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RESEARCH ARTICLE

Toxicological assessment of lambda-cyhalothrin and acetamiprid insecticides formulated mixture on hatchability rate, histological aspects, and protein electrophoretic pattern of *Biomphalaria alexandrina* (Ehrenberg, 1831) snails

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Abstract

Several formulated mixtures of pesticides are widely used in modern agriculture. Nevertheless, the agriculture runoff causes a serious damage to the aquatic ecosystem. Therefore, the present study aims to use *B. alexandrina* snails as bioindicators for 30 g/l lambda-cyhalothrin and 17 g/l acetamiprid as a formulated mixture insecticide. Results showed that it has a molluscicidal activity against snails at LC_{50} 7.9 mg/l. The hatchability percent of both treated 1-day-aged and/or 3-day-aged groups were less than that of the control group. The sublethal concentrations of the tested insecticide caused a remarkable abnormal necrosis in male and female gametogenic cells, besides a severe damage in both secretory and digestive cells. The results of SDS-PAGE protein profiles of treated snails showed that the least number of protein bands was noticed in snail groups that subjected to LC_{10} (6.6 mg/l) and LC_{25} (7.2 mg/l) concentrations when compared to control protein fractions.

Keywords Lambda-cyhalothrin and acetamiprid · Histopathology · Hatchability · Protein electrophoresis · *B. alexandrina*

Introduction

Nowadays, pesticides have been used in all over the world for centuries to decrease the number of pests or to help in the eradication of pests (El-Sheikh et al. 2012). They are considered as potent pollutants of the water environment with undesirable effects on non-target organisms such as fish and water animals (Guner 2016). They are commonly used in agricultural areas as single compounds or in mixtures and became a part of the water column via spray drift, runoff, and wastewater treatment plant effluent (Bakry et al. 2016).

Invertebrates represent a key indicator group for monitoring environmental change in many different ecosystems (Carew et al. 2013) and have been used for the detection and monitoring of the chemical pollution in coastal waters as they represent more than 90% of the aquatic species (Dixon et al. 2002). Among invertebrate animals, freshwater snails are commonly used in acute and chronic toxicity studies of chemicals which could potentially harm for all phyla of aquatic life as well as birds and mammals, including humans (Wilson-Sanders 2011). *Biomphalaria alexandrina* (Ehrenberg 1831) snails serve as sensitive biomarkers for aquatic ecosystem pollution (Ibrahim et al. 2018). The possibility of using *B. alexandrina* snails as biomonitors for aquatic pollution was reported by El-Deeb et al. (2017) and Silva et al. (2017). In addition, they are widely distributed and act as the intermediate host for *Schistosoma mansoni* in Egypt (Abd El-Ghany and Abd El-Ghany 2017).

Acetamiprid is an odorless neonicotinoid insecticide (Shaker et al. 2015) and is used for controlling of Hemiptera, especially aphids, Thysanoptera and Lepidoptera, by soil and foliar application. It is also a key pesticide in commercial cherry farming due to its effectiveness against the larvae of the cherry fruit fly. Raj and Joseph (2015) stated that Chronic exposure of *Oreochromis mossambicus* fish to lethal concentration (LC_{50}) of acetamiprid at 5.99 ppm increased activity of

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lactate dehydrogenase in liver, brain, and gill tissues during all the exposure periods when compared with the control and reasoned this significant increase in enzyme activity to the toxic effect of acetamiprid.

Lambda-cyhalothrin insecticide is a synthetic pyrethroid (IPCS 1990), used for pest control, especially mosquitoes, cockroaches, ticks, fleas, aphids, Colorado beetles, cutworms, and butterfly larvae (EXTOXNET 1996). It disrupts the nervous system in insects, causing paralysis and death in both insects and non-target organisms (Choi and Soderlund 2006). Also, it has adulticidal, ovicidal, and larvicidal activity (IPCS 1990). Guner (2016) confirmed that lambda-cyhalothrin contamination significantly affects the behavior of Mosquito fish, *Gambusia affinis* at three different doses 0.1, 0.5, and 0.75 ppm according to control group. Laboratory studies indicate that cyhalothrin has the potential to accumulate in fish (WHO 1990). El Bacha 47 E.C. insecticide is a formulated mixture of acetamiprid and lambda-cyhalothrin, which is used to control various kinds of insect pests effectively.

