Effect of Chlorpyrifos and Neem seed extract (Azadirechtein) on hepatopancreatic cellular structures of the freshwater crayfish *Procambarus clarkii*

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ABSTRACT

Histological and ultrastructural studies on the normal structures of hepatopancreas of *P. clarkii* showed three main cell types, secretory, absorptive and fibrillar cells. The ultrastructural alterations in the hepatopancreas of the red swamp crayfish *P. clarkii* exposed to sublethal concentrations of Chlorprifos and neem seed extract (Azadirechtein) pesticides were studied. LC_{25} of Chlorpyrifos (0.0075 ppm) produced deformation of the apical surface of absorptive cell, dispersedmicrovilli, mitochondrial swelling with ballooned cristae, deformed cisternae of RER, lytic and vacuolated cytoplasm and pyknosis of nuclei. On the other hand, LC_{25} of Azadirechtein (4.99 ppm) caused deformation of mitochondria with the sparce of their cisternae, fragmentation of microvilli, ruptured RER beside the presence of autophagic vacuole containing dark granules and finally bizzar nuclei having segregated nucleoli and irregular clumps of their chromatin. Chlorpyrifos was found to be more toxic than Azadirechtein on *P. clarkii*.

Keywords: *Procambarus clarkii*, Chlorpyrifos, Azadirechtein, hepatopancreas, ultrastructure.

INTRODUCTION

Procambarus clarkii was introduced in the early 1980′s into Egypt after a trial for its aquaculture that eventually failed and some them were thrown into the Nile. *P. clarkii* was greatly spread all over the River Nile, its branches and ditches through the Delta, Cairo and Giza (Ibrahim *et al*., 1995). They were leaved without control and caused a lot of damage to the fisheries of the Nile possibly by eating the eggs and young fish beside damaging the nets of fishermen as well ascausing serious damages to irrigation systems as a result of their burrowing activities (Soliman *et al*., 1998b). Considerable effort has been paid to control its dispersal with pesticides (Lang and Chang, 1967; Hobbs *et al*., 1989).

Chlorpyrifos is an organophosphorous pesticide that is commercially used to control foliar insects that affect agricultural crops and subterranean termites (Rusyniak and Nanagas, 2004; Rao *et al*., 2005). It has been used globally as an insecticide to control pests agriculturally and in the home because of its high insecticidal property, low mammalian toxicity, less persistence and rapid biodegradability in the environment (Tripathi *et al.,* 2000 and Singh *et al.*, 2010). Chlorpyrifoshas a very toxic effecton aquatic invertebrates especially crustaceans (Bharathi & Sandeep, 2005; Li *et al*., 2006). The toxicity of Chlorpyrifos is exerted through the inhibition of acetylcholinestrase (AchE). Repeated exposures to this insecticide, such inhibition may cause damage to aquatic organisms (Kwong, 2002; Barata *et al.* 2004).

Reduction of the environmental problems caused by the use of synthetic chemicals and the growing need for alternative methods of crayfish control that minimize this damage, there has been extensive research on pest control by natural

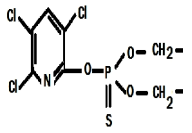
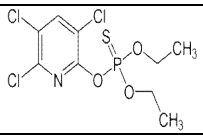
pesticides. Azadirechtein wasone of the most promising natural compounds extracted from theneem tree (*Azadirachtaindica*) that has antiviral, antibacterial and antifungal properties (Isman *et al*., 1990; Harikrishnan *et al*., 2003). It was generally considered to be less harmful to the environment than other more commonly used pesticides (Mordue and Blackwell, 1993). Azadirechtein has low toxicity against non-target organisms (fishes, mammals and beneficial invertebrates) and low persistence in the environment (Schmutterer, 1995; Wan *et al*., 1996; Isman, 1997 and Schaaf *et al.,* 2000). Its use is now considered for arthropod control (Jimenez *et al*., 1998). The hepatopancreas fulfills a key role in the temporary storage of exoskeletal calcium, phosphate, glycogen and lipids during the different phases of the moultcycleand represents a corner stone in the body metabolism (Mercado-Allen, 1991; Nicol *et al*., 1992; Scott-Fordsmand and Depledge, 1997; Wheatly, 1997). It is considered as the most sensitive organ to toxicants and the main site of accumulation and detoxification in crayfish bodies (Neff *et al*., 1976; Lauren & Rice, 1985 and Jaiswal & Sanojini, 1990). The hepatopancreatic tissue was chosen in the present study as a suitable and special model for studying the effect of Chlorpyrifos and Azadirechtein pesticides.

