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ORIGINAL ARTICLE

Assessment of the molluscicidal impact of extracted chlorophyllin on some biochemical parameters in the nervous tissue and histological changes in *Biomphalaria alexandrina* and *Lymnaea natalensis* snails

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Abstract
Biomphalaria alexandrina and *Lymnaea natalensis* snails are the intermediate hosts of schistosomiasis and fasciolosis. The aim of the present study is to evaluate the molluscicidal activity of chlorophyll extract as a photodynamic substance against these snails and how it affected its tissues and the biological system. Chlorophyllin was extracted from deep-frozen *Moringa oleifera* leaves, and then it was transformed into water-soluble chlorophyllin. The present results showed that it had a molluscicidal activity on *B. alexandrina* snails (LC_{50} 17.6 mg/l; LC_{90} 20.9 mg/l) and *L. natalensis* snails (LC_{50} 4.3 mg/l; LC_{90} 6.8 mg/l). Exposing *B. alexandrina* snails to the sublethal concentrations (LC_0, LC_{10}, and LC_{25}) resulted in a significant reduction in their survival rates. Regarding its effect on biochemical parameters, chlorophyllin significantly reduced the acetylcholinesterase activity, protein content, and alkaline and acid phosphatase activity in *B. alexandrina* nervous tissue compared to the control group. Histopathological changes occurred in the digestive gland of treated *B. alexandrina* snails where cells lost their nuclei, vacuolated, degenerated, and ruptured, and the lumen increased. Photosynthesizing materials like chlorophyllin are new approaches to control schistosomiasis and fasciolosis in developing countries by affecting their intermediate host. These materials were cheap and environmentally safe to replace the synthetic molluscicides for snail control.

Keywords *Biomphalaria alexandrina* · *Lymnaea natalensis* · Chlorophyllin · Survival rate · Enzymes · Histopathological changes

Introduction

Schistosomiasis and fasciolosis are widespread neglected tropical parasitic diseases that caused veterinary and human diseases and transmitted by snails (Chaturvedi et al. 2017; WHO 2017). About 200 million people worldwide are infected with schistosomiasis (Mahmoud et al. 2013), and about 250 million sheep and 350 million cattle are at risk of fasciolosis worldwide (Beesley et al. 2017). Human fascioliasis is considered now as a zoonosis of major global and regional importance (Soliman 2008) as it is affecting nearly 50 million people worldwide (Rahman et al. 2017).

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Freshwater snails act as the intermediate hosts for a huge number of trematode parasites in humans and animals (Lee et al. 2017; Chontananarth et al. 2017). Freshwater snails of *Biomphalaria* sp. are the intermediate hosts of *Schistosoma mansoni* in Egypt (Ibrahim and Abdalla 2017), and *Lymnaea* sp. snails were the main snail host for the liver flukes, *Fasciola hepatica* or *gigantica*, which are widely distributed in Africa (Moema et al. 2008). Several strategies were used to control snail populations (Abd El-Ghany and Abd El-Ghany 2017). One of these preventive ways was by the use of the chemical molluscicides (Abdel-Ghaffar et al. 2016), but because they were poisonous to nontarget organisms and had high cost (WHO 2014), it stimulated the interest to find suitable natural molluscicides (Elsareh et al. 2016).

Plant-derived molluscicides are promising choices for controlling these snails (Kiros et al. 2014). Phytotherapy of snails by photodynamic material like chlorophyllin is a new approach to control schistosomiasis and fasciolosis

in developing countries (Chaturvedi et al. 2017; Ragheb et al. 2018). The photodynamic substances are not toxic in darkness, but are activated by light. Upon reaction with oxygen, reactive singlet oxygen (ROS) is produced, which has highly cytotoxic effects (DeRosa 2002). ROS caused excessive oxidative stress in the cells, resulted in damage to cell membranes, proteins, DNA, and other cell structures. By simple chemical modifications, the hydrophobic chlorophyll can be made water-soluble in the form of chlorophyllin (Erzinger et al. 2011).

Water-soluble chlorophyllin exerts a pronounced photodynamic activity (Richter et al. 2014) that destroys parasites found in the aquatic ecosystems and acts as potent larvicides, cercaricides, and molluscicides against *L. acuminate* (Kumar and Singh 2016; Chaturvedi et al. 2017). Wohllebe et al. (2009) reported that water-soluble chlorophyllin when used at low concentrations was able to kill mosquito larvae and other small animals in the water body within a few hours under exposure of solar radiation. Mahmoud et al. (2013) reported that chlorophyllin even at low concentrations was able to kill *L. stagnalis, Biomphalaria spp.,* and *Physa marmorata* snails within a few hours under exposure of solar radiation. Besides, it had a killing effect by about 70% and 100% on the snails' eggs and the newly hatched snails, respectively, after 3-h exposure to solar radiation.