Pesticides produce numerous physiological changes and histological alterations in aquatic species (Regoli and Principato 1995) affect basic genetic materials (DNA and RNA), total protein, total free amino acids, and carbohydrates (Ansari and Kumar 1988). Proteins have important roles in DNA formation, growth, development, reproduction, and everyday functioning. One of the most simple and powerful technique for separation of proteins based on their molecular weight is sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bakry et al. 2013). This technique was used by several authors to detect the variation in protein patterns of different snail species (Mahobiya and Bhide 2013; Osman et al. 2014).

In Egypt, El Bacha 47 E.C. is a commercial mixture formulation of insecticide composed of 30 g/l of lambda-cyhalothrin and 17 g/l of acetamiprid; it applies for agricultural purposes and then became a part of the snail's habitat. Therefore, the present study aims to evaluate the toxic impacts of El Bacha 47 E.C. insecticide on *B. alexandrina* snails, development of its embryos, and histological and protein pattern in the tissues of these snails as bioindicators.

Materials and methods

The experiments were carried out according to the National Institute of Health Guide for Care and Use of Laboratory Animals (The authors declare that no experiments were performed on humans).

Experimental animals

Adult *B. alexandrina* snails (Ehrenberg, 1831) (9.45 ± 1.9 mm) were purchased from Schistosome Biological Supply Center (SBSC) at Theodor Bilharz Research Institute (TBRI) and maintained in plastic aquaria (16 × 23 × 9 cm). The aquaria were provided with dechlorinated aerated tap water (10 snails/l), at pH 7 and at temperature (28 ± 1 °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, a photoperiodicity of 13-h light/11-h dark. Pieces of polyethylene sheets were used for collecting egg masses.

Experimental materials

El Bacha 47 E.C. is one of the commercial mixture formulations of insecticide that composed of 30 g/l of lambda-cyhalothrin and 17 g/l of acetamiprid and plays a key role in agricultural purposes to control various kinds of insect pests effectively. It is manufactured by Modern Chemical Industries Company (Agripharm), Egypt, local registration number (757).

Bioassay tests

Distilled water was used for preparing a stock solution of the tested formulation. To calculate lethal concentrations, series of concentrations were prepared (10, 9, 8, 7, 6, and 5 mg/l). Three replicates were used, each of 10 snails (8–10 mm in diameter) for each one. The exposure period was 24 h at room temperature. Another group of snails was maintained under the same experimental conditions as a control group (WHO 1965). At the end of the exposure period, these snails were removed from each tested concentration, washed thoroughly with dechlorinated tap water and transferred to another container for a recovery period for 24 h. Then, dead snails were counted. LC_{50}, LC_{90}, and sublethal concentrations (LC_0, LC_{10}, and LC_{25}) were computed using SPSS (version 16.0).

Assay for egg development and hatchability

Egg masses of 1- and/or 3-day-aged were collected separately. About 100 embryo/three replicates were exposed to LC_0, LC_{10}, and LC_{25} concentrations of the tested formulation separately. After 5 days, egg masses were microscopically examined and photographed to recording the egg development, and then transferred to dechlorinated water to record the hatchability percent. Groups of normal egg masses from both 1- and 3-day-aged were maintained under the same experimental conditions.

Histopathological examinations

Snails (8–10 mm in shell diameter) were exposed to LC_0, LC_{10}, and LC_{25} sublethal concentrations of the tested formulation for two successive weeks. Thereafter, snails were

washed with water then dried. The digestive and hermaphrodite glands of each snail was separated gently from the soft parts, fixed Bouin's solution, embedded in paraffin wax, sectioned and stained with hematoxylin and eosin. Sections were examined under light microscope (Olympus System Microscope BX2 Series) and photographed by a Zeiss Video camera, Germany.

Tissue sampling

Snails of 8–10 mm were exposed to sublethal concentrations (LC_0, LC_{10}, and LC_{25}) of the tested formulation, separately for successive 2 weeks. Then, whole snail's tissue from each treated and control groups were washed with water then dried. Snail's shell was gently crushed between two glass slides and digestive gland was dissected out from 3 to 5 snails and pooled in 1-ml Eppendorf tube to which tissue-extracting buffer (Tris-buffer saline, 50 mM Tris-HCL, pH 7.5 containing 75 ml NaCl) was added in a ratio of 1:10 *w/v* according to (Bradford 1976). Homogenization was carried out in Eppendorf tube using small glass road, freezing-thawing method was performed to facilitate homogenization process. Centrifugation was carried out at 10,000 rpm for 15 min at 4 °C.

