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# Efficacy of biopesticide Protecto (*Bacillus thuringiensis*) (BT) on certain biochemical activities and histological structures of land snail *Monacha cartusiana* (Muller, 1774)

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## Abstract

**Background:** The investigation aimed to show the effect of two concentrations of (Protecto 9.4%) commercial formulation of *Bacillus thuringiensis kurstaki* (Bt) on some biochemical changes of the land snail *Monacha cartusiana* at three different experimental periods (24, 48 and 72 h). Also, some histobiological alterations of the snail at a lethal experimental period of 96 h were studied.

**Results:** Pathogenicity effect of two sub-lethal concentrations of biopesticide Protecto; LC<sub>20</sub> ( $6.72 \times 10^6$  IU/mg) and LC<sub>40</sub> ( $17.28 \times 10^6$  IU/mg) were studied on the land snail *M. cartusiana* at 3 different exposure periods (24, 48, and 72 h). Some biochemical parameters such as Alkaline phosphatase (ALP), Alanine amino transaminase (ALT), Aspartate amino transaminase (AST), and Total protein (TP) were investigated. These observations cleared that; a significant elevation of ALP, ALT, and AST increased with increasing the sub-lethal concentration of protecto (LC<sub>20</sub> and LC<sub>40</sub>) against the exposed snail. Also, the activity of enzymes significantly increased with increasing the time of exposure (24, 48, and 72 h), respectively. Conversely, the level of TP in the snail was significantly decreased under pathogenic exposure for both (LC<sub>20</sub> and LC<sub>40</sub>) concentrations of Protecto at the same three treated periods (24, 48 and 72 h). The histobiological examinations at LC<sub>20</sub> and LC<sub>40</sub> for the exposure period 96 h, showed that the digestive gland with vacuolated degenerated, ruptured digestive cells and hemocyte infiltration. Moreover, the foot was observed with necrotic changes, vacuolated connective tissue, as well as, deformation in muscle fiber, and rupture the outer layer.

**Conclusions:** Final results showed that protecto *B. thuringiensis* had a pathogenic effect on land snail enzymatic activities and histobiological structures of land snail.

**Keywords:** *Monacha cartusiana*—Protecto—*Bacillus thuringiensis* (Bt)—Land snails, Biochemical parameters, Histobiological alterations

## Background

Nowadays, the use of environmental safety microbial in pest management received more significant attention from many authors (Kumar et al. 2021). *B. thuringiensis* is

a Gram-positive soil bacteria that do not have a cytotoxic effect on mammalian cells (Ohba et al. 2009). Instead of infecting most invertebrates pests, and secrete either toxic proteins by bacteria or soluble toxins produced by vegetative cells, which are toxic (Malovichko et al. 2019). During the last years, several studies on biological control of land snails using microbial agents such as *B. thuringiensis* have been carried out (Said and Ali 2018). Land

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gastropods belonging to phylum Mollusca are a wide sector of the fauna around the world (Diaz et al. 2017). Recently, Land snails, considered the only one between the phylum Mollusca attacking most agricultural fields in Egypt, causing great damage to; greenhouses, nurseries, fruit crops, orchards, ornamentals, vegetables, medical, navel orange, and apple trees (Ali and Robinson 2020). *M. cartusiana* (Muller) is a widespread land snail in the Egyptian agricultural fields and caused economic losses (Rady et al. 2019).

The present work studied the effect of protecto (*B. thuringiensis*) on some biochemical changes of *M. cartusiana* land snail under laboratory conditions. Also, histobiological investigations of the land snail at sub-lethal concentrations after exposure to 96 h. were studied.

## Methods

### Experimented snails

Adults land snails *M. cartusiana* with a shell size about (9–12 mm) and (weight 3.1 g) were collected manually from infested field crops of Yosef El Sedek district, El Fayoum Governorate (29 22° N: 30 51° E), middle Egypt, during the spring season of 2020. The collected adult snails were transferred to the laboratory at Fayoum Agric. Res. Station, Agricultural Research Center, Egypt, in a muslin bags, then they were washed with distal water. The experimental snails were transferred into rearing plastic boxes, each (30 × 30 × 25 cm<sup>3</sup> in size), used as housing, filled with moist sterilized loamy soil at 25 ± 2 °C and 75% ± 5% soil moisture. The experimental housing was covered with a muslin cloth to prevent snails from escaping. The snails were fed on leaves of lettuce daily for 14 days.