The present study aimed to shed the light on the toxic effect of sublethal concentrations (LC_{25}) of Chlorpyrifos (0.0075ppm) and Azadirechtein (4.99ppm) for 7 days onthe cellular structure of hepatopancreas of *P. clarkii* compared with the control.

MATERIALS AND METHODS

1- Tested pesticides:

Table 1: List of pesticides, their trade names, activeingredients, chemical names and structural formula.

Pesticides	Trade name	Active ingredient	Chemical name	Structural formula
Chlorpyrifos	**Delta killer, Dursban, Lorsban**	**48% Chlorpyrifos**	**O, O-diethyl O-3, 5, 6-trichloro-2-pyridyl phosphorothioate**	
Neemseed extract (Azadirechtein)	**Margosan-O,Nimbecidine, Align, Azatin& Neemix**	**3.5 % Azadirechtein**	**$C_{35}H_{44}O$**	

2- Tested animals and acclimation procedures:

Adults male *P. clarkii* (25-34 gm. average weight and 9-11 cm total length) were collected from Bany Helal irrigation canal, Sharkia Governorate during September 2014. The collected specimens were transported alive to the laboratory in a well-areated large plastic containers (30 x50 x 30 cm).They were adapted for laboratory conditions at a temperature 25^{o}C and a 12:12 h light-dark regime. Water was changed every day and crayfish were fed on carrot.

3- Test solution:

Test solutions of Chlorpyrifos and Azadirechtein were prepared from stock solution which has been prepared by using distalled water as a solvent to give the following concentrations which were obtained by serial dilution of Chlorpyrifos 0.01, 0.015, 0.02, 0.04 ppm and for Azadirechtein 8, 10, 12, 14 ppm.

Three replicates per each concentration were used to determine the LC_{50}. Ten full mature male animals (9-12 cm) were placed in each aquarium. An equal number from males were left without treatment as a check control. Experiments were checked at 24 h intervals up to 96 hr. The dead crayfish were counted and reported. LC_{50} was determined according to Finney (1971) by the graphic method of the curve dose-effect, using the probit analysis. In the long – term exposure; half of the 96hrsLC_{50} of the tested pesticides was used and redosed every 4 days in a static renewal manner. Living animals, surviving the effect of the tested pesticides were sacrificed after 7 days of exposure. During chronic exposure times to the tested pesticides, feeding of animals was stopped.

Table 2: List of pesticides, their exposure time, LC50 and LC_{25}

Exposure period / Pesticides	96-hrs	7-days
	LC_{50}	LC_{25}
Chlorpyrifos	0.015	0.0075
Azadirechtein	9.98	4.99

4- Light microscopy preparations:

A group of *P. clarkii* specimens of control were dissected. The hepatopancreas of the dissected specimens were taken and fixedin 10% formalin for 24 hours and dehydrated through ascending series of ethanol. Tissues were embedded in paraffin wax andsections 4-6 μm thick were cut and stained with hematoxylin and eosinandmounted on a slide using Canda balsam and covered with a glass slip**.**

5- Transmission electron microscopy preparations:

Another group of both control and treated specimens were fixed in 2.5 % glutraldehyde in 0.05 M cocodylate buffer containing 0.15 sucrose at pH 7.2 for 2 hours. Specimens were post fixed in 1% Osmium tetroxide with the same buffer at 0-4^{o}C for one hour and washed again. The specimens were dehydrated in ascending ethanol series and finally embedded in Araldite of Epon. Blocks were sectioned with diamond ultramicrotome knifes. The semi-thin sections were stained with 1% toluidine blue. Ultra-thin sections stained with aqueous urinyl acetate and lead citrate and then examinedusing a Jeol Transmission Electron Microscopic at the regional center for Mycology and Biotechnology in El-Azhar University, Nasr city, Cairo, Egypt.

