The aquatic environment is confronted with the problems of pollution chiefly orchestrated through anthropogenic activities in and around water bodies. Municipal discharges, agricultural run-offs and industrial discharges are amongst the major sources of pollutants to the aquatic ecosystems. These varied discharges are gotten from both point and non-point sources which are either discharged directly or indirectly into water or ultimately through run-offs and seepages (Singh, 2007; Samuel et al., 2015). Organisms in aquatic environments are usually exposed to a complex mixture of chemicals including parent compound and their transformation products causing multiple damages at the organisms, population and ecosystem level, in organ function, reproductive stages and biological diversity (Vorosmarty et al., 2010; Ginebreda et al., 2014). The application of environmental toxicological studies on non-mammalian vertebrates is rapidly expanding, and for aquatic systems, fish have become the ideal indicators for the evaluation of the effects of noxious compounds (Ervenest, 2007; Khidr and Mekkawy, 2008).

Azadirachtin, a biopesticide derived from neem (Azadirachta indica) is used to control a wide variety of insect pests of agricultural and medical importance. The tissues of *Clarias gariepinus* liver was exposed to a sublethal concentration of 0.247ppm azadirachtin for a duration of 5, 10 and 15 days. The histological investigations showed high toxic effects which increased with increase in duration, alterations in the treated liver tissues showed hexagonal hepatocytes, haemorrhage, hepatocytic necrosis, capillary spillage, distortion and disintegration of hepatocytes, and occurrence of cell division without cell membrane which was devoid of nucleus resulting in loss of natural hepatocyte architecture. All these histopathological observations indicated that exposure to sublethal concentrations of azadirachtin caused destructive effect in the liver tissues of *Clarias gariepinus*.

Key words: *Clarias gariepinus*, liver, azadirachtin, histopathology
This fish was utilized as the model organism in this study, since it has a well-documented general biology; easy culture; fast growth rate; and year round reproduction since all these characteristics are to be considered as suitable test organism for toxicity test. This fish is more tolerant to environmental stresses and there is a paucity of experimental results about the histopathological impact of azadirachtin on its tissues. Histopathological alterations caused by plant based pesticides in fishes have been reported (Abraham et al., 2003; Mondal et al., 2007). The organ most associated with the detoxification and biomarker process is the liver due its function, position and blood supply. The corresponding author has reported the impact of biopesticide azadirachtin on its gill tissues (Samuel et al., 2008) and in continuation to the work, the present investigation was carried to study the impact of this biopesticide on the liver tissues of this fish.

MATERIALS AND METHODS

Clarias gariepinus was collected from Poondi reservoir, Tiruvallur district, Tamil Nadu, India (13°11’6’’N 79°51’36’’E) situated 60km away from the Chennai city. Fishes were acclimated for a couple of weeks to laboratory conditions and kept in aquaria (30 litres) under light-dark (12:12 hours) cycle and were fed ad libitum with artificial pellet feed in the Department of Advanced Zoology and Biotechnology, Loyola College, Chennai, Tamil Nadu, India. Before stocking the fish, the glass aquaria were thoroughly cleaned with potassium permanganate to free the aquaria from fungal infection. During the period of acclimatization and experimentation, the water used was clear dechlorinated ground water whose physicochemical characteristics were maintained throughout the experimental study as per APHA (1998) and was renewed on alternate days. On an average basis, the fish weighed 16.8 ±0.35g and their length was in the range of 12.1 ±0.8cm. Screening of fish was done often to detect any symptoms of disease, physical damage and mortality. The dead, injured and severely infected fishes were discarded immediately. Feeding of fishes were stopped 48 hours prior to the commencement of the experiment with a view to avoid any possible change in situ in the toxicity of the pesticide.

Commercial grade, azadirachtin (Star Neem 3% EC) was used in this study. Renewal toxic test methods (APHA, 1995) were done to find out the 96 hour LC_{50} concentration. Fishes maintained in pesticide free water served as control. Fishes for experimental purpose were exposed to 1/10th of the 96 hour LC_{50} for a period of 5, 10 and 15 days. The toxicant was added into the test tank containing ten litres of water and twenty fishes. Ten replicates were maintained. Fishes showing no signs of respiratory movement and response to tactile stimuli were considered dead and removed immediately. Percent mortality was calculated and the values were transformed into probit scale. Probit analysis was carried out as per Finney (1971). Regression lines of probit against logarithmic transformation of concentrations were made. Slope function and confidential limits (lower and upper) of the regression line was also performed (UNEP/FAO/IAE, 1987).

