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Toxicity of two biopesticidal plants aqueous leaf extracts to *Oreochromis mossambicus* –histopathology of gill, liver and intestine

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ABSTRACT

An investigation on the toxicity of two biopesticidal plants – *Carica papaya* and *Nerium oleander* aqueous leaf extracts to *Oreochromis mossambicus.* The LC_{50} value of *Carica papaya* and *Nerium oleander* aqueous leaf extracts was 700 ppm and 400 ppm respectively. The sub lethal concentration for *C. papaya* and *N. oleander* was 70ppm and 40ppm respectively. The fishes were exposed to the sub lethal concentration for a period of 14 days. At the end of 7^{th} and 14^{th} day the fishes were dissected and the gill, liver and intestine were removed from exposed and control group fishes. The tissues were processed and sectioned at 4µm and then were stained with Haematoxylin-Eosin. The observation of the slides was done under light microscope at 40x magnification and photographed. The exposed group showed histopathological changes in the tissues, such as: shrunken and narrow secondary gill lamellae, mild to moderate infiltration of inflammatory cells in the primary and secondary gill lamellae, vacuolar degeneration of epithelial cells of intestinal villi, massive infiltration of inflammatory cells throughout the base of the villi and disruption of epithelial cells. The liver showed swollen nucleus, hydrated and vacuolar degeneration of hepatocytes and mild pockets of infiltration of inflammatory cells.

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Key words: *Carica papaya, Nerium oleander, Oreochromis mossambicus,* gill, liver, intestine.

INTRODUCTION

Agrochemicals such as pesticides especially chlorinated hydrocarbons are routinely employed as part of the integrated farming practice to protect crops and animals from insects, weeds and diseases. Widespread use of pesticides on farm is now a worldwide phenomenon (Mitoyin *et al.,* 2006; Siememeon *et al.,* 2011). Plants may provide an alternative to currently used pesticides for the control of plant pests as they constitute a rich source of bioactive chemicals (Kim *et al.,* 2005; Daovbiet et al., 2005). Recent studies have demonstrated that insecticidal properties of chemicals derived from plants are active against specific target species; biodegradable to non-toxic products and potentially suitable for use in Integrated Pest Management (Markouk *et al.,* 2000; Tare *et al.,* 2004; Noriko *et al.,* 2010). Many plants contain chemicals which have traditionally been used to harvest fish and also to monitor various pests in almost all parts of the world (Siememeon *et al.,* 2011). Though the plant products are toxic they are degraded easily within 7-12 days and safe for users (Sudanshu, 2004; Chakroff, 1976). Several plants belonging to different families, having a number of compounds (saponins; tannins; alkaloids; alkenyl phenols; di and tri terpenoids; etc.) with high pesticidal activity are used to control predatory fish; disease causing insects such as mosquito larvae and harmful fresh water snails (Singh, *et al.,* 1996; Singh, *et al.,* 2000; Singh, *et al.,* 1993; Singh, *et al.,* 1998; Tiwari, 2003).

Nerium oleander Linn. (Apocynaceae) is indigenous to the indo-pak sub-continent. Al-Yahya *et al.* (2000) stated that *Nerium oleander* has shown insecticidal properties. The metabolism of Oleandrin, a cytotoxic component of N. *oleander* has been studied by Madden *et al.* (2002). The toxicity of crude extracts of *Nerium oleander*leaf and stem and bark to fresh water snail *Lymnea accuminata*; *Indo planorbis exutus* and the fresh water air breathing fish *Channa punctatus* has been established (Singh, 2000; Singh, *et al.,* 1996). Rao

(1957) and Hassan (1996) carried out laboratory experiments with *Nerium oleander* leaves and reported their insecticidal activity. *Carica papaya;* Linn; Family: Caricaceae leaves have been shown to contain many active components such as papain; chymopapain; cystatin; tocopherol; ascorbic acid; flavonoids; cyanogenic glucosides and glucosinolates. *Carica papaya* plants produce natural compounds inn leaf, bark and twig tissues that possess both highly anti-tumour and pesticidal properties (Noriko, *et al.,* 2010). It was suggested that a potentially lucrative industry based simply on production of plant biomass could develop anti-cancer drugs and natural (botanical) pesticides (Ayoola, 2010; Fathy, *et al.,* 2014). The high levels of natural self-defence compounds in the *Carica papaya* tree makes it highly resistant to insects and disease infestation (Joel, 2007).

