SOME PHYSIOLOGICAL AND BIOCHEMICAL STUDIES ON THE EFFECTS OF THE FRUITS OF A PISCICIDAL PLANT *Zanthoxylum armatum* DC. (*Z. alatum* Roxb.) ON FRESHWATER FISHES

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ABSTRACT

THESIS SUBMITTED IN FULFILMENT OF THE DEGREE OF
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ABSTRACT

Many toxins both of natural and synthetic origin are being used in the modern fisheries to clear ponds from unwanted aquatic organisms prior to using them for fish nursery purposes, and to catch and transport fishes. The toxins which are used for killing the fishes are called as piscicides. The synthetic piscicides when used show both acute and chronic effects on non-target aquatic organisms. Their biodegradability is slower and residual toxicity is higher compared to natural piscicides. Therefore, the use of natural piscicides have been prefered in fishery management. One of the natural piscicides commonly used throughout the world is a phytotoxin called rotenone. Rotenone is obtained from the plants mainly belonging to the genus Derris. In India, rotenone is not available easily and is being imported for fishery purposes. Therefore, studies have been initiated on various plants/plant products to develop suitable alternative indigenous natural piscicides.

More than hundred plants available in this country have been cited to have piscicidal property. Only a few of these plants and their parts have been investigated to establish their piscicidal potentiality. However, no systematic approach has been made to investigate scientifically the nature of toxicity and mode of action of any indigenous plant or plant material. Therefore, the present study was undertaken to find out the toxicity of some of the potential piscicidal plants used in native fisheries of North-Eastern India and the mode of action of the most potent plant part on certain freshwater fishes.

A survey of the plants used for fish killing by the natives of Meghalaya was conducted. Ten plants were collected, identified and their toxicity was screened on two species of gill-breathing (Puntius shalynius and Danio dangila) and one species of hardy air-breathing (Heteropneustes fossilis) fish. The results of the exploratory studies indicated that among the plants/plant parts screened the fruits of Zanthoxylum armatum DC. (= Z. alatum Roxb.) was most potent as a piscicidal agent with minimum effective concentration and lethal time. Therefore, further studies on extraction, bio-assay and mode of action of Z. armatum fruit were conducted. The extraction of the piscicidal factors of Z. armatum fruit was done by the Medical Chemistry Division of Central Drug Research Institute, Lucknow. The concentrated water soluble alcoholic extract prepared had retained about 95% toxicity as observed in the bio-assay experiments.

The bio-assay studies were done following standard static bio-assay method to find out the lethal dose of the toxicant using both crude powder and alcoholic extract on P. shalynius and only alcoholic extract on two different weight groups of H. fossilis. The results of bio-assay experiments showed that the toxicity of Z. armatum fruit extract varied with the behaviour and size of the fish. The air-breathing fish were more resistant than gill-breathing fish and among the air-breathing fish, the higher weight group was more resistant. The effect of alcoholic extract and crude powder on P. shalynius indicated that the alcoholic extract had sharper action than

crude powder. The LC_{50} values were calculated following three different statistical methods (by graphical interpolation using regression formula Y = a + bX, semi-logarithimic method and probit analysis) to reach a near accurate point. The 6 hr LC_{50} value of Z. armatum for P. shalynius with crude powder was found to be 95 ppm and with alcoholic extract 6.4 ppm. The average values of 3, 6, 12 and 24 hrs LC_{50} for H. fossilis were 18.9, 17.2, 14.00 and 14.00 ppm in 8 - 12 g weight group and 30.35, 20.00, 17.45 and 16.95 ppm in 13 - 20 g weight group respectively.

The fishes showed a typical behavioural pattern on treatment with the toxin. The fish became more active in the initial phase of treatment and then showed erratic movements. The opercular movement increased along with the activity of the fish. As the fish approached lethal phase, the opercular movement decreased, the fish failed to reach the surface, lost the equilibr and finally collapsed in an upside down posture at the bottom of the jar. If the fishes were transfered to freshwater at the beginning of lethal phase they recovered slowly.

The effect of the extract on the hematological paramters of H. fossilis was studied at different concentrations of the toxin. The relationship of hematological parameters with size of the fish were studied and was found to be quite variable. Therefore, it has been suggested that it is necessary to establish the intraspecific variations in hematological parameters in a particular species of fish before they are used for toxicity studies. The alcoholic extract of the fruits of Z. armatum did not show any effect on the hematological parameters studied in H. fossilis. Therefore, it was felt that the lethal

effect might be due to the damage done by the extract to some other physiological process(es).

The effect of the extract of the fruits of Z. armatum on oxygen consumption by the fish and the oxygen uptake at sub-cellular level were investigated. The experiments were conducted at organismal level using a weed fish Tilapia mossambica and at cellular level using liver and brain tissue homogenates of H. fossilis. The gross oxygen uptake was calculated from the changes in oxygen concentration of treated water estimated by Winkler's method. The effect on tissue oxygen uptake was studied in Warburg manometers using glucose as the substrate at two different temperatures. The results indicated that there was no effect of the extract on tissue respiration at sub-cellular level and the total oxygen consumption of fish was inhibited indirectly on treatment with the extract. The inhibition of oxygen uptake from water by the fish might be due to the paralysing effect of the toxicant on the different organs related to oxygen uptake. The general metabolic activity of the animal might have also been affected by the extract as total oxygen consumption of fish is indicative of general metabolism.

The results of the above experiments indicated that the extract of Z. armatum fruits might be affecting the nervous system and energy metabolism at sub-cellular level to result in different behavioural and physiological manifestations leading to death. Hence, studies on the effect of the extract on the activities of acetylcholinesterase (AchE), Mg^{2+} - and Na^{+}, K^{+} - ATPase in vivo and in vitro in different tissues of H. fossilis

were carried out. The activities of the enzymes were studied with relation to the concentration of the toxin and duration of treatment in different tissues of H. fossilis. The results showed definite inhibition of the enzymes studied by the extract. The inhibition were both related to the concentration of the extract and time of treatment. However, the maximum inhibition which was about 50% in the different enzymes studied did not change after a certain concentration of the extract. At sub-lethal concentration the enzyme activity showed gradual recovery to normalcy. Though the pattern of inhibition of different enzymes with concentration and time were similar, the initiation, the peak and the recovery were different for different enzymes and tissues. The AchE activity was inhibited maximally to about 40% in the three tissues. ATPase showed tissue specific variations in the rate of maximum inhibition in the following order (a) total ATPase gill > muscle > brain (b) Mg^{2+} ATPase - gill > muscle > brain (c) Na^{+}, K^{+} ATPase - gill > muscle > brain. In general, the activities of ATPase were maximally inhibited earlier than the AchE. However, at the initial stages the AchE was inhibited more than ATPase.

The in vitro studies with different concentrations of the extract indicated that the inhibition of the enzyme activities needed a higher concentration of the toxin than the in vivo inhibition. This could be due to the fact that the in vivo inhibition was the result of amplification of the effect of the extract through some other mechanism.

The kinetic studies on AchE and Mg^{2+} ATPase showed that the nature of inhibition was non-competitive for both the enzymes. However, the K_i of the extract for AchE was higher than for Mg^{2+} ATPase. The apparent K_m (Michaelis constant) of brain and muscle AchE for acetylthiocholine iodide was found to be 7.1×10^{-5}M and 8.85×10^{-5}M respectively. They decreased with eserine but did not change with Z. armatum fruit extract. The apparent K_m of brain, muscle and gill Mg^{2+} ATPase for ATP were found to be 9.72×10^{-4}M, 8.71×10^{-4}M and 7.1×10^{-4}M respectively. They decreased with KSCN but increased with Z. armatum fruit extract. The specific staining of AchE iso-enzymes on polyacrylamide gels showed only one band in brain and two bands in muscle of H. fossilis indicating that this tissue specific molecular variation in AchE might be the cause of the tissue specific differences in the enzymes activity and effect of the extract.

It is, therefore, concluded that the fruits of Z. armatum acts as a mild metabolic poison. It does not have significant effect on hematological parameters studied nor on cellular respiration process in fish. The primary modes of action seem to be through the blockade of neural transmission and supply of metabolic energy by inhibiting activities of AchE and ATPases. Though Z. armatum fruits showed a promise to be used as an indigenous substitute for rotenone, some work on its secondary toxicity and effects on plankton population are necessary before this could be tried in the field.

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Certified that the thesis entitled " SOME PHYSIOLOGICAL AND BIOCHEMICAL STUDIES ON THE EFFECTS OF THE FRUITS OF A PISCICIDAL PLANT Zanthoxylum armatum DC. (=Z. alatum Roxb.) ON FRESHWATER FISHES", submitted by Mr. S.N.RAMANUJAM for the degree of DOCTOR OF PHILOSOPHY in zoology of the North-Eastern Hill University, Shillong embodies the record of original investigations carried out by him under my supervision. He has been duly registered and the thesis presented is worthy of being considered for the award of the Ph.D. degree. This work has not been submitted for any degree of any other University.

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GENERAL INTRODUCTION

The development of civilization has led to the discovery of many new physical, chemical and biological products for meeting the different needs of human life in this world. In the process, industrialization, urbanization and modernization of agriculture have resulted in releasing intermittently a large varieties of new chemicals through industrial effluents, domestic sewages and agricultural chemicals at different concentrations to the environment. These chemicals ultimately find their way to the aquatic environment and gradually make them polluted. These substances which cause impairment of health or death of the organism due to their specific harmful properties when taken into the body are called as poisons or toxins. Some of these toxins released to the aquatic environment are degradable in nature whereas most others accumulate in the aquatic environment. Some toxins have shown biological magnification due to their progressive retention in the food chain. Such toxins, naturally, raise questions on the very existence of human life on this earth. It has been difficult to develop and to cope with the growing needs of the increasing population without the use of many such harmful chemicals. Therefore, the scientists all over the world are working towards the proper management of these harmful substances in this biosphere and also to find out alternate poisons or toxins which could be quickly degraded in the environment by some physico-chemical or biological means. The toxins can be classified broadly into two groups as synthetic and natural toxins.