### Tested bactericide

Protecto: a commercial formulation of *B. thuringiensis* about 32,000 I.U./mg, received from biocide, production Unit, Plant Protection, Research, Institute Agriculture, Research Centre, Dokki, Giza, Egypt. The active ingredient constituted 9.4%.

### Laboratory experiment

#### Bioassay

The preliminary studies were conducted to estimate the lethal concentrations of protecto on land snails *M. cartusiana*. 4 concentrations of the tested compound were prepared using dechlorinated tap water based on weight/volume (Osman et al. 2011; Osman and Mohamed 1991) as follows; (8 × 10<sup>6</sup>, 16 × 10<sup>6</sup>, 32 × 10<sup>6</sup>, and 48 × 10<sup>6</sup> IU/mg. The experiments took place by the leaf-dipping technique. About 30 snails were placed into rearing plastic boxes and starved for 2 days (Souza 2003) then feeding the snails by dipping the leaves of lettuce in the tested concentrations for 30 s (Ghamry 1994) then outside left

it for 1 min for dryness before treatment. Fresh lettuce was used as control, five replicates each of contained 30 snails were used for each concentration. The mortality percentages of snails were recorded and computed for 48, 72, and 96 h.

### Biochemical parameters

Snails were treated with calculated LC<sub>20</sub> and LC<sub>40</sub> of protecto at the periods achieved mortality between 20–80% then the tested snails from each group of control and both sub-lethal concentrations were removed and dissected out from the shell and quickly weighed, homogenized in saline (0.9%) at a ratio of 1:9 (w:v) (Yang et al. 2015), then centrifuged at 8000 × g for 10 min at 4 °C in the refrigerated centrifuge (El-Gohary and Genena 2011). The deposits were discarded, and the supernatants were used to determine the levels of biochemical parameters; ALP (U/L) was determined by commercially available diagnostic kits supplied by Biosystems company, Egypt, ALT (U/L) and AST (U/L) were estimated kinetically by Biomed using the method of (Young 2001), and TP (mg/dl) was determined by using Diamond company according to the method reported by (Burtis and Ashwood 1999).

### Histological studies

Adult snails of *M. cartusiana* were tested as control; another 2 groups were exposed to 2 sub-lethal concentrations of *B. thuringiensis* LC<sub>20</sub> and LC<sub>40</sub>. After 96 h of exposure, the tested snails were dissected out carefully and fixed in Boun's fluid, dehydrated in 70% ethyl alcohol, cleared in xylene, and embedded in paraffin wax. Histological Sections (4 μm) were cut and were stained with hematoxylin and eosin (H&E) stain (Banchroft et al. 1996). Finally, they were investigated by a light microscope and photographed.

### Statistical analysis

Statistical analysis was performed using IBM SPSS (Statistical Package for the Social Sciences, SPSS, version 20). ANOVA was applied to compare the activity of enzymes at the effect of 2 sub-lethal concentrations of *B. thuringiensis* concerning the corresponding control at each experimental period. Lethal concentration values (LC<sub>20</sub> & LC<sub>40</sub>) were determined according to probit statistical analysis (Finney 1971).

## Results

### Lethal concentrations

Effect of different concentrations of *B. thuringiensis* on the mortality percentages of the adult snail *M. cartusiana* at intervals times (48, 72 and 96 h) were determined according to probit statistical analysis. The results

**Table 2** Effect of *Bacillus thuringiensis* on some biochemical parameters of land snail *Monacha cartusiana* exposed to LC<sub>20</sub> and LC<sub>40</sub> at 96 h after interval periods of exposure