The objectives of the present research are to assess the molluscicidal activity of chlorophyllin as a photodynamic substance against *B. alexandrina* and *L. natalensis* snails and then to study the effect of its sublethal concentrations on some biological and biochemical parameters in the nervous tissue and the histological changes in *B. alexandrina* snails.

Materials and methods

Experimental animal

Adult *B. alexandrina* snails (Ehrenberg, 1831) (9.45 ± 1.9 mm) and *L. natalensis* (3.35 ± 0.30 mm) were maintained in medical malacology laboratory at Theodor Bilharz Research Institute (TBRI) and maintained in plastic aquaria ($16 \times 23 \times 9$ cm). The aquaria were provided with dechlorinated aerated tap water (10 snails/l), at pH 7 and at room temperature (22–25 °C) and covered with glass plates. Oven-dried lettuce leaves, blue green algae (*Nostoc muscorum*), and TetraMin (Fish food) were used for feeding. Water in aquaria was changed weekly with a photoperiodicity of 12-h light/12-h dark. Pieces of polyethylene sheets were used for collecting egg masses. Dead snails were removed immediately to prevent the water from being contaminated by decaying tissue.

Preparation of extracted chlorophyllin

The extraction was done according to Wohllebe et al. (2012), where chlorophyll was isolated from *Moringa oleifera* leaves using 100% ethanol at 55 °C for about 2 h. Then, 1 mg $CaCO_3$/gm plant material was added to prevent the transformation of chlorophyll into pheophytin (Rahmani and Csallany 1991). The extract was filtered using Whatman filter papers, and 50 ml petroleum benzene was added with shaking, so the chlorophyll moved into the lipophilic benzene phase and the two phases were separated in a separatory funnel. This crude chlorophyll could be further purified by two to three reprecipitations from ether–petroleum ether, and then about 1.0 ml methanolic KOH was added (to break the ester bond between the chlorophyllin and the phytol tail by saponification). After separation of the methanolic KOH phase and the benzene phase, most of the chlorophyllin was found in the methanolic KOH phase. The methanol was evaporated in darkness, and the chlorophyllin concentration was determined using a spectrophotometer (Erzinger et al. 2015). The extract was stored in a dark flask at room temperature.

Toxicity experiment

A fresh stock solution of 1000 ppm (1000 mg/l) was prepared from extracted chlorophyllin on the basis of V/V using dechlorinated tap water. *B. alexandrina* snails (5–7 mm) were subjected to a series of concentrations of extracted chlorophyllin (22, 20, 18, 15, and 13 mg/l) to calculate LC_{50} and LC_{90} at room temperature (22–25 °C) with a photoperiodicity of 12-h light/12-h dark. Another snail group of the same size (5–7 mm) was dipped in dechlorinated water only as the control. Three replicates were used, each of 10 snails for each concentration and the control group. The exposure period was 24 h; after that, the snails were removed from the experimental test solution and washed thoroughly with dechlorinated tap water and transferred to containers with fresh dechlorinated tap water for another 24 h of recovery, and then, the percentages of observed mortalities were recorded. No-observed-effect level (NOEL) is the greatest concentration or amount of a substance, found by experiment or observation, that causes no alteration of morphology, functional capacity, growth, development, or life span of the target organism distinguishable from those observed in normal (control) organisms of the same species and strain under the same defined conditions of exposure. Mortality of snails was recorded at 24 h (WHO 1983) and analyzed to obtain the lethal concentration values and slope value by probit analysis (Bull WHO 1965).

Effect on survival rate of snails

Biomphalaria alexandrina snails (8–10 mm) were divided into four groups (30 snails each); three groups were exposed to the extracted chlorophyllin at NOEL, LC_{10}, and LC_{25} for 24 h (exposure); then, the snails were removed, washed thoroughly with dechlorinated tap water, and transferred to containers with fresh dechlorinated tap water for 6 days of recovery, and this was done for 2 weeks and then followed by 2 weeks of recovery. The fourth group was left in dechlorinated water as the control, and all the experiments were repeated three times.