RESULTS

1- Light microscopy examination of normal hepatopancreas of *P. clarkii*:

The hepatopancreas was formed of numerous digestive tubules which communicate with the lumen of the mid gut and end blindly in the gland itself. The intertubular spaces are formed of connective tissues and blood vessels (Plate I, Fig .a). Each tubule has central lumen andwas formed of three types of cells, absorptive cell (AC), secretory cell (SC) and fibrillar cell (FC). Both secretory and absorptive cells were found to be more abundant than fibrillar cells (Plate I, Fig. b).

The absorptive cells were the most numerous cell types which were dark columnar in shape. The nucleus was centrally or basely located and has an apical brush border. The secretory cell was larger than absorptive cell and has have basely located nucleus (N). A large irregular vacuole (V) was found in its cytoplasm filled with acidophilic material (Plate I, Fig. b). The fibrillar cell was dark and scattered among absorptive and secretory cells but more concentrated among the secretory

cells. A larger basely located nucleus (N) and prominent nucleolus (NU) characterized the fibrillar cell (Fig.1.a and b).

2- TEM examinations of normal hepatopancreas of *P. clarkii*:

The apical surface of absorptive cell was modified for food absorption as a brush border composed of numerous microvilli (MV).A straight filament (F) extends downwards from each microvillus into the apical cytoplasm .The cytoplasm contain a number of irregularly shaped mitochondria (M), which concentrated just below the apical surface of the cell (Plate II, Fig. a). The cytoplasm between the nucleus and the apex of the absorptive cells usually contains very small apical vesicle containing dense material and parallel tubules of rough endoplasmic reticulum (Plate II, Fig .b and c). The cytoplasm of secretory cell contain very thin layer of rough endoplasmic reticulum (RER) found in a perinuclear position and small number of mitochondria (Plate II, Fig .d).

The apical surface of fibrillar cell lacks a brush border and less number of mitochondria than the absorptive cell, the rough endoplasmic reticulum (RER) is massive and fills almost all the cell and the chromatic material (CM) appears granular and scattered throughout the nucleus with a concentration towards the nuclear membrane (NM) (Plate II, Fig .e and f).

3- TEM examinations of hepatopancreas of *P. clarkia exposed to 0.0075 ppm* of Chlorpyrifos:

Plate III showed electron micrographs of hepatopancreas treated with 0.0075ppm Chlorpyrifos and it can be seen that deformed apical surface of absorptive cell with dispersed microvilli (Plate III, Fig a). Some rough endoplasmic reticulum (RER) has the form of short and thin cisternae, was in the form of concentric whorls and other are fragmented, presence of degenerated cellular debris and granules (G) encapsulated within small vesicles (V) in their cytoplasm (Plate III, Fig a). Mitochondria (M) of the absorptive cell were swollen with the sparse of their cisternae (Plate III, Fig b).The cytoplasm of absorptive cells become granular, lost its density and being highly vacuolated (Plate III, Fig c).The nucleus of secretory cells become pyknotic with segregated nucleolus and their chromatin material (CM) appeared as dense masses near the nuclear membraneand their cytoplasm become lytic and fill with many vesicles(Plate III, Fig d).The nucleus of the absorptive cellbecome pyknotic with irregular nuclear envelop and their chromatein materials (CM) appear as electron dense aggregates along the inner nuclear envelop (Plate III, Fig e).

4- TEM examinations of hepatopancreas of *P. clarkia* exposed to 4.99 ppm of Azadirechtein:

Electron micrographs show that microvilli of the absorptive cells are fragmented (Plate IV, Fig .a and b). Mitochondria were bizzar and characterized bythe sparse of its cisternae and lucent matrix (Plate IV, Fig .b). The cytoplasm become granular, losses its density and being highly vacuolated (Plate IV, Fig .a, b and c). It is also noted that the apices of cells contained degenerated cellular debris and granules (G) encapsulated within small vesicles (V) in their cytoplasm, autophagicvacuole containing dark vesicles was noted (Plate IV, Fig .d). The rough endoplasmic reticulum of the fibrillar cell has the form of short, thin cisternae and also has the form of concentric whorls while others were fragmented. The chromatin materials of the nucleus (CM) appeared as dense masses scatter in the nucleoplasm and as irregular clumps near the inner nuclear envelop; the nuclear envelop was ruptured (Plate IV, Fig .e).

