	0	0	0	0	0	0
			Malformed (%)	0	0	0	0	0	0	0
10.0	06	212	Dead (%)	94.3	100	100	100	100	100	100
			Non-hatched (%)							
			Normal (%)	5.7	0	0	0	0	0	0
			Malformed (%)	0	0	0	0	0	0	0
			Hatched (%)							
			Normal (%)	0	0	0	0	0	0	0
			Malformed (%)	0	0	0	0	0	0	0

on post-spawning day 14 (Table 4). Successful hatching and post-hatching survival of malformed embryos were seen mainly in snails showing shell and or cephalic (e.g. eye duplication) malformations. Embryos with hydropic-type malformations seldom hatched and when they did they died soon after hatching. Fig. 1E shows a swollen (hydropic) snail from a LAT-treated egg mass that exceptionally survived for a few days after hatching. Embryos with non-specific malformations, on the other hand, seemed to invariably evolve to death within the egg. The incidences of the different malformations in LAT-treated embryos can be seen in Table 5. The number of malformed embryos tended to decrease 6 days after spawning because some embryos with an abnormal morphological development died. It is of note that some embryos bearing shell-type malformations evolved to hydropic thereby increasing the

Table 4
Effects of *E. milii* latex (LAT) on the embryonic development of *Biomphalaria glabrata* snails. Egg-masses within 15 h of spawning were exposed to LAT dissolved in the assay water for 96 h and observed until 14 d post-spawning.

LAT ($\mu g\ L^{-1}$)	Egg masses	Eggs	Embryos	Days after spawning Exposure						
				1	4	6	8	10	12	14
0	09	228	Dead (%)	0	0.9	1.3	1.8	2.6	3.5	3.5
			Non-hatched (%)							
			Normal (%)	100	99.1	39.5	14.0	0	0	0
			Malformed (%)	0	0	1.7	1.7	0.9	0	0
			Hatched (%)							
			Normal (%)	0	0	57.5	82.5	96.5	96.5	96.5
			Malformed (%)	0	0	0	0	0	0	0
100	06	205	Dead (%)	0	0.5	1.0	3.4	4.4	4.9	4.9
			Non-hatched (%)							
			Normal (%)	100	99.5	95.6	17.1	0	0	0
			Malformed (%)	0	0	3.4	0.5	0	0	0
			Hatched (%)							
			Normal (%)	0	0	0	78.5	95.6	95.1	95.1
			Malformed (%)	0	0	0	0.5	0	0	0
250	07	206	Dead (%)	0	0	0	0	0.5	1.4	2.4
			Non-hatched (%)							
			Normal (%)	100	100	78.7	12.6	0.5	0	0
			Malformed (%)	0	0	1.4	1.4	1.0	0	0
			Hatched (%)							
			Normal (%)	0	0	19.9	86.0	98.0	98.6	97.6
			Malformed (%)	0	0	0	0	0	0	0
500	08	212	Dead (%)	0	0	0.5	0.9	1.4	3.3	3.8
			Non-hatched (%)							
			Normal (%)	100	100	52.3	1.4	0	0	0
			Malformed (%)	0	0	1.9	0.5	0.5	0	0
			Hatched (%)							
			Normal (%)	0	0	45.3	96.3	97.2	96.7	96.2
			Malformed (%)	0	0	0	0.9	0.9	0	0
1000	08	218	Dead (%)	0	3.7	5.5	6.4	7.8	11.5	18.3
			Non-hatched (%)							
			Normal (%)	100	96.3	57.4	1.8	1.4	0	0
			Malformed (%)	0	0	37.1	28.9	16.0	15.6	11.0
			Hatched (%)							
			Normal (%)	0	0	0	55.5	56.0	56.9	56.9
			Malformed (%)	0	0	0	7.4	18.8	16.0	13.8
2500	10	182	Dead (%)	0	0.5	2.2	7.1	16.5	26.4	27.5
			Non-hatched (%)							
			Normal (%)	100	99.5	25.8	1.1	0	0	0
			Malformed (%)	0	0	35.7	10.9	7.7	3.8	3.3
			Hatched (%)							
			Normal (%)	0	0	35.2	60.0	57.7	53.9	53.8
			Malformed (%)	0	0	1.1	20.9	18.1	15.9	15.4
5000	08	206	Dead (%)	0	1.9	2.9	6.8	9.7	20.9	29.1
			Non-hatched (%)							
			Normal (%)	100	98.1	7.3	0	0	0	0
			Malformed (%)	0	0	80.6	61.6	53.4	40.3	34.0
			Hatched (%)							
			Normal (%)	0	0	2.9	10.2	10.2	10.2	10.2
			Malformed (%)	0	0	6.3	21.4	26.7	28.6	26.7
10 000	10	227	Dead (%)	10.1	10.1	11.0	11.9	13.2	19.8	43.6
			Non-hatched (%)							
			Normal (%)	89.9	89.9	0	0	0	0	0
			Malformed (%)	0	0	88.1	74.4	56.8	50.2	21.1
			Hatched (%)							
			Normal (%)	0	0	0	0	0	0	0
			Malformed (%)	0	0	0.9	13.6	29.9	29.9	37.0
25 000	08	202	Dead (%)	13.9	19.3	21.3	25.2	33.7	37.6	54.9
			Non-hatched (%)							
			Normal (%)	86.1	0	0	0	0	0	0
			Malformed (%)	0	80.7	78.7	68.9	60.4	54.5	35.7
			Hatched (%)							
			Normal (%)	0	0	0	0	0	0	0
			Malformed (%)	0	0	0	5.9	5.9	7.9	9.4
50 000	11	227	Dead (%)	34.3	44.1	44.9	49.3	61.2	64.8	72.3
			Non-hatched (%)							
			Normal (%)	65.7	0	0	0	0	0	0
			Malformed (%)	0	55.9	55.1	50.7	38.8	30.8	23.8