Oreochromis mossambicus (Peters, 1852) displays many favourable attributes as culture species; on the basis of its general hardness, resistance to disease, high yield, potential and ability to grow on a wide range of natural and cheap artificial foods. Additionally it can also withstand low oxygen concentration, overcrowding, tolerates difficult ecological conditions and a wide range of salinities and still produces a highly acceptable flesh (El-Sayed, 2006). So, Tilapias are the second only to carps as the most widely farmed fresh water fish in world (FAO, 2010).

The present study evaluated the toxicity of *Carica papaya* and *Nerium oleander* aqueous leaf extract's on the histopathology of gill, liver and intestine in *Oreochromis mossambicus.*

MATERIALS AND METHODS

Fish

The fresh water fish *Oreochromis mossambicus* ranging in length 8-10±0.5 cms in total length and 6-7±0.25 grams in weight were collected using hand nets from Madinaguda tank, Hyderabad; Telangana; India. The fishes were transported to the laboratory and were stocked up in 500 litre capacity tank having dechlorinated tap water and were acclimatized for 15 days. The fishes were fed twice daily with commercially available fish feed pellets throughout the acclimatization period. The water was renewed after 24 hrs daily.

Preparation of aqueous leaf extract

Nerium oleander. Linn; Family: Apocynaceae and *Carica papaya*. Linn; Family: Caricaceae plant leaves were collected from Botanical Garden of Osmania University; Hyderabad; Telangana; India and were identified by plant taxonomist, Department of Botany; Osmania University; Hyderabad; Telangana; India. Fresh leaves of both the plants were collected and washed in tap water and dried in shade for ten days. After complete drying, the leaves were pulverised to fine powder in electric blender. 5% of aqueous leaf extract was prepared by dissolving 50grams of powdered leaves in 1 litre of distilled water and kept at room temperature for 24 hrs. with intermittent shaking. After 24 hrs the mixture was filtered and the extract was used immediately in the experiment (Saravanan, *et al.,* 2010).

Determination of 96 hr LC50 and sub-lethal toxicity testing:

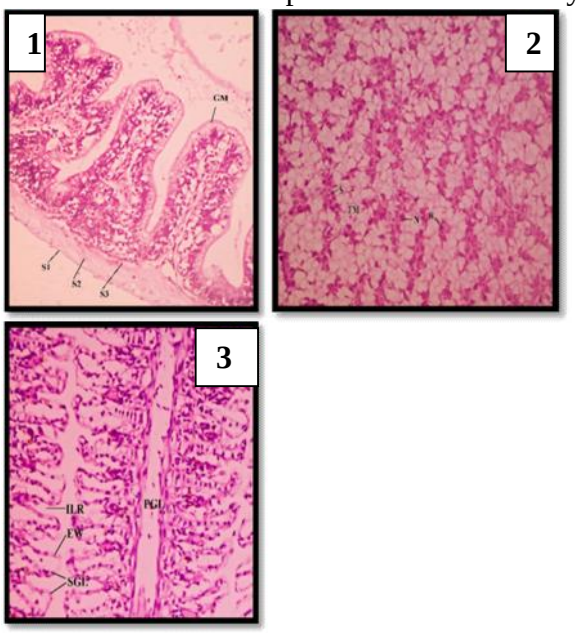
The fishes were divided into 12 groups, each group having 10 fishes in glass aquaria having 15 litres of dechlorinated tap water. 24 hrs before the commencement of LC_{50} testing the fishes were stopped giving feed. The groups 1-6 were used to study the LC_{50} of *Carica papaya* leaf extract. The concentration of leaf extract in aquaria 1-6 was 300ppm, 400ppm, 500ppm, 600ppm, 700ppm and 800ppm respectively. Groups 7-12 were used to study the 96 hrs LC_{50} of *Nerium oleander* aqueous leaf extract. The aquaria were having the leaf extract in the concentration of 100ppm, 200ppm, 300ppm, 400ppm, 500ppm and 600ppm respectively. The water was renewed after 24 hrs in semi-static method. Throughout the 96hr LC_{50} testing the fishes were observed for clinical signs like skin pigmentation, swimming patterns, response to stimuli and mortality. The 96 hrs LC_{50} value for both the leaf extracts were recorded and tested by probit analysis as described by Finney (1971).