Synthetic toxins : These include all chemical toxins and constitute the major part of the toxicants which are polluting our environment at present. Most of the fertilizers, insecticides, herbicides, molluscicides, fungicides, lampricides, algicides, piscicides and industrial pollutants come under this category.

Natural toxins : These constitute all those toxicants which are produced from living organisms. These include microbrial toxins, venoms produced by snakes, fish and other animals and other poisonous substances produced by animals or plants.

During the past decade considerable work has been done on the effects of synthetic toxins on physiological and biochemical parameters of organisms. The fish has been commonly used as the test animal in aquatic environment due to its economic importance. The effect of different agricultural chemicals and industrial pollutants ~~have~~ been studied on nervous system, respiratory system, circulatory system and metabolic pathways of fish and a few other aquatic organisms.

There are several reports on the effects of toxicants on fresh water fish~~es~~. Popova (1970), Holden (1972), Schnick (1972, 1974a, 1974b), Shimada (1973), McKim et al (1973, 1974, 1975, 1976), Abel (1974), Hunka (1974), Hamilton (1974a, 1974b), Nelson (1974), Weiner (1975), Sprague (1976) and Brungs et al (1977) have reviewed the works done on the effects of toxicants (agriculture chemicals, industrial pollutants and domestic wastes) on fresh water fish~~es~~.

Morphological and histological changes were studied in the earlier days to evaluate the effect of a toxin on an organism. Grischchenko (1970) from his pathomorphological studies in the gill and liver of carp and the pattern of mortality seen on treatment with pesticides, suggested that the pesticides might be acting [illegible] by influencing both central and peripheral nervous system. Schulz (1971, 1972) observed degenerative and inflammatory changes in liver, kidney, heart and gonad of carp treated with herbicides. He also observed a decrease in RNA content in the pancreatic tissue of the treated fish. Kamarovskii (1972) exposed several fish species to herbicide, Monurox for 75 days and observed morphological changes in erythrocytes and leucocytes. Abel (1976) from the electron microscopic studies showed that the anionic detergent caused acute inflammation of the gill tissue and extensive detachment of epithelium which are associated with lysosome formation. Higher concentration of the detergent caused very rapid lysis of cells resulting in complete disruption of cellular and tissue structure. Histopathological changes like necrosis of liver tissue, inflammation of postal areas, hardening of connective tissue, shrinkage of nuclei and septa formation studied by Sastry and Gupta (1978) using lead nitrate led them to take up some enzymological studies. Physiological changes such as loss of appetite and changes in colour accompanied by behavioural changes such as uncoordinated movements were reported by Hattula and Karlong (1972) in gold fish (Carassius auratus) treated with polychlorinated biphenyls.

Dubale and Shah (1979) have shown that necrosis of liver tissue occuring in Channa punctatus on exposure to cadmium. The effect of synthetic toxins such as agricultural chemicals and industrial effluents on different physiological and biochemical parameters in fish have been studied by various authors. A brief review of the same has been presented here.

Agricultural chemicals

(a) Hematological parameters : The pesticides and herbicides are generally known to affect different hematological indexes such as RBC count, Hb concentration, packed cell volume (PCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and blood oxygen carrying capacity (BOC). Popova (1970) and Svabodova (1971) have studied the effect of chronic administration of pesticides on hematological parameters of blood of ide (Leviscus idus) and carp (Cyprinus carpio) respectively. Their studies indicated that the pesticide increased MCV and PCV significantly and decreased MCHC. Sakaguchi (1972) observed in blood of carp exposed to organophosphates a significant increase in transaminases, AchE, glucose, triglycerides and total cholesterol level in the serum. The liver glycogen was, however, reduced. Grant and Mehrle (1973) exposed mature rainbow trout (Salmo gairdneri) to sublethal doses of endrin and found an increase in the mobilization of liver glycogen and osmolality in the blood serum. Blood glucose was increased by 50%. This was accompained by

inhibition in their growth and ultimately led to death of the fish. They have concluded that the physiological dysfunctions caused by endrin are due to its effect on the above biochemical parameters. Shakoori et al (1976) have shown that the serum protein increased in the blood of Channa punctatus treated with insecticides at chronic doses. Kawatsu (1977) reported a decrease in RBC number and Hb concentrations in blood of fishes treated with herbicides. This has also been shown to cause hemorrhagic anemia. Verma et al (1979d) have shown pesticide induced hematological changes such as decrease in RBC number and Hb concentration, and increase in MCHC in a fresh water fish Saccobranchus fossilis.

(b) Respiratory changes : The impact of agricultural chemicals on respiratory parameters of fish has been studied to a lesser extent than on hematological parameters. The uptake of labelled ^{14}C-DDT and its effect on oxygen uptake of Gambusia affinis was studied by Murphy and Murphy (1971). They reported a linear relationship between ^{14}C-DDT uptake and oxygen consumption of the fish. Kamler (1972) observed the inhibition of respiration by the influence of a herbicide, 2-4 dichlorophenol acetic acid (2-4D (Na)) on early developmental stages of the carp. Hiltibran (1974) studied the effect of 16 insecticides on in vivo oxygen uptake by blue gill and found that all insecticides except for dieldrin and carbofuran inhibited oxygen uptake. The effects of methoxychlor on survival, oxygen consumption and

activity of white sucker (Catastomus commersoni) were investigated by Waiwood and Johansen (1974). They found that just before the lethal concentrations were reached the oxygen consumption of fish increased by 2 to 3.5 times the control level. Luhn et al (1976) observed a gradual increase in the rate of respiration of trout with increasing pesticide concentration. The effect of lethal concentration of thiodon on Mystus vittatus was observed by Reddy and Gomathy (1977). They found an increase in aquatic respiration of the fish. Natarajan (1980) found no change in total oxygen uptake of Anabas scadens on treatment with sumithion though there was decrease in gill respiration.

(c) Nervous system : Most of the work on pesticide effect on biochemical parameters are concerned with the changes occurring in key enzyme of neurotransmission, acetylcholinesterase (AchE). Carter (1971) investigated sub-lethal effects of organophosphate and carbamate insecticides on AchE in channel cat fish. Hogan (1971) examined the action of carbamate, eserine, organophosphate and some selective inhibitors on the activity of AchE in the brain of cutthroat trout (Salmo clarki). Coppage (1972) reported that inhibition of brain AchE beyond 17.7% by the inhibitory effects of organophosphate pesticides lead to the death of minnow (Cyprinodon variegatus). Gibson and Ludke (1973) reported that treatment of Lepomis cynellus with the N-alkyl compounds produced a significant proctection against inhibition of brain AchE by parathion. Benke and Murphy (1974) studied the

time course of onset of inhibition and recovery of AchE in mice and pumpkinseed sunfish exposed to methyl parathion, parathion and azinphosmethyl at concentrations which caused moderately toxic symptoms. Post and Leasure (1974) studied the sublethal effect of malathion on both brain AchE and sustained activity of brook trout, rainbow trout and coho salmon. The data indicated that a loss of 20 to 30% of stored AchE activity resulted in less than 5% decrease in physical ability. Coppage and Braidech (1976) reported that the fish collected from a river below the discharge point of effluents from a factory producing organophosphate and carbamate pesticides had significantly lower level of AchE activity than the fishes collected upstream. Thirugnanam and Forgesh (1977) studied the environmental impact of mosquito pesticide, chloropyrifos on Fundulus heteroclitus. The pesticide inhibited brain AchE both in vivo and in vitro. In vivo pesticide inhibition of muscle esterases of mosquito fish Gambusia affinis has been reported by Whitemore and Hodges (1978).

(d) Metabolic alterations and changes in other enzymes : Davis and Wedemeyer (1971) observed that the organochlorine pesticides such as dicofol, endosulfan and DDT inhibited Na^+, K^+ activated and Mg^{2+} dependent ATPase activity in vitro in gill, brain and kidney microsomal preparations from the yearlings of rainbow trout (Salmo gairdneri). Cutkomp et al (1972) used the brain homogenates of blue gill sunfish to demonstrate the in vitro inhibitory effects of

several chlorinated hydrocarbon pesticides to mitochondrial Mg^{2+} and Na^{+}, K^{+} ATPase. Chambers and Yarbrough (1973) found that organochlorine pesticide resistant fish had higher level of microsomal mixed function oxidase (MFO) activity. MFO are involved in degradation of pesticides. Ghosh and Konar (1973) showed that phosphomidon inhibited gut invertase activity in cat fish (Clarias batrachus). McCorkle and Yarbrough (1974) studied the in vivo effects of mirex on SDH activity in mosquitofish and green sunfish. Mirex concentrations, even at 10^{-6}M inhibited SDH activity in mitochondrial preparations from non-resistant individuals of both species. Desaiah and Koch (1975a, 1975b, 1975c) examined the effects of kepone and its reduction product on the in vivo activity of brain ATPase in channel catfish. They found inhibition of all three ATPase tested. The oligomycin sensitive (mitochondrial) Mg^{2+} ATPase of brain showed the greatest sensitivity to both inhibitors. In vitro inhibition of ATPase activity in the brain of channel catfish by aldrin, dieldrin, photodieldrin and aldrin transidol were also investigated. Christensen and Tucker (1976) studied in vitro activity of channel catfish carbonic anhydrase. The effect of in vivo exposure of endrin on the activity of acid, alkaline and glucose-6-phosphatase in liver and kidney of Ophiocephalus punctatus was studied by Sastry and Sharma (1978). Koundinya and Ramamurthy (1980) studied the effect of organophosphate pesticide, sumithion on some aspects of carbohydrate metabolism in Sarotherodon mossambica. At LC_{50} dose blood glucose level and phosphorylase activity increased and hepatic

glycogen level decreased. This indicated that the observed hyperglycemia was due to breakdown of hepatic glycogen. Schulz (1971) studied the effects of the herbicide, dowpan on carp and found a decrease in RNA content of pancreatic tissue. Bostrom and Johansson (1972) reported that pentachlorophenol (PCP) *in vitro* and *in vivo* altered the activity of the liver enzymes involved in energy metabolism of the eel (*Anguilla anguilla*). McBride and Richards (1975) used an isolated perfused gill method to examine the effects of atrazine and trifluralin on sodium uptake and fluid flow rates in carp (*Cyprinus carpio*). They found that these chemicals decreased gill sodium uptake and had no effect on fluid flow rates.