Electrophoretic analysis

A total protein of *B. alexandrina* snails' tissue was performed using SDS-PAGE according to the protocol of (Boswell et al. 1987). Total protein was separated on 8% resolving gel and 3.75% stacking gel using electrophoresis apparatus (Bio-Rad USA vertical mini gel, double side).

Gel analysis

Similarity of the polypeptide profile between the different groups was assessed from Dice similarity coefficient (Dice 1945), which is still valid and used to solve many genetic problems in animal and plant species (Ravelo et al. 2003). Similarity equation is ($S = 2a \ / \ 2a + b + c$) where S is the degree of identity, a is the number of common shared bands in two compared samples, b is the number of excess bands in the first compared sample, and c is the number of excess bands in the second compared sample. An "S" value of 1.0 denotes complete identity in the electrophoretic profile of both groups, while a value of < 1.0 indicates a variation in the polypeptide profile between the two compared samples.

Statistical analysis

Lethal concentration values were defined by Probit analysis (Finney 1971). Percentage of egg hatchability was analyzed by chi-square values of contingency tables using SPSS v. 17.0 for Windows (SPSS Inc. 2008).

Results

The calculated lethal concentrations (LC_{90} and LC_{50}) on *Biomphalaria alexandrina* snails after exposure of the El Bacha 47 EC for 24 h were 9.2 and 7.9 mg/l, respectively as shown in Table 1.

The present results showed that egg hatchability percent of both treated 1-day-aged and/or 3-day-aged groups were less than that of the control group with a significant difference at $P < 0.001$ as shown in Fig. 1. In addition, the hatchability percent of 3-day-aged eggs was higher than 1-day-aged after exposed to LC_{10} and LC_{25} concentrations. The results attributed to fetal malformations that observed in 1-day-aged eggs that suffered from delay in growth and degeneration in embryos as shown in Fig. 2c, d.

The normal hermaphrodite gland of *B. alexandrina* comprises of the different stages of spermatogenesis: primary spermatocytes, secondary spermatocytes, and sperms and oogenesis; primary oocytes, secondary oocytes, and mature ova as shown in Fig. 3a (1). Although the exposed snails to LC_0 concentration showed a moderate effect such as a degeneration of certain mature ova and deformation of others (Fig. 3a (2), the exposed snails to LC_{10} and LC_{25} concentrations have marked morphological changes in both male and female gonadal cells. Most of the spermatocytes became scattered. In addition, mature ova and secondary oocytes suffered from disintegration and deformation (Fig. 3a (3 and 4) that results to completely reproductive dysfunction.

The normal digestive gland of *B. alexandrina* snails consists of two main cell types: the digestive cells which are columnar with round apices, their nuclei are oval and lie in basal region and secretory cells which are pyramidal in shape

Table 1 Lethal and sublethal concentrations of El Bacha 47 EC on *B. alexandrina* snails after 24 h of exposure

Tested materials	LC_{50} (mg/l)	Confidence limits of LC_{50} (mg/l)	LC_{90} (mg/l)	Slope	LC_0 (mg/l)	LC_{10} (mg/l)	LC_{25} (mg/l)
El Bacha 47 EC	7.9	7.04–8.73	9.2	1.135	2.03	6.6	7.2