(*continued on next page*)

Table 4 (*continued*)

LAT (μg L^{-1})	Egg masses	Eggs	Embryos	Days after spawning Exposure						
				1	4	6	8	10	12	14
			Hatched (%)							
			Normal (%)	0	0	0	0	0	0	0
			Malformed (%)	0	0	0	0	0	4.4	3.9
100 000	11	231	Dead (%)	39.8	59.3	63.2	69.3	83.1	85.3	86.6
			Non-hatched (%)							
			Normal (%)	60.2	0	0	0	0	0	0
			Malformed (%)	0	40.7	36.8	29.8	16.1	12.5	11.2
			Hatched (%)							
			Normal (%)	0	0	0	0	0	0	0
			Malformed (%)	0	0	0	0.9	0.9	2.2	2.2
200 000	08	210	Dead (%)	30.9	62.4	67.1	74.8	94.8	97.1	97.1
			Non-hatched (%)							
			Normal (%)	69.1	0	0	0	0	0	0
			Malformed (%)	0	37.6	32.9	25.2	5.2	2.9	2.9
			Hatched (%)							
			Normal (%)	0	0	0	0	0	0	0
			Malformed (%)	0	0	0	0	0	0	0

occurrence of this type of malformation at a later post-spawning day (Table 5). The NOEC for LAT-induced developmental toxicity in *B. glabrata* was set at 500 μg L^{-1}.