Biochemical alterations in the nervous tissue

Acetylcholinesterase (AChE)

Acetylcholinesterase activity was measured according to the method of Ellman et al. (1961) as modified by Singh and Agarwal (1991). The nervous tissue of *B. alexandrina* was taken around the buccal mass and homogenized in 1.0 ml of 0.1 M phosphate buffer pH 8.0 for 5 min in an ice bath and then centrifuged at 1000*g* for 30 min at 4 °C. The supernatant was used as an enzyme source. The enzyme activity was expressed in mg tissue unit (µmol/mg).

Acid and alkaline phosphatase (ACP/ALP)

Phosphatases were measured by the method of Bergmeyer (1985) modified by Singh and Agarwal (1991). Tissue homogenate (2%, w/v) was prepared in ice cold 0.9% NaCl and centrifuged at 5000*g* for 20 min at 4 °C. The activity of both phosphatases was expressed in mg tissue unit (µmol/mg).

Protein

Estimation of protein was made according to the method of Lowry et al. (1994). The results have been expressed as µg/mg tissue.

Histological study

Adult snails (8–10 mm) were exposed to the extracted chlorophyllin at (NOEL, LC_{10}, or LC_{25}) for 24 h (exposure); then, the snails were removed from the experimental test solution, washed thoroughly with dechlorinated tap water, and transferred to containers with fresh dechlorinated tap water for another 24 h of recovery, and this was done for 2 weeks. To study the changes in the histology of the digestive gland of treated snails compared with control snails, the digestive gland was dissected, fixed in Bouin's solution, embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin (Mohamed and Saad 1990). The sections were examined under the light microscope (Olympus System Microscope BX2 Series) and photographed by a Zeiss Video camera, Germany.

Statistical analysis

Lethal concentration values were defined by probit analysis (Finney 1971), and the data were analyzed using the Graph Pad Prism 6.04 software for Windows (Graph Pad Software, San Diego, California, USA; 1992–2014).

Results

The molluscicidal activity of the extracted chlorophyllin for adult *B. alexandrina* and *L. natalensis* snails after 24 h of exposure followed by another 24 h for recovery is represented in Table 1 and Fig. 1. These results showed that it was toxic to *B. alexandrina* snails at LC_{50} 17.6 mg/l and to *L. natalensis* snails at LC_{50} 4.3 mg/l.

By studying the effect of the sublethal concentrations (NOEL, LC_{10}, and LC_{25}) on *B. alexandrina* snails, the survival rates of these snails were highly significantly reduced ($p < 0.001$) compared to the control group and this reduction was concentration dependent (Fig. 2).

Regarding the biochemical alterations in the nervous tissue of *B. alexandrina* snails exposed to the sublethal concentrations (LC_{25}) of chlorophyllin for 2 weeks of the exposure, there was a significant ($p < 0.05$) decrease in acetylcholine

Table 1 Molluscicidal activity of chlorophyllin for adult *B. alexandrina* and *L. natalensis* snails after 24 h of exposure followed by another 24 h for recovery

Snails	NOEL (mg/l)	LC_{10} (mg/l)	LC_{25} (mg/l)	LC_{50} (mg/l)	Confidence limits of LC_{50} (mg/l)	LC_{90} (mg/l)	Slope
Biomphalaria alexandrina	1.8	14.3	15.93	17.66	15.01–20.1	20.95	1.1
Lymnaea natalensis	0.4	1.7	2.9	4.3	2.23–6.1	6.8	1.7

Done by probit analysis; number of snails used = 30 snails

esterase activities, acid and alkaline phosphatase levels, and protein content (Table 2).

The normal digestive gland of *B. alexandrina* snails consists of two main cell types: the digestive cells which are columnar with round apices and the secretory cells which are pyramidal in shape (Fig. 3a). Histopathological examinations showed that there were deleterious effects in the snails that were exposed to LC_{25}, which are represented by a deformation in the secretory cells, and a rupture of connective tissue between tubules and disintegration in the digestive cells (Fig. 3d), while less and moderate effects were observed in the digestive gland of snails groups that were exposed to NOEL and LC_{10}, respectively, when compared to the control group (Fig. 3b, c).

