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Biochemical and Histological Alterations in the Digestive Gland of the Land Snail *Helicella vestalis* (Locard, 1882) Exposed to Methiocarb and Chlorpyrifos in the Laboratory

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Abstract: ***This study investigated the impact of two pesticides namely: Methiocarb and Chlorpyrifos against the biochemical and histological aspects of the helicid land snail, Helicella vestalis at Sharkia Governorate, Egypt. The activities of three vital enzymes, total protein (TP) and total lipid (TL) were laboratory tested. These enzymes were Aspartate amino transaminase (AST), Alanine amino transaminase (ALT) and Alkaline phosphatase (ALP). Results showed that all tested pesticides lead to increase the activity of AST, ALT and ALP in the tissue homogenate of the digestive gland of the land snail Helicella vestalis. On the other hand, the levels of total protein and total lipid were increased after treatment with all tested pesticides. In general, the two pesticides were significantly affected on the activities of enzymes, total lipid and total protein compared with control when applied against the tested snails. Meanwhile, many histological changes were observed in the digestive gland of H. vestalis after exposure to sublethal concentrations of both Methiocarb and Chlorpyrifos. These alterations included severe tubular disruption, vaculation, nuclear pyknosis and necrosis of tubules. Moreover, this study revealed that Chlorpyrifos was much more toxic to the tested snail than Methiocarb.***

Keywords: (ALP), (ALT), (AST), (TL), (TP), Chlorpyrifos, Digestive gland, *Helicella vestalis*, Methiocarb.

1. Introduction

Many growers of fruit trees, vegetables and ornamental plants as well as, most of the home gardeners in Delta region, complain of certain land snails. These animals have been considered as serious pests of major economic importance in Egypt, being incriminated in considerable damage and great losses in various vegetations [1-6]. The land snail, *Helicella vestalis* is a dangerous agricultural pest to several vegetations including orchard trees, vegetable crops and ornamental plants causing damage to all plant parts [2, 7]. It is well known that successful control methods of terrestrial molluscs depend greatly on the broad base of knowledge of their biological and ecological aspects [6, 8, 9]. Therefore, it warrants detailed investigations aiming to provide as much information as possible that could be of some value in the control of these harmful organisms. [10] reported that the optimum period for good control of three land snails, *Monacha cartusiana* , *Theba pisana* , and *Eobania vermicula*ta were in Autumn months where the snails were active and the population was very low. The control of land snails on different crops is heavily dependent on the use of pesticides that limit the effect of these pests below damage level. Hence, the synthetic pesticides are the most effective means available at present for the control of terrestrial gastropods [12-17]. Bait formulation of molluscicides was the most effective applied method in the field for controlling terrestrial gastropods rather than any other technique. The effect of Methomyl and Copper Sulphate on *Eobania vermiculta* and *Helicella vestali*s snails under laboratory and field conditions was evaluated at Sharkia Governorate. Results under laboratory conditions revealed that, the mortality percentages increased with increasing the concentration values and the duration of exposure [19].

Carbamates are known to act as nervous toxins by inhibition of cholinesterase. On the other hand, metaldehyde pesticides cause an excessive increase of fluid excretion in the soft snail body, leading to snail death [20]. Both carbamate and organophosphates pesticides are successfully used in Egypt and many other countries for controlling land snails [12]. Transaminase enzymes , acetylcholine esterase , total proteins and total lipids are important biological indicators for the tested land snails [14].

Methiocarb is a carbamate pesticide which is used as a bird repellent, insecticide, acaricide and molluscicide since the 1960s. It acts as contact and stomach poison on mites and has neurotoxic effects on molluscs [21]. When Methiocarb is applied to rats at a dose of 50 ppm, it gives a reduction of brain cholinesterase by 14% and 5% in males and females, respectively [22]. Acute exposure to Chlorpyrifos can be toxic to bees, with an oral LD50 of 360 ng/bee and a contact LD50 of 70 ng/bee [23]. Chlorpyrifos is highly toxic to freshwater fish and aquatic invertebrates. Cholinesterase inhibition was observed in acute toxicity tests of fish exposed to very low concentrations of this insecticide [24]. The toxic action of methomyl and methiocab on *Eupania* digestive gland is evaluated [25], methomyl was more toxic than Methiocarb and the digestive gland tissues suffered from hemocytes infiltration, bizarre nuclei ranged from karyolysis to severe karyorrhexis and complete pyknosis. The effect of Thiometoxam on the histology and total carbohydrates, proteins and lipids of the hepatopancreas of *Helix aspersa* is studied [26]; they found a significant decrease in total proteins, lipids, and carbohydrates as well as degeneration of the digestive tubules and breakdown of the basement membrane.

This research was planned to determine the biochemical effects of two pesticides namely, Methiocarb and

Chlorpyrifos on the land snail *H. vestalis* on the activities of three vital enzymes. Also, Total Proteins (TP) and Total Lipids (TL) were measured to spot a light on the toxicity of such chemicals. The enzymes measured in this study were; Alanine amino transaminase (ALT), aspartate amino transaminase (AST), and Alkaline phosphatase (ALP). Besides, the effect of sublethal concentrations of the two pesticides on the histology of the digestive gland using light microscopy has been studied.