4. Discussion

All molluscicidal substances tested in this study proved to be toxic to *B. glabrata* snail embryos. Concentration–response curves for the three developmental toxicity endpoints, however, differed considerably among the molluscicides. Although exhibiting a weak embryo-lethal effect, *E. milii* latex, at concentrations ⩾1000 μg L^{-1}, markedly impaired the development of *B. glabrata* embryos. The percentage of LAT-exposed embryos that were unaffected (normal) and successfully hatched until post-spawning day 14 decreased from 96.2% at 500 μg L^{-1}, through 56.9% at 1000 μg L^{-1} to 10.2% at 5000 μg L^{-1}. At concentrations of LAT ⩾10 000 μg L^{-1}, all exposed snails surviving to day 14 were malformed and most of them failed to hatch. The synthetic molluscicides NCL and TPTH were potent embryolethal compounds with 96-h LC_{50}s as low as 70 μg L^{-1} and 0.8 μg L^{-1}, respectively, and LC_{50}s determined 10 days after spawning (10d-LC_{50}) as low as 70 μg L^{-1} and 0.3 μg L^{-1}, respectively (Table 6). $CuSO_4$ and LAT were less potent embryolethal agents with 96-h LC_{50}s of 3670 μg L^{-1} and 69 440 μg L^{-1}, respectively, and 10 d-LC_{50}s of 2190 μg L^{-1} and 34 030 μg L^{-1}, respectively (Table 6). While NCL and $CuSO_4$ did not induce increases in the rate of malformations with increased concentration, a slight teratogenic effect was noted at intermediate concentrations of TPTH (0.001–0.1 μg L^{-1}) (Tables 1–4). *E. milii* latex, on the other hand, was a strong snail teratogen. At concentrations ⩾1000 μg L^{-1}, LAT markedly enhanced the occurrence of malformations (Table 4). Most malformed embryos found in LAT-treated egg masses either died or failed to hatch before post-spawning day 14. All snails exposed to LAT concentrations ⩾10 000 μg L^{-1} and surviving to post-spawning day 14 possessed at least one type of malformation. Non-specific malformations invariably evolved to death without hatching while hydropic embryos generally died within the egg or soon after hatching. Owing to this fact, the percentage of embryos that successfully hatched and did not exhibit any morphological abnormality (normal hatched) on day 14 decreased in a concentration-dependent manner at concentrations >500 μg L^{-1}. These results therefore demonstrated that *E. milii* latex was by far the weakest embryolethal (ovicidal) agent among the four molluscicides tested in this study. Owing to its prominent teratogenic action, however, LAT-NOEC for developmental toxicity 10 d after spawning was equal to $CuSO_4$-NOEC (500 μg L^{-1}) and only 20 times the NCL-NOEC (25 μg L^{-1}).

It has been shown that LAT is a highly potent plant molluscicide against *Biomphalaria* snails (48-h LC_{50} = 0.12 mg L^{-1}) with newly hatched (⩽1 mm shell diameter) and young snails (3–8 mm shell) only slightly less susceptible than older snails (10–25 mm shell) (Oliveira-Filho et al., 1999; Oliveira-Filho and Paumgartten, 2000). The mechanism(s) by which LAT kills snails and exerts its teratogenic action is (are) still unclear. Nonetheless, a possible explanation for the difference between the low susceptibility of embryos exposed within the eggs and the high vulnerability of hatched snails is that egg membranes and the gelatinous egg mass are efficient barriers against penetration of toxic substances present in *E. milii* latex. *Biomphalaria* eggs are covered with perivitelline fluid, synthesized and secreted by the snail albumen gland, and encapsulated by a membrane produced by the *pars contorta*. The packaged eggs are subsequently surrounded by secretions from the muciparous and oothecal glands to form an egg mass that is deposited by the snail on smooth surfaces in water environment. It has been demonstrated that the protein components of the egg mass possess antimicrobial activity, thereby protecting the offspring inside the eggs from bacteria, fungi and other potential pathogens (Hathaway et al., 2010). Little is known, however, about their role in protecting the snail embryos against hazardous chemical substances found in the aquatic environment. In contrast to LAT, the synthetic molluscicides NCL and TPTH seemed to easily penetrate the gelatinous egg mass and rapidly reach embryolethal concentrations inside the egg. It is of note that other plant-derived products such as extracts from *Stevia rebaudiana* and *Laurus nobilis* tested on *B. glabrata* egg masses exhibited a similar pattern of developmental toxicity, i.e., a weak embryo-lethal effect and marked teratogenic and hatching retardation effects (Kawano and Simões, 1986; Ré and Kawano, 1987). As far as *E. milii* latex is concerned, molluscicidal activity has been attributed to the presence of diterpene esters of ingenol type such as milliamines A-G (Marston and Hecker, 1983) and milliamine L (Zani et al., 1993). Some milliamines isolated from *E. milii* latex are rather potent molluscicidal compounds and Zani et al. (1993) reported that milliamine L, a di-anthroniloyil peptide ester of ingenol, killed *B. glabrata* adult snails at concentrations as low as 4 nM. Apparently, the gelatinous coat of the egg mass prevents to some extent the penetration of these highly potent plant-derived biocide com-

