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TOXIC AND HISTOPATHOLOGICAL EFFECT OF *NEEMAX* ON *ANABAS TESTUDINEUS (PISCES)*

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An experiment was set up to determine the 48 hr sublethal concentration (LC_{50}) of commercial biopesticide *Neemax* (powdered seeds of *Azadirachta indica* A. Juss) on *Anabas testudineus* (Bloch). Histopathological examination revealed serious lesions in liver of *Neemax* treated fishes. Significant histopathological lesions observed were hypertrophy, hyperplasia, lifting up of the epithelium, rupture of hepatic cells, scattering and necrosis of nucleus, appearance of pyknotic nucleus, aggregation of cells/nucleus, hepatic ulcers, vacuolization, degeneration, excess mucous secretion, infiltration of lymphocytes and overall necrosis of the tissue. Results indicate that comparatively low concentration of *Neemax* is enough to elicit major histological changes in liver of *Anabas testudineus*.

Key words: toxicity, histopathology, liver, Neemax, Anabas testudineus, lesions, sublethal concentration, biopesticide.

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ТОКСИЧНІСТЬ ТА ГІСТОПАТОЛОГІЧНИЙ ВПЛИВ *NEEMAX* НА *ANABAS TESTUDINEUS (PISCES)*

В експерименті визначалася 48-годинна сублетальна концентрація комерційного біопестициду Neemax на тестовий об'єкт – *Anabas testudineus*. Гістопатологічні дослідження виявили серйозні пошкодження печінки риб, які піддавалися впливу Neemax. Виявлені такі пошкодження печінки: гіпертрофія, гіперплазія, підвищення епітелію, розрив клітин, дроблення та некроз ядра клітин, поява пікнотичних ядер, гепатичних виразок, об'єднання ядер, спостерігається вакуолізація, дегенерація, зайве виділення слизу, інфільтрація лімфоцитів та поверхневий некроз тканин.

Ключові слова: токсичність, гістопатологія, печінка, Neemax, біопестициди.

Neem (*Azadirachta indica* A. Juss) is a traditional and highly esteemed medical tree for the people of Indian sub-continent. Biological activities and medical properties of neem have been extensively reviewed by Biswas *et al*. (2002). Azadirahctin (a tetranotriterpenoid) is one of major components (Kraus *et al*., 1981; Broughton *et al*., 1986; Saxena, 1990) of neem, which have pesticide property (Anjaneyulu and Mishra, 1998). The neem product, «*Neemax»* is made from pure neem seed powder and is used in agriculture as organic manure with insecticide properties. It is a potential source of organic manure and is rich in many plant nutrients *viz* nitrogen (2–3 %), phosphorous (1 %) and potassium (1,4 %). Recently neem based pesticides are popularised due to their effectiveness, cheaper price and comparatively safe for users, which is used widely in several states of India (Anjaneyulu and Mishra, 1999).

Attri and Ravi Prasad (1980) and Deshmukh and Pariyal (1992) have reported toxic effect of *Neemax* on *Tilapia mossambica* and *Gambusia* sps respectively. Anjaneyulu *et al*. (1998) has reported 24 and 96 hr LC_{50} values of *Nimin* and Neem oil in carps, *Labeo rohita* and *Cirrhinus mrigala.* They have studied the bioassay of neem oil on *Puntius ticto*. Even though *Neemax* has insecticide properties, extensive use in agricultural fields especially in paddy fields results in pesticide pollution, which may lead to bioconcentration as well as biomagnification. Hence the bioassay of *Neemax* and its histopathological effect on liver was investigated on *Anabas testudineus* (Bloch), which is a freshwater table fish with very high environmental resistance and common in paddy fields of Kerala.

MATERIALS AND METHODS

Test Animal, Collection and Handling

Climbing perch, *Anabas testudineus* (Bloch) is hardy and partially air breathing fish, very common in paddy fields, ponds, rivers and inland water bodies, satisfies the qualities for experimental fish as suggested by Butler *et al.* (1971). Test fishes collected from unpolluted freshwater areas of Kottayam district of Central Kerala was brought to the laboratory in

plastic containers without disturbances and injury. Healthy fishes of uniform size (8 ± 0,75 cm; 4 ± 0,50 gm) were acclimatised to the laboratory conditions and subjected to a gut evacuation period of four days before the experiment.