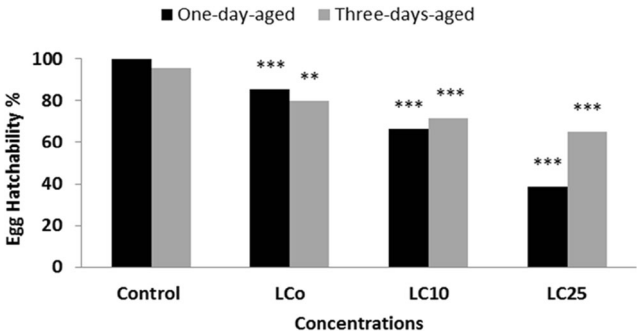

Fig. 1 Hatchability percent of *Biomphalaria alexandrina* snail's eggs exposed to sublethal concentrations of El Bacha 47 EC for successive 5 days

and contain large nuclei (Fig. 3b (5). Histopathological examinations showed that the highest deleterious effects were in the digestive gland of snail group that was exposed to LC_{25}, which represented by deformation in the secretory cells, rupture of connective tissue between tubules, and disintegration in the digestive cells (Fig. 3b (8). Meanwhile, the less and moderately effects were observed in the digestive gland of snail groups that were exposed to LC_0 and LC_{10}, respectively, when compared to the control group (Fig. 3b (6 and 7).

Concerning protein electrophoretic pattern of the exposed snails to El Bacha 47 EC, results showed various effects on protein synthesis, which yielded a complex pattern of polypeptides after 2 weeks of the exposure period. Also, many bands were disappeared in exposed snails and appeared in control and vice versa (Fig. 4).

Generally, two bands (83.74 and 24.69 kDa) were common in both control group and snail group that were exposed to LC_0 concentration. Although two bands (142.9 and 39.80 kDa) were shared between snail groups that were exposed to LC_0 and LC_{25} concentrations, one band (27.87 kDa) was shared between snail groups that were exposed to LC_{10} and LC_{25} concentrations. Regarding the similarity index, results showed that the highest similarity index was 0.45 between control and snail groups that were exposed to LC_0 concentration. Meanwhile, the similarity index for the snail groups that were exposed to LC_{10} and LC_{25} concentrations recorded 0.24 with the control group as shown in Table 2.

In numerical analysis, bands were scored for their presence (1) or absence (0); each group was characterized by the presence of numbers of unique bands. Tissue extract from snails that were exposed to LC_0 concentration showed two unique bands (68.42 and 34.03 kDa). Although snails that were exposed to LC_{10} concentration showed six unique bands (138.25, 85.35, 70.57, 55.64, 35.34, and 11.54 kDa), snail groups that were exposed to LC_{25} concentration have four unique bands (69.24, 57.39, 13.50, and 10.98 kDa). The remaining bands that were shared by more than one group were polymorphic bands (Table 3).

