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SOME BIOCHEMICAL AND HISTOPATHOLOGICAL CHANGES IN *PROCAMBARUS CLARKII* EXPOSED TO ACUTE TOXICITY OF NEEM EXTRACT, NEEMIX.

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Abstract

The main objective of the present study was to evaluate the acute toxicity of Neemix and biochemical parameters (haemolymph glucose, total protein and triglyceride). The 24h LC_{50} of Neemix to the adults crayfish, *Procambarus clarkii* were evaluated. The LC_{50} values were 431 and 493 ppm for adults male and female, respectively. Adults *P.clarkii* were subjected to acute lethal concentration (431ppm) of Neemix for 96 h. The results showed a gradual increase in haemolymph glucose within 72h. The total protein levels decreased at the first 48h then followed by a sudden increase after 72 h. While, the triglyceride value decreased only after 24h. The levels of the haemolymph glucose, total protein and triglyceride have reached more or less the control values by the end of experiment. The increase of the basal (regenerative) cells, the extensive mucification of the inner lining of the gland and the hypertrophy of almost all absorptive and secretory cells were the most observed pathological changes.

INTRODUCTION

The Lousiana red swamp crayfish, *P. clarkii* (Girard, 1852) was introduced into Egypt in the early 1980s mainly as food value (aquaculture) (Ibrahim, *et al*. 1995). Since then, crayfish increased without control, invading most of wetland areas and rice fields, causing serious damages to drainage systems and rice crop as consequence of its digging activities (Burton, *et al.*1980; David, 1994 and Soliman, *et al.* 1998).

During the last decade the use of cleaner technologies for insect pest control has greatly increased. Plant extracts from the Indian neem tree, *Azadirachta indica* are used extensively in various neem-based formulations and have a good potential pest control. These natural pesticides are known to have strong antifeedant, growth regulatatory, metamorphosis, repellency and sterility effects on insects (Guerrini and Kriticos, 1998; Su and Mulla, 1998; Koul, 2003 and Lucantoni, *et al.* 2006).

Because of low toxicity of neem to fishes, mammals and beneficial invertebrates (Schmutterer, 1995; Wan, *et al.* 1996 and Isman, 1997) and its broad spectrum control to insects, Neemix is used now days on a wide scale as an arthropods pesticide in rice fields (Jimenez, *et al.* 1998). Moreover, Neemix has been found to be rapidly and easily degraded by sun light and completely hydrolyzed in water (Mordue and Blackwell, 1993 and Isman, 2006).

Most of the studies dealt with toxicity of neem seed extracts were carried out on water fleas and fish (Zebitz, 1987; Saucke and Schmutterer, 1992; Schmutterer, 1995; Kreutzweiser, 1997; Scott and Kaushik, 1998). Jimenez, *et al.* (2003) showed that crayfish was most sensitive to all products after 96 hours and azadirachtin (AZA) was the most toxic to crayfish among the tested compounds. Moreover, Goktepe (2004), showed that the lethal concentration of Neemix ^{TM} and Bioneem^{TM} for juveniles of *P. clarkii* ranged from 4.71 to 6.60 µg AZA/mL. The lethal concentration for pure AZA was greater than the highest concentration used (>1 µg AZA/mL).

The current study aimed to test acute lethal concentration (LC_{50}) responses of some biochemical changes take place in the haemolymph and histopathological changes in hepatopancreas of *P.clarkii* exposed to the commercial Neemix (4.5%).

MATERIALS AND METHODS

1- Tested pesticide:

Trade name: Neemix.

Common name: Azadirachtin from the neem tree, *Azadirachta indica*

(Meliaceae)6(2,4-dinitrophenylaminohexanyl)-22,23dihydroazadirachtin.

Empirical formula: C35 H44 O16. (Wan, 1994)

Formulation: 4.5%.

This compound was obtained by Agri Dyne technologies INC.

2. Test animals and acclimation procedures:

Adults of red crayfish *P. clarkii* both sexes, (22- 29 gm average weight and 8.5 to 9 cm total length) were collected in the spring of 2009 from Abou-Kabir district, Sharkia Governorate. The collected specimens were transported alive to the laboratory in a well-aerated large plastic containers (30x50x30 cm). They were adapted for laboratory conditions at a temperature of 21-23, a photo period of 12h light and housed in 10 cm deep aged tap water previously aerated for 48h to remove chlorine. Water was changed once every two days and crayfish were fed on cucumber during acclimation and not during testing period.