Test Material and Preparation

Commercial grade insecticide cum organic manure, *Neemax* or powdered seeds of neem (*Azadirachta indica* A. Juss) manufactured by Ecomax Agro System Ltd., Mumbai, India was procured for the present study. Stock solution was prepared by soaking different amounts of «*Neemax*» in water for about 3 hours. It was filtered first through cotton cloth and later through filter paper. The filtrate was a saturated solution of *Neemax* and sub-dilutions were prepared from this stock solution.

EXPERIMENTAL SET UP

Animals were transferred from holding tanks to experimental troughs after the acclimation and gut evacuation period. Clean, acid washed plastic troughs with a capacity of 10 litres of water were used for bioassay studies. Control and experimental/exposure systems were set up and the experiment was run in triplicate.

a) LC_{50} Determination

For the estimation of LC_{50}, values, the static test method, (Doudoroff, 1951) was adopted in the present study. A range finding test was carried out to find out the mean tolerance limit. Test solution was renewed every 24 hrs by fresh solution of the same concentration during the experiment as suggested by Sprague (1973). Selected concentrations in narrow range from the results of range finding test were used to determine the LC_{50}. Observations were taken at 24 hour and continued for a period of 48 hrs. Cumulative mortality at 48 hr was determined and LC_{50} values for 48 hour were calculated by Regression analysis by plotting cumulative mortality against respective concentration. The regression equation between concentrations (x) and the cumulative mortality (y) was found out by using equation, $y = bx + c$ (Finney, 1971).

b) Histopathological Examination

After 48 hours of exposure, the liver of test fishes exposed to sub-lethal concentration and control fishes were dissected out and preserved in 10 % Neutral Buffered Formalin (NBF). They are processed for histological examination adopting the standard methods (Gurr, 1959) such as fixation, dehydration, embedding, sectioning and staining. NBF is used as the fixative and the tissues were dehydrated by treating them in various grades of alcohol ranging from 30 % to 100 %. The tissues were embedded in paraffin wax and were cut at 5m thickness using the microtome. The sections were stained with Harris Haematoxylin and Eosin (H & E). The stained slides were examined with the help of compound microscope and photographs were taken.

RESULTS

The behavioural and morphological responses of *Anabas testudineus* varied according to the test concentrations. The air gulping behaviour, swimming behaviour, resting behaviour, grouping behaviour etc were found to be changed extremely according to the concentration gradient. Fishes exposed to the toxicant also showed morphological lesions. Skin/scale lesions, tail and fin rot, excess mucous secretion and haemorrhages were noted at the final stage of exposure.

LC_{50} Determination

Based on the range of concentration obtained from range finding test, four test concentrations selected as 0 gm/l (Control), 2, 3, 4, 5 and 6 gm/l were used for bioassay. Observations on mortality were taken at 24 hr and continued for a period of 48 hours. *Neemax* concentrations for sublethal exposure and percentage cumulative mortality are presented in table 1.

Probit analysis was made by using regression analysis taking x as toxicant concentration and y as cumulative mortality at 48 hr. Regression coefficient (r) was 0,99, which showed high significance between the variables (Table 2). The regression equation was obtained as

$$y = 24{,}285x - 45{,}714.$$

LC_{50} value was estimated as 3,942 gm/l from the equation by substituting 50 for y. Hence the sublethal concentration (LC_{50}) of *Neemax* on *Anabas testudineus* at laboratory conditions was estimated as 3,942 gm/l (Fig. 1).

Table 1

Cumulative mortality at 48 Hrs of Anabas testudineus exposed to different concentration of Neemax

Concentration, gm/l	Fishes Exposed	Cumulative Mortality	Percentage Mortality
2	7	0	00,00
3	7	2	28,57
4	7	4	57,14
5	7	5	71,42
6	7	7	100,00

Table 2

Residual Regression Statistics (ANOVA) of cumulative mortality against concentration

	df	*SS*	*MS*	*F*
Regression	1	5897,6	5897,6	288,8*
Residual	3	61,26	20,42	
Total	4	5958,9		