Industrial pollutants

Different industries produce various types of waste products which are usually discharged into the nearby water bodies. Depending on the industry, the effluents include mainly different organic chemicals, metal ions and polychlorinated biphenyls etc. Studies are being done on the effect of various industrial effluents and their components on different physiological and biochemical parameters of fish. A brief review of the work done is presented here.

A lot of work has been done on the effect of the individual metal ions on the different physiological parameters in fish. Christensen (1971-72) studied the *in vitro* alteration in the activities of GOT and LDH in the blood plasma of white sucker (*Catostomus commersoni*) treated with different metal ions.

Brafield and Matthiessen (1976) studied the rate of oxygen uptake by stickle back (Gasterosteus aculeatus) exposed to zinc. Oxygen uptake showed an increase and then became extremely erratic before declining as death approached. The activity of delta aminolevulinic acid dehydratase in blood of rainbow trout exposed to lead was measured by Hodson (1976a). The enzyme was inhibited after 4 weeks of exposure to lead. Gould and McInnes (1977) studied short term effects of two silver salts on tissue respiration and enzyme activity in cunner (Tontogoiabrus adspersus). Hiller and Perlmutter (1971) reported that zinc stimulated viral growth and caused infectious pancreatic necrosis in trout at the exposure concentration of 10 ppm. The effects of copper or zinc on the adaptability of premigrant yearling of coho salmon to sea water, ATPase activity and effect of copper on down stream migration were examined by Lorz and McPherson (1976). Exposure of coho salmon for 144 hours to zinc in fresh water had almost no effect on Na^+, K^+ activated ATPase activity in gill microsomes and on the adaptability of the fish to sea water.

Effects of some industrial pollutants and factory effluents on kidney peroxidase activity in two fresh water fish (Ophiocephalus punctatus and Clarias batrachus) were studied by Mukherjee and Bhattacharya (1975). Peroxidase activity was greatly inhibited indicating that sub-lethal doses of these toxicants may cause drastic changes in physiological systems. In some cases, peroxidase activity increased well above control level after 5 and 27 hour of exposure, but declined towards the end of the test by 96 hours. Bhattacharya and Mukherjee (1976) investigated

hepatopancreatic protease and esterase activity in fish exposed to industrial pollutants which inhibited both enzymes. Effect of mercuric bichloride on some biochemical characteristics of brain, blood and liver of Rutilus rutilus were examined by Dokholyan and Akhmedova (1978). Significant metabolic disturbances in brain and decrease in blood protein were observed.

Hattula and Karlong (1972) studied the toxicity of PCB to gold fish (Carassius auratus). Behavioural and physiological changes noted were loss of appetite, changes in colour and uncoordinated movements. The influence of polychlorinated biphenyls (Aroclor 1248) on aquatic organism was studied by Ito (1973a, 1973b) and Ito and Murata (1974). Their finding suggests that the plasma β-glucuronidase activity of fish may be used as an index of PCB pollution of water bodies.

Hunn (1972) studied the effect of a lampricide, thanite, on certain blood characteristics of carp. The effects were similar to those in cyanide poisoning. Kawatski and McDonald (1974) showed that TFM significantly reduced oxygen consumption in homogenates of brain and liver of white sucker, large mouth bass and blue gill sun fish.

Natural toxins

Studies on physiological and biochemical effects of natural toxins are much limited in comparison to those of chemical toxins. Various types of naturally occurring chemicals derived from different organism have been taken as natural toxins. They are either microbial toxins, zootoxins or phytotoxins depending on their source of origin. Many of these toxins are

being used conventionally by human being without understanding their actual mode of action. Recently, attention of scientists has been directed to study systematically such natural toxins for their utilisation. The chemists extract the active principles and derive their chemical structure whereas the biologists establish their effects on different organisms.

(i) Microbial toxins : These include the toxins obtained from lower organisms such as microbes, algae and fungi. Bacterial toxins are primarily proteinacious in nature. There are different types of bacterial toxins depending on their mode of action. A detail study of bacterial toxins is given by Ajil et al (1973). The main groups of bacterial toxins studied are botulinum toxin, tetanus toxin, diphtherial toxins and enterotoxins. Botulinum toxin is thought to act by inhibiting the release of acetylcholine packaged in presynaptic vesicles of motor end plates in response to nerve action potential (Koelle, 1965). Tetanus toxins are known to interfere with transmission of impulses in cholinergic nerves. Fal and Czerchawski (1963) have shown that the acetylcholine liberation is enhanced by tetanus toxin. Diphtherial toxin is a metabolic inhibitor of protein synthesis. Enterotoxins acts on the nervous system of enteric tract and cause emetic activity.

Blue green algal blooms in water bodies are known to be toxic to fish and wild life (Hashimoto et al, 1968), Mackenthum et al (1948) reported that the primary cause of

fish mortality associated with decomposing algal blooms was due to depletion of oxygen supply. The toxic elements released from decomposing algae were of secondary importance. Phytoflagellates like Prymnesium are known to have hemolytic, cytotoxic and bacteriolytic activity and damage gill tissues resulting in specifically the loss of their selective permeability. Several toxins from algal sources have been investigated for their effects on neuromuscular junction. Gymnodinium veneficum toxin potentiates nerve transmission before blocking and acts as a depolarizing agent (Abbot & Ballantine, 1957). The toxin of Gonyaulax cantenella (Burke et al, 1960), and saxitoxin produced by Saxidinus giganteus block axonal and muscle conduction without depolarization and show very little effect on neromuscular junction (Kao & Nishiyama, 1965). Saxitoxin obtained from contaminated shell fish is reported to cause vasodilation by direct relaxant action on the vascular muscle at lower doses and by an additional blockade of vasoconstrictor nerves at higher doses (Nagasawa et al, 1971).

The toxins produced by fungi are called mycotoxins. Different species of Aspergillus, Pencillium, Fusarium which are found associated with food products are known to produce many plant toxins. Aflatoxins are a closely related group of secondary fungal metabolites and are produced by various species of Aspergillus and Pencillium (Detroy et al, 1971). Aflatoxins inhibit the incorporation of labelled precursors

into DNA (De Recondo et al, 1966), RNA (Sporn et al, 1966) and protein, especially into the inducible enzyme proteins both in vivo (Wogan & Friedman, 1965) and in vitro (Smith, 1963). Inhibition of nucleic acid synthesis might be due to the direct action of aflatoxins with the enzymes involved or might be due to the toxin-DNA binding modifying the DNA template (Clifford & Rees, 1966). The aflatoxins are also known to be carcinogenic causing cancer in liver.

(ii) Zootoxins : Production of substances that may be poisonous or repulsive to other animals is well known among the animals. These are special products of metabolism and serve as chemical defensive mechanism in different organisms. Most of them are effective only after they have been taken orally or introduced by some special puncturing or stinging device into the body. Some are effective as contact poisons. Venoms of both vertebrates and invertebrates are mixtures of the recognised pharmacologically active substances and are products of nitrogen metabolism. They some-times contain enzymes such as hyaluronidase and phospholipase which soften the tissues and quickly spread the venom. It is probable that various components of venom act synergistically and their cumulative action accounts for the severity of the effect. These toxicants act directly upon central nervous system initiating spontaneous electrical activity followed by complete cessation of it. Besides neurotoxic action, venoms from different sources act either by blocking respiration or causing hemolysis or by causing muscular distrophy (Ownby et al, 1976; Tu et al, 1976). Venoms from some invertebrates

contain toxic carbohydrates and saponins which cause hemolysis. These are also found to be secondary plant products (Gardiner, 1972). Scorpions, bees, wasps and some ants inject their poison by means of stings. Scorpion venom induce contraction of smooth muscle, spastic paralysis and increased excitability of central nervous system tested in perfusion systems in vitro. Venoms from Centruoides sculpturatus, North-American species of scorpion, is known to cause paralysis of neuromuscular junctions and partly of spinalcord (Welsh, 1964). Venom from Indian scorpions have been shown to contain several enzymes, such as proteases, 5-nucleotidase and phosphodiesterases (Adams & Weiss, 1958). Scorpion venoms, in general, have some of the characteristics of snake venoms and may be equally fatal to humans. Snake venoms which are also enzymatic in nature are generally known to be either neurotoxic or cardiotoxic in nature (Meldrum, 1965). Some snake venoms are known to block acetylcholine receptors at post synaptic membrane (Low et al, 1976). Some are also known to have the nephrotoxic action such as venoms of rattle snake and sea snake (Schmidt et al, 1976). Besides their toxic effects some venoms are known to /possess therapeutic properties. Venoms from different sources like snake, toad and spider are known for their analgesic, hemostatic and antirheumatic applications (Deklobusitzky, 1971).