3. Determination of mortality rate and LC_{50} value of Neemix.

To determine the median lethal concentration (LC_{50}) of Neemix, stock solution was prepared by using dechlorinated tap water as a solvent to achieve desired concentrations.

Adult crayfish (males and females) were exposed to four concentrations of Neemix (400, 500, 600 and 700 ppm). These concentrations were chosen on the basis rang finding tests. The experiment was repeated 3 times, which exposed (10x3) *P. clarkii* to each test concentration, plus control (10x1), for 96 h.

The test containers were checked every 2 hrs over 96 hrs to record and remove dead animals. The criterion for death was the failure of crayfish specimen to respond to prodding with a blunt glass probe. The test time for Neemix was 24h, because by 48h all the crayfish tested started recovery. The concentration causing 50% mortality of the tested animals (LC_{50}) and 95% confidence limits were calculated using the method of Litchfield and Wilcoxon, (1979). The animals were exposed to lethal LC_{50} (431 ppm) for 96h.

4-Biochemical studies:

Effect of acute lethal concentration (431ppm) of Neemix 4.5% on some haemolymph constituents of adults *P. clarkii* for 96h were studied. Glucose, total protein and triglycerides concentrations determined by colorimetric method using commercial kits supplied by Caraway and Watts, (1987), Henry, (1964) and Young and Pestaner, (1975), respectively.

5. Histological studies:

For histological examination, hepatopancreas from adult LC_{50} Neemix treated males *P. clarkii* were separated at the end of the experiment (96h) and immediately fixed in 10% formalin or Bouin,s solutions. Graded dehydration of the tissue was done by 70-100% alcohol in subsequent steps. Fixed specimens were then embedded in paraffin wax and cross sectioned at 5µ and stained with haematoxylin and eosin.

RESULTS AND DISCUSSION

1- Toxicity test:

Neemix was highly toxic at high concentrations (700 ppm), but this activity declined progressively as the concentration decreased (Table 1). At concentration above 600 ppm of Neemix formulation, 100 and 90 % of mortality occurred for adult males and females of *P. clarkii*, while at lower concentration (400ppm) the mortality was 40 and 20 % for both

sexes. Total mortality of all crayfish was recorded only within the first 24 h. of Neemix exposure indicated the high toxicity of the product, while in surviving samples the effect of Neemix started to decline from the second day (48h.) approaching the control by the end of the experimental period (96h of exposure). The median lethal concentration (LC_{50}) of Neemix after 24h. were 431ppm (95% Confidence Limits(CL): 3.58–15.96) and 493 ppm (95% CL: 3.04–13.18) for adult males and females of *P. clarkii*, respectively. Similar toxic effects were found by Jimenez, *et al.* (2003) who tested neem seed extract (0.3% Azadirachtin) on *P. clarkii* (7 cm total length).They reported that Azadirachtin was the most toxic to crayfish with an LC_{50} 0.057mg/l when compared with other commonly used synthetic insecticides. Also by used other commercial neem-based insecticide (Bioneem™ 0.09% AZA and Neemix™ 0.25% AZA) and pure AZA similar effects have been observed on juvenile of *P. clarkii* (3-4 weeks old). Goktepe, (2004) found that LC_{50} ranged from 4.71 to 6.60 µg AZA/mL (5.18 µL Bioneem™ or 2.64 µL Neemix ™/mL) and for pure AZA was greater than the highest concentration used (>1 µg AZA/mL). Although, 5% of neem seed kernel extract was toxic to Nile bolti, *Oreochromis niloticus*; Fernandez, *et al*. (1992) showed that 50% of neem oil at 3ml/litre was harmless. But, a dose of 302 ppm of neem oil was found to represent the LC_{50} of carp, *Cyprinus carpio* after 24h of treatment. On the contrary, neem seed kernel extract and neem oil were not toxic to the common rice field Toad when used below 0.1 % concentration (Jayaraj, 1992). Lack of toxicity was observed in rats orally administrated neem in a dose of 1500 mg/Kg/day for 90 days (Razada, *et al.* 2001). Whereas Mousa, *et al.* (2008) found that neem leaf extract was toxic to juvenile's *Oreochromis niloticus* and African cat fish, *Clarias gariepinus* at LC_{50} 1.8 and 4 g/l after 96-h., respectively.

