1. INTRODUCTION

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Introduction :

In recent years natural toxins, mostly of plant origin, are used to clear the ponds from the weed fishes and other animals, prior to their use in pisciculture. Rotenone (derived from Derris root) is most commonly used for this purpose all over the world and its mode of action has been studied in detail (Horgan et al., 1968). With the rapid increase in pisciculture it has become necessary to find out alternative piscicides because rotenone supply is not enough to meet its fast growing need. In India more than hundred plants have been reported to contain piscicidal components (Chopra et al., 1949; Babu, 1965; Bhuyan, 1967, 1969; Chakraborty et al., 1972; Nandy and Chakraborty, 1976; Kanasuki and Ohata, 1976; Ramanujam and Ratha, 1980; Nanaware and Harold, 1987a-c; Harold, 1987; Bhosale, 1988 and Patil, 1988).

Most of the earlier investigators have studied the impact of synthetic and natural piscicides on the mortality and histology or histochemical alterations of the mucosubstances in the target tissues of the fishes. But haematological and enzymological studies using the natural piscicides of the plant origin are meagre. The following survey on this aspect gives the exact idea and correct status of such studies in the toxicological literature.

A) A brief survey of haematological and enzymological studies on the fish during intoxication due to synthetic and natural piscicides :

1) Haematological studies

i) Metal toxicity :

It is well documented fact that the metal toxicity in fish produces alterations in the various haematological parameters. Among the metals, mercury toxicity has been investigated by number of investigators. Hilmy et al. (1980) have investigated haematological responses to mercury toxicity in the marine teleost Aphanis dispar. They noted a significant decrease in RBC count, Hb content and haematocrit values. There was greater increase and quite a different pattern of leucocyte number as compared with RBC. Erythrocyte fragility was not changed during exposure. In this study increased clotting time and TWBC count were indicative of stress created due to exposure to Hg. Mercury toxicity initially produced fall in the RBC count and Hb content but after 2-3 weeks an increment occurred in both the indices in another teleost fish Punctius conchorius (Gill and Pant, 1981). The incipient decrease was found due to haemolysis whilst subsequent recovery and then a rise in the RBC number was due to enhanced erythropoisis which was triggered as a typical stress response. Mercury salt ($HgCl_2$) at sublethal (0.1 ppm) as well as at lethal (3.5 ppm) doses affected haematological variables like RBC number, Hb and packed cell volume in fresh water teleost Tilapia mossambica and marked a significant decrease in them in experimental fish (Naidu et al., 1984). Such decreased values in TRBC count, Hb content and small lymphocyte count were also observed recently in

fish Heteropneustes fossilis on exposure to mercury sulphate (Banerjee, 1986). But in this study, total leucocyte count along with large lymphocytes and basophil counts was increased. And the clotting time in acclimatized fish was considerably decreased after exposure to 3.0 mg/l (24 h LC_{50}) mercuric sulphate for 24 hrs.

Cadmium sulphate ($CdSO_4$) at sublethal concentration (100 mg/l) evoked lymphocytosis, thrombocytosis, with concomitant hyper-coagulability of whole blood and anaemia with simultaneous regeneration of red blood cells after 90 hrs post treatment in fish C. fasciatus (Srivastava and Mishra, 1979).

The effect of lead on erythropoiesis and ultrastructural changes of erythroblast in the catfish Ictalurus melas has worked out by Carla and Martelli (1979). The changes in various haematological parameters led to establishment of microcytic, hypochronic anaemia. Ultrastructural investigations revealed that the initial effect of Pb toxicity was the fragmented and reduced cristae of erythroblast mitochondria, an increase in ribosome density and decrease in polyribosome density. At 1 M concentration of Pb, intermediate and late erythroblasts were the most damaged elements which evidently proved to slow maturation of erythroblast. According to Tishinova (1982) Pb at 1 and 8 mg/l concentration for 1.5 and 15 days produced erythropenia and morphological changes in erythrocytes in the peripheral blood of a carp. The lymphocyte count was decreased even after 1 day treatment. Whereas neutrophils were increased at 8 mg/l and 1 mg/l concentration of Pb after 1 day and 15 days, respectively.

Expósure of fresh water fish Colisa fasciatus to manganese sulphate at 2500 mg/l for 90 hrs produced a significant decrease in TEC as well as in number of erythrocyte of all the types. The exposure also evoked leukocytosis due to an increase in the number of small lymphocytes. Other haematological characteristics i.e. total count of large thrombocytes, distribution of immature red blood cells, haematocrit, erythrocyte sedimentation rate, haemoglobin and clotting time did not differ significantly from the control (Agrawal and Srivastava, 1980).