2. Material and Methods

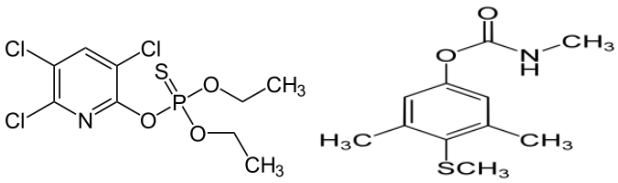
2.1 Tested Snails

Adult land snails *Helicella vestalis* were collected from infested fields in Belbais City, Sharkia Governorate, Egypt, during the spring of 2014. The collected snails were transferred to the laboratory, and then reared in plastic containers (40x30x30cm) with soil base (10 snails/ jar) and fed on lettuce for two weeks to be acclimatized.

2.2 Tested Chemicals

Two pesticides belonging to two different groups namely, Methiocarb 3,5-Dimethyl-4-(methylthio)phenyl methylcarbamate (carbamate) and Chlorpyrifos *O*,*O*-Diethyl *O*-3,5,6-trichloropyridin-2-yl phosphorothioate (organophosphate), were chosen to examine their toxic effects on this serious land snail.

Chlorpyrifos Methiocarb



2.3 Tested Solution

Serial concentrations of the two chemicals were prepared from stock solution by diluting with distilled water. These concentrations were 0.01, 0.02, 0.04 ppm for Chlorpyrifos and 10, 12, 14 ppm for Methiocarb. Three replicates per each concentration were used to determine the LC_{50}. Ten snails of the same size were placed in each glass container. An equal number was left without treatment as a check control. Experiments were checked at 24 h intervals up to 96 hr. The dead snails were counted and recorded. LC_{50} was determined according to Finney [27] by the graphic method of the curve dose-effect, using the probit analysis. In the long –term exposure, half of the 96hrs LC_{50} of the tested pesticides was used and redosed every 4 days in astatic renewal manner. Living animals, surviving the effect of the tested pesticides were sacrificed after 7 days of exposure.

2.4 Biochemical Measurements

Snails digestive glands were dissected out and homogenized in distilled water (50 mg mL^{-1}). The homogenates were centrifuged at 8000 rpm for 15 min at 5°C in refrigerated centrifuge. The deposits were discarded and the supernatants were kept in a deep freezer till use to determine the activities of Alkaline phosphatase (ALP), Alanine amino transaminase (ALT), Aspartate amino transaminase (AST) enzymes and Total lipids (TL), Total proteins (TP).

The activity of AST and ALT was determined according to [28]. While, Alkaline phosphatase (ALP) was determined according to [29]. The Total proteins (TP) were calorimetrically determined according to [30] while total lipids (TL) were assayed by the method of [31].

2.5 Histological Studies

The digestive gland of *H. vestalis* snails was dissected and fixed in Bouin's fluid. Paraffin sections of 5*u*m thickness were deparaffinized, hydrated and then stained in Ehrlich' hematoxylin for 15 minute, washed rapidly in water then counter stained with 1% eosin solution for 2 minutes. Stained sections were dehydrated in alcohol and cleared in xylene, mounted on clean microscope glass slides with Canada balsam and covered by thin cover slides.

2.6 Statistical Analysis

Data were calculated as Mean ±SD and analyzed using analysis of variance (ANOVA) followed by Least Significant Difference (LSD) to check significance among the means. Probability of 0.05 or less was considered significant. All statistical analysis was done with [32] .

3. Results

3.1 Determination of the LC50 and LC25

The LC50 and LC25 values of Chlorpyrifos and Methiocarb were presented in Table (1).

3.2 Biochemical studies

The biochemical effects of three concentrations of the two pesticides on the activity of AST,ALT,ALP enzymes were presented in Table (2) and illustrated in Fig.(1). Also, total proteins (TP) and total lipids (TL) were recorded in Table (3) and illustrated in Fig. (2).

3.3 Activity of Aspartate amino transaminase (AST)

The obtained data showed that the two tested pesticides increased the level of (AST) except at 0.01ppm of Chlorpyrifos which decreases the level of AST enzyme when applied against *H. vestalis*. Data also illustrate that there was no significant increase in case of Methiocarb at 10ppm and 12 ppm in the activity of AST more than control. Methiocarb at 14ppm caused the highest increase in the activity of (AST). On the other hand, 0.02ppm and 0.04ppm Chlorpyrifos increased the level of (AST). While Chlorpyrifos at 0.01ppm decreased the level of AST Table (2) Fig. (1).