Biochemical parameters	Sub lethal Concentrations (IU/mg)	Exposure period								
		24 h		48 h		72 h				
		Control	Mean	Change %	Control	Mean	Change %	Control	Mean	Change %
ALP (U/l)	6.72 × 10 <sup>6</sup>	38.48 ± 1.61	40.38 ± 1.28	(+ 4.94%) <sup>1</sup>	38.08 ± 1.18	48.38 ± 0.82 <sup>aA</sup>	(+ 27.05%) <sup>1</sup>	38.28 ± 0.66	51.5 ± 0.82 <sup>aA</sup>	(+ 34.5%) <sup>1</sup>
	17.28 × 10 <sup>6</sup>		55.02 ± 1.98 <sup>ab</sup>	(+ 42.98%) <sup>1</sup>		56.5 ± 1.42	(+ 48.37%) <sup>1</sup>		66.4 ± 1.81 <sup>abAB</sup>	(+ 73.46%) <sup>1</sup>
ALT (U/l)	6.72 × 10 <sup>6</sup>	63.1 ± 0.97	72.4 ± 1.5 <sup>a</sup>	(+ 14.7%) <sup>1</sup>	63.5 ± 0.63	84.0 ± 1.3 <sup>aA</sup>	(+ 32.3%) <sup>1</sup>	64.3 ± 0.61	112.9 ± 2.0 <sup>aAB</sup>	(+ 75.6%) <sup>1</sup>
	17.28 × 10 <sup>6</sup>		84.3 ± 1.5 <sup>ab</sup>	(+ 33.6%) <sup>1</sup>		97.7 ± 0.91 <sup>abA</sup>	(+ 53.9%) <sup>1</sup>		118.6 ± 1.9 <sup>abAB</sup>	(+ 84.4%) <sup>1</sup>
AST (U/l)	6.72 × 10 <sup>6</sup>	603.3 ± 1.80	616.2 ± 6.32	(+ 2.1%) <sup>1</sup>	604.7 ± 1.66	626.1 ± 4.05 <sup>a</sup>	(+ 3.5%) <sup>1</sup>	603.3 ± 1.28	738.6 ± 5.42 <sup>aAB</sup>	(+ 22.4%) <sup>1</sup>
	17.28 × 10 <sup>6</sup>		684.1 ± 4.12 <sup>ab</sup>	(+ 13.4%) <sup>1</sup>		787.0 ± 6.93 <sup>abA</sup>	(+ 30.1%) <sup>1</sup>		871.8 ± 6.15 <sup>abAB</sup>	(+ 44.5%) <sup>1</sup>
TP (mg/dl)	6.7 × 10 <sup>6</sup>	1.16 ± 0.067	0.77 ± 0.018 <sup>a</sup>	(- 33.62%) <sup>1</sup>	1.16 ± 0.040	0.75 ± 0.041 <sup>a</sup>	(- 35.34%) <sup>1</sup>	1.14 ± 0.051	0.62 ± 0.058 <sup>a</sup>	(- 45.61%) <sup>1</sup>
	17.28 × 10 <sup>6</sup>		0.59 ± 0.026 <sup>ab</sup>	(- 49.13%) <sup>1</sup>		0.38 ± 0.019 <sup>abA</sup>	(- 67.24%) <sup>1</sup>		0.35 ± 0.024 <sup>abA</sup>	(- 69.30%) <sup>1</sup>

Data are represented as a mean ± SE

**a, b:** In the same column, the significant difference of enzyme activity at LC<sub>20</sub> (6.72 × 10<sup>6</sup> IU/mg) and LC40 (17.28 × 10<sup>6</sup> IU/mg) in comparison to the corresponding control

<sup>1</sup> In the column, percentage of change in relation to control

**A, B:** In the same row, the significant difference compared to the pathogenic time 24 and 48 h of the experimental periods at α = 0.05 (P < 0.05)

Percentage of change in enzyme activity % =  $\frac{\text{level of enzyme activity in treated snail} - \text{level of enzyme activity in control snail}}{\text{level of enzyme activity in control snail}} \times 100$

**Table 1** Mortality percentage of *M. cartusiana* land snail against *Bacillus thuringiensis* after different interval periods of exposure