Very recently, the effect of hexavalent chromium on the haematological parameters in fish Clarias batrachus has studied by Agarwal et al. (1985). The results indicated the significant decrease in RBC and TL C leading to erythropenia and leucopenia, decrease in Hb and PCV after chromium treatment and the fish suffered from acute anaemia.

ii) Chemical toxicity :

Exposure to pentachlorophenol (PCP) produced a significant increase in the haematocrit and haemoglobin values in fish Angula angula (Holmberg et al., 1972). These changes were habitat dependent. In sea water, increased values persisted for long time during the entire recovery period. But in freshwater increased haematocrit value persisted after transfer to clean water only. Very significant observations were recorded by Madhyastha and Nayak (1979) on the effect of sodium lauryl sulfate (SLS) on the haematological parameters of Rasbora daniconius. No changes were found in TRBC and TWBC counts after 7, 14 and 21 days exposure to SLS. However, significant decrease in the number of mature RBCs and increase in number of immature RBCs, smudge cells, small lymphocytes

and large lymphocytes were observed in treated fish after 14 days. These changes were also observed after 21 days exposure and in addition to this increase in neutrophils and some changes in the morphology of RBC were evident. Exposure for 28 days marked a significant increase in immature RBCs, smudge cells and differential counts of neutrophils and large lymphocytes and decrease in differential counts of the small lymphocytes and leucocytes. At this stage, distinct morphological changes in the RBCs were observed and fish contained many microcytes and elliptocytes and suffered from haemolytic anaemia.

Erythrocytic abnormalities were also observed due to urea stress in Cirrhinus mrigala fingerlings (Srivastava and Sriwastawa, 1980). The abnormalities included the change in shape of RBCs from oval to spherical the nuclear hypertrophy, bursting of RBC due to which segregation of pink nuclei and agglutination of erythrocytes. During the process of rupture, the erythrocytes were acquired different shapes and changes in colour of their nuclei ranging from dark brown red to pink.

The static exposure to malachite green showed chronic leucopenia in rainbow trout Salmo gairdneri (Hcauek and Bulkley, 1981). During this study, the investigators recorded the major changes in the fish including decline in total leucocytes-thrombocytes counts. Exposure of fish to higher concentrations, developed thrombocytosis during the first 24 hrs and lymphopenia and neutrophilia after the 4th post-treatment day. According to them, the changes in the leucocytes were probably due to a nonspecific vertebrate stress syndrome, rather than of a specific leucocytotoxic effect of this chemical.

The effects of sublethal concentrations of pesticides- rogar, benomyl, pomuran and domatol on haematological parameters were studied by Frank (1981). 96 hrs LC_{50} values of rogar, benomyl and pomuran could produce higher RBC count, haematocrit and haemoglobin concentration but decrease in the leucocyte count. Alterations in the erythrocyte shape were also observed in this study.

Increase in the TLC, MVC, MCH and decrease in erythrocyte count and Hb percentage were observed after exposure to alachlor in fresh water fish C. batrachus (Goel et al., 1984). But the effect of sublethal concentration of metasystox led to decrease in WBC count, MCV, MCH and increase in RBC and PCV in another fresh water fish Channa striatus (Natrajan, 1984).

Banerjee and Verma (1988) have reported the effect of three pesticides-thimet, elsan and bazanon on erythrocyte and leucocyte morphology of Channa punctatus. In erythrocyte, observed hypochromasia, vacuolization and deformities in shape and in its nucleus were taken as immediate effect of these pesticides. Hypochromasia was also observed in leucocytes but there was no pronounced change in its shape or in the shape of its nucleus.

Very recently, the effect of BHC on haematology of an air breathing fish C. batrachus was investigated by Thakur and Pandey (1989). Exposure to various sublethal concentrations of BHC led to decrease in erythrocytes diameter, Hb concentration, PCV, morphological variations in RBCs and haemolysis after 96 hrs intoxication.

iii) Plant product toxicity :

Certain piscicidal compounds in the plant derivatives have been reported to affect the different haematological parameters like erythrocyte number, haemoglobin concentration, packed cell volume, mean corpuscular volume, erythrocyte haemoglobin and haematocrit (Anonymous, 1966; Svobodova, 1971). Saponin is one of the plant derived piscicides known to cause haemolysis and consequently affecting the haematological parameters in fish (Anonymous, 1952; Chopra et al.,1958). Ramanujam and Ratha (1979) did not find haemolytic factor such as saponin or rotenone in the chemical analysis of fruits of Zanthoxylum armatum. Therefore, the values like RBC number, Hb concentration and Hb/RBC ratio of control and treated fish were not changed in fish H. fossilis after exposure to alcoholic extract of Z. armatum fruits. But dietary T-2 toxin at the levels of 72.5 mg/Kg depressed the haematocrit and blood haemoglobin concentrations in the rainbow trout Salmo gairdneri (Coffin and Combs, 1982).