3.4 Activity of Alanine amino transaminase (ALT)

The data obtained showed that the two tested pesticides increase the level of (ALT) except at 0.01ppm of Chlorpyrifos which decreased level of ALT enzyme when applied against *H. vestalis.* Data also illustrated that there were no significant increase in case of Methiocarb at 10ppm and 12 ppm in the activity of ALT respectively. Methiocarb at 14 ppm caused the highest increase in the activity of (ALT). On the other hand, 0.02 ppm and 0.04 ppm Chlorpyrifos increased the level of (ALT) while, 0.01ppm Chlorpyrifos decreased the level of ALT Table (2) , Fig.(1).

3.5 Activity of Alkaline phosphatase (ALP)

ALP activity was increased in the land snail after treatment with all tested pesticides concentrations. There were significant differences between all treatments and control. Methiocarb at 12ppm and 14ppm caused the highest increase in ALP activity respectively. Methiocarb at 10ppm caused the lowest increase in the level of this enzyme in comparison with control. ALP activity increased significantly in the land snail after exposure to all molluscicides concentrations. Chlorpyrifos at 0.04ppm and 0.02 caused the highest increase in ALP activity. Chlorpyrifos 0.01ppm showed the lowest increase in the level of this enzyme compared to the control Table (2) and Fig. (1).

3.6 Total lipids

The tested pesticides concentrations increased the level of total lipids in land snails and there were insignificant differences between all treatments and control except at 14 ppm Methiocarb that caused highest increase in total lipids followed by 12 ppm and 10ppm. Chlorpyrifos at 0.02 ppm and 0.04ppm increased the level of total lipids when applied against the snail. Chlorpyrifos at 0.01ppm decreased the level of total lipids fewer than control Table (3) and Fig. (2).

3.7 Total proteins

Methiocarb at 12ppm and 14ppm increased the level of total proteins when applied against the snail. Methiocarb at 10ppm decreases the level of total proteins fewer than control. Chlorpyrifos at 0.02ppm and 0.04ppm caused an increase in the level of total proteins. On the other hand, Chlorpyrifos at 0.01ppm decreased the level of total proteins fewer than control Table (3) and Fig. (2).

3.8 Histopathological studies

3.8.1 Histology of untreated digestive gland

The digestive gland of normally feeding untreated *H. vestalis* consists mainly of digestive tubules (DT) separated by intertubular connective tissue (CT) containing hemolymphatic sinuses and hemocytes (HE). Each tubules surrounded by circular muscle layer (ML). Three different cell types are observed in the epithelium lining the digestive gland tubules Fig. (3). The cells are differentiated into, digestive cells (DC), calcium cells (CC) and excretory cells (EC).

3.8.2 Digestive cells

Digestive cells constitute the most abundant cellular component of the digestive gland tubular epithelium. Digestive cells are simple columnar epithelium. The basally located nuclei (N) of digestive cells are rounded or oval.

3.8.3 Calcium cells

Calcium cells are fewer than digestive cells, occur either singly or in groups in the corners of the tubules. They have pyramidal shape with pointed distal end. Calcium cells possess apical excretory granules (EC) and large rounded nuclei (Figs. 4 & 5).

3.8.4 Excretory cells

Excretory cells have a rounded shape. They are characterized by the presence of a single large vacuole (V) Figs. (4 & 5).

3.8.5 After treatment with LC25 of Methiocarb

Few tubules with severe atrophy were mingled in some places. Hemocytic infiltration (HI) was frequently observed and the basement membrane of tubules appeared ruptured. The digestive cells show accumulation of large numbers of dark granules (DG) and appear to undergo extensive breakdown into membrane bound vesicles. Calcium cells were packed with enlarged calcium spherules and exhibited pyknotic nuclei (PN). The cytoplasm of most calcium cells was replaced by large vacuoles containing darkly stained granules. Excretory cells showed increased number of excretory granules with cellular debris and necrotic areas (NA). (Figs.6, 7 and 8).

3.8.6 After treatment with LC25 of Chlorpyrifos

Destruction of most tubule was evident, the basement membrane and the tubular connective tissue (BM) showed severe structural disruption. Digestive cell cytoplasm appeared to be nearly devoid of cytoplasmic granules and there was a marked reduction in number of calcium cells and the size of calcium spherules. In addition, calcium cells contained an increased deposition of calcified dark granules (DG) with vacuolated cytoplasm (V) and karyolitic nuclei. Most excretory vacuoles of excretory cells appear with highly decomposed cellular elements Figs. (9, 10 and 11).

4. Discussion

The present study revealed that both Methiocab and Chlorpyrifos caused an increase in the activities of AST, ALP and ALT in the land snail, *H. vestalis.* The transaminases enzymes; AST, ALP and ALT were not solely located in hepatocytes but rather are found in many body organs. Also, the increase in their activities could be due to a variety of conditions including muscle damage, intestinal and hepatic injury and toxic hepatitis [28]. On the other hand, the decrease in activities of AST and ALT might be due to either leakage of enzymes into extracellular compartments or due to actual enzymes inhibition by these molluscicides. Thus, the deviation of both enzymes activities out of the normal range could lead to biochemical impairment, lesions of the tissues and cellular functions [33]. Accordingly, the elevations or reductions in the activities of AST and ALT enzymes in tissues of both E. *vermiculata* and *M. cantiana* treated with molluscicides

might be partially due to cell injury of their different organs and this might lead to disturbances in their enzymatic systems [12, 34, 35]. These results are supported by the findings of [33] who's found that carbamate compounds led to significant elevation of the activity of AST and ALT when applied against the land snail, *Theba pisana*.