Concentrations (IU/mg)	Mortality percentages (%)		
	48 h	72 h	96 h
8 × 10 <sup>6</sup>	20.0	23.33	23.33
16 × 10 <sup>6</sup>	26.67	33.33	36.67
32 × 10 <sup>6</sup>	36.67	46.67	53.33
48 × 10 <sup>6</sup>	50.0	56.67	66.67
LC <sub>20</sub>	8.96 × 10 <sup>6</sup>	7.04 × 10 <sup>6</sup>	6.72 × 10 <sup>6</sup>
LC <sub>40</sub>	32.64 × 10 <sup>6</sup>	22.08 × 10 <sup>6</sup>	17.28 × 10 <sup>6</sup>

presented in Table 1 cleared that the mortality percentage of the snail increased with increasing the concentration of *B. thuringiensis* and the time of exposure. Also, the results indicated that the LC<sub>20</sub> and LC<sub>40</sub> values were 6.72 × 10<sup>6</sup> IU/mg and 17.28 × 10<sup>6</sup> IU/mg, respectively, at the experimental exposure period of 96 h.

#### Biochemical parameters

In order to investigate the biochemical activities of (ALP, U/l), (ALT, U/l), (AST, U/l), and (TP, mg/dl) of *M. cartusiana* exposed to 2 sub-lethal concentrations of *B. thuringiensis*, LC<sub>20</sub> and LC<sub>40</sub> were analyzed and computed by one-way ANOVA for the 2 concentrations comparing to the tested control at each corresponding exposure period. A significant difference at (P < 0.05) was confirmed as shown in Table 2, at 24 h, where the activity of ALP has non-significant difference between LC<sub>20</sub> and the tested control but it was significantly increased at the tested concentration LC<sub>40</sub> than the control. After 48 and 72 h, the activity of the enzyme was significantly increased at the LC<sub>20</sub> value (48.38 ± 0.82, and 51.5 ± 0.82, respectively) and at LC<sub>40</sub> value (56.5 ± 1.42 and 66.4 ± 1.81, respectively) than the corresponding control (38.08 ± 1.18 and 38.28 ± 0.66). In addition, the activity of ALP was not only computed by one-way ANOVA but also by Post hoc analysis which compared the activity of the enzyme at the 3 different periods; which increased at 48 and 72 h in relation to 24 h. Furthermore, ALT activity was significantly high at exposure the snail to LC<sub>20</sub> and LC<sub>40</sub> comparing to control at the different tested periods (Table 2). Additionally, the activity of the AST enzyme increased significantly after exposing the snail to LC<sub>40</sub> value as compared those of control at the 3 exposure times. As well as, after exposing to LC<sub>20</sub> values, the enzyme activity significantly increased only at the exposure periods (48 and 72 h) as compared to those of control. For comparison, the enzyme activity at the three periods, 24, 48, and 72 h, post hoc analysis cleared that it was significantly increased at 72 h than the periods of 24 and 48 h after

LC<sub>20</sub> and LC<sub>40</sub> values' treatments. On the other hand, the total protein (TP) level was significantly decreased after LC<sub>20</sub> and LC<sub>40</sub> exposure compared to control at the 3 different exposure times. The post hoc analysis showed that the level of TP at lethal concentration LC<sub>40</sub> had a significant reduction at 48 and 72 h with relation to lethal time 24 h only.

#### Histological studies

##### Digestive gland

In the present study, the digestive gland of the control *M. cartusiana* snail showed a normal structures without histopathological alterations under a light microscope (Fig. 1a). It consists of highly—branched numerous, compressed digestive tubules (DT) separated by loose connective tissues. Each tubule has a narrow lumen (L), all surrounded by a circular thin muscle layer consisting of 3 different cells; Digestive cells (DC) with columnar shape and containing basally nuclei, Calcium cells, Excretory cells (EC). Histological alterations were observed in *M. cartusiana* treated with LC<sub>20</sub> of *B. thuringiensis* after 96 h of exposure observed in (Fig. 1b), the lumen of the digestive tubule enlarged and increased, the cells lining the tubules were irregularly arranged and extremely indistinguishable. The excretory cells become vacuolated (V), rupture and degeneration of digestive cells were reported. Additionally, histopathological observations were observed at exposure to LC<sub>40</sub>, hemocyte infiltration (HI), the digestive tubule, and their constituents lining cells; digestive, calcium, and excretory cells showed complete rupture (Fig. 1c).