DISCUSSION

The hepatopancreas represents the most important organ in the body metabolism of crayfish. It is considered to be the most sensitive organ to pollutants and toxicants and the main site of accumulation and detoxification in crayfish bodies (Neff *et al*., 1976; Lauren &Rice, 1985 and Jaiswal & Sanojini, 1990).

The present study showed clearly that there are three main cell types forming the digestive tubules of *P. clarkii*. These cells were the absorptive, secretory and fibrillar cells. This findings are in agreement with Abdel- Atti (2002) but disagree with Davis & Burnett (1964), Bunt (1968), Stanier *et al*. (1968), Gibson & Barker (1979), Papathanassiou & King (1984), Trinadaha Babu *et al*. (1989) and Zilli *et al*. (2003) who mentioned that hepatopancrase has four types of cells: E-cells (embryonic cells), R-cells (absorptive cells), F-cells (fibrillar cells) and B-cells (secretory cells). R-cells (absorptive cells) are the most abundant cell type and are involved in lipid, glycogen and calcium storage and in nutrient absorption. The F-cells play an important role in the synthesis and secretion of digestive enzymes into the lumen (Paquet *et al*., 1993). It is generally assumed that B- cells (secretory cells) and F- cells (fibrillar cells) types are mostly concerned with the synthesis and secretion of digestive enzymes (Gibson and Barker, 1979; Dall and Moriarty, 1983). The present study showed many alterations in the hepatopancreatic cells of *P. clarkii* after being exposed to sublethal concentrations (LC_{25}) of Chlorpyrifos and Azadirechtein using TEM. The TEM studies showed that Chlorpyrifos produce severe degeneration of cellular organelles including deformation of the apical surface of absorptive cell with dispersed microvilli, mitochondrial swelling with ballooned cristae, deformed cisternae of RER, lytic and vacuolated cytoplasm and pyknosis of nuclei. This finding is in agreement with Abd El-Atti (2002) and Chiodiboudet *et al.* (2015) who found that Mercury and Cadmium induced destructive effects on the ultrastructure of the hepatopancreas of *P. clarkii* and *Palaemonetes argentines,* respectively. Desouky *et al*. (2013) revealed severe pathological changes in hepatopancreatic cells of *P. clarkia* after exposure to Ethionincluding vacuolation, degeneration and distinct cellular damage; these histopathological alterations may be responsible for the high toxicity of Ethion to the crayfish. Azadirechtein causeddeformation of mitochondria with the sparce of their cisternae, fragmentation of microvilli, ruptured RER beside the presence of autophagicvacuole containing dark granules and finally bizzar nuclei having segregated nucleoli and irregular clumps of their chromatin. Similar changes were reported by Aly (2000) and Abdel-kader (2011) who demonstrated that jojoba seed oil and Neemix (Azadirechtein) caused hydropic degeneration of digestive cells of the hepatopancrease of *P. clarkii.* These changes may be attributed to direct toxic effects of toxicant on hepatopancreatic cell because it is the main site of detoxification of all type of toxins. Heterochromatin condensation and marginalization have observed in this study may be due to progressive inactivation of nuclear component (Braunbeck, 1990). Treated hepatopancreatic cells showed the presence of vesicles containing cytoplasmic debris distributed all over the cytoplasm. These vesicles probably arisen to digest the destructed cellular organelles as a result of treatment with Chlorpyrifos and Azadirechtein. Asztalos *et al*. (1988) proposed that the focal development of empty vacuoles might be the starting point of cellular autolysis process. The presences of autophagicvacuole in this study improve the detoxification role of hepatopancreatic cells against pesticides. The fragmentation of RER might be a consequence of final hyperactivity prior to cell necrosis (Roncero *et al*., 1992).Also, in the present study, mitochondrial swelling was noted in some hepatopancreatic cells

of chlorpyrifos treated specimens. Mitochondrial swelling might be due to pollutant-induced inhibition of Na+/H+ transporter and impairment of the overall osmoregulatory process of the cell (Vilella *et al*., 1991), thus swelling reflects the entry of solutes and water into mitochondrial matrix (Ghadially, 1985; Cheville, 1994). It can conclude that Chlorpyrifos has much toxic effect on the red swamp crayfish *P. clarkii*.