* $p < 0{,}01$

Histopathological Examination

The normal and exposed fishes were sacrificed; liver tissue procured was processed for histopathological examination. The normal and exposed liver tissues were microphotographed and are presented in the plate I with figures No. 2 to 9. The normal liver (Fig. 2) of the *Anabas testudineus* consists of liver lobule as basic unit. The lobule is constructed around a central vein. Radiating out from the central vein are the hepatic cells arranged in the form of plates called hepatic cell plates. Each cell plate is generally of two cells thick. Between adjacent cells lie minute bile canaliculi that empty of into terminal bile ducts lying at the periphery of the lobule. Also lying in the periphery, small portal vesicles that receive blood and on the other side to bile canaliculi. In addition to these, hepatic arterioles are also present at the periphery of the lobule. They also empty into the hepatic sinusoids. The sinusoids are lined by two types cells, typical endothelial cells and larger phagocytic cells called Kupffer cells.

Neemax treated liver showed significant histopathological lesions and the damages were apparent as the exposure prolonged. Different types of pathological lesions can be noticed in *Neemax* treated liver of *Anabas* (Plate I: Fig. 3 to 9).

Hyperplasia and hypertrophy of hepatic cells were the most notable features in treated fishes (Fig. 3). Cells proliferated as well as the size of each cells increased abnormally, which is noted all over the liver tissue. The lifting up of epithelial cells along the surface of liver is another noted lesion in treated fish. The epithelial layer is found to be lifted up from the liver tissue. Fig. 4 shows the lifting up of epithelium of liver along its tissue border. Rupture of epithelium can be very well noted in fig. 6.