2. Enzymological Studies :

i) Metal toxicity :

Lead nitrate treatment to fish H. fossilis caused marked inhibition in the activities of glucose-6-phosphatase, lactic dehydrogenase, pyruvate dehydrogenase and succinate dehydrogenase in most of the tissues (Sastry et al., 1987).

ii) Chemical toxicity :

Koundinya and Ramamurthi (1982) observed increase in the alkaline phosphatase activity after exposure to sumithion in fish S. mossambica. The increase was recorded in gill, kidney, liver, muscle, intestine and brain. Sumithion also induced hyperglycemia as a result of glycogenolysis. According to these two investigators such changes were to meet energy requirements in order to counter the stress. Natrajan (1984) reported that metasystox treatment to air breathing fish, C. striatus decreased activities of selective oxidative enzymes, like succinic dehydrogenase and increased activity of lactic dehydrogenase in gill, brain, muscle, liver and kidney. Exposure of fish Brachydanio rerio to melathion decreased acid and alkaline phosphatase activities of the liver (Kaushal and Ansari, 1986).

iii) Plant products toxicity :

Ramanujam and Ratha (1982) noted the concentration dependent inhibition due to fruit extract of Z. armatum in the activity of acetylcholine estarase (AchE) in the brain and muscle of H. fossilis and P. shalynius. However, the rate of inhibition of AchE activity in vivo reached a platue at higher concentrations of the extract and was tissue specific. During lethal phase of the treated fish, the maximum inhibition of enzyme was 54% in the brain and 38% in the muscle.

3. Other Studies :

i) Behavioural responses :

Exposure to DDT caused evoked and spontaneous activity in the rainbow trout due to disturbances in the lateral line nerve (Bahr and Bali,

1970). After 1 hr exposure, fish became hyperexcitable and demonstrated periods of violent unco-ordinated swimming movements with very apparent erratic eye and jaw movements. These symptoms increased severity for about three hours. By the end of 6 hr exposure period fish demonstrated erratic eye movements, opercular trembling and occasional trembling in the trunk. Lal and Vohra (1974) also observed the effect of DDT but on fish P. titco. They observed marked momentary excitation, after which a long period quiesency. Quiesency was accompanied by a gradual retardation in the rate of opercular movements. The quiesency was followed by fish which attempted to move on the surface of the water and swam with the belly upwards. Body became inverted and the fish floated on the surface for sometime and finally settled down at the bottom of the trough. Fish was completely anaesthetized and did not exhibit any response towards stimuli of exterior movement, sound and touch. The moribundity was visualized by the absence of any motion and respiration. Mouth and operculum remained distended. Ramanujam and Ratha (1982) reported the behavioural responses in H. fossilis and P. shalynius after treatment of fruit extract of Z. armatum. In the initial phase of treatment, the fish became more active and then showed eratic movements. The opercular movements also increased along with the activity of fish. As the fish approached lethal phase, their opercular movements decreased, lost their equilibrium and finally collapsed in an upside down posture at the bottom of jar.

Recently, a group of researchers from this department has done considerable amount of work on the behavioural responses of fish

S. mossambica during intoxication due to natural piscicides from the plant origin. Harold (1987), Bhosale (1988) and Patil (1988) have shown altered feeding responses, decreased sensitivity to external stimuli like light, touch, sound, vibrations, etc., change in skin colour, erratic movements and changed respiratory movements after exposure to ethanol extracts of leaves of Lasiosiphon eriocephalus, fruits of Sapindus laurifolius and the fruits of Acacia concinna, respectively.

ii) Pathological alterations :