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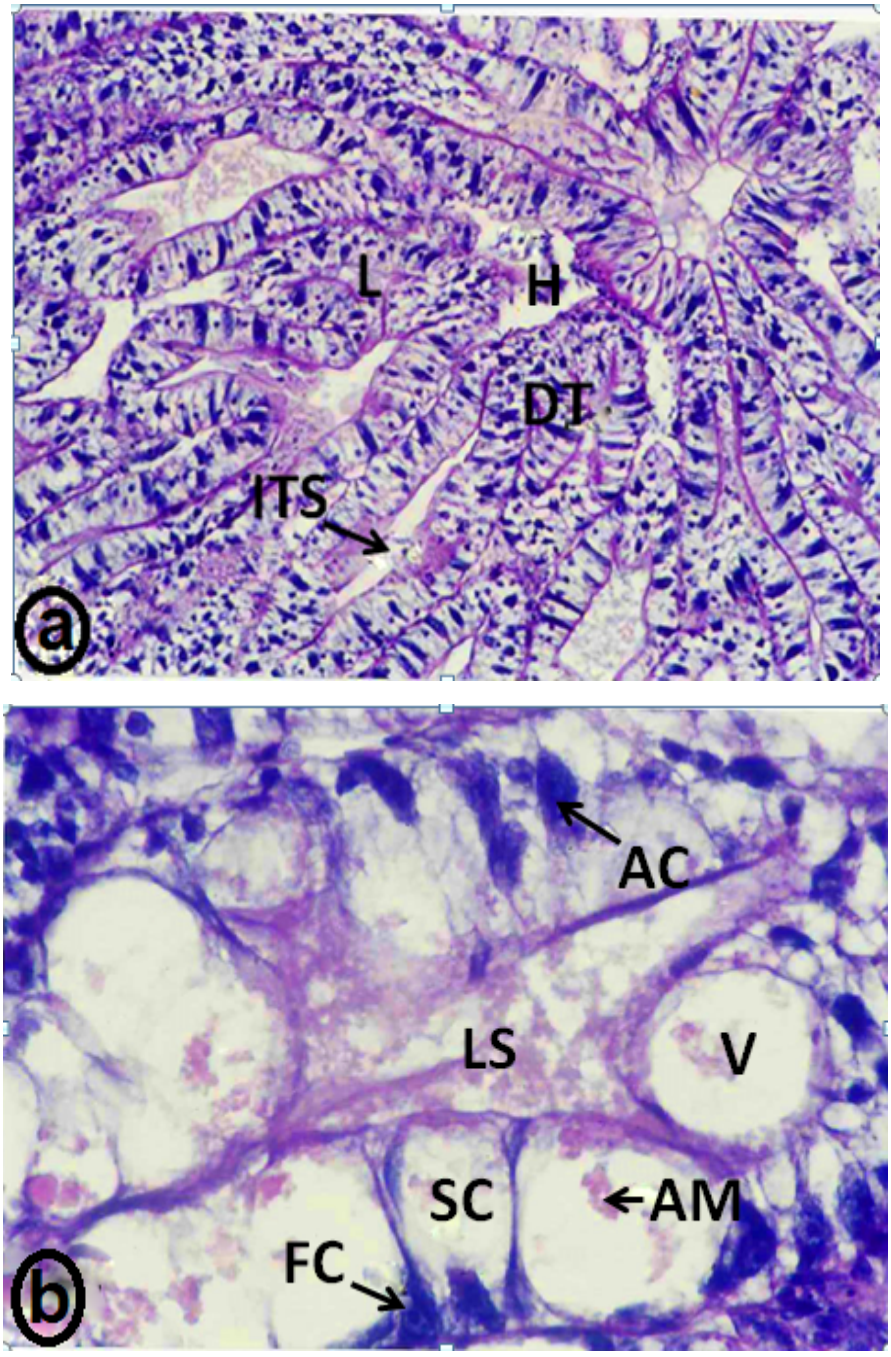

Plate I: (a-b) Histological sections of control hepatopancreas of *P. clarkia* (a) DT: Digestive tubules, H: Haemocyte, ITS: Intertubular space (original mag. X=100). (b) AC: Absorptive cell, AM: Acidophilic material, FC: Fibrillar cell,LS: Lumen secretion, SC: Secretory cell, V: Vacuole (original mag. X=400).