Any comparison of our data with the previous studies is difficult because there is several commercial formulation of neem with different concentration of active ingredients of the products. However, previous

studies indicated that the effectiveness of neem-based pesticides may vary among life stages, species (even closely related species can differ markedly in susceptibility to the same plant extract or pure allelochemicals (Isman, 1993; Akhtar and Isman, 2004)) and formulations (Isman, 1997).

2-Biochemical studies:

2-1-Effect on haemolymph glucose

Haemolymph analysis (glucose, protein and triglyceride) of *P.clarkii* after acute exposure to Neemix are shown in Table (2) and Fig.(1). In this acute phase a gradually increase in the haemolymph glucose (15.15±1.10) was observed during 48 hrs till reaching the maximum value of 21.21± 2.48 after 72h while at the end of the experiment (after 96h of exposure) the value was 15.15 ±1.08 mg/dl tendency to reach the control value (13.33±1.82). Neemix-induced hyperglycaemia was revealed in *P.clarkii*. The source of such hyperglycaemia may be due to enhanced glycogen breakdown in hepatopancreas under the effect of hypoxia and/or the high water and sediment (residue) pollutant concentrations. Raja, *et al*. (1992) suggested that this hyperglycaemia caused by pesticide treatment may indicate disrupted carbohydrate metabolism due to enhanced in adrenocorticotrophic and glucagon hormones and/or reduced insulin activity. Moreover, Gupta, (1974) mentioned that the hyperglycemia induced by any toxicant might be explained by the inhibition of the neuro-effector sites in the adrenal medulla leading to hyper secretion of adrenaline, which stimulates glycogen breakdown. Similar observations were also reported in the crab, *Scylla serrata* (Ghosh and Shrotri, 1992) and *P.clarkii* **(**Salah El-Deen, *et al.*2001) in response to endosulphan and malathion, respectively.

In our work, glucose levels might elevate to cope with the increased energy demand during pesticide stress as important pathways

for the recovery (Schreck, 1981 and El-Danassouri, *et al.* (1997)). The present results in agreement with the findings of Winkaler, *et al.* (2007) who reported that the increase in blood glucose of freshwater fish *Prochilodus lineatus* can be viewed as a part of stress response triggered by the presence of neem leaf extract in water.

2-2-Effect on haemolymph protein:

The next alternative source of energy is protein to meet the increased energy demand. In the present study, there were noticeable changes in the total protein levels in all Neemix-exposed crayfish during the time of exposure. A slight decrease (3.82±0.82) after 48h then a sudden increase (6.00±1.04) was observed 72h of acute exposure, followed by a slight decrease (3.82 ± 0.63) nearly reaching the control levels (4.40±1.11) at the end of the experimental period (96h). The decrease in total proteins could be attributed in part to the damaging effects of pesticide and metal on liver cells (Gluth and Hanke, 1985and Yang and Chen, 2003). The same observations were recorded by Torreblanca, *et al.* (1991), Rawi, (1995) and Salah El-Deen, *et al.*(2001) in *P.clarkii* exposed to Al, Cd and malathion, respectively. Tiwari and Singh, (2006) reported that the decrease in protein level in the liver and muscle of fish exposed to neem extract resulted from high protein hydrolytic activity due to elevation of protease enzyme in both tissues. Rivarola and Balegno, (1991) attributed the reduction in plasma protein in pesticides treated animals, to the changes in protein and free amino acid metabolism .Furthermore, the decrease in blood protein maybe due to reduced protein synthesis or increased proteolytic activity and degradation (Shakoori, *et al.* 1990 and Koul, *et al.* 1996). On the other hand, the increase in the total protein could be attributed to increased biosynthesis process occurred by high enzyme stress (Khater, *et al.* 1990).Also the increase in the total protein level was reported by Radhakrishnaiah, *et al.* (1995) in the prawn *Macrobrachium*

malcolmsonii exposed to endosulphan. A decline and increasing in total protein level was reported in the desert locust *Schistocerca gregaria* which injected with azadirachtin (Annadurai and Rembold, 1993). They concluded that overall levels of protein synthesis are reduced; some protein bands disappear while others appear and some remain the same. Azadirachtin has more than one mode of action. Firstly, azadirachtin alters or prevents the formation of new assemblages of organelles or cytoskeleton resulting in disruption of cell division, blocked transport and release of neurosecretory peptides. Secondly, it inhibits protein synthesis in cells, which are metabolically active and have been switched on to produce large amounts of protein (Mordue, 2004).