After exposure to azadirachtin, the fishes were sacrificed and the liver tissues of the control and experimental groups were dissected out and dropped in aqueous Bouin’s fluid for 24 hours which were then subjected to histological techniques. (Pearse, 1985). Sections of 4-6µm thickness were prepared from paraffin blocks using a rotary microtome and sections were fixed on albuminoid slides for 24 hours, and treated for staining with Ehrlich’s haemotoxylin and as per procedure and mounted with Distyrene Plasticizer Xylene (DPX). Tissues of liver were examined and photographed at lower and higher power of magnification using Leica photomicroscope.

RESULTS AND DISCUSSION

The susceptibility of Clarias gariepinus to the toxic effect of azadirachtin observed as percent mortality increased with increase in concentration. Mortality in controls was absent. The 96 hour LC_{50} was 2.47ppm (95% confidence limit ranged from 1.6255 to 3.7532ppm). The microscopic study of the control liver revealed its normal architecture characterized by normal blood capillaries and hepatocytes. The treated liver exhibited formation of hexagonal hepatocyte followed by haemorrhage and hepaticoytic necrosis after five days of exposure. Incessant necrosis and haemorrhage occurred highlighting capillary spillage and distortion and disintegration of hepatocytes after ten days of exposure. After fifteen days of treatment, cell division occurred without cell membrane, devoid of nucleus. Necrosis prolonged resulting in loss of natural hepatocyte architecture (Fig. 1).

Aquatic toxicology still is an evolving discipline which has resulted from concern for the safety and preservation of the aquatic environment. The evaluation may include the potential human health posed by contamination of commercially important aquatic species with pesticides. Biopesticides have
been one of the most effective weapons discovered by man to protect agricultural produce from the attack of insect pests. But its extensive usage has been a constant threat at the aquatic life by impacting the habitat, behaviour pattern, growth and nutrition value, reproductive potential, cellular morphology and physiology (Bagchi et al., 1990; Rahman et al., 2002). Although there are considerable research activities in the field of biopesticides there is a wide variation in the information available concerning the effect of particular biopesticides on selected non-target organisms. Among those studies, fish have drawn more attention as they are ideal biological indicators of the aquatic environment alterations induced by pollution and because of their economic importance.

Toxicity is a characteristic feature of an individual organism’s response to a chemical at a particular concentration for a specific period of time. Acute and chronic toxicity tests are mostly used to assess the toxicity of chemicals on non-target organisms (Santos et al., 2010). The 96 hour LC50 is customary for evaluating the toxic effects of pollutants and to represent the lethality of a test species in terms of mortality and time (Nwani et al., 2015). Data obtained in acute toxicity tests are generally used to compare the susceptibility of different fish species and the potencies of different toxic pesticides using LC50 values and to derive safe level concentrations (Haber and Schmitt, 1988). In general, toxicity of chemicals to aquatic organisms has been reported to be affected by temperature, pH, dissolved oxygen, size and age, strain of species, water quality, concentration and formulation of test chemicals (Nwani et al., 2010; Boran et al., 2012; Rauf and Arain, 2013). The magnitude of toxic effects of pesticides also depends on the length and weight, corporal surface/body weight ratio and breathing rate (Murty, 1986). The toxicity and LC50 values differ from species to species for the same and for different pesticides due to their mode of action on fishes (Applegate and King, 1962) and in the present study, the LC50 values of azadirachtin ranged from 1.37 to 2.47ppm for 24 to 96 hours. Further, differences in LC50 values may be understood in terms of altered physiochemical properties of the holding water and size of the test animal (Stendahi and Sprague, 1992) and species specificity (Rand and Petrocelli, 1985).

Histopathological studies help to establish casual relations between exposure to contaminants and various biological responses and have proven to be a sensitive tool used in detecting direct effects of chemical compounds within target organs of laboratory experiments (Altinok and Capkin, 2007; Boran et al., 2012). The importance of the study of histological changes brought about under the stress of different toxicants in the different organs and organ systems have been well documented (Konar, 1970) and from the present study, it is evident that the biopesticide induced many lessons in the liver tissues of Clarias gariepinus because pesticides enter into the blood circulation of the fish through gills of mucous epithelium of the mouth and finally finds their way to different tissues of the body where they affect normal metabolism. Histopathological changes in the liver of freshwater fishes caused by pesticides/insecticides intoxication have been well recorded by Gill et al.,(1980) which corroborates with the present study. Abraham et al., (2003) studied the histopathological effect of neemax (neem based biopesticide) on the liver of Anabas testudineus. The histopathological lessons elucidated by neemax were hypertrophy, hyperplasia, rupture and lifting of the epithelium, appearance of pyknotic nucleus, aggregation of hepatic cells, infiltration of lymphocytes and overall necrosis of the tissue. Further, Siddiqui et al., (2013) reported that a neem based biopesticide altered the biochemical concentrations of Glutamate Oxaloacetate Transaminase (GOT) and Glutamate Pyruvate Transaminase (GPT) enzymes in the liver of Clarias batrachus. Eller (1971) reported vacuolization in liver cells when exposed to biopesticides and other changes included liver cord disarray, damage of connective tissue, enlargement of liver cells and their nuclei. The typical response of hepatic cells to toxic compounds is indicated by the appearance of vacuoles in the cytoplasm and hypertrophy of hepatic nuclei which was observed in the present study. In general, liver is a target organ due to its large blood supply that causes noticeable toxicant exposure and accumulation and also its clearance function and its pronounced metabolic capacity (Mohamed, 2009) and numerous categories of liver pathology are present as reliable biomarkers of toxic damage (Marchand et al., 2009; Deore and Wagh, 2012; Reddy and Baghel, 2012).