$1/10^{th}$ of the 96hr LC_{50} value was taken as the sub-lethal concentration, i.e. 70ppm for *C. papaya* and 40ppm for *N. oleander leaf extract.* The fishes were exposed to both the leaf extracts sub-lethal concentration in group of 5 fishes for a period of 14 days. Throughout the sub-lethal exposure period the

fishes were fed with the commercial fish feed pellets twice daily and the water was renewed after 24 hrs. One group of fish did not receive any leaf extract and this group served as the control. After the completion of 7 and 14 days the fishes from both the exposure groups and also the control group were dissected and the gill, liver and intestine were carefully removed, washed in 0.9 % saline and were then fixed in 10% formalin for 24 hrs. After 24hrs the tissue were dehydrated, embedded in paraffin and sectioned at 4µm and stained with Mayer's Haematoxylin-Eosin stain. The slides were observed under light microscope at 40 x magnification and were photographed with Olympus digital camera attached to the microscope.

RESULTS AND DISCUSSION

The LC_{50} values of *Carica papaya* and *Nerium oleander* aqueous leaf extracts for the fish *Oreochromis mossambicus* after 96 hours in semi-static system was 700ppm and 400ppm respectively. $1/10^{th}$ of the LC_{50} value i.e. 70 ppm and 40 ppm were taken for *Carica papaya* and *Nerium oleander* aqueous leaf extracts respectively, to see the histopathological changes in the gill, liver and intestine after the completion of 7 and 14 days.



Figures 1-3. Normal gill (1), liver (2) and intestine (3) of *Oreochromis mossambicus*. PGL-Primary gill lamellae; S-Sinusoids; S1-Serosa; SGL-Secondary gill lamellae; H-Hepatocytes; S2-Submucosa; EW-Epithelial Wall, **SR:** Size Reduction, **DSGL:** Dilation of Secondary Gill Lamellae, **II:** Infiltration of inflammatory Cells, **V:** Vacuolation, **Dis:** Distortion, N-Nucleus; S3-Mucosa; ILR- Inter Lamellar Region; FM-Fatty Mass; GM-Gastric Mucosa Distortion, N-Nucleus; S3-Mucosa; ILR- Inter Lamellar Region.

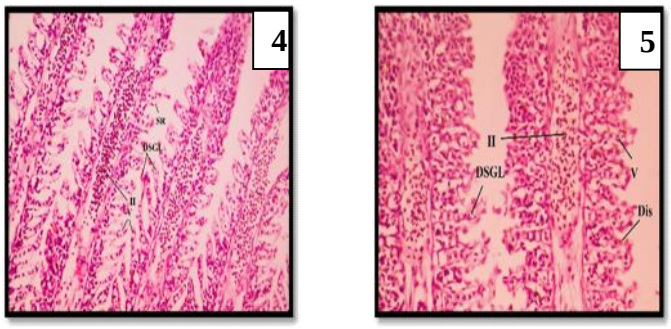


Fig 4. Gill exposed to *C.papaya*, 7 days (7) and 14 days (5).