(iii) Phytotoxins : Many plant products have toxic effect on humans and animals. Various types of toxins are produced by different groups of plants. A brief account of different plant toxins and their actions are given below. Many higher plants produce toxic chemicals like saponins, tanin, resin, essential oils and alkaloids (brucine and strychnine). These are very strong protoplasmic poisons which have a stupefying effect on peripheral, sensory and motor nerves and muscles of fish (Brandt, 1972). Harborne (1977) has classified the active principles from higher plants into Nitrogenous toxins and Non-nitrogenous toxins and characterised them as follows. Nitrogen based toxins are widely present in plants and may be acting directly as antimetabolites like aflatoxins. There are many non-protein aminoacids in this group of toxin. These aminoacids may be incorporated into proteins of the treated organism to produce wrong proteins. These proteins cannot function properly and thus lead to the death of the organism. The toxicity of some of these compounds have been worked out on higher group of animals such as mammals and birds. Non-nitrogenous toxins include different classes of compounds like cardiac glycosides, saponins, flavonoids, quinones and polyacetylenes which do not contain nitrogen atom in their structure. Glycosides like oubain are known to be cardiac poisons probably acting by inhibiting Na^+, K^+ ATPase. Another non-nitrogenous toxin

is monofluroacetic acid which cause respiratory arrest through inhibition of TCA cycle. Some like quinones are notable for causing photosensitization in farm animals. This group of plant toxins includes some of the insecticides and piscicides such as pyrethrin and rotenone (flavonoid) which are not very much poisonous to higher group of animals.

Use of toxins in fisheries management

In modern fisheries management, for clearing ponds and water bodies before using them for pisciculture, many toxins both of chemical and natural origin have been used. Some of these have also been used as anaesthetics while transporting large fish. U.S. Department of the Interior, Fish and Wild-life Service have listed about 33 chemicals and their current status in fisheries management in its report in 1978. These include herbicides like copper sulphate, diquat, diuron, silvex and also anaesthetics like MS 222 and quinaldine sulfate. However, the use of chemical toxins ~~have~~ has not been very much favoured due to their severe toxicity and many side effects. The efforts have been made always to use moderately toxic plant toxins.

Plant toxins : Plant toxins are more readily degradable than the synthetic chemical toxins. Therefore, rotenone which is produced from the roots of the plant species of genus Derris, has been extensively used for fisheries management in all parts of the world. At present, rotenone is not easily available in this country and is being imported. Therefore, there is need for

finding out a suitable substitute which will have nearly same potency as rotenone in their piscicidal activity. There are many plants and plant parts reported to have been tried in fish nursery management. Chopra et al (1941) listed about 164 varieties and species of plants found in India which have been reported to contain insecticidal and piscicidal properties. Only a few of them have been investigated for their usefulness in fisheries management. Babu (1965) observed the toxicity of the seeds of Croton tiglium on predatory and weed fishes. Bhuyan (1967) studied the eradication of unwanted fish from ponds by using an indigenous poisonous plant Milletia pachycarpa. The use of the seeds of Croton tiglium as a fish poison in ponds was reported also by Bhuyan (1968). Das (1969) investigated action of Derris trifoliata roots with reference to its usefulness in fish nursery practice. Chakraborty et al (1972) studied the action of crude powder of roots of Barringtonia acutangula as a fish poison. Certain plants from Japan such as Justicia hayatai (Ohta et al, 1969) and Marchantia polymorpha (Kanasaki & Ohta, 1976) have been reported to have piscicidal action and isolation of their active principles is being carried out.

Many plant products have also been studied for their usefulness as fish poisons. Konar (1968) studied piscicidal effect of nicotine. The usage of seed cake of tea (Camellia sinesis) in China has been reported by Hora and Pillay (1962). This has been shown to contain 7-8% saponin. Choudhury (1968) and Chakraborty et al (1972) have reported the use of oil cake

from mahua (Madhuca indica) in fish nursery management in India. Nandy and Chakroborty (1976) reported the toxicity of unripe fruits of Randia dumetorum to freshwater fishes. Juglone is another plant derived piscicide obtained from walnut trees. Marking (1970) studied the toxicity of juglone on rainbow trout. Pyrethrin obtained from the genus Chrysenthimum is reported to have lethal effect on fish (Mauck et al, 1976) inhibiting the ATPase activity in nervous system (Desaiah et al, 1973). Buchanan et al (1976) have reported the toxicity of spruce (Picea stichensis) and hemlock (Tsugo heterophylla) to esturine organisms in South-Eastern Alaska.

Rotenone which is most extensively used piscicide is mainly derived from Derris roots. Some other plants such as Milletia pachycarpa, Milletia piscidia, Randia dumetorum, Piscidia erythrima are also known to contain rotenone and are used as fish poisons. The action of rotenone and its derivative rotenoids on fish was studied as early as 1933 by Danneel and Eagle. Bianco (1943) has compiled the plants used in Venezuala for fish poisoning. He reported that those plants contain substances that paralyse the respiratory center of fish and also damage the nervous system. Simon (1948) reported conjuctivitis and scleritis in the fishes treated with derris powder and pyrethrum. Petard (1951) studied icthyotoxic plants of Polynesia and reported the presence of rotenone in them. According to Oberg (1959), the rotenone poisoned fish had a normal rate of blood flow through gill lamellar capillaries. Lindahl and Oberg (1960) first demonstrated that

rotenone treated gill filaments of fish showed reduced oxygen uptake. Horgan et al (1968) studied the action of rotenone at sub-cellular level and reported that it acts principally by blocking the reduced nicotinamide adenine dinucleotide segment in mitochondrial electron transport chain. Fromm (1970) later demonstrated that rotenone reduced perfusion flow rate in isolated gill arches. Spitler (1970) analysed the rotenone treatment for elimination of fish population in Southern Michigan lakes during 1957-67 and has given the optimum conditions of physico-chemical factors under which rotenone could be best effective. Rowe-Rowe (1971) discussed the tolerance of rotenone on some fresh water fishes of Natal. Fabacher and Chambers (1972a) have shown tolerance of mosquito fish to rotenone. They reported that rotenone tolerance was solely due to the increase in the activity of mixed function oxidase enzyme. Mors et al (1973) studied the ichthyotoxic activity of plants of genus Derris and compounds isolated from them. They have reported that the toxicity of Derris to fish cannot be due solely to the presence of rotenone as some of the species which contained no rotenoids were as toxic as the plants which contained rotenone. Cumming et al (1975) have studied the control of grass carp with rotenone and other chemical piscicides like antimycin and thanite. Perry and Conway (1977) have indicated that primary rotenone toxicity develops because of reduced oxygen withdrawal from the blood by tissue capillary beds. Nath et al (1980) have suggested that the inhibitory activity of different rotenoids depends on their structural configurations.

Chemical constituents of some piscicidal plants have been studied. Hauschild (1936a, 1936b) reported a highly active fish poison from the bark of Piscidia erythrina. The toxic substance present in the plant is assumed to contain compounds similar to rotenone and death of the animal has been reported to occur due to respiratory stand still. The dried fruits of Animirta cocculus has been used as fish poison. The active principle isolated from it is picrotoxin (Araceli & Alfredo, 1941). Russel and Kaczka (1944) have extracted rotenone like material from Ichthyomethia piscipula. Maiti and Barua (1957) have isolated triterpenoids from Barringtonia acutangula. Prista et al (1962) have done some phytochemical studies on the roots of Albizzia adianthifolia and shown that it contains saponins. Agarwal et al, (1963) have isolated triterpenes from the bark of Myrica esculenta. Kawazu and Tetsuo (1967a, 1967b) have isolated callicarpone a fish killing component from Callicarpa inophyllum which is commonly used as fish poison in South-East Asia. Ohta et al (1969) studied the piscicidal compounds of Justicia hayatai and isolated the piscicidal component, Justicidin. Kanasaki and Ohta (1976) isolated the piscicidal component of Marchantia polymorpha and identified it as costunolide.

Fishing with plant poison has been particularly prevalent in tropical areas like Asia and South America. Fish poisoning by ichthyotoxic plants are usually done in small water bodies, upto 500 hectares or in bays or arms of larger lakes. In running waters, barriers are installed down stream from the place where the poison is to be used in order to catch the more or less floating helpless

fishes. Poisonous plants or/ and plant parts are usually crushed, cut to pieces or pulverized and sprinkled on water or added to bait for the purpose of poisoning. The fish is thereby killed, narcotised or at least so affected that they raise to water surface and can be easily captured. Fishes are seen some times to recover from these effects when brought into fresh water.

Zanthoxylum armatum DC. (= Z. alatum Roxb.): The plants of this species belonging to the family Rutaceae grow as shrub or small trees in the hot valley of sub-tropical Himalayas from Trans-Indus area eastwards to Bhutan upto an altitude of 7,000 feet, also in the Khasi and Naga Hills between 2,000 and 3,000 feet and in the hills of Ganjam and Vizagapatam at babout 4,500 feet (Chopra et al, 1958). Very little work has been done on the chemistry and toxicity of this plant. Essential oils from the seeds of Z. alatum was first isolated by Simonsen and Rao (1929). They reported 85% of it as hydrocarbons, a small quantity of linalol and in addition an unidentified sesquiterpene. Nazir and Handa (1961) investigated the chemical components of roots of Z. alatum. The eluents of petroleum ether and benzene on fractionation gave a pungent odour distillate which was seen to possess strong insecticidal properties. Mathur et al (1961) reported the insecticidal property of bark powder of Z. alatum. They observed a 100% mortality of nymphs of Locusta migratoria within 24 hours. Deb et al (1962) isolated dictamine from stem bark of Z. alatum. Abrol and Chopra (1963) found that the volatile oil obtained on

steam distillation of fruits of Z. alatum was highly active. 0.5 ml dose exhibited knock down mortality of 90% against house flies, the 24 hours kill being 95%. Singh (1969) studied the antihelminthic property of fruits of Z. alatum against ascarids. Dubey and Purohit (1970) further fractionated the chemical components of the essential oil derived from the seeds of Z. alatum by thin layer chromatography and found β-myrcene, d-limonene, methyl cinnamate and citral besides the constituents reported by earlier workers. Lamba (1970) listed Z. alatum as a useful piscicidal plant. The antibacterial activity of Z. alatum was studied by Jain and Kar (1971). The antihelminthic activity of essential oils from leaves of Z. alatum and reported it to be as most potent one (Kakote & Varma, 1971). Shah and Gupta (1976) have listed Z. alatum as a useful medicinal plant of Ranikhet. All these studies mentioned here are only on the gross toxicity of the plant or plant parts and on some of their chemical analysis. However, no report is seen on the mode of action of this plant or its fruits which are comparatively more toxic as a piscicide.