Table 5
Occurrence of different types of malformations in *Biomphalaria glabrata* embryos exposed to *Euphorbia milii* latex (LAT). Egg-masses within 15 h of spawning were treated with LAT dissolved in the assay water for 96 h and observed until 14 d post-spawning.

LAT (μg L^{-1})	Eggs	Type of malformation	Days after spawning Exposure						
			1	4	6	8	10	12	14
0	228	Living embryos	228	226	225	224	222	220	220
		Shell	0	0	0	0	0	0	0
		Hydropic	0	0	3	3	2	0	0
		Shell + cephalic	0	0	0	0	0	0	0
		Non-specific	0	0	1	1	0	0	0
100	205	Living embryos	205	204	203	198	196	195	195
		Shell	0	0	0	0	0	0	0
		Hydropic	0	0	7	2	0	0	0
		Shell + cephalic	0	0	0	0	0	0	0
		Non-specific	0	0	0	0	0	0	0
250	206	Living embryos	206	206	206	206	205	203	201
		Shell	0	0	0	0	0	0	0
		Hydropic	0	0	0	0	0	0	0
		Shell + cephalic	0	0	0	0	0	0	0
		Non-specific	0	0	3	3	2	0	0
500	212	Living embryos	212	212	211	210	209	205	204
		Shell	0	0	2	2	2	0	0
		Hydropic	0	0	1	1	1	0	0
		Shell + cephalic	0	0	0	0	0	0	0
		Non-specific	0	0	1	0	0	0	0
1000	218	Living embryos	218	210	206	204	201	193	178
		Shell	0	0	47	42	39	39	30
		Hydropic	0	0	32	36	37	30	24
		Shell + cephalic	0	0	0	0	0	0	0
		Non-specific	0	0	2	1	0	0	0
2500	182	Living embryos	182	181	178	169	152	134	132
		Shell	0	0	35	33	29	20	18
		Hydropic	0	0	26	23	16	16	16
		Shell + cephalic	0	0	0	0	0	0	0
		Non-specific	0	0	6	2	2	0	0
5000	206	Living embryos	206	202	200	192	186	163	146
		Shell	0	0	92	85	78	53	53
		Hydropic	0	0	84	83	86	89	72
		Shell + cephalic	0	0	0	0	0	0	0
		Non-specific	0	0	3	3	1	0	0
10 000	227	Living embryos	204	204	202	200	197	182	128
		Shell	0	0	137	118	110	99	61
		Hydropic	0	0	56	75	80	79	63
		Shell + cephalic	0	0	4	4	4	2	2
		Non-specific	0	0	5	3	3	2	2
25 000	202	Living embryos	174	163	159	151	134	126	91
		Shell	0	0	2	5	18	12	17
		Hydropic	0	133	139	130	114	113	74
		Shell + cephalic	0	0	2	2	0	0	0
		Non-specific	0	30	16	14	2	1	0
50 000	227	Living embryos	149	127	125	115	88	80	63
		Shell	0	21	0	23	23	8	8
		Hydropic	0	0	105	79	57	69	53
		Shell + cephalic	0	86	6	5	3	3	2
		Non-specific	0	20	14	8	5	0	0
100 000	231	Living embryos	139	94	85	71	39	34	31
		Shell	0	0	0	4	16	11	2
		Hydropic	0	0	52	48	10	14	22
		Shell + cephalic	0	63	12	10	7	6	5
		Non-specific	0	31	19	7	4	1	0
200 000	210	Living embryos	145	79	69	53	11	6	6
		Shell	0	0	0	0	0	0	0
		Hydropic	0	0	57	42	9	4	4
		Shell + cephalic	0	70	4	3	2	2	2
		Non-specific	0	9	8	8	0	0	0