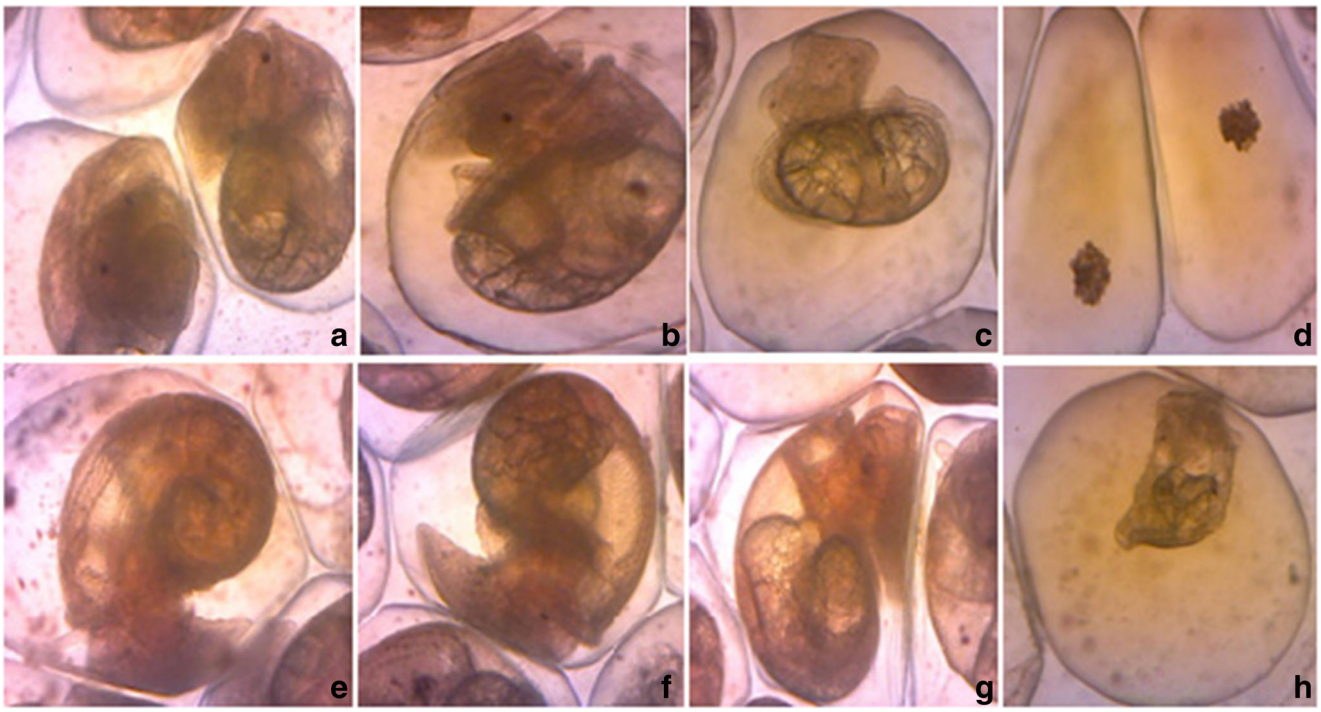

Fig. 2 Developmental stages of embryo of *B. alexandrina* after successive 5-day exposure to El Bacha 47 EC. **a** Normal embryo of 1-day-aged after 5 days. **b** Embryo treated with LC_0 of 1-day-aged. **c** Delay in growth of embryo treated with LC_{10} of 1-day-aged. **d** Degenerated embryo treated with LC_{25} of 1-day-aged. **e** Normal embryo of 3-day-aged. **f** Treated embryo with LC_0 of 3-day-aged. **g** Treated embryo with LC_{10} of 3-day-aged. **h** Treated embryo with LC_{25} of 3-day-aged