Though lot of work has been done on the effect of different synthetic toxins like pesticides, herbicides and other industrial pollutants on physiological and biochemical aspects of fish, comparatively very little work exists on the mode of action of natural toxins on animals and more specially on fishes and lower vertebrates. Recently, many plant derived products have been tried as insecticides and piscicides. They are found to be more useful than the synthetic toxins in keeping the aquatic system less polluted. Rotenone has been extensively used as a good natural toxin in fisheries management as its physiological

effects on fish and aquatic organisms have been worked out to some extent.

Plan of work

Zanthoxylum armatum DC. (Z.alatum) and few other plants are used in this region to catch fish from different water bodies by local tribal people. There has been no systematic studies on the nature of these toxic plants and their mode of action at cellular or sub-cellular levels. The indiscriminate use of these piscicides without knowing their mode of action might cause adverse effects on the aquatic fauna in this region and also can cause secondary toxic effects on the human beings consuming the treated fish and treated water. A proper scientific investigation might prove any one of these plants to be a potent indigenous piscicide for fish nursery management with less side effects. The mechanism of action of these toxins would give a measure of its impact on fish and other aquatic fauna. Keeping these objectives in view, the following studies were carried out and the findings have been presented in the four different chapters of this thesis.

Chapter I presents (a) the results of the survey of the locally available and used piscicidal plants, (b) the results of the relative toxicity tests with the parts of these plants on certain fresh water fishes and (c) the observations on the bio-assay studies on Puntius shalynius and Heteropneustes fossilis using both the crude powder and alcoholic extract of the fruits of Z. armatum. (Among the screened plants/parts, Z. armatum fruit was found to be most effective and was also easily available. Therefore, detailed studies were conducted on the fruits of Z. armatum).

Chapter II deals with normal hematological parameters of H. fossilis and the effects of the alcoholic extract of the fruits of Z. armatum.

Chapter III presents the observation on the effect of the alcoholic extract of Z. armatum fruit on (a) the oxygen uptake by Tilapia mossambica from treated water and (b) the oxygen uptake by brain and liver homogenates of H. fossilis in vitro.

Chapter IV presents the studies on the effect of the alcoholic extract of Z. armatum fruits on the activity and kinetics of Acetylcholinesterase, Mg^{2+} ATPase and Na^{+}, K^{+} ATPase in brain, muscle and gill tissues of H. fossilis both in vivo and in vitro.

CHAPTER I

BIO-ASSAY

INTRODUCTION

Any foreign substance, either a pure chemical or a chemical product from natural origin, will have certain effect when added to aquatic environment. The effect of these substances may or may not be harmful to aquatic organisms. The substances which have an harmful effect have to be quantified on various aquatic organisms to find out its range of effects on aquatic ecosystem. To define toxicity of any substance, usually laboratory set experiments under controlled conditions known as "Bio-assay" are conducted before using them in natural conditions. Different bio-assay procedures have been developed to quantify aquatic pollutants (Dudoroff et al,1951; Sprague, 1971; European Inland Fisheries Advisory Commission Technical Report, 1975). A bio-assay is a test in which the quantity or strength of a substance is determined by the reaction of a living organism to it (Sprague, 1973). The effects of toxins on living organisms can be broadly classified, into two categories as (i) acute toxicity which is an immediate effect and usually lethal and (ii) chronic toxicity which occurs due to prolonged treatment and may be lethal or sub-lethal. Acute toxicity involves those stimuli which are severe enough to bring about a quicker response, usually within four days in case of fish. Chronic toxicity involves the stimuli which are less severe than acute and produces the response(s) in a comparatively longer time. The toxicity of a substance as obtained by bio-assay procedures

was usually expressed as the median lethal concentration (LCm). The current trend, however, is to use the symbol LC_{50} (lethal concentration for 50 percent of the individuals), a notation which is used in most fields of biological testing. The symbol TL_{50} (formerly known as TLm), the median tolerance limit, has been commonly used by the fisheries workers in United States. The two terms, however, have the same numerical value. A time period is always specified while expressing LC_{50} values, for example 12 hrs LC_{50}. The LC_{50} is merely a convenient reference point for expressing the acute lethal toxicity of a given pollutant to the average or typical organisms. The "safe" concentration which permits successful reproduction, growth and other normal physiological processes in fish is much lower than the LC_{50} value (Warren, 1971). Generally, safe concentration is calculated by multiplying LC_{50} value for a known period with an application factor. In Holland, Germany and Switzerland application factors of 0.05 to 0.1 for concentration lethal to 50 percent of test animals in 20 days are generally considered acceptable in receiving water (Warner, 1967). The National Technical Advisory Committee (1968) under the auspices of the Federal Water Pollution Control Administration of United States has recommended application factors of about 0.002 to 0.1 for protecting aquatic organisms from acutely toxic substances. The term median lethal dose (LD_{50}) is seldom valid for aquatic pollutants, since it means the amount of drug or toxicant which is actually getting into the body.

Two types of bio-assays are in general use. They are the static bio-assay with the fish in a container of standing test water and the continuous flow or flow-through bio-assay in which the test solution is renewed continually or added periodically to maintain a particular concentration of the toxicant.

Various factors like size of the fish, and oxygen level, temperature and quality of diluent water play important roles in bio-assay tests. It is generally known that the larger fishes have better survival capacity than the smaller ones in any toxin (Cairns & Scheier, 1958). Therefore, it has been suggested that in an experimental group of fish the larger one should not be more than 1.5 times the size of the smallest fish (Sprague, 1973). Lloyd (1961) demonstrated that the toxicity of several substances to fish increased almost proportionately with the reduction of oxygen concentration of the water. He proposed that these increases in toxicity were primarily due to increased respiratory irrigation bringing more of toxicants to gill surface. At lower oxygen concentration a toxicant may be absorbed more rapidly or may damage respiratory structures. In one of the reports Lloyd (1962) however, presented that the resistance of rainbow trout Salmo gairdneri to copper, lead and zinc was inversely related to the ambient oxygen levels. Warren (1971) therefore, said that this might not be applicable in all cases. Voyer(1975) studied the effect of dissolved oxygen concentration on acute toxicity of cadmium to Fundulus heteroclitus at selected

salinities and reported that it was not influenced by reduction in dissolved oxygen. Temperature variations, well within a harmless range, can greatly influence the tolerance of most aquatic organisms for lethal agents (Warren, 1971). Hodson and Sprague (1975) reported that increased temperature accelerated metabolic processes and rate of gill irrigation in fish. In consequence, the aquatic animals are usually killed by toxicants more rapidly at moderately elevated temperature than at lower temperature. The two week LC_{50} of zinc for Atlantic salmon (Salmo salar) was also shown to increase with the increase in acclimation temperature. The effects of temperature and hardness of water on toxicity of naphthenic acid to common blue gill sun fish (Lepomis macrochirus) were studied by Cairns and Scheier (1962). Brungs et al (1976) showed that hardness and such other characteristics as pH, acidity and alkalinity of diluent water affected both acute and chronic toxicity of copper to fathead minnow. pH values between 5.0 and 9.0 are generally not acutely lethal for most fish species (Doudoroff & Katz, 1950; European Inland Fisheries Advisory Commission Technical Paper, 1969).

~~The~~ fish show the avoidance reaction immediately after treatment with the toxin. Various types of behavioural manifestations occur in the fish on exposure to different types of stress (Whitemore et al, 1960; Hoglund, 1961; Sprague, 1964). The observations on the behaviour of the treated fish help in speculating the possible mode of action of the toxins at the physiological level.

Many plants are used as piscicidal agents by native fishermen in many parts of the world (Bradt, 1972). A few of them such as Croton tiglium (Babu, 1965, Bhuyan, 1968), Milletia pachycarpa (Bhuyan, 1967), Justicia hayatai (Ohta et al, 1969), Derris trifoliata (Das, 1969), Barringtonia acutangula (Chakraborty et al, 1972), Randia dumetorum (Nandy & Chakraborty, 1976) and Marchantia polymorpha (Kanasaki & Ohta, 1976) have been tried in modern fishery management. However, a systematic study on the effect of these plants have not been done except for rotenone, produced from Derris root. Some species of plants are used by the local tribes of this region for fish catching in freshwater bodies. Therefore, an effort was made to find out these plants and their effectiveness as piscicides. Before taking up detailed physiological and biochemical effects of these piscicidal plants on fish their bio-assay and behavioural studies were carried out.

Plan of work

This chapter deals with our studies on the screening and bio-assay of some of the locally available piscicidal plants as given below:

(1) Screening of the plant and their parts with regard to their toxicity on two species of gill-breathing fish (Danio dangila) and (Puntius shalynius) and one especies of air-breathing fish (Heteropneustes fossilis).

(From this it was found out that the Zanthoxylum armatum fruit was comparatively more toxic and this was used for detail studies)

(2) Effect of Z. armatum fruit powder on some physico-chemical factors like pH, conductivity and oxygen content of diluent water.

(3) Bio-assay of crude powder and alcoholic extract of Z. armatum on P. shalynius and H. fossilis to find out the LC_{50} values.

(4) Observations on the behavioural changes in the fish treated with Z. armatum fruit extract.