2-3-Effect on haemolymph triglyceride

The storage of lipid reserves mostly triglyceride acts as a main source for energy utilization. The sudden depressed in triglyceride (35.71± 4.56) in the haemolymph of *p.clarkii* was observed only after 24h of acute lethal concentration of Neemix compared with the control value (42.86± 3.89). Then the level returned to the normal value at 48h of exposure time reflecting a tendency to overcome the toxic effects of Neemix. This decrease might be attributed to increase hormonal secretions in the haemolymph_that enhance the metabolic rate which in turn reduces the metabolic reserves of the triglyceride (Turner and Bagnara, 1976) or may be due to decline in lipid synthesizing capacity and /or increase in the hydrolysis of hepatic lipid to combat the stress conditions as reported by Saxena, *et al.* (1989). Similarly, decrease in haemolymph total lipid have been reported in *P. clarkii* exposed to malathion (Salah El-Deen, *et al.* 2001)

3- Histopathological studies:

The hepatopancreas has been chosen, as pesticides had been found to accumulate in this organ in several crustaceans (Jaiswal and Sanojini 1990).

3-1-Histological structure of hepatopancreas (digestive gland):

The gland is consists of a numerous digestive tubules (DT) with stellate shape lumen (L) Fig. (2) and separated by inter-tubular connective (ITC) tissue. Each tubules is formed of four types of cells, absorptive cell (AC), secretory cell (SC), fibriller cell (FC) Fig.(3) and embryonic (regenerative) cells (EC) Fig.(4). The long columnar absorptive cells has rounded apices, irregular basal nuclei and containing numerous small vacuoles near the lumen. The shorter pyramidal secretory cells has wedged apices, basal nuclei and filled with secretory granules inside vacuoles. The columnar fibriller cell is dark and scattered among absorptive and secretory cells. The nucleus is spherical and has large nucleolus. The embryonic cells (EC) are undifferentiated cells which can be transforming into either of hepatopancreatic cells.

3-2-pathological finding:

In the present study, few signs of pathological changes were observed in the hepatopancreas of *p.clarkii* treated with Neemix, which were characterized by forming the mucous film covering the internal lining epithelium of the whole gland tubules (Fig.4). Mucus acts as a trap for active radicals of the Neemix extruding it outside the cells and /or as a barrier that prevent the entery of other undesirable radicals. Moreover an increase of the small rounded basal regenerative, cells (hyperplasia) was observed in between the other of hepatopancreas cells as indicator for recovery of *P. clarkii* (Fig.4). Also hypertrophy of several absorptive cells and secretory cells can be observed (Fig.5). This finding were in disagreement with those of (Nasiruddin and Mordue, 1993) working on locust.

Table (1): Showed the effect of different concentrations of Neemix against adult males and females of *P. clarkii*.

Conc. ppm	No. of animals		Mortality of adults/h.										Total mortality		% mortality	
			Males					Females								
			2	4	8	12	24	2	4	8	12	24				
	♂	♀											♂	♀	♂	♀
400	10	10	2	0	2	0	0	0	0	0	0	2	4	2	40	20
500	10	10	3	1	2	1	0	0	2	1	1	2	7	6	70	60
600	10	10	3	3	0	3	0	3	1	3	0	0	9	7	90	70
700	10	10	5	3	2	0	0	4	0	2	0	3	10	9	100	90
Control (0)	10	10	0	0	0	0	0	0	0	0	0	0				

Table (2): Showed the effect of lethal concentration of Neemix (431 ppm) on haemolymph glucose,total protien and triglyceride of adults *P. clarkii*.