Elimination of the absorbed pesticide may occur simultaneously with its absorption. Gill, liver and intestine are the main sites of metabolism and filtration, and play a major role in degrading these pesticides through different metabolic activities (Allison et al., 1963). Liver is a vital and is the largest visceral (internal) organ of the digestive system present in fish (Romer and Parsons, 1977). It is the principle site for
metabolism and detoxification in vertebrates (Bhattacharya and Mukherjee, 1976) and is principally involved in biotransformation of xenobiotics (Lerapetritou et al., 2009). Liver has a wide range of functions, including detoxification, protein synthesis and production of biochemical necessary for digestion. Nero et al., (2006) reported that the liver tissue damage occurs when it is constantly exposed to toxicants and it is more liable to injury from toxicants. One of the important functions of liver is to eliminate toxicants through metabolism. Hence, the liver becomes hyper-active to eliminate the toxicants. 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. Similarly, the liver tissue will try to avoid such intoxicant being absorbed for which the epithelial tissues will lift up to avoid the toxicants. Olurin et al., (2006) reported that the shrinkage of the hepatic cells can result in contraction of the blood vessels, thereby impeding the portal flow through the liver which negatively affect the normal physiological necessary for the functioning of the liver. Osmoregulatory dysfunction from toxins has been been responsible for necrosis of hepatocyte. Further, the length of exposure of toxicant to an organism determines the severity of injury on the organs of the fish (Ferguson, 2006). Hence, exposure of Clarias gariepinus to azadirachtin beyond 96 hours may therefore inflict further noticeable pathological changes in the hepatocyte.

Amongst various physiological responses, respiratory distress is one of the early symptoms of pesticide poisoning in case of an aquatic organism like fish. Exposure to sublethal concentrations of pesticides have been reported to increase respiratory activity resulting in increased ventilation and hence increased uptake of the pesticide. Increase in swimming, gasping for air, loss of balance and reflexes are some of the behavioural characteristics which can serve as biosensor in fish under the influence of a toxicant (Chindah et al., 2004; Bobmanuel, 2006). These toxicity induced signs were observed in this study and may have resulted from the adaptive response of the fish to the toxic stress imposed by azadirachtin in the present study. The severity of the symptoms was in accordance with the nature of the toxicant, dose and duration of exposure (Galat et al., 1985; Tilak et al., 2005). Hence, impairment of organs by the effect of the pathological changes in the exposed fish will have grave consequences with respect to the normal fish as reported by Fatma (2008). All these histopathological observation indicated that exposure even to sublethal concentrations of azadirachtin caused destructive effects on the liver of Clarias gariepinus.

CONCLUSION

The aquatic environment provides a sink for many environmental contaminants some of which have the potential to cause oxidative stress in aquatic organisms (Amaeze et al., 2014). Biopesticides when used close to waterways are usually washed into the aquatic environment where they hamper aquatic life with high residue accumulation in tissues of aquatic organisms (Ojutiku et al., 2012; Gill and Garg, 2014). However, plant based insecticides can suitably edge out their synthetic counterpart due to their biodegradability (Ajani and Ayoola, 2010).

REFERENCES

HISTOPATHOLOGICAL RESPONSES OF LIVER TISSUES OF THE AFRICAN CATFISH CLARIAS GARIEPINUS


Fig. 1. Histopathological changes in *Clarias gariepinus* liver tissues exposed to azadirachtin
A: Liver control; B & C: Liver treated 5 days;
D & E: Liver treated 10 days; F & G: Liver treated 15 days.
Bic: Blood capillaries; He: Hepatocytes; Hh: Hexagonal hepatocytes; Ha: Haemorrhage;
Hen: Hepatocytic necrosis; Ne: Necrosis; Cas: Capillary spillage;
Ddh: Distortion and disintegration of hepatocytes; Cdc: Cell division without cell membrane;
Nfa: Nucleus formation absent; and Nhal: Natural hepatocytic architecture lost.