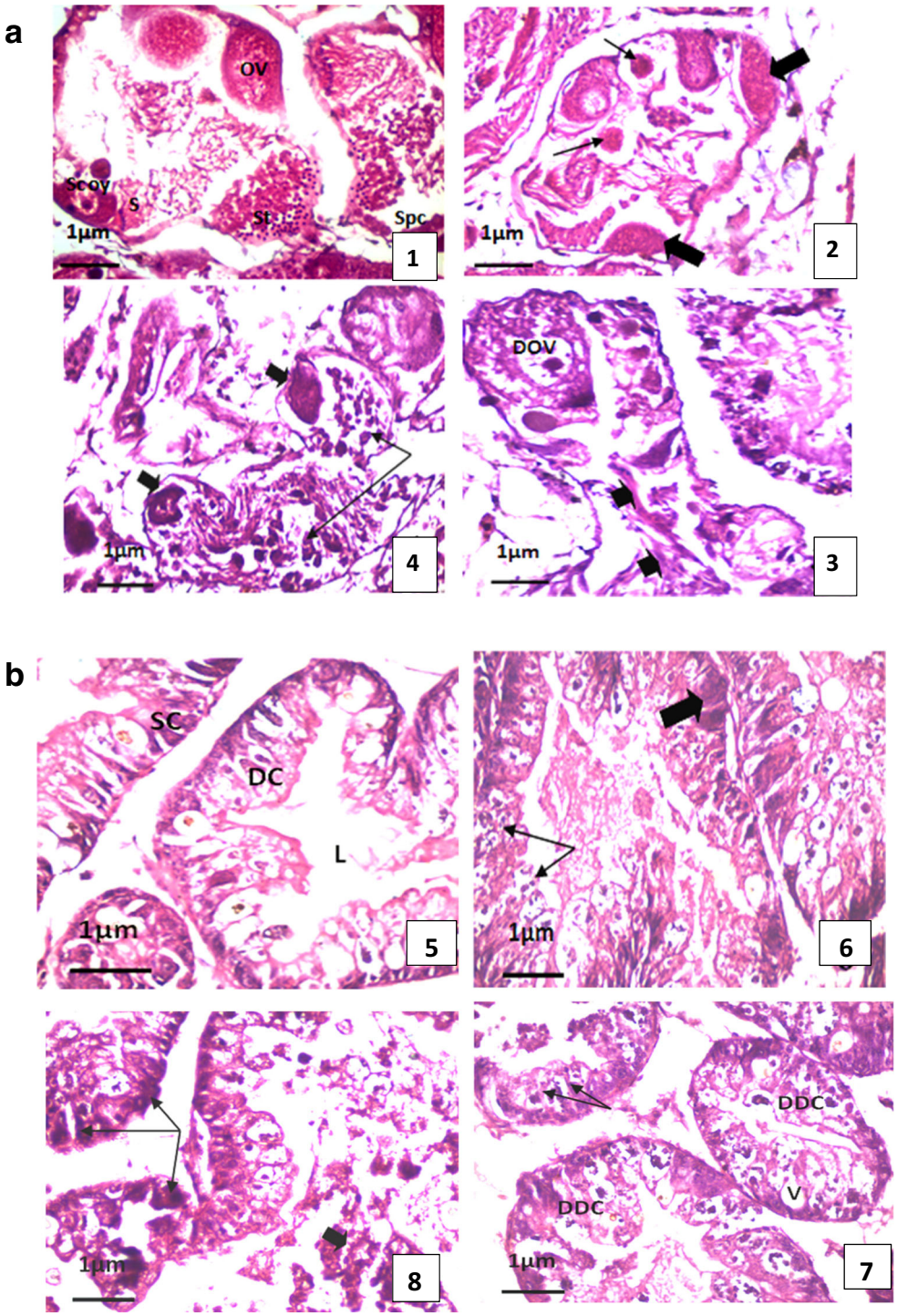

Fig. 3 **a** Section of hermaphrodite gland (hematoxylin and eosin stain) of normal *B. alexandrina* snails (1) showing secondary oocyte (Sc oy) and mature ovum (OV), spermatocytes (Spc), spermatids (St), and sperms (S). Snails exposed to LC_0 (2) showing degeneration of certain mature ova (thin arrow), deformation of others (thick arrow). Snails exposed to LC_{10} (3) showing lysis in all stages of spermatogenesis (thick arrow) and disintegrated mature ovum (DOV). Snails exposed to LC_{25} (4) showing scattering of spermatocytes (thin arrows) and deformation of secondary oocytes (thick arrow). **b** Section of digestive gland (hematoxylin and eosin stain) of normal *B. alexandrina* snails (5) showing columnar digestive cells (DC) and pyramidal secretory cells (SC) surround the lumen (L). Snails exposed to LC_0 (6) showing dense enlarged secretory cells (thick arrow) and ruptured digestive cells (thin arrows). Snails exposed to LC_{10} (7) showing cell membrane of some digestive cells disappeared which led to vacuole (V) formation and degenerated digestive cells (DDC). Snails exposed to LC_{25} (8) showing deformation in secretory cells (thin arrows) and disintegration in digestive cells (thick arrow)

Discussion

Pesticides are toxic to the living organism at the particular dose and few studies on pesticide formulation mixture were done. In the present study, the half lethal concentration (LC_{50}) of the El Bacha 47 EC (30 g/l lambda-cyhalothrin and 17 g/l acetamiprid) on *B. alexandrina* was 7.9 mg/l after 24 h. This could be attributed to the active constituents (Abdel-Ghaffar et al. 2016) and its mode of action on the target organ (Etges and Gilbertson 1966). Specifically, lambda-cyhalothrin as synthetic pyrethroid insecticide penetrates the organism tissues, disrupting nerve conduction which leads to cessation of feeding, loss of muscular control, paralysis, and eventual death (He et al. 2008), while acetamiprid is one of neonicotinoids insecticide that reduces feeding rates,

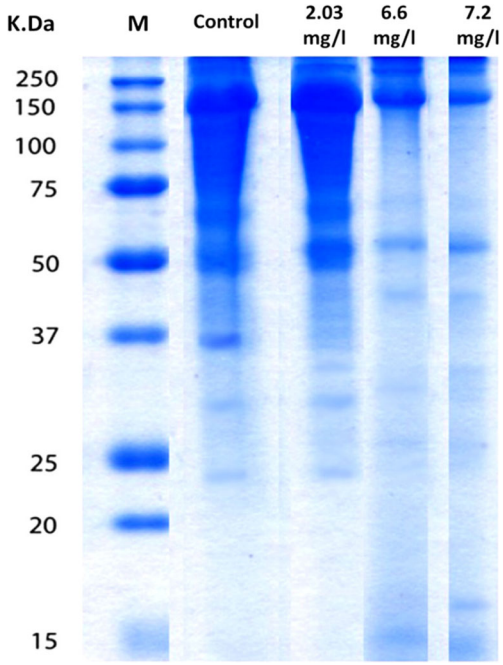

Fig. 4 SDS-PAGE proteins patterns of *Biomphalaria alexandrina* snails' tissue exposed to sublethal concentrations of El Bacha 47 EC after 2 weeks of exposure. M marker

movement, fecundity, developmental rates, and growth in aquatic insects (Colombo et al. 2013).