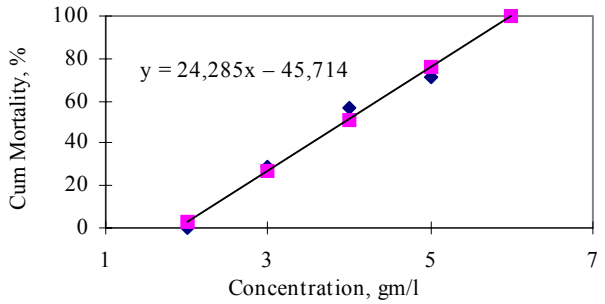

Fig. 1. **LC_{50} of Anabas testudineus exposed to Neemax**

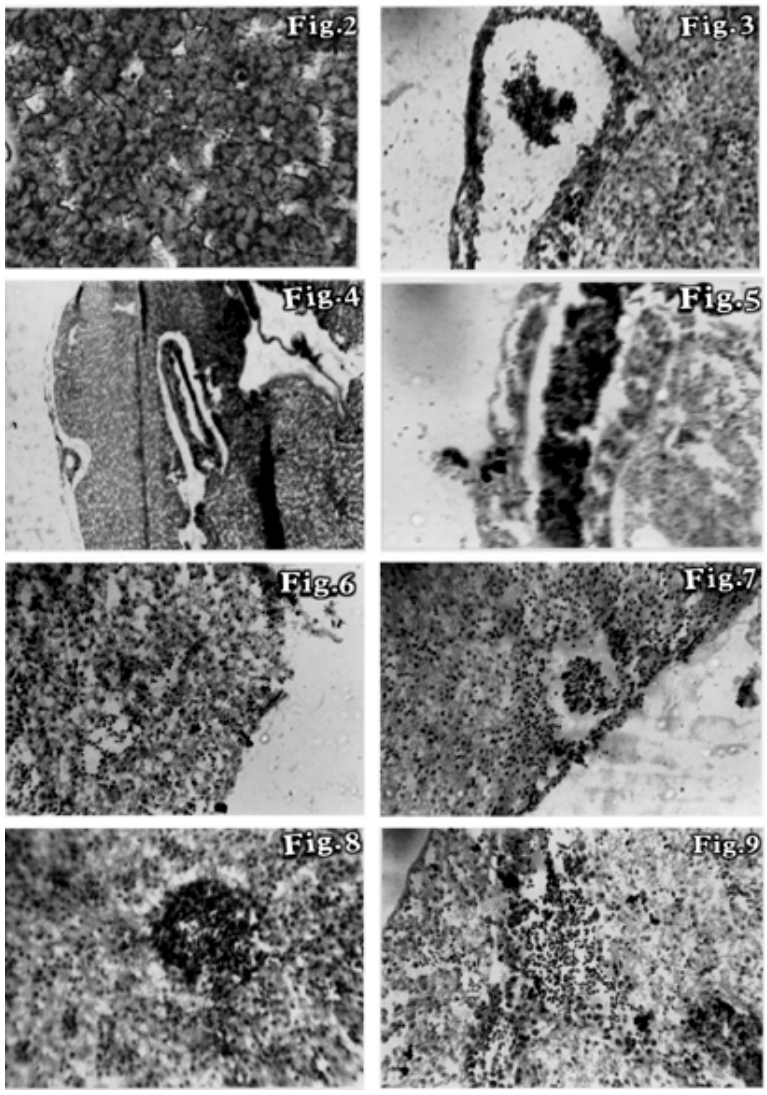

PLATE 1. **Microphotographs of treated and normal liver of *Anabas*** *(Figs. 2 to 9)*:

2 – micro-photograph of liver (5 μ) of control *Anabas testudineus (Normal tissue showing Hepatic cells, Canaliculii, Veins/arteries etc.)*, × 1000;

3 – microphotograph of liver (5 μ) of *Neemax* exposed *Anabas testudineus* (Lesions: Hyperplasia, Hypertrophy, Lifting up of epithelial cells), × 450;

4 – microphotograph of liver (5 μ) of *Neemax* exposed *Anabas testudineus(Lesions: Lifting up of epithelial cells, Vaculations)*, × 100;

5 – microphotograph of liver (5 μ) of *Neemax* exposed *Anabas testudineus (Lesions: Scattering of cells & nuclei, degeneration of cells, Lifting up of epithelial cells)*, × 450;

6 – microphotograph of liver (5 μ) of *Neemax* exposed *Anabas testudineus(Lesions:, Scattering of cells & nuclei, degeneration of cells, Lifting up of epithelial cells)*, × 450;

7 – microphotograph of liver (5 μ) of *Neemax* exposed *Anabas testudineus* (450X)*(Lesions: Pyknotic nuclei, aggregation of cells, degeneration of cells, Excess mucous secretion)*;

8 – microphotograph of liver (5 μ) of *Neemax* exposed *Anabas testudineus(Lesions: Pyknotic nuclei, aggregation of cells, degeneration of cells)*, × 450;

9 – microphotograph of liver (5 μ) of *Neemax* exposed *Anabas testudineus (Lesions: Infiltration of phagocytic cells, Vaculoation, necrosis)*, × 450

The hepatic cells are found to be ruptured and scattered over in the treated gills (Fig. 5). The rupture of hepatic cells is yet another lesion noted in *Neemax* treated liver which lead to degeneration of cells as well as tissues (Figs. 5 and 6). Figures 6 to 9 show a notable feature as appearance of pyknotic nuclei. Nucleus in treated fishes appeared enlarged and formed pyknotic by absorbing more stain. Again, nucleus becomes scattered in treated fishes (Fig. 7). A prominent lesion observed was aggregation of nuclei to form granular appearance in liver tissue (Fig. 8).

Infiltration of phagocytic lymphocytes is an important feature of *Neemax* treated liver. The phagocytic lymphocytes can be very well noted in figure 9. Excess mucous production by liver cells with in the tissue and out of the tissue can be noted in *Neemax* treated fishes (Fig. 7 and 9). Vacuolisation in liver tissue, congestion of veins/ducts, formation of hepatic ulcers and degeneration of cells/tissues can be observed in *Neemax* treated fishes. A general necrosis of the cells and tissue can also be noted in all over the liver.

Histopathological lesions elucidated by *Neemax* in liver of *Anabas testudineus* are hypertrophy, hyperplasia, rupture and lifting up of the epithelium, rupture of hepatic cells, scattering and necrosis of nucleus, appearance of pyknotic nucleus, aggregation of cells/ nucleus, hepatic ulcers, vacuolisation, degeneration, excess mucous secretion, infiltration of lymphocytes and overall necrosis of the tissue.

DISCUSSION

Biological activity of neem is reported for the crude extracts and their different fractions such as leaf, bark, root, seed and oil etc. Various parts of neem tree have been used as traditional Ayurvedic medicine in India. Neem oil, bark and leaf extracts have been used as medicine to control, leprosy, intestinal helminthes, respiratory disorders etc. Nearly 60 % mortality was observed in white leghorn chicks with a day feeding powdered ripe neem berry aqueous extract. Mild to severe changes in kidney, liver, spleen, intestine and heart of chicks was also reported. An aqueous extract of neem seed kernel produces trypsin inhibitory activity in weanling rats. Retardation of spermatogenesis was observed by feeding neem seed cake to rats. Calves fed with the neem seed cake showed reduced hemoglobin content in blood, along with depression (Biswas *et al*., 2002).