MATERIALS AND METHODS

The work was started with the collection of informations from the local people on the plants and plant parts they used for killing ~~the~~ fishes from the streams and other fresh water systems. Ten species of such plants were collected and identified with the help of ~~the~~ taxonomists from Department of Botany of our School. ~~The~~ plant parts were collected fresh, air-dried in shade and finely powdered. These powders were used to assess their comparative piscicidal activity on both gill-breathing and air-breathing fish. Z. armatum fruit powder which was found to be potent toxin was further purified to get alcoholic extract following the method given in Indian Pharmacopoeia (1966). ~~The~~ alcoholic extract was prepared by the Central Drug Research Institute, Lucknow. This preparation refered to as 'extract' here after was used in all experiments reported in this thesis to find out its mode of action in fish. Three species of fish used in different experiments include one species of air-breathing and two species of gill-breathing fish. The species of air-breathing fish was the hardy cat fish,

Heteropneustes fossilis (Bloch). The two species of gill-breathing fish were Puntius shalynius (Yazdani & Talukdar) and Danio dangila (Ham.) available commonly in local streams. H. fossilis were purchased from the market at Gauhati, Assam and brought to Shillong. P. shalynius and D. dangila were collected locally from the streams and ponds in and around Shillong, Meghalaya. The fish were maintained in the laboratory according to the procedure suggested by European Inland Fishery Advisory Commission Technical Paper (1975). They were acclimatized to 20 ± 2°C in glass aquaria for about 3 weeks before they were used in experiments. The gill-breathing fish were aerated to keep the dissolved oxygen level in the aquaria about 6 ppm during acclimatization. P. shalynius and D. dangila were fed on commercial fish food (obtained from Shalimar Company, Bombay, India) and H. fossilis were fed on dried fish powder (purchased locally) on alternate days. No food was given 24 hrs before the experiments were started.

Three main parameters pH, conductivity and oxygen concentration of diluent water, before and after the treatment of the toxin, were studied. The pH and conductivity were measured by a pH meter and conductivity bridge respectively. The oxygen concentration of water was measured according to Winkler's method (Welch, 1948).

The toxicity screening of the plant parts were done using all the three species of fish. However, the bio-assay of Z. armatum fruit powder and extract was done in P. shalynius and H. fossilis.

The length and body weight of the three species of fish used in the experiments were as given below. The effect on H. fossilis was analysed in two size groups to compare the relative toxicity with size.

	Weight(g)	Length(cm)
P. shalynius	1.5 - 2.0	4.5
D. dangila	2.0 - 3.0	4.5
H. fossilis Gr. I.	8.0 - 12.0	8-10
Gr. II.	13.0 - 20.0	11-16

The method used for bio-assay tests was that described for static tests in European Inland Fishery Advisory Commission Technical Paper (1975). The bio-assay of the toxin was carried out in 10 litre glass jars containing 6 litre of diluent water with 6 fish in each jar. A minimum of three replicates were run at each concentration with the control set containing 6 litre of diluent water and 6 number of fish. Fish were removed from the experimental jars soon after their death. Mortality rates were recorded after 3, 6, 12 and 24 hours of treatment. Percent mortality for each time point was calculated from actual data. The LC_{50} values for different time were calculated following three different standard methods as given below.

1. By drawing regression lines based on percent mortality and concentration using the formula $Y = a + bX$, where Y represents the percent mortality and X the concentration of the fruit extract, 'a' is intercept and 'b' is regression coefficient obtained by calculation (Bailey, 1965).

2. By using straight line semilog graphical interpolation as given by Dudoroff et al (1951).

3. By using Probit analysis (Goulden, 1962).

The behavioural changes such as irratic body movement and opercular movement were observed visually in the fish after treatment with the toxin.

RESULTS

The list of the piscicidal plants collected by us and their parts commonly used in this region are given in Table 1. The minimal lethal concentrations and lethal time for the three species of fish tested with crude powders of different plant materials are given in Table 2. It could be seen from ~~the~~ Table 2 that the Z. armatum fruit was most effective as an piscicide followed by Myrica esculenta, Randia dumetorum, Polygonum hydropiper and Potentilla fulgens. ~~The~~ Studies on gill-breathing and air-breathing fish indicated that the concentration of the toxicant required to produce a visible response on air-breathing fish was more ~~compared to~~ than that of gill-breathing fish. The lethal time was also higher in air-breathing fish than gill-breathing fish. The lower range of time given in Table 2 is for gill-breathing fish and the higher range for air-breathing fish.

The effect of Z. armatum fruit powder on pH, conductivity and oxygen content of water is given in Table 3. It could be seen that the treatment of toxin caused no significant change in the quality of the diluent water.

The percent mortality of P. shalynius and H. fossilis at different concentrations of crude powder and extract of Z. armatum have been shown in the Tables 4-7. In general, it could be seen that the percent mortality of fish increased with concentration and duration of treatment of the toxicant.

The lethal threshold concentration (LTC) for P. shalynius was found to be 60 ppm with crude powder (Table 4) and 6 ppm with extract (Table 5). The survival time of P. shalynius in crude powder was also comparatively more than that in the extract.

The percent mortality of H. fossilis in the extract which was analysed in two weight groups are given in Table 6 and 7. The LTC for two weight groups of H. fossilis were different with 12 ppm for 8-12 g wt. group (Table 6) and 15 ppm for 13-20 g wt. group (Table 7). It could be seen from Tables 6 & 7 that the maximum mortality of fish occured within 12 hrs of treatment. There was not much change in the percentage of mortality between 12 and 24 hrs.

The LC_{50} values calculated from drawing regression lines (Figs. 1-4), from semilog graphical interpolation (Figs. 4-7) and from probit analysis (Figs. 8-10) have been presented together in Table 8. The three methods used for calculation of LC_{50} gave similar values. It could be seen that the LC_{50} values for different time are higher for air-breathing fish than for gill-breathing fish.

The behavioural responses observed after the treatment of the crude powder and the extract of Z. armatum fruit were of similar pattern. The fish were seen to become more active in the initial phase of treatment and then showed erratic movements. The opercular movement also increased along with the activity of the animal. As the fish approached lethal phase, the opercular movement decreased, the fish lost its equilibrium and finally collapsed in an upside down posture at the bottom of the jar.

DISCUSSION

A list of piscicidal plants of this region collected during the initial phase of the present study and their piscicidal properties have been already reported (Ramanujam & Ratha, 1980). This gives an idea of varied types of plants and parts which can be used in fishery management. Out of the plants collected in our study, toxicity experiments were conducted with five plants on three species of fish. The results of exploratory studies on them (Table 2) indicated that these plant materials can serve as potent piscicides both on air-breathing and gill-breathing species of fishes. Thus, it could be suggested that such exploratory studies on many other plants which have not been investigated may help to find out a suitable substitute for rotenone which is commonly used at present as a piscicide in fish nursery management and is being imported to this country. In the present study Z. armatum fruit powder was most potent, among the plant parts studied, with minimum effective concentration and least lethal time. Thus,

Z. armatum shows the promise to be an effective indigenous natural piscicide to substitute rotenone and was chosen for further studies after a step of purification by extracting with alcohol and concentrating the product.

There are reports indicating that certain toxicants such as industrial pollutants (Warren, 1971) and chemicals (Cardwell et al, 1976) added to water change different physico-chemical factors like pH, O_2 level and conductivity. Changes in water quality under the influence of environmental factors have been shown to affect aquatic organisms directly or modify the effect of different toxicants on them. Increase in temperature is usually accompanied by greater metabolic activity and higher rate of gill irrigation. The two week LC_{50} of zinc for Altantic salmon (Salmo salar) has been shown to increase with temperature (Hodson & Sprague, 1975). Cairns and Scheier (1962) studied the effect of temperature and hardness of water on toxicity of naphthenic acid to common bluegill sunfish. They found that the variation in temperature did not exert any effect upon the tolerance of fish. However, the difference in the dilution of water altered the toxicity of naphthenic acid. Brungs et al (1976) showed that pther factors of diluent water such as pH, acidity and alkalinity affected both active and chronic toxicity of copper to fathead minnow. There are conflicting reports about the effect of dissolved oxygen on the tolerance of the fish to a toxicant (Lloyd, 1961, 1962; Warren, 1971; Voyer, 1975). Asphyxiation and lower dissolved oxygen levels have been

reported to show marked physiological changes in fish (Saivio et al, 1973; 1974; Ratha & Bhagowati, 1981).

In the present study the observations on water quality (Table 3) indicated that there was no significant change in dissolved oxygen levels till 24 hours after addition of four concentrations of the crude powder. The experiments were conducted at the same time and the temperature was kept under control. It was also seen that there was no change in pH and conductivity of diluent water on addition of the toxin. These results clearly show that there was no ionic disturbance in diluent water during the bio-assay experiments and the action of the toxin was solely due to its direct effect of the active principle on the fish.

A comparative account of action of crude powder and alcoholic extract of Z. armatum fruits on P. shalynius (Table 4 & 5) showed that the crude powder LTC was 70 ppm and 6 hr LC_{50} was 95 ppm where as the alcoholic extract LTC was 6 ppm and 6 hr LC_{50} was 6.4 ppm. The alcoholic extract showed 96% recovery of activity compared to crude powder which indicated that almost complete toxic fraction was recovered and concentrated in alcoholic extract. The survival time of H. fossilis was longer than to that of P. shalynius. There was almost a cent percent mortality of P. shalynius in alcoholic extract within 6 hrs above 7 ppm concentrations. The death of fish was 80-90% within 6 hrs. Those which survived after 6 hrs were either not at all affected by the toxicant or gradually acclimatized to the prevailing conditions after the initial stress. The survival time of P. shalynius was longer in crude powder than in alcoholic extract. It prolonged upto

12 hrs and the fish which lived longer showed the phenomenon of tolerance. Such phenomenon leading to acclimatization of the fish to different toxicants like synthetic detergents (Degens et al, 1950, Lemke & Mount, 1963), hydrogen ions (Jordan & Lloyd, 1964), ammonia (Lloyd & Orr, 1969), cyanide (Neil, 1957) and zinc (Edwards & Brown, 1967) have been reported. The extent of acclimatization is a function not only of the concentrations of the factors to which the animal is exposed but also of the duration of exposure.