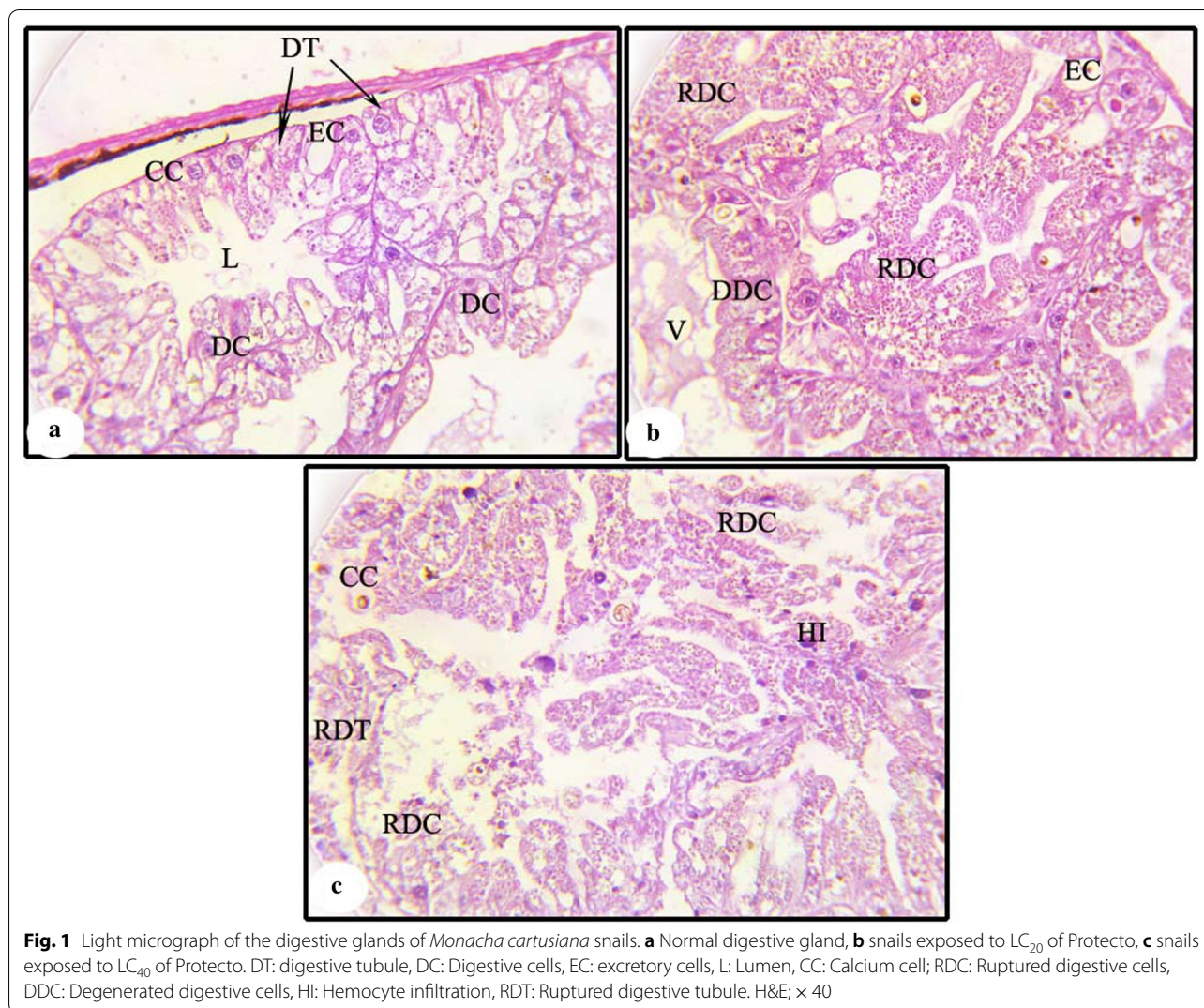
##### Foot

The foot tissue of control *M. cartusiana* snail was found composed of a simple columnar epithelium layer (E), followed by a layer of connective tissue containing mucous gland (MG). The innermost layer was formed of muscle fiber (MF), as illustrated in (Fig. 2a). The foot tissue of snail exposed to LC<sub>20</sub> was observed with necrotic changes in the mucous gland (N), the connective tissue containing empty spaces vacuoles (V). Additionally, the muscle fiber layer suffered from the cells' deformation (Fig. 2b). Meanwhile, at snail exposure to LC<sub>40</sub>, the foot tissue contained vacuoles in the muscle fiber layer as long as deformation occurs in this layer. Also, the outer epithelial layer contained undifferentiated necrotic epithelial cells and ruptured the outer layer (Fig. 2c).