As regards to the development of *B. alexandrina* embryos, all treated egg groups were less in hatchability percent than that of the control group. In addition, the hatchability percent of 3-day-aged eggs was higher than 1-day-aged after exposed to LC_{10} and LC_{25} concentrations; these results may be attributed to fetal malformations that were observed in early eggs that suffered from delay in growth and degenerated embryos. These results are in agreement with Hasheesh et al. (2011), who stated that the early-aged eggs were more sensitive to all concentrations of difenoconazole fungicide than the older eggs and attributed that to fungicide constituents passing through the egg wall, causing malformation in developmental stages of embryo or mix with yolk layer changing its constituent leading to forbid the embryos from developing or hatching, while at older stage, the embryo is already completed and became able to hatch even with a little percent. Seidenglanz et al. (2011) stated that the pyrethroids (lambda-cyhalothrin, alpha-cypermethrin) and neonicotinoids (acetamiprid, thiacloprid) showed some ovicidal and larvicidal effects, where they significantly decreased survival rates of *Bruchus pisorum* L. (Coleoptera: Chrysomelidae) eggs.

Table 2 Electrophoretic separation of *B. alexandrina* tissue soluble protein groups exposed to sublethal concentrations of El Bacha 47 EC for 2 weeks of exposure

Bands	Marker		Control		LC_0 (2.03 mg/l)		LC_{10} (6.6 mg/l)		LC_{25} (7.2 mg/l)	
	kDa	%	kDa	%	kDa	%	kDa	%	kDa	%
1	250	2.0								
2	150	1.6								
3					142.93	41.9			142.93	30.4
4			140.24	34.8						
5							138.25	38.8		
6	100	1.9								
7							85.35	4.7		
8			83.74	8.1	83.74	7.3				
9	75	4.8					70.57	20.7		
10									69.24	21.4
11					68.42	33.4				
12			65.40	29.7						
13									57.39	6.8
14	50	5.2					55.64	8.2		
15			45.79	14.8						
16					39.80	2.2			39.05	12.2
17							35.34	7.5		
18					34.03	6.6				
19	37	3.1	33.62	6.1						
20							27.87	4.4	27.87	12.1
21	25	5.6	24.69	6.5	24.57	6.1				
22	20	3.2								
23	15	2.0							13.50	7.4
24							11.54	12.9		
25									10.98	8.3
No. of bands	9		6		6		7		7	
Similarity index					0.45		0.24		0.24	

Table 3 SDS-PAGE analysis of *B. alexandrina* tissue soluble proteins groups exposed to sublethal concentrations of El Bacha 47 EC for 2 weeks of exposure

Bands	Molecular weight (kDa)	Control	LC_0 (2.03 mg/l)	LC_{10} (6.6 mg/l)	LC_{25} (7.2 mg/l)	Total	Polymorphism
1	142.93	0	1	0	1	2	Polymorphic
2	140.24	1	0	0	0	1	Unique
3	138.25	0	0	1	0	1	Unique
4	85.35	0	0	1	0	1	Unique
5	83.74	1	1	0	0	2	Polymorphic
6	70.57	0	0	1	0	1	Unique
7	69.24	0	0	0	1	1	Unique
8	68.42	0	1	0	0	1	Unique
9	65.40	1	0	0	0	1	Unique
10	57.39	0	0	0	1	1	Unique
11	55.64	0	0	1	0	1	Unique
12	45.79	1	0	0	0	1	Unique
13	39.80	0	1	0	1	2	Polymorphic
14	35.34	0	0	1	0	1	Unique
15	34.03	0	1	0	0	1	Unique
16	33.62	1	0	0	0	1	Unique
17	27.87	0	0	1	1	2	Polymorphic
18	24.69	1	1	0	0	2	Polymorphic
19	13.50	0	0	0	1	1	Unique
20	11.54	0	0	1	0	1	Unique
21	10.98	0	0	0	1	1	Unique
Total		6	6	7	7	26	