The present data indicated that the two tested compounds increased the level of ALP in H. vestalis. These compounds presented an increase of total lipids (TL) and total proteins (TP) in the digestive gland. The current results are coincide with the findings of [14] who observed that Niclosamide increased the level of total lipids and total proteins more than control after 24, 48, 72 and 96h of exposure when applied against the land snail, E. vermiculata. Chlorpyrifos at 1% decreased the level of total proteins and total lipids less than control. Methiocarb 1% decreases the level of total proteins.

The decrease in the level of both TP and TL might be partly resulted from imbalance between the rate of synthesis and the rate of degradation. [9] reported that the depression in total lipids might be due to decline in lipid synthesizing capacity or to an increase in the hydrolysis of hepatic lipids to combat the stress conditions. The harmful effect of chemical compounds could be attributed to enhancement of energy utilization or destruction of cells organelles of treated snails that may lead to inhibition of protein synthesis [36].

Also, the present results proved that these chemicals caused alterations in some biochemical elements which could lead to serious metabolic and cellular damage. In general, the two molluscicides adversely affected the activities of three vital enzymes, total lipids and total proteins when applied against the tested land snail. However further, studies are needed to clearly elucidate the most effective mode of action of these chemical compounds on such land snails.

The present study revealed also many histological changes in the digestive gland of *H.vestalis* after exposure to sublethal concentrations (LC25) of both Methiocarb and Chlorpyrifos. These alterations included severe tubular disruption, vaculation; pyknotic nuclei and necrosis of digestive tubules .These findings are in agreement with [25] who found that Methomyl was more toxic than Methiocarb on some land snails. They also reported that their digestive gland tissues suffered from hemocytes infiltration, bizarre nuclei ranged from karyolysis to severe karyorrhexis and complete pyknosis. These results are in agreement also with [26] who studied the effect of Thiometoxam on the histology and total carbohydrates, proteins and lipids of the hepatopancreas of *Helix aspersa.* They found a significant decrease in total proteins, lipids, and carbohydrates as well as degeneration of the digestive tubules and breakdown of the basement membrane.

The present histopathological and biochemical results revealed that Chlorpyrifos has much more toxic effects than Methiocarb on the land snail *H. vestalis*.

5. Competing Interests

The authors declare that they have no competing interests.

6. Authors' Contributions

Hesham M. Sharaf , Mohamed A. Salama and Mahmoud S. Abd El-Atti are the main investigators, performed all experimental work and paper writing, carried out data analysis the authors read and approved the final manuscript.

7. Acknowledgement

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Appendix

Table 1: LC50 and LC25 Chlorpyrifos and Methiocarb Pesticides with their exposure time.

Pesticides \ Exposure period	96-hrs	7-days
	LC_{50}	LC_{25}
Chlorpyrifos	0.010	0.005
Methiocarb	8.8	4.4

Table 2: Biochemical components (means ± S.D) of the digestive gland of the land snail, *H. vestalis*, exposed to sublethal concentrations of Methiocarb and Chlorpyrifos:

Character \ Treatment		AST (U x 10^3/ mg)	ALT (U x 10 3/ mg)	ALP (U x 10^3/ mg)
Control		202.69±13.18	144.89 ^{a} ± 5.88	55.16 ^{a} ± 1.69
Methiocarb	10pmm	216.39 ± 27.56	168.70 ^{d} ± 12.00	61.06 ^{d} ± 2.96
	12ppm	270.36 ± 1.43	242.08 ^{c} ± 4.81	94.63 ^{c} ± 1.74
	14ppm	424.04 ± 50.13	283.11 ^{b} ± 8.95	108.42 ^{b}± 12.83
Chlorpyrifos	0.01ppm	159.19 ± 9.33	139.14 ± 4.17	74.29 ± 0.31
	0.02ppm	236.38 ± 104.04	189.73 ± 2.49	81.23 ± 5.76
	0.04ppm	275.00 ± 28.07	241.39 ± 19.39	118.94 ± 3.00

Different superscripts differ from each other significantly at P < 0.05).
AST = Aspartate aminotransferase, ALT = Alanine aminotransferase, ALP = Alkaline phosphatase.
Values followed by the same letter (s) are not significantly different at the 0.05 level according to Duncan's test.