Histopathological alterations in the target tissues of fish like liver, kidney, gill and brain in response to synthetic and natural piscicides have been recorded. Dalela et al. (1979) have reported histological damage of gill such as separation of epithelium from the basement membrane, fusion of adjacent gill lamellae and loss of cell membrane in C. gachua or subacute exposure to endosulfan. The effect of dietary pyrrolizidine (Senecio jacobaea) alkaloid led to severe hepatic lesions including megalocytosis, necrosis and fibrosis in centrotubular and hepatic veins in the liver and significant thickning of glomerular basement membranes in the kidney of S. gairdneri (Hendricks et al., 1981). Toxic effects of cadmium in aquatic medium on the kidney of C. punctatus depended on dosase and period of exposure. In such treatment the cells of proximal tubules were affected first and then the injury spread to the glomeruli, hematopiotic tissue and other parts of tubules (Dubale and Shah, 1982). At sublethal and lethal concentrations, $HgCl_2$ affected the liver and intestinal tissue of S. mossambica and caused enlargement of blood sinusoids, vacuolization, granular degeneration of hepatocytes, edema,

focal necrosis and proliferation of fibroblasts (Naidu et al., 1983). Kumar and Pant (1985) reported the effects of monocrotophos on kidney of P. conchonius. According to them acute toxicity of this compound led to swelling and enlargement of glomeruli and tubules within 24 hr and caused necrosis at some places in the renal tubules. Similar to above observations histopathological alterations during intoxication due to natural piscicides have been noted in liver, kidney, gill (Harold, 1987; Bhosale, 1988 and Patil, 1988) and intestine (Bhosale, 1988 and Patil 1988) of freshwater undesirable fish T. mossambica.

iii) Histochemical mucin changes :

The distribution and chemical nature of acidic and neutral mucins in the various parts of the alimentary canal, liver, kidney, gill and brain of fish have been investigated by many investigators (Sastry and Sharma, 1979; Sastry and Malik, 1979; Joshi and Desai, 1982; Chatterjee et al., 1983; Sinha et al., 1988 and Mane, 1989). These investigators have studied the toxic effects of different pesticides on various tissues of fish as revealed by histopathological and histochemical studies. Some investigators have revealed the mucin changes in response to different seasons or during the growth of fish (Sinha et al., 1988; Mane, 1989). Other group of investigators have studied the alterations of mucosubstances in the liver, kidney and gill of T. mossambica, especially in relation to the natural piscicides present in the leaves of L. eriocepalus (Harold, 1988), fruits of S. laurifolius (Bhosale, 1988) and fruits of A. concinna (Patil,1988). But very few investigators have paid their attention to find out the enzyme changes in different target organs of fish during intoxication due to natural piscicides.

B) Reasons that led us to take up this problem and analysis of problem :

From the survey it seems that comparatively more information is available on histopathological changes induced by several plant derivatives, whereas, very less information exists on the haematological studies and enzyme studies on fresh water fishes. Therefore, the present study was aimed to find out the impact of alcoholic extract of the leaves of L. eriocephalus on the major haematological parameters and one important enzyme, alkaline phosphatase in the target organs like liver, kidney, gill and brain of a locally available undesirable freshwater teleost fish Sarotherodon mossambica.

1) Choice of the plant :

Harold (1987) from this laboratory has noted that an alcoholic extract of the leaves of L. eriocephalus possesses piscicidal compound. The leaves of this plant are the most toxic part which exhibited significant toxic effect on T. mossambica (Harold, 1987). This fish exhibited low tolerance to this compound and therefore it can be used as a selectiive fish eradicant. Availability in abundance of this indigenous plant material in Western Ghats also stimulated us for its further use in the toxicological studies. Harold (1987) also found that it induced histopathological and histochemical changes in many target organs of fish. Hence it was thought desirable to study in detail different haematological parameters during intoxication due to this compound.

2) Choice of the haematological parameters and enzyme :

Many toxins are known to have a direct effect on the haematological parameters (Svobodova, 1971). Chakraborty et al. (1972) worked on Barringtonia acutangula and found that it caused haemolysis. The crude powder of Gymnodinium breeve affected RBCs and haemolysis took place in Mugil cephalus (Kim et al., 1964). Lasiosiphon latifolium poisoning resulted in lymphocytopenia. In acutely intoxicated bull calves haemorrhages took place. In chronic cases, lymph nodes and spleen showed lymphocytic degeneration with cellular depletion in the follicles (Kipton et al., 1982). Svobodova (1971, 1975) and Ramanujam and Ratha (1980) observed that the piscicidal components in the plant extract affected the Hb concentration. Therefore, in the present study different parameters like total R.B.C. count (TRBC), total W.B.C. count (TWBC), Hb percentage (Hb%) and clotting time (CT) were selected.