Parameter / Time interval	Glucose (mg/dl)	Total protein (g/dl)	Triglyceride (mg/dl)
(Control)	13.33 ± 1.82	4.40 ±1.11	42.86 ± 3.89
(24h)	15.15 ±1.10	4.55 ± 0.70	35.71 ± 4.56
(48h)	18.18 ± 2.04	3.82 ± 0.82	42.86 ± 3.68
(72h)	21.21± 2.48	6.00 ± 1.40	42.86 ± 2.92
(96h)	15.15 ± 1.08	3.82 ± 0.63	42.86 ± 3.22

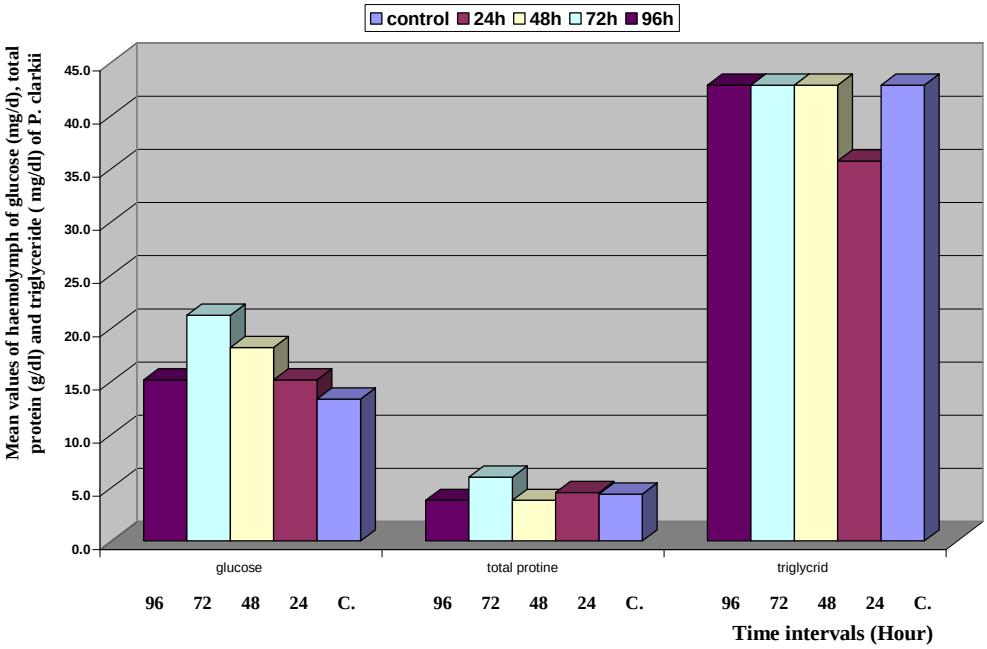

Fig. (1): Showed the mean Values of haemolymph glucose, total protein and triglyceride of *P.clarkii* after being exposed to lethal concentration of Neemix (431 PPm).

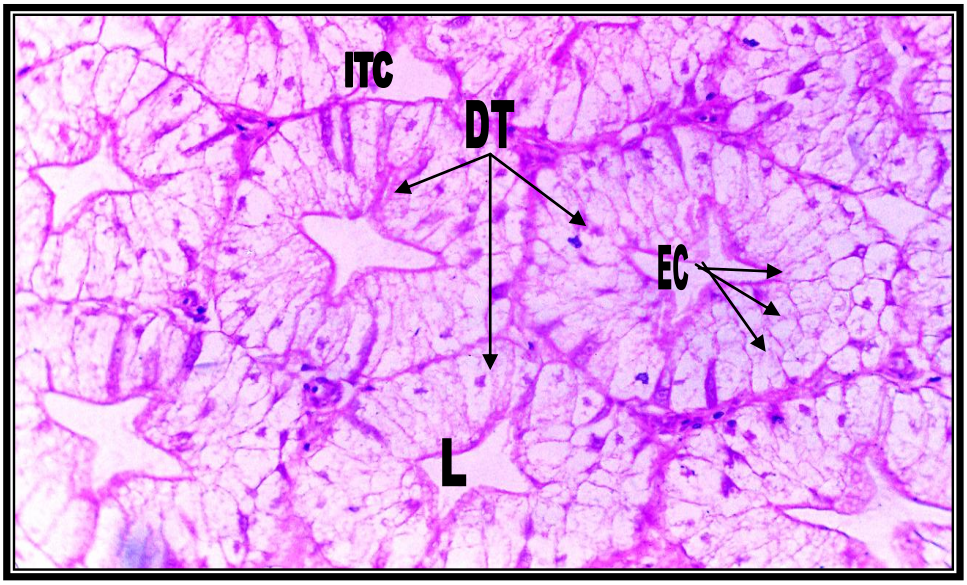

Fig. (2)