It was seen in the present studies that the active principle extracted in alcoholic extract showed more acute toxicity than the crude powder. This might be due to the fact that alcoholic extraction is the first step in purification of active principle from plant materials. It was not possible to select the concentrations of toxicant in geometric propprtion due to a very short survival time and the phenomenon of tolerance exhibited by the fish. Therefore, it was not possible to calculate the LC_{50} values for certain periods by regression plot and probit analysis.

Size and habitat of fish play an important role in toxicity tests (Sprague, 1973). The fish of lower weight group, in general, have lesser resistance capacity to any toxicant than the fish of higher weight group. Different types of habitat like complete dependence on dissolved oxygen or an air-breathing habitat are known to influence the degree of toxicity. It was seen that hardy air-breathing fish showed more resistance to mercury toxicity than the sensitive gill-breathing fishes (Das et al, 1980). In this study differences in LC_{50} values of

P. shalynius and H. fossilis could be accounted in part to their differences in habitat. H. fossilis is a comparatively hardy and an air-breathing fish whereas P. shalynius is purely aquatic and very sensitive to disturbances in the media. There could be difference in LC_{50} values in the same species of fish with different weight groups. This was observed in our experiments on H. fossilis of two weight groups viz., 8-12 g and 13-20 g. The percent mortality in 8-12 g weight group was almost double compared to 13-20 g weight group at 15 ppm concentration of the extract (Table 6 & 7). This could be due to the fact that the larger fishes ~~are having~~ better adaptability to the changes in their immediate environment ~~than the smaller ones.~~

The average values of 3,6, 12 and 24 hrs LC_{50} for H. fossilis by the three methods adopted for calculating were 18.9, 17.2, 14.00, and 14.00 ppm. in 8-12 g weight group and 30.35, 20.00, 17.45 and 16.95 ppm in 13-20 g weight group respectively. The LC_{50} values calculated by the three different methods were similar. Studies on the mode of action of a toxicant are usually done at LC_{50} concentration. This is because at higher concentrations the action will be very acute and at lower concentrations there may not be enough response to bring about measurable changes in physiological processes in the organism. Hence, 20 ppm of Z. armatum fruit extract which is around 6 hr LC_{50} value for H. fossilis (13-20 g) was chosen for further studies on physiplogical and biochemical parameters.

The behavioural responses of fish to various piscicidal plant materials have been observed visually by different workers

(Bhuyan, 1967, 1968; Babu, 1965; Konar, 1969; Charkraborty et al, 1972; Nandy & Chakravarty, 1976). It has been reported, in general, that most of the piscicidal plant materials produce immediate irritant response in fish. It was observed in the present study that both gill-breathing and air-breathing fish showed avoidance reaction on addition of toxin. The fish became more active in the initial phase of treatment when treated above the threshold concentration. The frequency of the opercular movement in gill-breathing fish increased at the initial phase and gradually decreased towards the lethal phase. As the fish became more active, sporadic erratic movements were observed. The frequency of surfacing of the air-breathing fish, H. fossilis , increased and showed convulsive movements. Finally, the fish collapsed at the bottom of the jar turning upside down. The time taken for death after reaching the lethal phase was ~~relatively~~ less for gill-breathing fish ~~compared to~~ than that of air-breathing fish. Some predictions on the mode action of the toxin could be made from the observations of behavioural studies. The prominent convulsive movement of both air-breathing and gill-breathing fish suggests that the toxin or the active principle present in Z. armatum may be acting as a neurotoxic agent. The increase in frequency of opercular movement and surfacing may be due to the stress caused by the lack of availability of energy and hence the fish is trying to increase oxygen uptake. Apparently, it was observed that there was no bleeding either in the opercular region or in any other part of the fish both inside and outside the body. This eliminated, partially, any speculation on severe action of the toxicant

on circulatory system causing haemorhage. However, morphological symptoms does not completely reveal the physiological changes occuring in an organism.

The behaviour of fish, especially air-breathing, suggests that the toxin might be acting on general metabolism of the fish. As the lethal phase approached, the active fish gradually slowed down their surfacing activity and settled at the bottom. ~~It was~~ They were not in a position to move. This type of behaviour could be due to the effect of the toxicant in decreasing the production of metabolic energy for different physiological processes. The fish might have also lost its ability to move to the surface of water to get oxygen due to insufficient production of ATP or block in the release of energy from ATP and therby settled at the bottom and succumbed.

It could be speculated from the above behavioural studies on the effect of the toxicant on the gill-breathing and air-breathing fish, that the toxicant might be acting either on the nervous system to paralyse different organs or on the circulatory system causing the failure of proper distribution of essential and waste products of metabolism or on the respiratory process of the animal causing the failure of energy production or on the release of stored metabolic energy thus affecting all active physiological processes in the body or combination of more than one of these or on some other processes causing severe stress leading to the death of the fish.

In view of the above, further detailed studies on hematological parameters, oxygen uptake both *in vivo* and *in vitro* the activities of key enzymes in neural transmission, acetylcholin-

esterase (AchE) and in metabolic energy release ATPase were carried out in fishes treated with the Z. armatum fruit extract. These studies have been sequentially discussed in subsequent chapters to findout the possible mode of action of the toxicant present in Z. armatum fruit.

CHAPTER II

HEMATOLOGICAL STUDIES

INTRODUCTION

Studies on conventional bio-assay experiments, of any toxicant provide the lethal toxicity data for a particular species of fish. The measurement of the strength of the toxicant in such studies is done using death as indicator. However, many toxicants are known to cause serious physiological damages at very low concentration. In recent years, measurements of sublethal changes in physiological functions have been attempted and assessment is, often, possible before these changes become irreversible. It has been, therefore, necessary to select such parameters which are very sensitive to a particular toxin and can show immediate measurable effects. Various behavioural and physiological parameters like flickering response, opercular rhythmicity, osmoregulation, oxygen consumption and hematological parameters have been used to know the effect of a toxicant. The hematological parameters have been studied most commonly for evaluation of toxicity of any toxicant. The alterations in hematological parameters like red cell counts, hemoglobin content, hematocrit, blood oxygen carrying capacity, blood clotting time etc. are taken as the direct effect of a toxicant.

It has been reported that various physical and biological factors like habitat (Anthony, 1961; Albritton, 1952; Haws & Goodnight, 1962; Cameron, 1970), sex (Slicher, 1961; Banarjee, 1966; Mulachy, 1970),

age (Lysaya, 1951; Ostrumova, 1960), seasons (Preston, 1960; Yamashita, 1969), body size (Dombrowski, 1953; Preston, 1960; Pradhan, 1961; Pandey et al, 1976) and temperature (Anthony, 1961; Platner, 1950; Griggs, 1968; Syrov, 1970; Pandey, 1977) cause variation in hematological parameters in fish.

Many types of environmental stress like hypoxia and hyperoxia (Randall & Jones, 1973; Kirk, 1974; Saivio & Oikari, 1976; Smith & Hattingh, 1978), hypercapnia (Hans, 1976), mechanical stress (Casillas et al, 1977) and temperature (Houston & Rapert, 1976) are also known to alter blood pH, PCO_2, PO_2, glucose, hematocrit and hemoglobin concentration.

The effect of different chemical factors in the aquatic system on the hematological parameters in fish have been studied widely. Some herbicides (Popova, 1970; Kawatsu, 1977), insecticides such as organophosphate (Svabodova, 1971, 1975; Sakaguchi, 1972; Shakoori et al, 1976), organochlorides (Lone & Javaid, 1976; Fourie & Hattingh, 1979), and carbamates (Winteringham, 1966) and many metal ions like lead, cadmium, nickel, mercury, zinc and copper (Gardner & Yevich, 1970; Christensen et al, 1972; Whanger, 1973; Edward & Lessler, 1974; O'Connor, 1975; Hodson, 1976a; Johanson & Larsson, 1978; Agarwal et al, 1979; Srivastava & Agarwal, 1979; Srivastava & Mishra, 1979; Mishra & Srivastava, 1979) have been shown to alter the hemoglobin concentration, RBC number, blood serum enzymes and other hematological parameters to a considerable extent in different fishes. Acute exposure of adult rainbow trout (Salmo gairdneri) to chlorine (Zeitoun et al, 1977) resulted in hemoconcentration, hemolysis of

erythrocytes, increase in hemoglobin and slight increase in methemoglobin in its blood. Hemolysis and anemia were found in fingerlings of coho salmon (Oncorhynchus kisutch) chronically exposed to chlorine (Buckley et al, 1976). Kristoffersson et al (1974) reported that the industrial pollutants like phenol significantly increased the activities of LDH, GOT and GPT in the blood plasma of northern pike. The chemical piscicides like thanite and malachite green have been shown to influence blood chemistry of coho salmon, Onchorhynchus kisutch and channel cat fish, Ictalurus punctatus (Bill & Hunn, 1976; Grizzle, 1977).

Studies on the effect of piscicidal plant toxins on hematological parameters of fish are scanty. Rotenone, the only plant material which is extensively used as a piscicide, has been studied in some detail. Rotenone decreases oxygen dissociation from the blood at tissue capillary beds (Perry & Conway, 1977). Iwama et al (1976) studied the changes in some hematological characteristics of coho salmon (Oncorhynchus kisutch) in response to acute exposure of one of the several naturally occuring resin acids, dehydroabietic acid (DHAA). This is extracted from soft wood trees during the kraft processing. They found that the clotting time increased and WBC decreased in the treated fish. However, there was no change in RBC and hematocrit. Saponin obtained from plant materials is known to cause hemolysis in fish (The Wealth of India, 1952; Chopra et al, 1958).