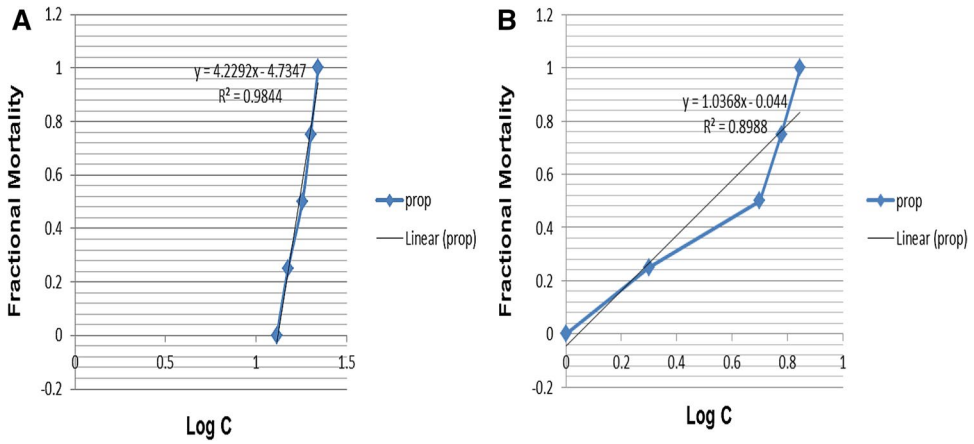

Fig. 1 Molluscicidal activity of the extracted chlorophyllin against: **a** adult *B. alexandrina* and, **b** *L. natalensis* snails after 24 h of exposure followed by another 24 h for recovery

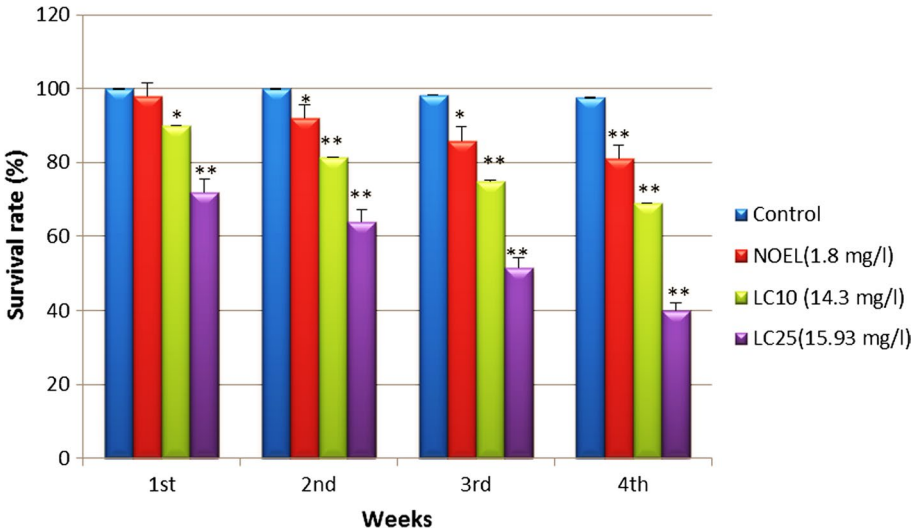

Fig. 2 Survival rate of *B. alexandrina* snails exposed to sublethal concentrations of chlorophyllin for 2 weeks of exposure followed by 2 weeks of recovery. *< 0.05 and **< 0.01; the error bars are standard errors

Table 2 Biochemical alterations in the nervous tissue of *B. alexandrina* snails exposed to the sublethal concentrations of chlorophyllin for 2 weeks of exposure; number of snails used = 10 snails

Group	Acetylcholinesterase (µmole/mg)	Alkaline phosphatase (µmol/mg)	Acid phosphatase (µmol/mg)	Protein (µg/mg)
Control	0.174 ± 0.02	3.59 ± 0.45	3.77 ± 0.12	12.87 ± 0.4
NOEL	0.172 ± 0.01	3.04 ± 0.5	3.15 ± 0.03	12.1 ± 0.07
LC_{10}	0.147 ± 0.03	2.24 ± 0.19*	3.07 ± 0.1*	11.7 ± 0.3*
LC_{25}	0.07 ± 0.01*	1.9 ± 0.2*	2.9 ± 0.04*	10.7 ± 0.1*