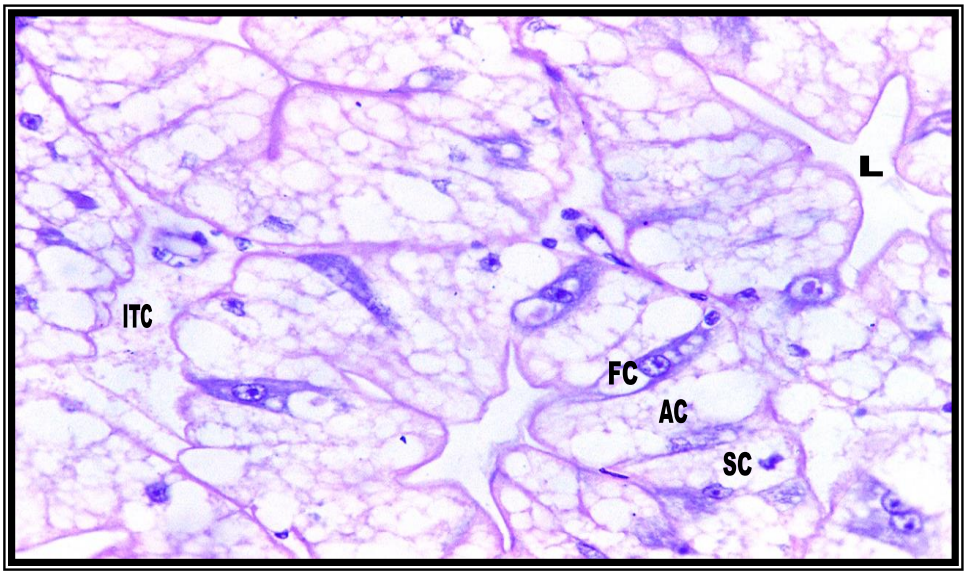

Fig. (3)

Fig. (2 and 3): Showed sections of normal hepatopancreas of *P.clakii* showing four types of cells (AC Absorpative cell; Sc: Secratory cell; FC: Fibrillar cell; EC: Embryonic cell); DT: Digestive tubules; L: Lumen and ICT. Intertubular connective tissue. X= 200 and 400

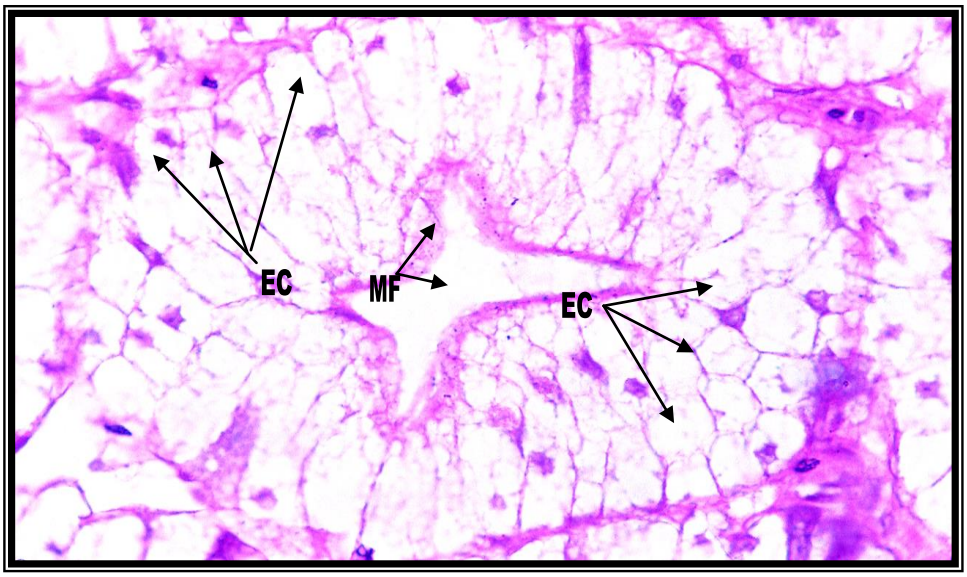

Fig. (4): Showed sections of hepatopancreas of *P.clakii* after 96h.of exposure to lethal concentration of Neemix (431ppm) showing thing mucous film (MF) with hyperplasia of regenerative cells (EC). X= 400