In the present study, morphological and behavioural changes in exposed fishes were noted. Air gulping behaviour was increased rapidly and tend to decrease after a short period. Similarly active swimming was reduced after an active stage. Grouping behaviour was found to be distorted. As the period of exposure and test concentrations increased, subsequently the activity became progressively lethargic and they died. Morphological lesions were also shown by fishes exposed to the toxicant. Skin/scale lesions, tail and fin rot, excess mucous secretion, haemorrhages were noted at the final stage of exposure. Holden (1973) states that acute toxicity primarily damages the central nervous system resulting in instability, respiratory difficulties and sluggishness.

Earlier reports shows LC_{50} values of neem products to different fish species. LC_{50} values of *Neemax*, neem oil extract and *Nimin* were calculated for *Tilapia mossambica, Gambusia* and *Labeo rohita* respectively. LC_{50} of *Anabas* was found to be comparatively high for *Neemax*, which may be due to the hardy nature of the fish.

Histological or cellular changes elucidated by toxicants are significant as cell forms the basic unit of life. According to Fower *et al*. (1983), histopathological studies become essential to understand the mechanism of cell damage resulting from exposure to toxicants and the extend for which chemically diverse group of trace metals regulate metabolic processes by altering organelle structure. Several histopathological investigations were reported on the toxic effect of different kinds of pollutants like heavy metals and pesticides (Hemmaid and Kaldas, 1994; Brock, 1998; Abraham and Radhakrishnan, 2002). Shibu Vardhanan (1998) reported exhaustively the histopathological changes by heavy metals and organic pesticide in crab, *Paratelphusa hydrodromus*.

Liver being the main metabolic factory of the body, serves several very basic functions such as metabolism, storage, and the secretion of bile. Liver accumulates more toxicants than other organs of body. Liver is the organ, which metabolises the toxicants and excrete it out. Since metabolism of proteins, fats and carbohydrates and detoxification of endogenous waste

products and drugs take place in liver, it is more liable to injury from toxicants. In aquatic organisms, liver is greatly affected by pesticidal contaminants (Konar, 1970; Eller, 1971). Binding of toxic substance with plasma proteins affects the excretion. Intracellular binding proteins are important in accumulating and storing toxicants with in the liver. Metallothionin, a binding protein in liver and kidney binds metals. Considering the importance of liver, several studies were undertaken to explore the histopathological changes in liver and reported cellular level complexities and reasons for mortality (Konar, 1970; Jana and Bandhopadhaya, 1987; Hemmaid and Kaldas, 1994; Brock, 1998; Vurk and Sharma, 1999). Most of the histopathological lesions observed in the present study are similar to those reported in earlier studies on other fishes with different toxicants.

One of the important functions of liver is to eliminate toxicant through metabolism (Konar, 1970; Eller, 1971). Hence the liver becomes hyper-active to eliminate the intoxicants. Due to the hyper activity and accumulation of compounds, the cells may become larger in size and to meet the requirement, cells proliferate much faster, which may be the reasons for hyperplasia and hypertrophy (see fig. 3). Similarly the liver tissue will try to avoid such intoxicant being absorbed for which the epithelial tissues will lift up (see fig. 6) to avoid the toxicants. Toxicants will affect cells badly that the properties of cells are lost. The nucleus become larger and Richmonds and Dutta (1989) reported such condition in malathion treated Blue-Gill, *Lepomis macrochirus*. Jamila (1992) and Abraham and Tresa Radhakrishnan (2002) also reported such conditions in aquatic crustaceans.

The results of the present study indicate that the biopesticides such as *Neemax* also cause far reaching consequences in the aquatic system. Even the sublethal concentration of *Neemax* is enough to elicit significant changes in liver histology of fishes like *Anabas*. The results further suggest that even smaller concentrations of any toxicant in the environment can induce major histological changes and more care and vigil is needed before dumping *Neemax* as biopesticides or organic/inorganic manure in to agricultural fields or environment.

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