pounds into the egg and, therefore, the embryolethal concentrations of LAT (10d-LC_{50} 34 030 μg L^{-1}) are much higher than those levels that kill newly hatched and older snails (LC_{50}s in the range of 100 μg L^{-1}) (Oliveira-Filho et al., 1999). Although not lethal to the embryo, constituents of LAT that penetrated the egg mass barrier disrupted embryo morphogenesis and retarded hatching. NCL and TPTH seemed to easily cross the egg mass barrier thereby being more potent ovicidal compounds. Concentration–response

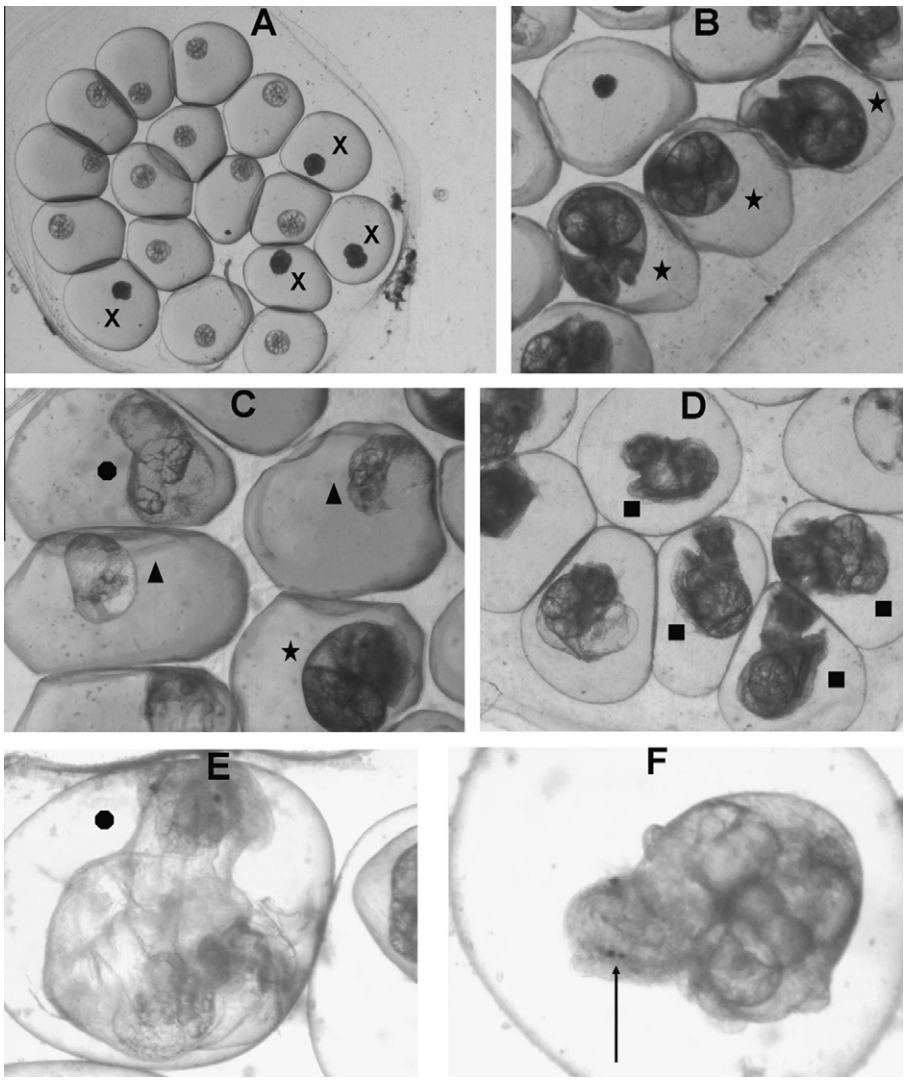

Fig. 1. Toxic effects of *Euphorbia milii* latex and synthetic molluscicides on *Biomphalaria glabrata* embryos. Egg masses were exposed to plant and synthetic molluscicides for 96 h (days 1–4) and observed daily until day 14 after spawning. Embryolethality; A: (magnification 12×) snail eggs 48 h after spawning; an X indicates dead embryos; B: (magnification 32×) control eggs 7-d-old; stars indicate normal embryos; Teratogenicity C: (magnification 32×) 7-d-old embryos displaying non-specific malformations (triangles); a circle indicates a "hydropic" embryo and a star indicates a normal embryo; D: (magnification 32×) 7-d-old embryos with a malformed (irregularly shaped) shell; marked with squares; E: (magnification 40×) 10-d-old "hydropic" embryo; F: (magnification 40×) 10-d-old embryo with double eyes on one side (indicated by an arrow).

Table 6
Effects of the molluscicides niclosamide (NCL), copper sulphate ($CuSO_4$), triphenyltin hydroxide (TPTH) and *Euphorbia milii* latex (LAT) on the embryonic development of the snail *Biomphalaria glabrata*. Values are expressed in $\mu g\ L^{-1}$.

Molluscicide	Embryolethality			Hatching delay	Teratogenicity	Embryotoxicity NOEC*
	24 h-LC_{50}	96 h-LC_{50}	LC_{50}*	IC_{50}*	EC_{50}*	
NCL	80.0 60.0–110.0	70.0 60.0–80.0	70.0 60.0–80.0	+	#	25.0
$CuSO_4$	7000 6910–7080	3670 3030–4350	2190 950–3300	+	#	500.0*
TPTH	7.00 6.30–7.80	0.80 0.58–1.20	0.30 0.27–0.34	+	#	0.1*
LAT	>200 000	69 440 54 290–88 800	34 030 30 450–3804	5180 3290–8290	2040 1170–3470	500.0*