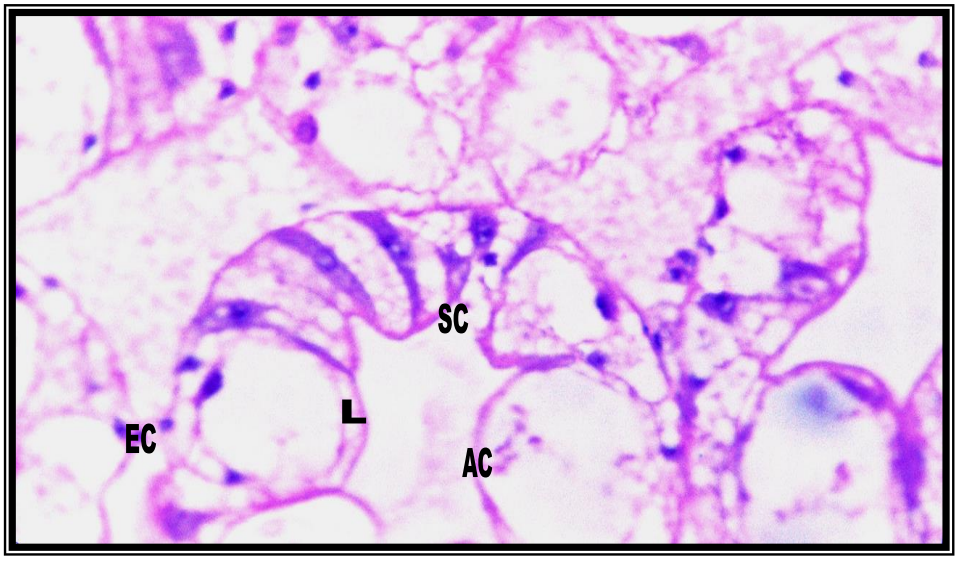

Fig. (5): Showed sections of hepatopancreas of *P.clakii* after 96h.of exposure to lethal concentration of Neemix (431ppm) showing hypertrophy of absorptive cells (AC) and secretory cells (SC). X= 400

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دراسة التاثير السام لمستخلص النيم (النيمكس) على بعض التغيرات البيوكيميائية والهستوباثولوجيه لأربيان المياه العذبه بروكمبارس كلاركى

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الملخص العربى

تم إجراء هذه الدراسة بغرض تقييم سمية مركب النيمكس النباتى الاصل على البروكمبارس كلاركى حيث وجد ان قيم التركيزات النصف المميتة للنيمكس بعد ٢٤ ساعة كانت٤٣١ ، ٤٩٣ جزء فى المليون للذكور والاناث على التوالى. وتمت دراسة التاثيرات الناجمة من استخدام الجرعة المميتة لنصف العدد على بعض المحتويات البيوكيميائية داخل الهيموليمف للحيوان وكذلك الهستوباثولوجى على الغدة الهاضمة لمدة ٩٦ ساعة.

وقد اسفرت هذة الدراسة عن ارتفاع تدريجى فى جلوكوز الهيموليمف خلال ٧٢ ساعة بينما البروتين الكلى قل خلال ٤٨ ساعة الاولى ثم صاحبة ارتفاع مفاجى بعد ٧٢ ساعة. وعلى الجانب الاخر فان ثلاثى الجليسريد قل فقط بعد ٢٤ ساعةوقد تبين عند نهاية التجربة أن تركيز المحتويات البيوكيميائية الثلاثة عادت تقريبا الى نسبها الطبيعية. اما بالنسبة للتغيرات الهستوباثولوجية للغدة الهاضمة_فقد لوحظ زيادة فى عدد الخلايا الجنينية مع انتفاخ فى معظم الخلايا الماصة والهاضمة وكذلك زيادة فى الافرازات للغشاء المبطن للغدة والتى تعمل على طرد او منع دخول اى_عناصر غير مرغوبه الى داخل انبيبيات الغدة وهذة كلها علامات تدل على ان لهذا الحيوان القدرةعلى استعادة حالتة الطبيعية.

ولهذا يوصى باستخدام ذلك المركب والذى اثبتت الدراسه فاعليتة خلال ٢٤ ساعة كوسيلة للحد من انتشار هذا الحيوان فى محافظة الشرقية.