*Significant compared to control at $p < 0.05$

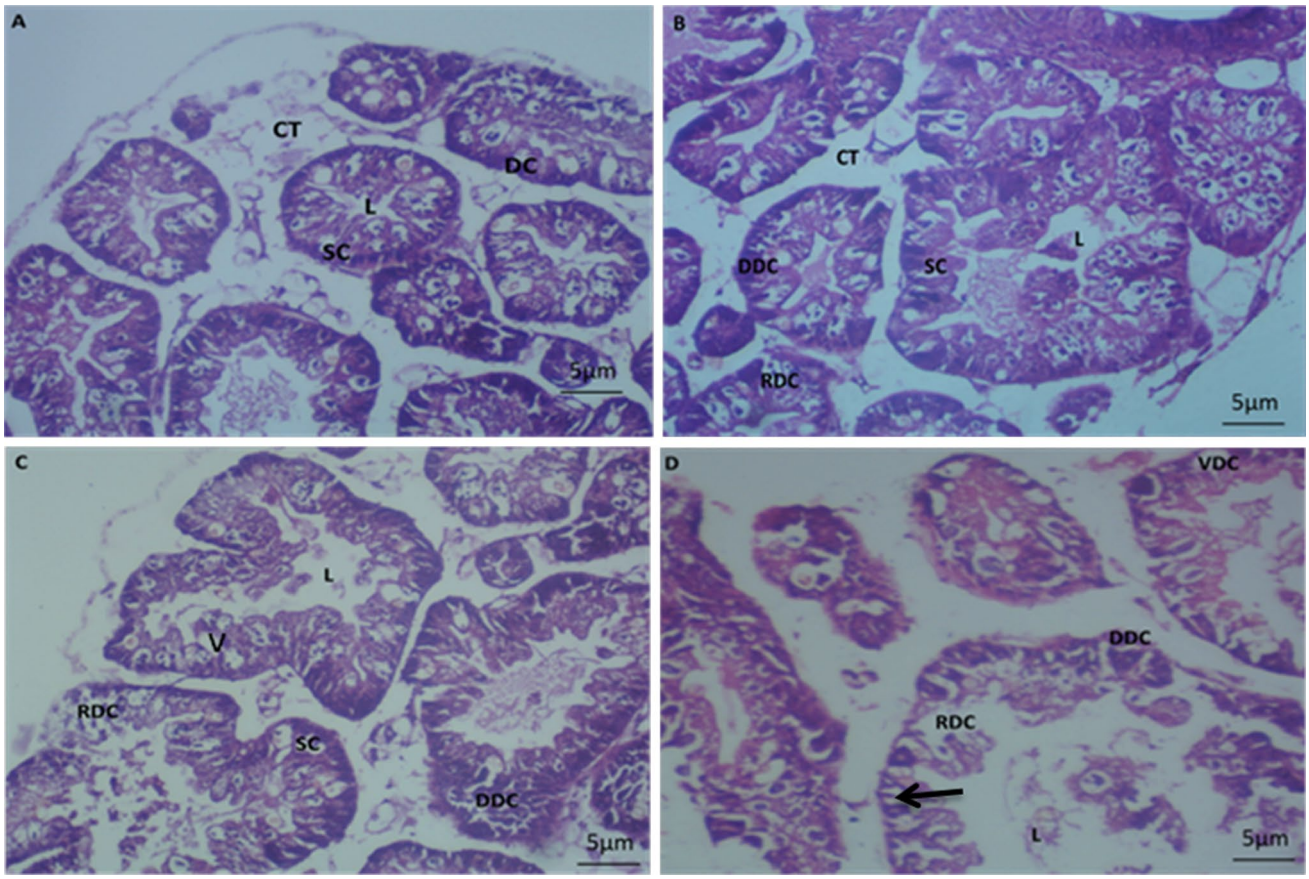

Fig. 3 Sections of the digestive gland (hematoxylin and eosin stain) of normal (control) *B. alexandrina* snails **a** columnar digestive cells (DC) and pyramidal secretory cells (SC) surround the lumen (L) and presence of connective tissues (CT). Snails exposed to NOEL, **b** dense enlarged secretory cells (SC) and ruptured digestive cells (RDC). Snails exposed to LC_{10}, **c** cell membrane of some digestive cells disappeared which led to vacuole (V) formation and degenerated digestive cells (DDC). Snails exposed to LC_{25}, **d** deformation in secretory cells (thin arrows) and disintegration in digestive cells (DDC)

Discussion

Water-soluble chlorophyllin can act as a molluscicidal agent (Chaturvedi et al. 2017). It is a photodynamic active substance that is excited by light (Singh et al. 2010). The present study showed that the calculated molluscicidal activity of the extracted chlorophyllin on *B. alexandrina* snails was LC_{50} 17.6 mg/l and that on *L. natalensis* snails was LC_{50} 4.3 mg/l. These results agreed with that of Elhadad et al. (2018) who stated that the exposure of *B. alexandrina* snails to LC_{50} of chlorophyllin (50 mg/l) greatly increased the mortality of these snails after 4 h of sunlight exposure to exert its photodynamic action. Erzinger et al. (2015) indicated that under short-term exposure for 24 h (one light/dark cycle), chlorophyllin can be used to control the vectors of parasitic diseases and that *Astyanax bimaculatus* fish showed a greater resistance (EC50 values of 29.96 mg/l).