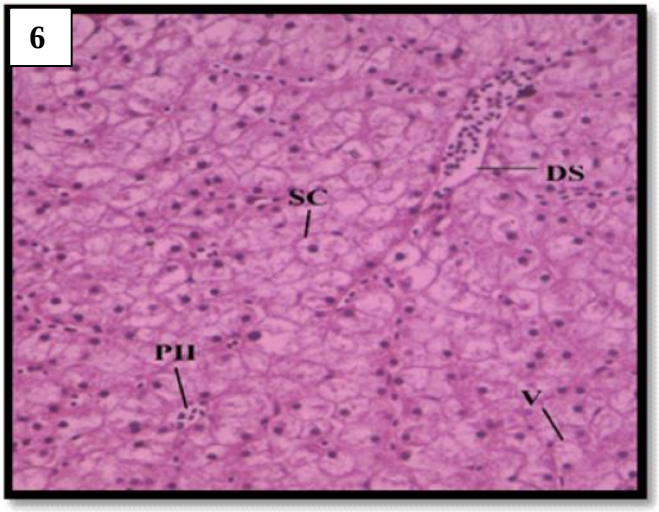
Below the operculum are found four branchial arches (Fig. 1). Each branchial arch bears two hemi-branches consisting of two rows of tapered and flattened primary gill lamellae (PGL), which lies parallel to one another and perpendicular to the arch. On the upper and lower surfaces of each PGL are a series of flattened leaf like structures called secondary gill lamellae (SGL) which form the respiratory surface. The epithelial wall (EW) of SGL is held apart and supported by pillar cells between the two adjacent SGL lies the inter-lamellar region (ILR). The normal liver shows hepatic cells arranged in cords (Fig. 2).

Hepatocytes are polygonal in shape with distinct round and centrally located nucleus. Vacuolar areas are predominant with fatty mass which is common in fish liver. Blood Sinusoids are present between the hepatic cords. Outermost layer is the serosa (S1) consisting of single layer of epithelial cells. Sub serosa or Muscularis layer (S2) consists of smooth muscle fibres arranged in definite pattern, the outer being longitudinal and inner circular. Sub-mucosa (S3) consisting of connective tissue fibres blood vessels and nerve endings. Gastric mucosa (M) is folded into a number of finger like processes- the intestinal villi, having columnar epithelium (Fig. 3).

Histological alterations in *Carica papaya* aqueous leaf extract treated fish.

After the 7 days exposure the following changes were seen (Fig.4) – dilation of secondary gill lamellae (DSGL), size reduction of SGL, massive infiltration of inflammatory cells in the primary gill lamellae and secondary gill lamellae. Vacuolation of SGL epithelial cells, degeneration of epithelial cells of SGL. The 14 day exposure to sub-lethal

concentration showed the following changes (Fig.5). Mild infiltration of inflammatory cells in PGL, mild dilation of SGL, mild vacuolar degeneration, distortion and inflammation of SGL.



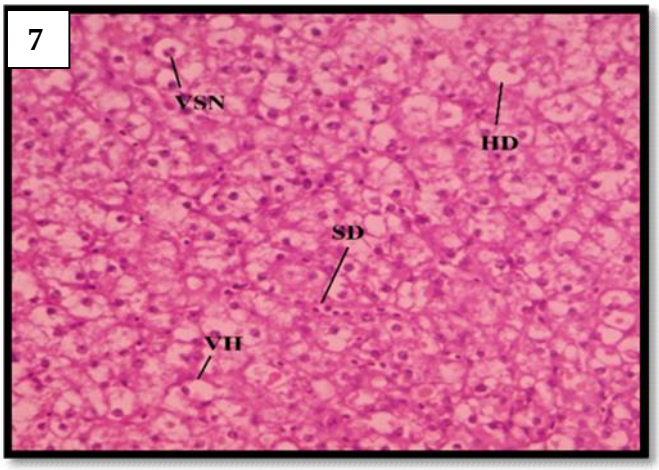
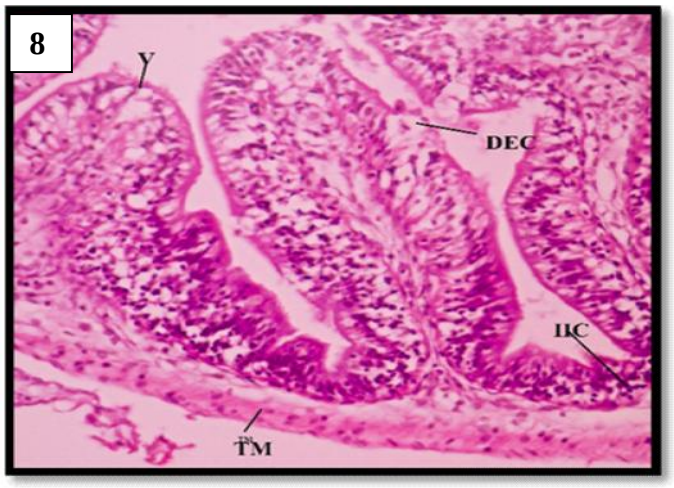
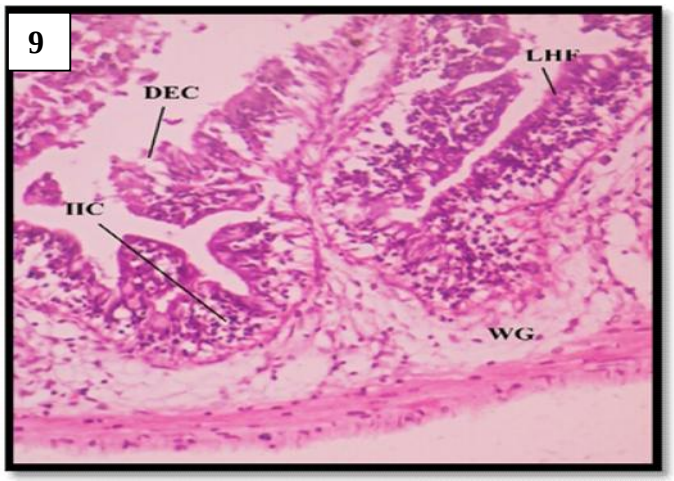