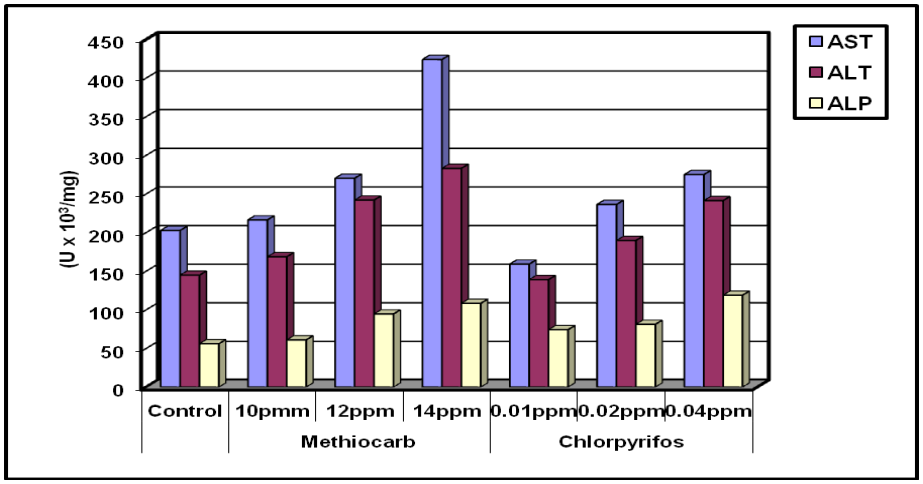


Figure 1: AST, ALT and ALP values in the digestive gland of *H. vestalis* exposed to sublethal concentrations of Methiocarb and Chlorpyrifos.

Table 3: Biochemical components (means ± S.D) of the digestive gland of the land snail, *H. vestalis*, exposed to sublethal concentrations of Methiocarb and Chlorpyrifos:

Character \ Treatment		TL (U x 10^3/ mg)	TP (U x 10^3/ mg)
Control		19.0 ± 0.33	1.03 ± 0.08
Methiocarb	10ppm	19.08 ± 0.63	0.88 ± 0.08
	12ppm	19.46 ± 0.31	1.08 ± 0.02
	14ppm	20.64 ± o.14	1.29 ± 0.34
Chlorpyrifos	0.01ppm	17.08 ± 0.75	0.97 ± 0.04
	0.02ppm	19.96 ± 0.34	1.47 ± 0.08
	0.04ppm	21.35 ± 0.59	1.53 ± 0.29

TL = Total lipids and TP = Total proteins, Total proteins (T.P): (mg/ snail).
Values followed by the same letter (s) are not significantly different at the 0.05 level according to Duncan's test.

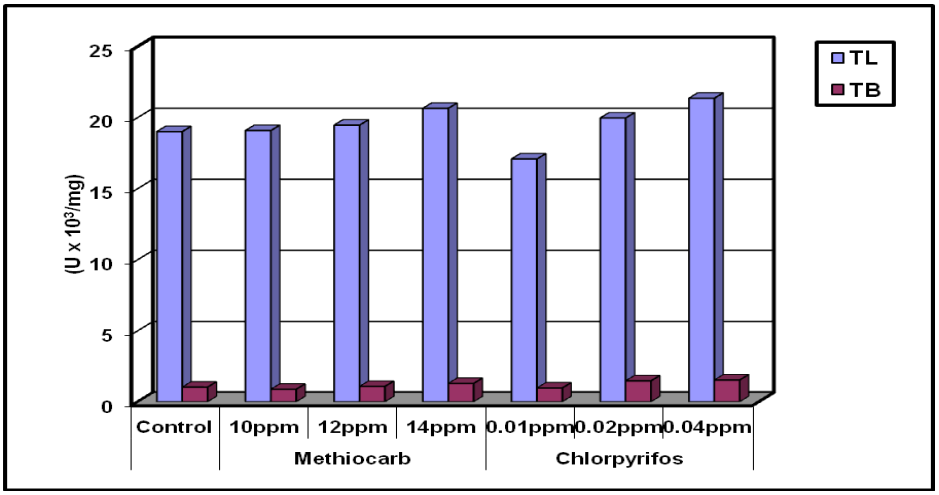


Figure 2: Total proteins and Total lipids values in the digestive gland of *H. vestalis* exposed to sublethal concentrations of Methiocarb and Chlorpyrifos.