#### Discussion

Obtained data indicated that the mortality of the snail *M. cartusiana* after protecto treatments increased with increasing the concentration and the exposure time. The

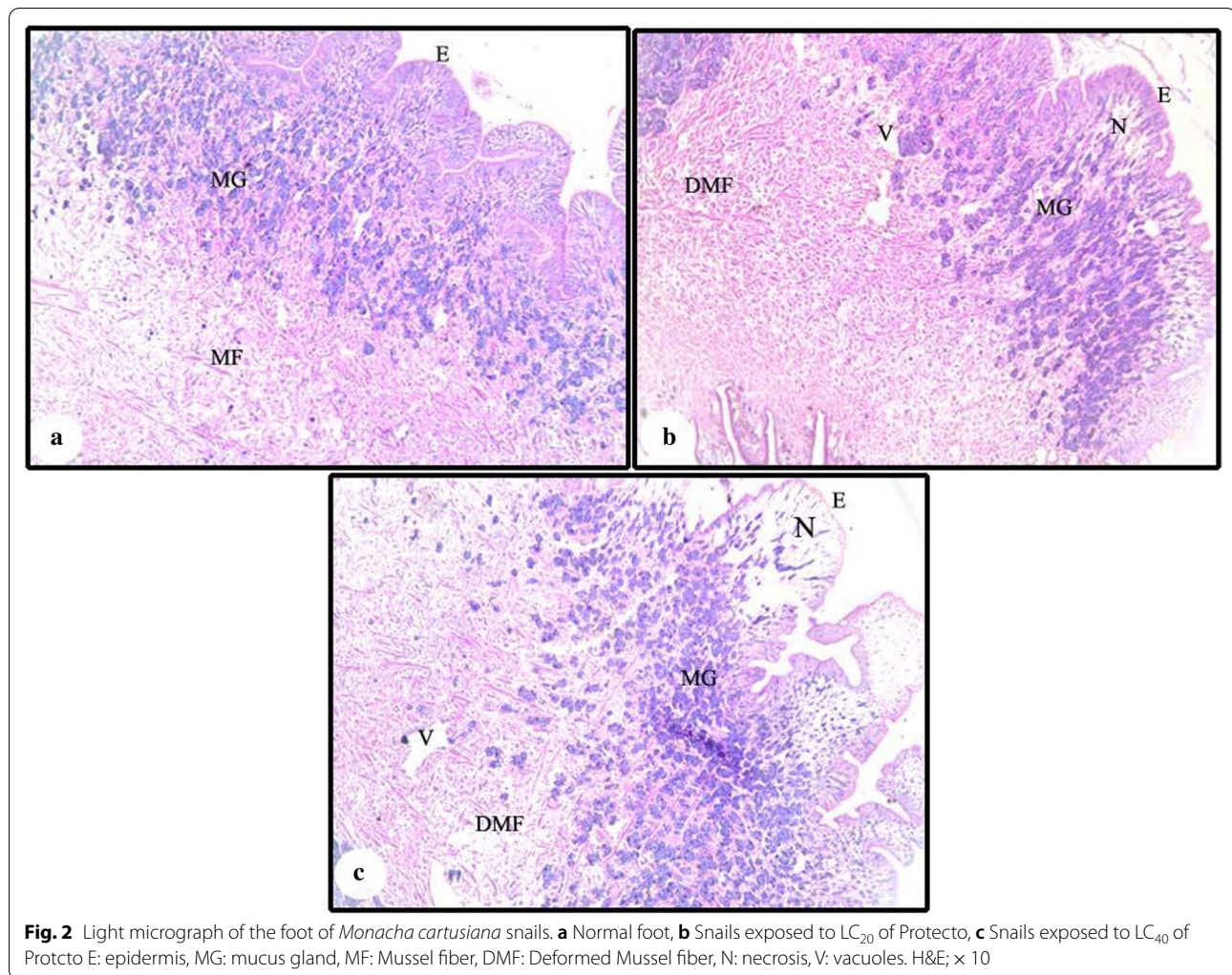




sub-lethal concentrations (LC<sub>0</sub>, LC<sub>10</sub>, and LC<sub>25</sub>) reduced the survival and growth rates of *S. mansoni* snails after 12 weeks of *B. thuringiensis* treatment in relation to the corresponding control (Osman et al. 2011). The snail *Physa marmorata* was found more sensitive to the lethal concentration of the biocide *B. thuringiensis* (LC<sub>50</sub>) at 24, 48, and 72 h (Mansouri et al. 2013). This finding was in accordance with that obtained by Genena and Mostafa (2008), who found out that *B. thuringiensis* showed high pathogenicity against land snail *M. cantina*, with mortality increasing with an increase in the concentration of bacteria.

The gained results in this study indicated that the commercial formulations of tested biopesticide protecto increased the activity of enzymes ALT, AST, and ALP at the two sub-lethal concentrations LC<sub>20</sub> and LC<sub>40</sub> at the 3 different tested periods 24, 48 and 72 h in comparison to the control. Also, the results showed a significant

decrease in the level of total protein in the snail at both concentrations for 3 lethal times. Those enzymes are located in hepatocytes and in many organs, lungs, and hurt (Abd-El-Haleem et al. 2021). The commercial formulations of *B. thuringiensis* var *kurstaki* "Agerin, Dipel 2X and Dipel DF" significantly affected activities of ALT and AST enzymes of larvae of cotton leafworm *Spodoptera littoralis* (Kamel et al. 2010). Also, (Kramarz et al. 2007) showed that *Bt* toxin does not affect the survival rates of *Helix aspersa* snails. The obtained results are incorporated with those cleared by (Abdel-Halim et al. 2006), who showed that the activities of AST and ALT were decreased in *M. cartusiana* snail exposed to LC<sub>50</sub> and 0.5 LC<sub>50</sub> of *B. thuringiensis* with different intervals time. Also, (El-Gohary and Genena 2011) revealed that the molluscicides, Gastrotov, Molotov, and Mesurol caused a significant decrease in ALT activity. At the same time, they have an insignificant increase in AST activity



and TP level tested on land snail *E. vermiculata*. Also, they increased the AST and ALT activities but decreased TP level in land snail *M. cantina*. The results agree with (Banaee et al. 2016), who cleared that ALT, AST, and ALP activities increased significantly in carp exposed to sub-lethal concentrations of paraquat but decreased the level of total protein of the carp at the same conditions. The activities of AST, ALT, and ALP of *C. punctatus* increased during toxic exposure to Triazophos concentrations. The total protein level decreased during toxic exposure to sub-lethal concentrations at time intervals may be attributed to involving the proteins in the metabolic purposes of the cell, which led to the breakdown of protein for utilization in metabolism (Naveed et al. 2010). The enhanced activities of AST and ALT in land snails caused by the toxic stress of *B. thuringiensis* may be attributed to insufficient additional energy that resulted from the deprivation of snails from food (Samanta et al. 2014). The activities of hemolymph enzymes AST and ALT of

*E. vermiculata* when exposed the snail to sub-lethal concentrations of Lannet were increased significantly than control, but ALP activity and TP level were decreased at all tested concentrations (Khalil 2016). These results support the finding of (Awadalla et al. 2017) which showed that the total protein decreased highly in the larvae of *S. littoralis* treated with Protecto. The sub-lethal concentration LC<sub>25</sub> of Ginger extract caused AST and ALT enzymes elevation in *M. cartusiana* land snail (Abd El-Atti et al. 2019).