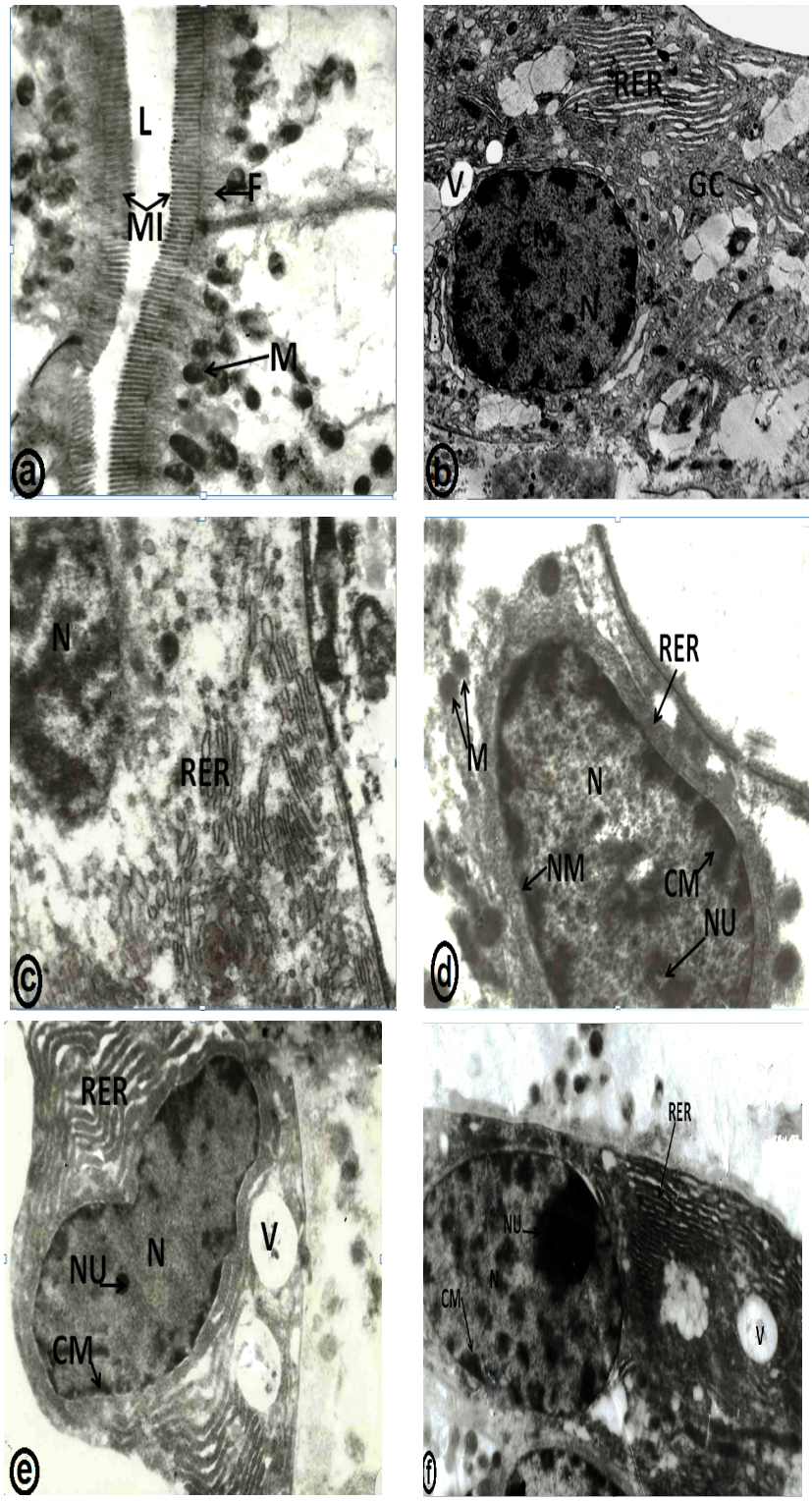

Plate II: (a) Electron micrograph showing the apical portion of control hepatopancreatic absorptive cell of *P. clark*ii. F: Filaments, L: Lumen, M: Mitochondria, MI: Microvilli (original mag. X=16000).(b and c) The basal portion of control hepatopancreaticabsorptive cell, CM: Chromatin materials, G C: Golgi complex, M: Mitochondria, N: Nucleus ,RER: Rough endoplasmic reticulum,(original mag. (b) X= 8000 (c)X=28000).(d)Secretory cell ,CM: Chromatin material, M: Mitochondria, N: Nucleus, NU: Nucleolus, NM: Nuclear membrane, RER: Rough endoplasmic reticulum(original mag. X=20000).(e and f)Fibrillar cell, CM: Chromatin materials, NU: Nucleolus, N: Nucleus, RER: Rough endoplasmic reticulum, V: vacuole (original mag.X=20000).