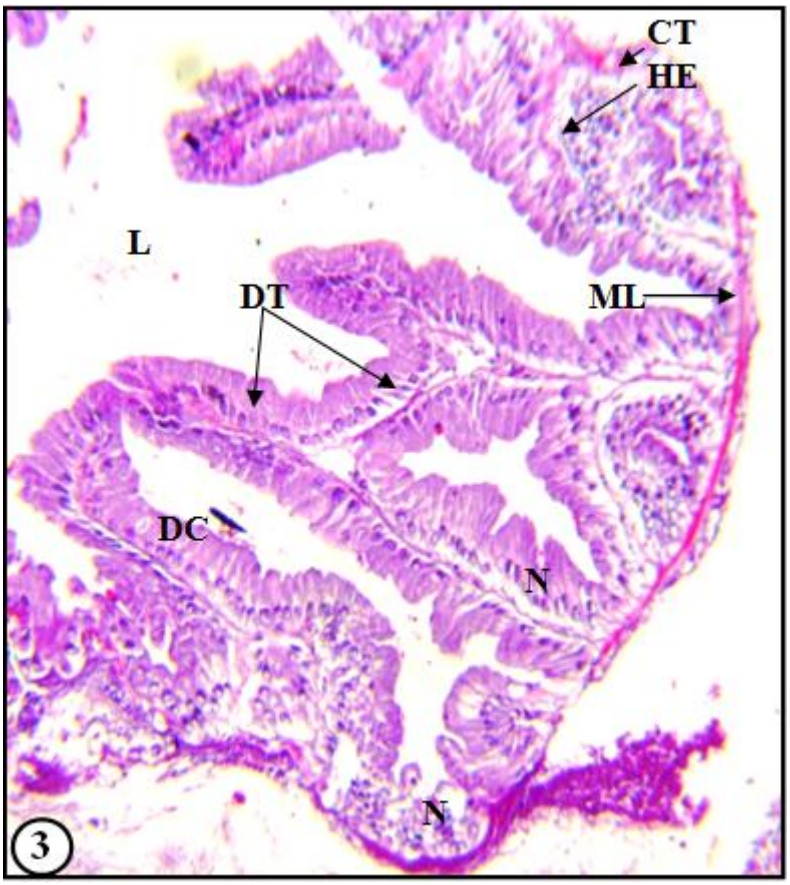


Figure 3: Photomicrograph for T.S of untreated digestive gland of *Helicella vestalis* showing: Digestive tubules DT, Digestive cell DC, Excretory cell EC, Intertubular connective tissue CT, Hemocytes HE, Lumen L, Muscle layer ML and Nucleus N. H&E. X20