Fig 6 and 7. Liver exposed to *C. papaya*, 7 days (7) and 14 days (7). **SC:** Swollen Cells, **DS:** Dilation of Sinusoids, **PII:** Pockets of Infiltration of Inflammatory Cells, **VSN:** Variously shaped Nuclei, **HD:** Hydrobic degeneration, **VH**: Vacuolar Hepatocytes.

The 7 day exposure shows (Fig.6) dilation of Sinusoids, swelling of cells with dark nuclei and nuclear abnormality, vacuolated hepatocytes, mild pockets of infiltration of inflammatory cells. The 14 day exposure shows (Fig.7) vacuolated hepatocytes with various shaped nuclei, Sinusoids showing mild dilations more of hydrobic degeneration (nucleus moved to corner with granular cytoplasm).

Intestine





Figs 8, 9. Intestine exposed to *C. papaya*, 7 days (8) and 14 days (9). IIC: Infiltration of Inflammatory Cells, TM: Thin Muscularis, V: Vacuolation, DEC: Disruption of Epithelial Cells, WG: Wide Gap, LHF: Loss of Histological Features.

The 7 day exposure (Fig.8) showed thin muscularis, inflammatory cells, moderately present at the base of the villi, disruption of epithelial cells, and moderate change in tip of villi. The 14 day exposure shows (Fig.9) wide gap between muscularis and villi, disruption of epithelial cells, loss of histological features and mild infiltration of inflammatory cells.