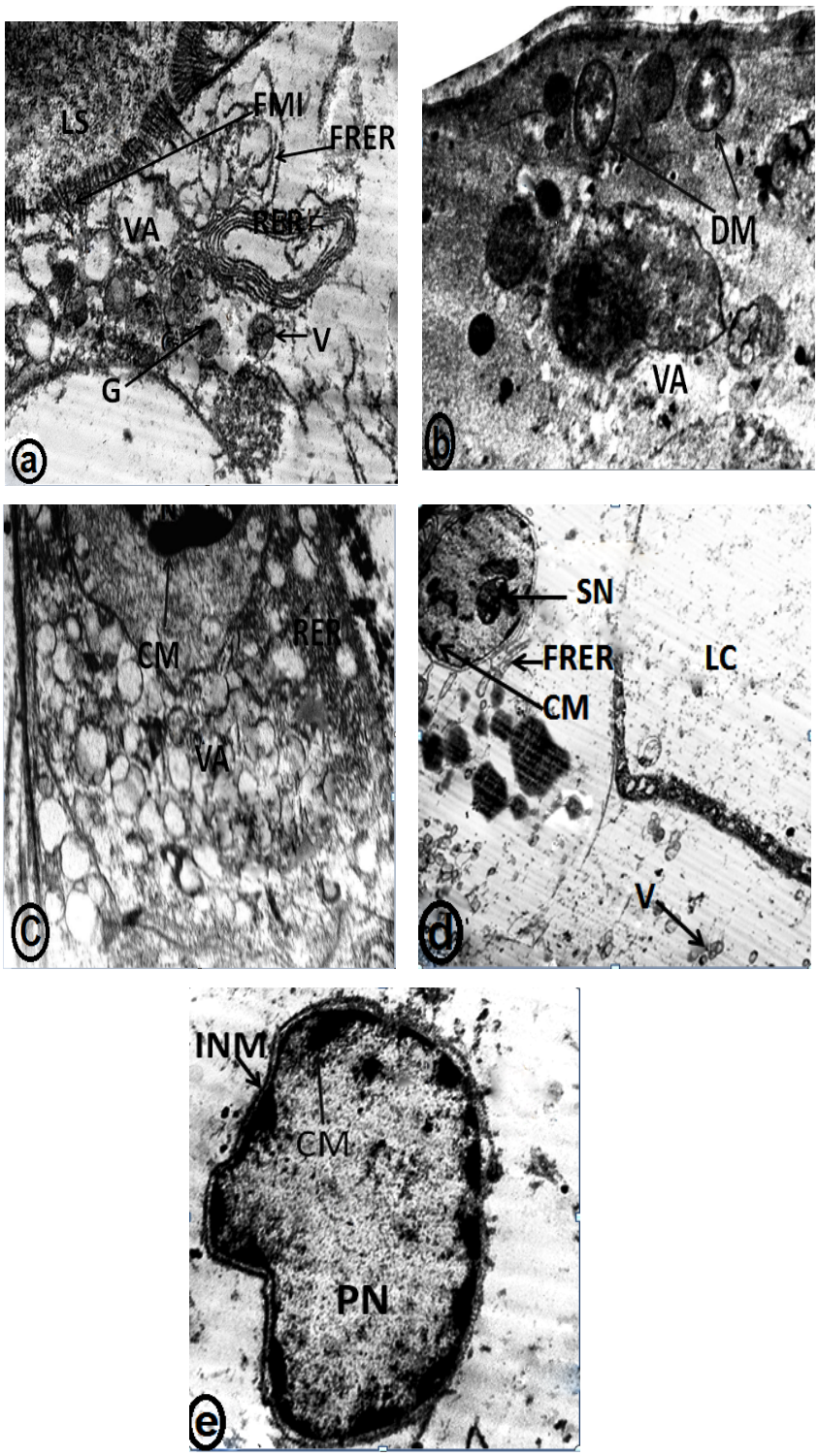

Plate III: (Fig. a) Electron micrograph showing the apical region of hepatopancreatic absorptive cell of *P. clarkii* treated with 0.0075 ppm Chlorpyrifos. DMI: Disordered microvilli, FRER: Fragmented rough endoplasmic reticulum,G: Granules, LS: Lumen secretion, RER: Concentric whorls of rough endoplasmic reticulum, V: Vesicles containing dark granules, VA: Vacuolated cytoplasm,(original mag. X =10000) (b) Basal region of an absorptive cell, DM: Deformed mitochondria, VA: vacuolated cytoplasm(original mag. X=20000) (c) CM: Chromatin material, N: Nucleus, RER: Rough endoplasmic reticulum, VA: Vacuolated cytoplasm (original mag.. X=12000). (d) Secretory cell CM: Chromatin material, DM: Disordered microvilli, FRER: Fragmentedrough endoplasmic reticulum, LC: Lytic cytoplasm, SN: Segregated nucleolus V: vesicle (original mag. X=4000). (e) Nucleus of hepatopancreatic absorptive cell, C: Lightened cytoplasm, CM: Chromatin material, INM: Irregular nuclear membrane, (KNu): Karyorrexic nucleolus, PN: Pyknotic nucleus (original mag.X= 12000)

ARABIC SUMMARY

تأثيرات الكلوربيريفوس ومستخلص بذور نبات النيم على التراكيب الخلوية للكبدالبنكرياسية لإستاكوزا المياه العذبة (*بروكمبارس كلاركى*)