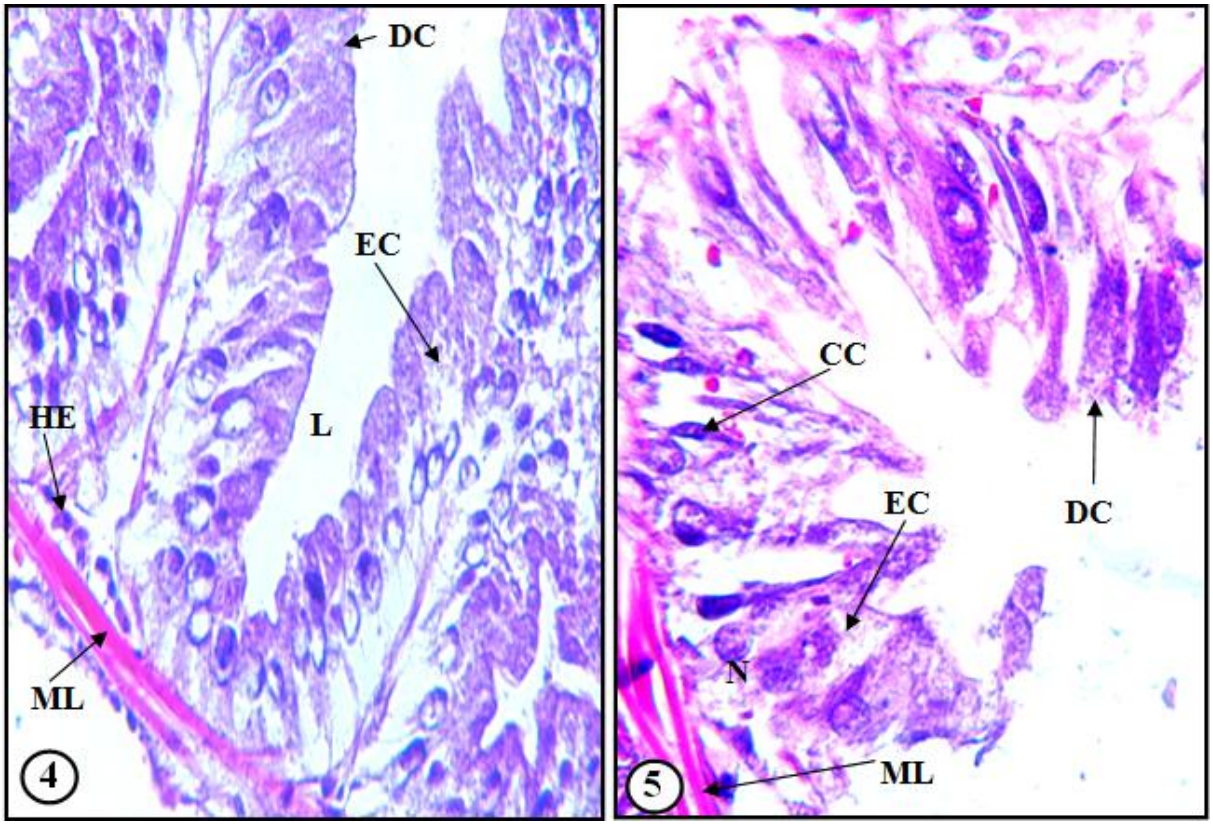
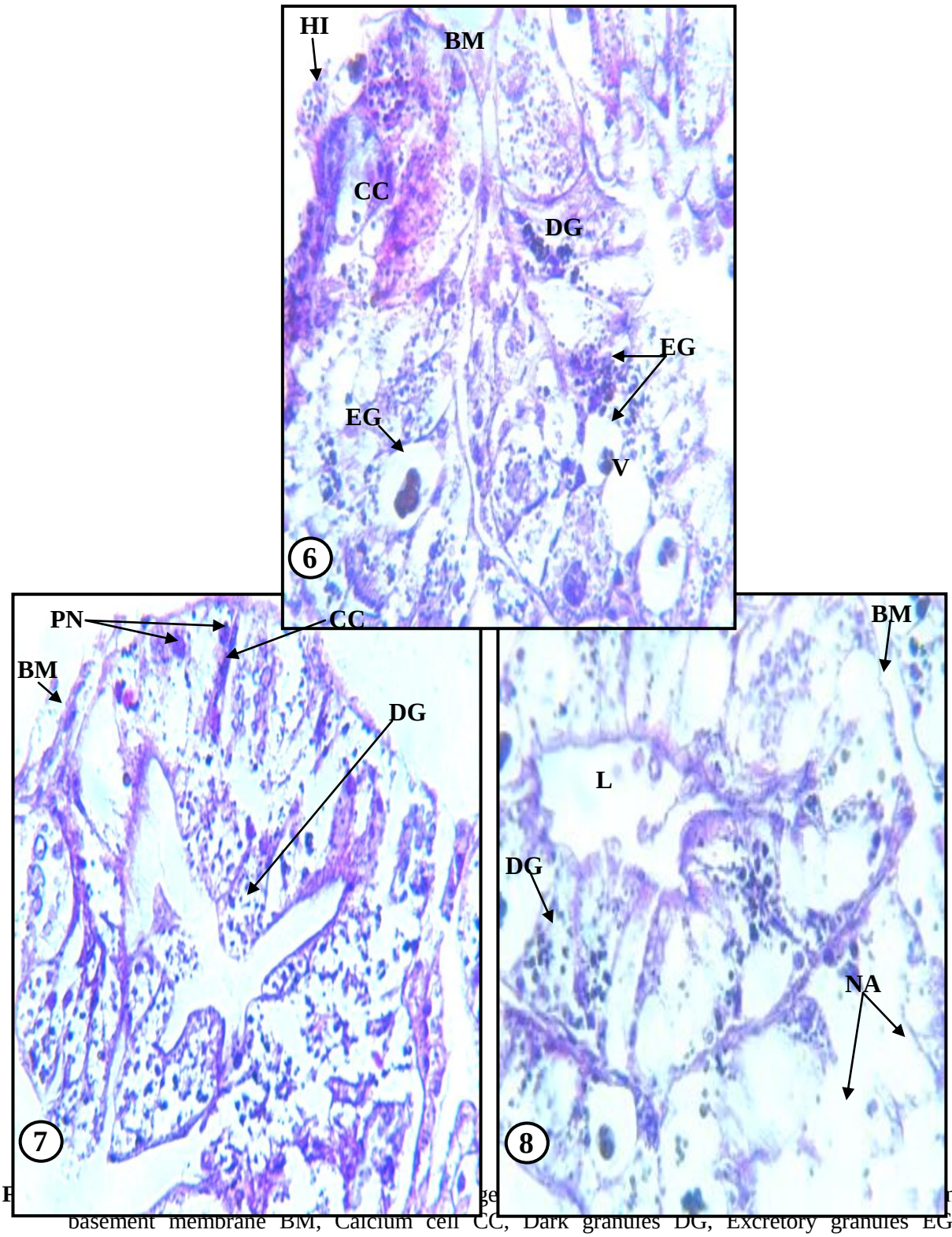
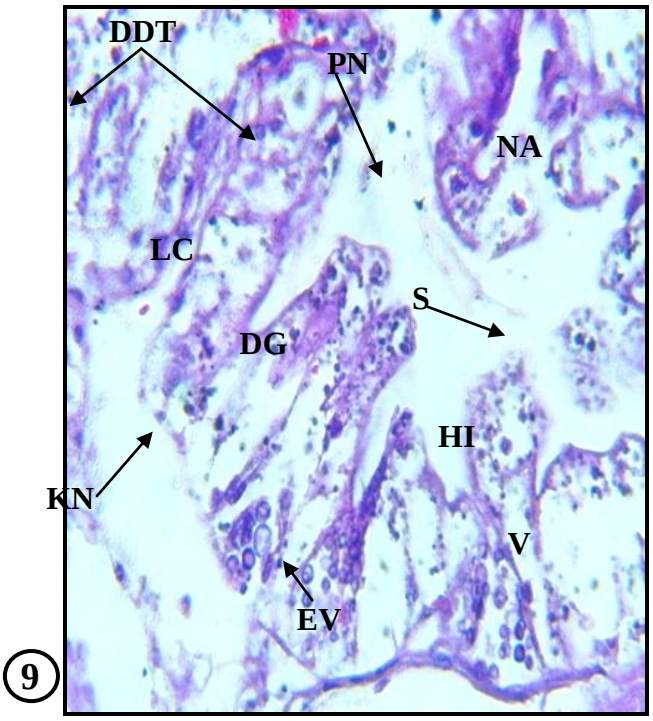