Obtained results cleared that *B. thuringiensis* caused histological alterations in *M. cartusiana* tissues; digestive glands, foot, and mantle at both sub-lethal concentrations LC<sub>20</sub> ( $6.72 \times 10^6$  IU/mg) and LC<sub>40</sub> ( $17.28 \times 10^6$  IU/mg). The digestive cells lining the tubule are irregular and indistinguishable. The cell contained vacuoles, degenerated and ruptured. Those observations agree with that cleared by (Said and Ali (2018), who stated that the digestive tubule has fragmentation



cells and disorganization in cells components in Land snail *Eobania vermiculata* treated by *B. thuringiensis*. (Attia et al. 2021) exposed *E. vermiculata* to inorganic fertilizer and observed histological alterations in the digestive gland as; distinguished the epithelium lining and increased in a number of excretory cells. Caselio (plant fertilizer) has a pathological effect on the freshwater snail, *Lanistes carinatus*, which causes necrosis for the basement membrane of the digestive tubule, degenerated, and disintegration for connective tissue in the digestive gland (Sheir 2015). Ali and Said (2019) stated that the epithelial cells in the digestive tubule of *Monacha obstructa* land snail lost their normal shape after exposure to UV A radiation. Cofone et al. (2020) observed that the outer epithelial layer in treated *E. vermiculata* foot showed darker in color, a significant decrease in mucus gland, and an increase in vacuoles number within a sub-epithelial layer of the tissue. The foot of *B.alexandrina* snails occurring in pollutants aquatic environment was observed with shrinkage in unicellular glands, atrophy, and increased vacuoles. Also, the digestive gland shows histological alterations (El-Khayat et al. 2018). The histological alterations in the digestive gland were necrotic changes of digestive cells and loss of cell organizations. The alterations in the foot were disruption of muscle fibers and decreased in Mucous cells and protein cells (Cengiz et al. 2005).

Furthermore, the foot of *M. cartusiana* study showed histological changes as; deformed in Mussel fiber, necrosis, and increased in vacuoles numbers. These observations were in congruence with the results of (Parvate and Thayil 2017), who recognized that the foot in *Achatina fulica* land snail with increasing the vacuoles and infiltration in myoepithelial cells layer at exposed to clove oil. Furthermore, the foot of snails had histological alterations under toxic exposure, shrinkage, rupture of muscular tissue, distortion of epithelial covering, and accumulation of the pigment cells (Abdel-Rahman 2020).

## Conclusions

It was concluded that Protecto had a significant pathogenic effect on mortality and biochemical activities of land snail *M. cartusiana* at both sublethal concentrations of exposure, LC<sub>20</sub> and LC<sub>40</sub> values at 3 different experimental periods (24, 48, and 72 h). Also, the bactericide had a lethal effect on the histological structures of the snail.

## Abbreviations

BT: *Bacillus thuringiensis*; LC<sub>20</sub>: Lethal concentration of protecto required to kill 20% of exposed snails; LC<sub>40</sub>: Lethal concentration of protecto required to kill 40% of exposed snails; ALP: Alkaline phosphatase; ALT: Alanine amino transaminase; AST: Aspartate amino transaminase; TP: Total protein; DT: Digestive tubule; DC: Digestive cells; EC: Excretory cells; L: Lumen; CC: Calcium cell;

RDC: Ruptured digestive cells; DDC: Degenerated digestive cells; HI: Hemocyte infiltration; RDT: Ruptured digestive tubule; H&E: Hematoxylin and eosin; E: Epidermis; MG: Mucus gland; MF: Mussel fiber; DMF: Deformed Mussel fiber; N: Necrosis; V: Vacuoles; ANOVA: Analysis of variance; SE: Standard error.

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## Author contributions

AA and OA designed the study. OA and FK conducted the experiment. GE, AA and HM provided research material and helped in conducting the experiments. OA, AA and HA helped in reviewing and editing the manuscript. All authors read and approved the final manuscript.

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## Availability of data and materials

All data generated or analyzed during this study are included in this published article.

## Declarations

### Ethics approval and consent to participate

All of the authors participate in preparing the manuscript and agree to submit the manuscript.

### Consent for publication

All of the authors agree to submit the manuscript in the Egyptian Journal of Biological Pest control.

### Competing interests

The authors declare that they have no competing interests.

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