The present study demonstrated that histopathological changes of the digestive and hermaphrodite glands and the extent of damages increased with increasing the concentrations, where the highest deleterious effects were in the digestive gland of snail groups that were exposed to LC_{25} (7.2 mg/l), which was represented by deformation in the secretory cells, rupture of connective tissue between tubules, and disintegration in the digestive cells. These findings are in agreement with Sharaf et al. (2015) who revealed severe histopathological alterations in the digestive gland of *Helicela vestalis* after exposure to sublethal concentrations (LC_{25}) of both methiocarb and chlorpyrifos, and these alterations included severe tubular disruption, vacuolation and necrosis of digestive tubules. Also, Abdel-Ghaffar et al. (2016) reported the same observations in the digestive gland of *B. alexandrina* post 2 weeks of exposure to LC_{25} of glyphosate isopropylammonium, butralin, and pendimethalin herbicides where the tips of digestive cells ruptured, and most of the cells were degenerated. Vacuoles and lumens inside tubule increased, and the secretory cells became denser in color and increased in number, and the connective tissue between digestive tubules shrank.

In the present work, the histological examination of the hermaphrodite gland in treated snails showed marked morphological changes in both male and female gonadal cells. Most of the spermatogenesis stages were lysis and spermatocytes became scattered. In addition, mature ova and secondary oocytes suffered from disintegration and deformation. These results agree with Osman et al. (2008) who found that exposure of *B. alexandrina* snails to sublethal concentrations of the herbicides Roundup and/or Topik showed complete destruction of gametogenic cells. This also agrees with Mossalem et al. (2013) who studied the molluscicidal properties of the antihelminthic plant derivative, dihydro-artemisinin methyl ether (artemether) against some histological and histochemical parameters of *B. alexandrina* snails, and stated that there is a complete destruction of gametogenic cells and severe damage of hermaphrodite gland tissues, especially when the exposure period increased. These results coincide with the findings by Abdel-Ghaffar et al. (2016), where the histological examination of the hermaphrodite gland in treated snails with glyphosate isopropylammonium and pendimethalin herbicides showed loss of connective tissue, irregular sperm, and mature ova appeared dense and irregular in shape and some are degenerated.

As regards SDS-PAGE patterns of tissue soluble protein extract of *B. alexandrina* snails, it was found that the exposed snails showed various effects on the synthesis of protein which yielded a complex pattern of polypeptides after 2 weeks

of the exposure period. The highest number of protein bands was observed in snail groups that subjected to LC_{10} and LC_{25} concentrations of the tested insecticide; therefore, the similarity index was 0.24 with the control group. Meanwhile, the highest similarity index was 0.45 between control and snail groups that were exposed to LC_0 (2.03 mg/l) concentration. These results confirm the former observations of the highest deleterious effects on both digestive and hermaphrodite tissues when snails were exposed to LC_{10} and LC_{25} concentrations. Also, this report was previously recorded by Bakry et al. (2011) who found that deltamethrin and malathion had qualitative and quantitative effect on the protein patterns of *Helisoma duryi* snails. Also, (Bakry et al. 2016) stated that *B. alexandrina* snails subjected to LC_{10} of diazinon (1.9 ppm) and profenfos (0.75 ppm) showed differences in number and molecular weights of protein bands than the control group. The fractionation of native proteins into bands were different from that of the control may be attributed to changes occurring in the DNA of the treated snails (El-Sayed 2006) or due to production of additional proteins, or to disturbance of polypeptide metabolism (El-Deeb et al. 2015).

In conclusion, the present insecticide mixture had a remarkable effect on protein synthesis in digestive and hermaphrodite tissues, as well as important for the development of gametogenesis stages and development of embryos. Therefore, studying the effects of pesticide mixtures, it is important to appreciate the interactions that exist when chemicals are mixed. An understanding of such interactions will provide an insight into the overall effects of pesticides on aquatic biota.

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Compliance with ethical standards

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.

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

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