The present investigation indicated that the survival rate of *B. alexandrina* snails exposed to these sublethal concentrations was highly significantly reduced ($p < 0.05$) compared to the control group and this reduction was concentration dependent. This is in agreement with Ragheb et al. (2018) who showed a marked reduction in the survivorship of *B. alexandrina* exposed to LC_{10} (5×10^{-6} mol/l) copper and magnesium chlorophyllin in the light. Also, Wohllebe et al. (2009) stated that water-soluble chlorophyllin under exposure of solar radiation was able to kill mosquito larvae and other small animals within a few hours. (LD50 value in *Culex* sp. larvae was 6.88 mg/l, and that in *Chaoborus* sp. larvae was about 24.18 mg/l.) Chaturvedi et al. (2017) confirmed that chlorophyllin enters the snail's body through its surface which causes effective killing of *L. acuminata* snails.

Acetylcholine is a neurotransmitter that regulates the animal's behavior. The acetylcholinesterase enzyme is used as a biomarker in various toxicological studies (Matozzo et al. 2005). This enzyme is involved in cholinergic neurotransmission of impulses by breaking acetylcholine into acetic acid and choline (Brien 1976). So, the inhibition in this enzyme will lead to the accumulation of acetylcholine at the nerve synapses, producing paralysis and general lack

of coordination in neuromuscular system and eventual death (Jaiswal et al. 2010). Alkaline phosphatase is useful in protein synthesis and other secretory activities (Ibrahim et al. 1977) in gastropod; its inhibition may result in reduction in protein level. Acid phosphatase (ACP) is a lysosomal enzyme (Aruna et al. 1979) which has an important role in autolysis, pathological necrosis, and overall catabolism (Abou-Donia 1978).

The present results showed that there were significant ($p < 0.05$) decreases in acetylcholinesterase, acid and alkaline phosphatase, and protein levels in the nervous tissue of *B. alexandrina* snails exposed to LC_{25} of chlorophyllin. These results are in accordance with Kumar and Singh (2016) who showed that chlorophyllin bait caused strong inhibition in the activities of acetylcholinesterase and cytochrome oxidase with a dose-dependent manner in the nervous tissue of *Lymnaea acuminata* with exposure of sunlight and red light. Also, Chaturvedi et al. (2017) stated that treatment of *L. acuminata* snails with 80% of 4-h LC_{50} (264.80 mg/l) of chlorophyllin caused maximum reduction in protein, amino acid, DNA, RNA and enzymes acetylcholinesterase, alkaline phosphatase and acid phosphatase activity in their nervous tissue. The reduction in protein levels may be due to the direct interference of the chlorophyllin with the protein biosynthesis (Kumar and Singh 2016; Chaturvedi et al. 2017) and the decrease in alkaline phosphatase.

Histopathological examinations showed that there were deleterious effects in the digestive gland of *B. alexandrina* snails that were exposed to LC_{25}, which are represented by a deformation in the secretory cells, and a rupture of connective tissue between tubules and disintegration in the digestive cells, while less and moderate effects were observed in the digestive gland of snails groups that were exposed to NOEL and LC_{10} when compared to the control group, and these effects were mirrored on their decreased survival rates. Elhadad et al. (2018) stated that the digestive gland of *B. alexandrina* snails treated with LC_{50} of chlorophyllin (50 mg/l) showed degenerations in the digestive cells with shrinkage of the supporting connective tissue, while the tubular epithelial cells lost their regular shape and have ruptured cell tips.

Conclusion

Chlorophyllin is a cheap substance that is found in every green plant and causes deleterious effects on snails that transmit diseases like *B. alexandrina* and *L. natalensis.* So, it can be used as a molluscicidal agent as it is a natural and biodegradable material.

Author's contributions AMI and FAB conceived and designed the study. AMI performed the experiments and analyzed the data. AMI wrote the first draft, and FAB revised and edited it. Both authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

Ethics approval and consent to participate Ethical approval had been granted approval by the Ethics Committee of Theodor Bilharz Research Institute (TBRI).

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