Histological alterations in Nerium oleander aqueous leaf extract treated fish

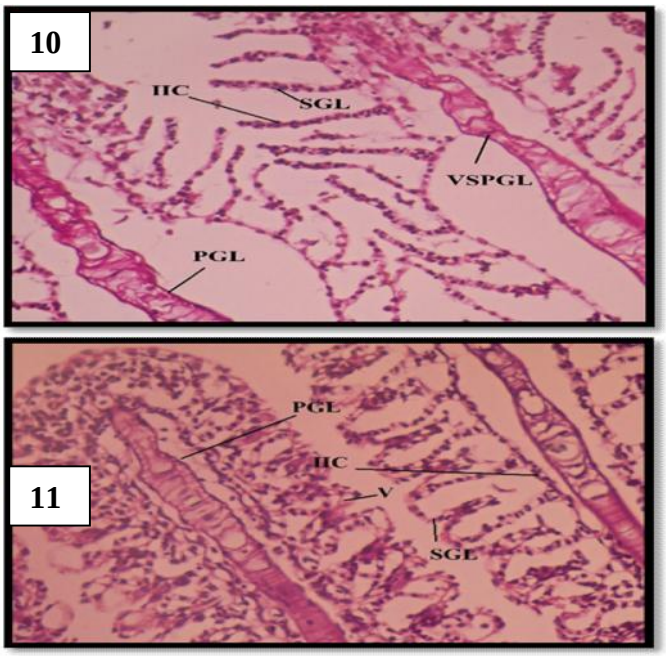


Fig 10 and 11. Gill exposed to *N. oleander*, 7 days (10) and 14 days (11). **IIC:** Infiltration of Inflammatory Cells, **PGL:** Primary Gill Lamellae, **SGL:** Secondary Gill Lamellae, **VSPGL:** Vacuolated and shrunken Primary Gill Lamellae, **V:** Vacuolation.

The 7 day exposure showed (Fig.10) massive loss of architecture of gill lamellae, primary gill lamellae shrunken and vacuolated, massive dilation between the central cord of PGL and SGL, mild infiltration of inflammatory cells throughout SGL. The 14 day exposure shows (Fig.11) vacuolar degeneration in the centre of PGL, severe vacuolar degeneration in SGL and massive infiltration of inflammatory cells in SGL.

Liver

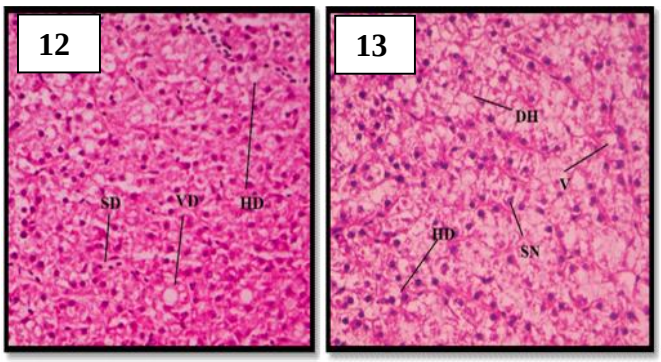


Fig 12. Liver exposed to *N. oleander*, 7 days (12) and 14 days (13). **HD:** Hydrobic Degeneration, **VD:** Vacuolar Degeneration, **SD:** Sinusoid Dilation, **DH:** Disruption of Hepatocytes, **SN:** Swollen Nuclei.

The 7 day exposure shows (Fig.12) moderate to severe vacuolar and hydrobic degeneration of hepatocytes and sinusoids dilation. The 14 day exposure shows (Fig.13) cells with swollen nuclei and multinucleated cells, complete disruption of hepatocytes and vacuolar degeneration.