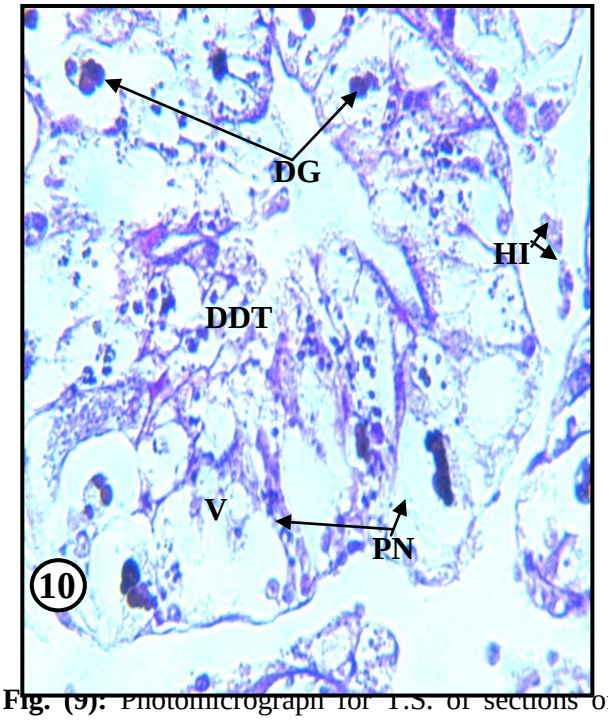
Figure 4 and 5: Higher magnification for T.S. of the digestive gland showing: Digestive cell DC, Excretory cell EC, Calcium cell CC, Hemocytes HE, Lumen L, Muscle layer ML, vacuoles V and Nucleus N. H&E X40



F ge ng: Ruptured basement membrane BM, Calcium cell CC, Dark granules DG, Excretory granules EG, Hemocyte infiltration HI. H&E. X20

Figs. (7 and 8): Higher magnification for T.S. of the digestive gland treated with Lc25 Methiocarb showing: Ruptured basement membrane BM, Necrotic areas NA, Pyknotic nuclei PN, Calcium cell CC, Dark granules DG, Hemocyte infiltration HI. H&E X40





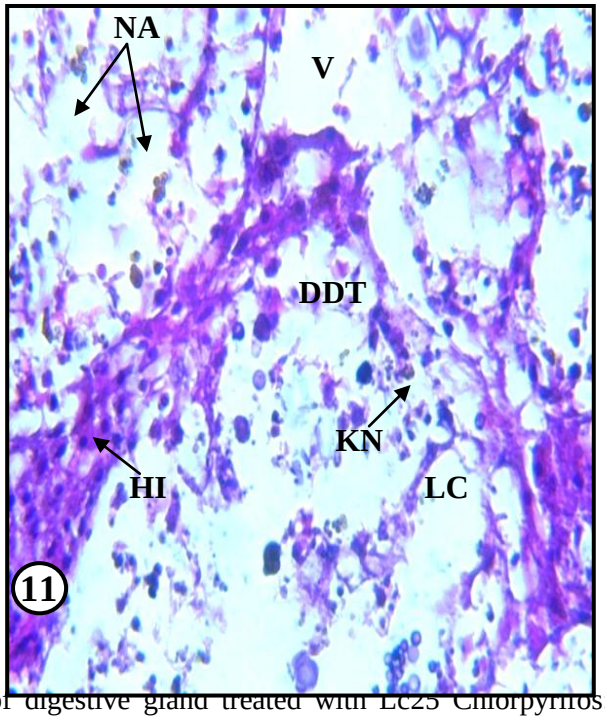


Fig. (9): Photomicrograph for T.S. of sections of digestive gland treated with Lc25 Chlorpyrifos: Destructed digestive tubules DDT, Dark granules DG, Excretory vesicles EV, Hemocyte infiltration HI, Karyolytic nuclei KN, Lytic cells LC, Necrotic areas NA, Pyknotic nuclei PN, secretions S, Vaculation V. H&E. X20

Figs. (10 and 11): Higher magnification for T.S. of the digestive gland treated with Lc25 Chlorpyrifos showing: Destructed digestive tubules DDT, Hemocyte infiltration HI, Karyolytic nuclei KN, Lytic cells LC, Necrotic areas NA, Pyknotic nuclei PN, secretions S, Vaculation V. H&E. X40.