LC_{50}: lethal concentration, IC_{50}: inhibitory concentration 50%, EC_{50}: effective concentration 50%, confidence intervals 95% (95% CI) are shown below LC_{50}, IC_{50}, EC_{50} values. + IC_{50} was not calculated owing to the high lethality and small number of surviving non-hatched embryos. # EC_{50} for teratogenic effect was not determined owing to the low incidence of malformed embryos. NOEC – no-observed effect concentration: Highest tested concentration at which no effect was observed.
* Determined 10 d after spawning, i.e., 6 d after the end of exposure.

curves for embryo-lethal effects of NCL and TPTH were steep and no malformed embryos were noted at non-lethal concentrations. Within the lethal concentration ranges of NCL and TPTH, only a few malformed embryos were observed. $CuSO_4$ was weaker than NCL and TPTH and stronger than LAT as an ovicidal substance and produced almost no malformed embryos.

5. Conclusions

In conclusion, results from this study showed that, although exhibiting a weak ovicidal effect during the first 24-h of exposure, *E. milii* latex impaired the embryonic development of *B. glabrata* snails at concentrations $\geqslant 1000\ \mu g\ L^{-1}$. Among the tested substances, *E. milii* latex was the only molluscicide that produced a high incidence and a full range of malformations. In contrast to *E. milii* latex, niclosamide and triphenyltin hydroxide proved to be rather potent ovicidal compounds that induced no malformation at non-lethal concentrations and very few anomalies among the embryos surviving lethal concentrations. Copper sulphate did not exhibit a clear teratogenic effect and was less potent than niclosamide and triphenyltin hydroxide as an ovicidal compound. Data presented here therefore suggest that, although not rapidly killing the embryos after an exposure through the egg mass, *E. milii* latex is teratogenic to *B. glabrata*, a Planorbidae snail, an effect that is likely to contribute to its effectiveness as a plant molluscicide.

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