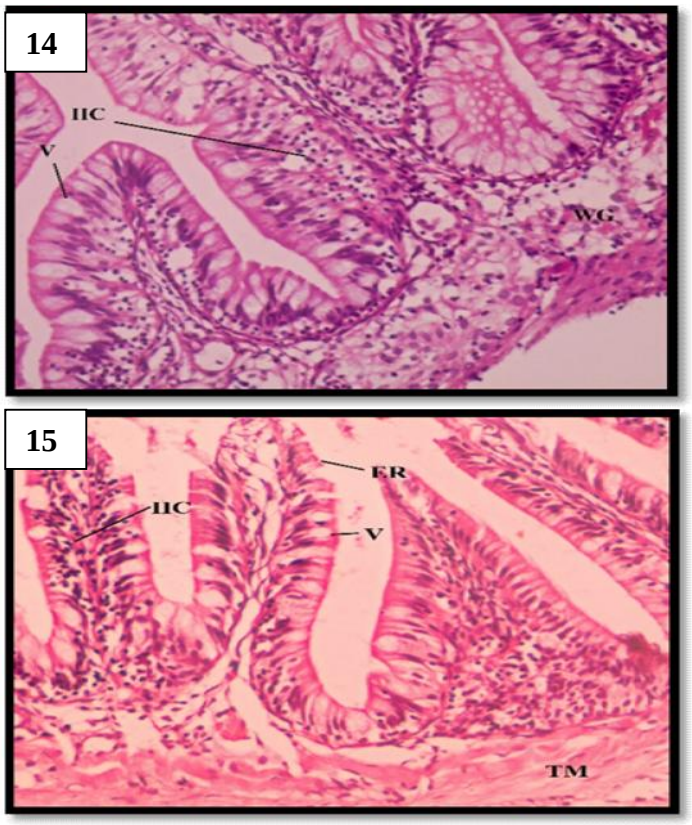


Fig 14 and 15. Intestine exposed to *N. oleander*, 7 days (14) and 14 days (15). **V:** Vacuolation, **IIC:** Infiltration of Inflammatory Cells, **WG:** Wide Gap, **ER:** Epithelial Rupture, **TM:** Thick Muscularis.

The 7 day exposure shows (Fig.14) wide space between muscularis and villi and infiltration of inflammatory cells in the epithelium. The 14 day exposure shows (Fig.15) thick and muscularis and infiltration of mild fibrous tissue at the base of villi. Severe vacuolar degeneration of epithelial cells of villi. Gill is the first site of osmoregulation and respiration in aquatic vertebrates. It is the main target organ which gets affected easily when the organism is exposed to any pollutant or toxic substance. Pathological changes like dilation of SGL infiltration of inflammatory cells and vacuolar degeneration of SGL epithelium increased at the 14 day of exposure in both the cases of *Carica papaya* and *Nerium oleander* aqueous leaf extract and was more severe in case of *N. oleander* leaf extract exposure. This type of similar transformation in the gills were also noted by Pandey, *et al.* (1993) DDT

treated *Liza parsia*, Ramamurthy, *et al.* (1987) methyl parathion treated *Cyprinus carpio*, by biopesticide Azardirachtin in *Clarius gariepinus* (Tennyson, *et al.*, 2008) and endosulfan in mosquito fish *Gambusia affinis* (Cengiz and Unlu, 2002). The secondary gill lamellae became shapeless and were broken at some places. The observation were in agreement with the results of Tilak and Yacobu (2002) fenvalerate treated *C. idellus*.

Liver is the first organ to face any foreign molecule through portal circulation and is subjected to more damage (Jayanth Rao, 1982). Liver is an important organ of detoxification which breaks down toxic substances and metabolites of administered substances. This breakdown is carried out by endoplasmic reticulum of hepatocytes. Due to this reason probably the hepatic cells are severely damaged. The liver includes several pathological changes like degeneration of blood vessels, sinusoid dilation, vacuolization, hydrobic degeneration, necrosis, variously shaped nuclei and swollen nuclei. The present study showed dilation of sinusoids, vacuolated hepatocytes, hydrobic degeneration, swollen nuclei and variously shaped nuclei. These changes were seen to lesser extent in 7 day exposure and were prominent in 14 day exposure of both the leaf extracts. The investigations by many other workers showed the above changes in the liver like the work done by Pandey, *et al.* (1996) in the liver of estuarine mullet, *Lizaparsia* exposed to DDT, in *Puntius ticto* by Sahai and Suneeta Singh (1989) due to BHC, lindane and malathion toxicity have been reported. Magar and Afsar Shaikh (2013) observed several histological changes in hepatic cells in *Channa punctatus*.

Histopathological changes in intestines due to pesticides and other toxicants are observed by a number of workers. The present study showed vacuolated epithelial cells inflammation of epithelial cells and disruption of epithelial cells. The changes were severe on the 14^{th} day in both the cases. The changes in the intestine were in agreement with the observations of Wagh and Throat (2001) and Banaee, *et al.* (2013). Suchismita and Abhik Gupta (2013) have also observed similar pathological changes in the intestine of Malathion treated *Esomus danricus*.

The present study showed that histopathology is a useful biomarker for environmental contamination. The plant extracts being used as molluscicides and pesticides and also as piscicides are toxic to the freshwater fishes which constitute the non-target organisms, causing large number of changes in the gill, liver and intestine. *Nerium oleander* was more toxic than *Carica papaya*. Therefore the present study might be of help to establish the safer usage of aqueous extracts of *C. papaya* and *N. oleander* in the agricultural field and aquaculture farms.

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