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أظهرالفحص النسيجى والتركيبى الدقيق وجود ثلاثة أنواع مختلفة من الخلايا التي تتركب منهاالانيبيبات الهضميه للكبد البنكرياسية في استاكوزا المياه العذبه(*بروكمبارس كلاركي*) وهم المفرزه والماصه والليفيه.تم دراسة التغيرات التركيبية الدقيقة التى حدثت فى الكبد البنكرياسية لاستاكوزاالمياة العذبة بعد تعرضها لتركيزات تحت مميتة لكل من مبيد الكلوربيريفوس ومستخلص بذور نبات النيم.أحدث تركيز ٠.٠٠٧٥جزء في المليون من مبيد الكلوربيريفوس حيث ظهر تشوة فى الحواف القمية للخلايا الماصة وخلل فى انتظام خملاتها. وكذلك انتفخت الميتوكوندريا، تمزقت الشبكة الاندوبلازمية الخشنة وأصبح السيتوبلازم فجويا ومتحللا وانكمشت الأنوية.من الناحية الاخرى اثر تركيز ٤.٩٩ جزء في المليون من مستخلص بذور نبات النيم على عضيات الخلايا وأحدث تغيرات فى تركيب الميتوكوندريا وتمزق الشبكة الاندوبلازمية والخملات مع ظهورفجوات بلعمية داخل السيتوبلازم والتى تحتوى على حبيبات داكنة اللون.هذا بالاضافه الي ظهور انويه متحلله النويات وتركز الكروماتين في بؤر حول الغشاءالنووي. ولقد أظهرت الدراسة أن مبيد الكلوربيريفوس أكثرسمية من مستخلص بذور نبات النيم على التركيب الخلوى للكبد البنكرياسية لاستاكوزا المياة العذبة.