It is known that NAD-dehydrogenase, a mitochondrial enzyme activity was found affected by the piscicides from the plant origin (Lindal and Oberg, 1960 and Horgan et al., 1968). L. latifolium toxicity decreased aspartate aminotransferase, creatine phosphokinase and alkaline phosphatase from the fish blood (Kipton , 1982). In fish H. fossilis and P. shalynus enzyme acetylcholinesterase of brain and muscle was greatly inhibited (Ramanujam and Ratha, 1982). Alkaline phosphatase activity has been shown increased in the testis of white rats (Toro, 1984) after administration of Vinca rosea and Butea monosperma (Awati, 1985). Whereas, Shah (1985) has reported decrease in the alkaline phosphatase activity after treatment of Daucus carota seed extract. Therefore, for the present

investigation an enzyme, alkaline phosphatase has been selected. This enzyme has widespread occurrence and known to play significant role in the physiology of many organs.

3) Choice of fish :

The fish S. mossambica (formerly it was called as T. mossambica) creates number of problems in the pisciculture. This fish has a greatest capacity of reproduction. It attains sexual maturity at early age and even about 2 to 3 months old fish can reproduce (Chimitz, 1955). The females of this fish can produce 100-300 or more young fishes per spawn and breads throughout the year at intervals of about 30-40 days. Thus due to high rate of reproduction, at an early age and at small size, the pond is soon fills with spawned fry, all competing for food in the pond. The medium sized fishes are more destructive. S. mossambica, when cultured with major carps in ponds feeds on carp spawn even in the presence of natural food. Therefore, it is observed that rohu and common carp population falls when they are stocked in the combination of S. mossambica. In case of rohu as well as common carp the presence of S. mossambica in the culture seemed to have very poor growth and they attain only about half of the weight that in controls. Jhingran (1968) has mentioned that under Indian conditions this fish is unsuitable for culture along with the Indian major carps, since it adversely affects the growth and production of carps. Therefore, it is strongly recommended that this fish species not to be introduced into commercial fish farms, and to control this species in ponds is a big problem in the pisciculture. Therefore, in order to find out the suitable natural piscicide to control S. mossambica the present investigation was undertaken.

4) Choice of the methods :

In earlier haematological investigations, the RBC, WBC counts, percentage of haemoglobin and clotting time have been shown greatly affected during toxicity due to metals, chemicals, pesticides, etc. Therefore, these four haematological parameters have been chosen for present study.

In the present investigation both histochemical and biochemical techniques have been employed to study the alkaline phosphatase activity. The histochemical techniques are of immense use in the study of cellular localization of the enzyme but they give little information on variations in the enzyme levels. The biochemical techniques on the other hand give most reliable information on the enzyme activity in exact mathematical units but fail to give information on the distribution of the enzyme in various cellular elements in organs under study. Hence alkaline phosphatase enzyme has been studied by employing both histochemical and biochemical assay techniques. For the enzymatic investigations important target organs which are commonly affected during intoxication like liver, kidney, gill and brain have been chosen for the present study.

C) Outline of the present study :

Some of the above mentioned problems are being undertaken for detail analysis in the Department of Zoology, Physiology Laboratory, Shivaji University, Kolhapur. The present dissertation concerns with the studies on the impact of natural piscicide from the leaves of L. eriocephalus on the behaviour, RBC count, WBC count, clotting time and

haemoglobin percentage and on alkaline phosphatase activity of the liver, kidney, gill and brain of freshwater teleost fish S. mossambica.

The present investigation was proposed to carry out in the following manner :

1) Extraction of leaves of the indigenous plant L. eriocephalus from Western Ghat area of Maharashtra employing mainly solvent fractionation methods.

2) To study the impact of the ethanol extract of the leaves of L. eriocephalus on the behaviour of S. mossambica.

3) To study the impact of the ethanol extract of the leaves of L. eriocephalus on the haematological parameters like RBC count, WBC count, clotting time and percentage of haemoglobin of S. mossambica.

4) To study biochemically and histoenzymologically the impact of the ethanol extract of the leaves of L. eriocephalus on the alkaline phosphatase activity of liver, kidney, gill and brain of S. mossambica

For the convenience of the present investigation and ease of understanding, the next part of the dissertation is divided into four chapters. The second chapter presents the information on the material and methods employed in the present dissertation. The third chapter gives the observations and discussion on the haematological parameters. The fourth chapter describes the observations and discussion on the alkaline phosphatase activity in the liver, kidney, gill and brain. In both these

chapters (3 and 4) the observations have been discussed in the light of existing information. The fifth chapter gives the general summary of the study and certain conclusions which have been arrived at. The last chapter is followed by bibliography.