Plan of work

The present study was conducted to know whether the alcoholic extract of fruits of Zanthoxylum armatum, has any effect on blood parameters like RBC number, hemoglobin concentration, blood oxygen carrying capacity and mean corpuscular hemoglobin. It was thought necessary to establish the normal hematological parameters of the experimental fish as various factors like habitat, sex, size etc. are known to cause variation in hematological parameters. The different hematological parameters were established in the experimental fish (H. fossilis) to compare the effects of the toxin on the treated fish.

The effect of toxicant on hematological parameters ~~were~~ was studied in two weight groups (9-14 g and 15-20 g) as there were considerable variations in their blood parameters in the two weight groups and the LC_{50} studies were also done in similar weight groups.

MATERIALS AND METHODS

Heteropneustes fossilis were used for experiments without discriminating their sex. The fish were purchased from market at Gauhati, Assam and were acclimatized to laboratory conditions at Shillong. They were maintained in aquaria with alternate day feeding at 20 $\pm$ 2°C for three weeks before commencing the experiments. Their weights ranged from 8 to 22 g and length from 10 to 15 cm.

The fish were sorted into two groups for experimentation on the basis of their weight as 9-14 g and 15-20 g. The alcoholic extract of fruits of Z. armatum was prepared by C.D.R.I. as

mentioned in Chapter I. The toxicity experiments were carried out in 10 litre glass jars containing 6 litre of tap water with six experimental fish at a time in each jar. A jar with 6 fish in tap water (diluent water) was used as control.

In all fish, the blood was sampled from the caudal vein and RBC number/mm^3 and g % Hb were measured using the hemocytometer and hemoglobinometer respectively following Brown (1973). The blood oxygen carrying capacity (BOC) was computed by multiplying the hemoglobin content with the oxygen combining power of 1.25 ml of oxygen per gram of hemoglobin (Johansen, 1970). The data on Hb and RBC were utilised to calculate the different ratios like g% Hb/g wt., RBC number/g wt. and mean corpuscular Hb (MCH). Correlation between the body weight and different parameters in control fish were calculated and regression lines drawn as per Bailey (1965).

The fishes were treated with four different concentrations of the toxin (15, 20, 30 and 35 ppm). The blood samples were taken from fish treated with 15 ppm after 12 hours, 20 and 30 ppm after 6 hours and 35 ppm after 3 hours, since the threshold effect and survival time were lesser at higher concentrations.

RESULTS

The data for different hematological parameters with reference to the body size of the fish are given in Table 9. It could be seen from the table that RBC/mm^3, g % Hb and BOC

increased significantly ($p < 0.05$) along with the body size of the fish upto 12 g and remained almost unchanged till 15 g wt. Again from 15 g to 16 g there was a significant increase ($p < 0.02$) in all three parameters following which there was no significant change upto 22 g wt. It was observed that there was no significant correlation between MCH and weight of the fish with the 'r' (correlation coefficient) value as 0.2888. The 'r' values for different parameters in relation to body weight were observed as follows: weight and length = 0.8520, weight and RBC number = 0.7829, weight and Hb g % = 0.7670, weight and RBC/g wt, = - 0.8514, weight and Hb g % g wt. = -0.6842 and weight and BOC = 0.7436. The actual and estimated values of different parameters in relation to body weight have been shown in Fig. 11. The rate of increase in length and different hematological parameters along with the weight followed in order of length ($b = 0.2653$) > BOC ($b = 0.2328$) > Hb ($b = 0.1836$) > RBC ($b = 0.0816$). The rate of decrease of Hb g %/g wt. was about twenty times more than the rate of decrease of RBC number/ g wt. with increase in body weight.

The hematological values like RBC number, Hb concentration, MCH and BOC of control and treated fish of the two weight groups are given in Tables 10 and 11. The four different concentrations of the extract used in the experiment showed no significant effect ($p > 0.05$) on any of the hematological parameters studied in both the weight groups of fish.

DISCUSSION

1. Variation in hematological parameters of *H. fossilis* with size :

The observations on the hematologic parameters have shown that in *H. fossilis* there was an increase in RBC number and Hb concentration with the increase in size of fish (Table 9; Fig. 11). It can be said from regression analysis that the rate of increase of Hb g % ($\hat{b} = 0.1836$) and BOC ($\hat{b} = 0.2328$) was more than twice compared to that of RBC ($\hat{b} = 0.0816$) in relation to the weight of the fish. These results are similar to the findings of Dube and Munshi (1973) in *Anabas* and Pandey *et al* (1976) in *H. fossilis*. They have shown that the percentage of hemoglobin concentration per erythrocyte increased with increasing body weight. This also might be the condition in this fish resulting in higher increase of hemoglobin concentration with body weight. It has been shown that there is a close relationship between Hb content and BOC of five species of marine fishes (Putnam and Freel, 1978). The level of BOC is correlated with high levels of activity of the fish which in turn require high metabolic rate of an organism. In the present study it was observed that the rate of increase in BOC with weight was higher than rate of increase in total RBC. BOC is directly related to Hb content of fish. The length of fish was more related to BOC and Hb than RBC.

The significant increase ($p < 0.05$) of Hb g % and erythrocyte number upto a body weight of 12 to 13 g may be attributed to the exponential growth phase of the fish.

During the active growth period the rate of metabolism of any animal increases. Thus, the higher Hb concentration with elevated BOC observed in our study might be an adaptation for higher metabolic rate during the growth phase.

The gradual decrease in Hb and RBC values between 13 to 15 g weight of the fish and a significant sharp increase ($p < 0.02$) between 15 to 16 g weight may reflect the process of maturation of the fish during which the gonadal development takes place. Gutierrez (1967) reported in blue fin thuna (Thunnus thymus) that during spawning season there was an increase in RBC number and Hb concentration of both sexes and decrease of these values in post-spawning period. It was observed in H. fossilis with weight above 16 g that there was significant change in length, RBC number and Hb concentration. The RBC number and Hb concentration were observed to be directly related with the length of the fish with RBC/cm and Hb/cm remaining almost constant and the ratio RBC/g wt. and Hb/g wt. decreasing significantly. This suggests that probably there is a maximum limit of length upto which the total RBC number and Hb concentration of H. fossilis increase along with weight and there after reach a platue. Similar results were obtained by Dube and Munshi (1973) in Anabas testudineus, and Joshi and Tandon (1977) in Clarias batrachus. It is possible that as the fish grows old, it becomes less active and the metabolic rate is retarded resulting in increased weight.

Among the three ratios of hematological parameters studied it was seen that g % Hb/g wt. and RBC number/ g wt.

showed decreasing trend with increasing weight of fish. The rate of decrease of Hb g %/g wt. was twenty times greater than the decrease of RBC number/g wt. with increasing weight. This suggests that variation in Hb content is more sensitive to age than RBC number. However, the mean corpuscular hemoglobin, in general, remained constant with the mean value $59.4 \pm 7.96 \times 10^{-9}$ g and at 95% confidence limit. This appears to be species specific constant which needs confirmation in a larger population from different environments. It has been suggested that the air-breathing fishes have higher Hb concentration (Lenfant & Johansen, 1972) and higher oxygen holding capacity (Dubale, 1963) than the water-breathing fishes. Comparison of the Hb concentrations and BOC as observed in the present studies on H. fossilis and by others in marine fishes (Hall & Gray, 1929; Putnam & Freel, 1978), in Anabas testudineus (Dube & Munshi, 1973; Singh et al, 1979), in Ophiocephalus gachua, Anabas scandens and Mystus vittatus (Ramaswamy & Reddy, 1978), in Clarias batrachus (Joshi & Tandon, 1977) and in H. fossilis and Mystus vittatus (Joshi & Tandon, 1976) reveal that H. fossilis has higher level of Hb and BOC. Thus, it could be suggested that H. fossilis is more dependent on atmospheric oxygen and is more evolved among amphibious fishes studied.

2. Effect of Z. armatum fruit alcoholic extract on hematologic parameters of H. fossilis:

The effect of different concentrations of the alcoholic extract on hematologic parameters of H. fossilis was studied

in two weight groups of 9-14 g and 15-20 g. The results have been presented in Tables 10 and 11. The concentrations used were chosen to fall on either side of LC_{50} value i.e. 20 ppm. The different hematological parameters have been reported to be influenced by different pesticides, herbicides, heavy metals and chemical piscicides.

Pesticides like sumithion and sevin are known to change hematological parameters in fresh water fish Sarotherodon mossambicus (Koundinya & Ramamurthi, 1980). The total RBC, packed cell volume and Hb content were decreased at 48 hr LC_{50} concentrations of sevin. Sub-lethal concentrations of sumithion and sevin increased mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration. This has been attributed to the hypoxic condition in water created by the pesticide. It is known that hypoxic condition increased RBC and Hb content (Vanliere & Stickney, 1963). Other types of stress like hyperoxia, hypercapnia and thermal shocks also increased blood hematocrit, hemoglobin and other hematological parameters (Kirk, 1974; Casillas et al, 1977; Hans, 1976; Smith & Hattingh, 1978; Green, 1977). However, in the present study there was not much change in oxygen content and other water qualities like pH, conductivity and temperature (Table 3). Further, experimental fish H. fossilis being predominantly on air-breather, would not be much influenced by oxygen concentration of water.

In the present study, no significant change could be obtained in Hb, BOC, RBC and MCH of the fish treated with four

different concentrations of the extract (Ramanujam & Ratha, 1980a). Certain active principles like saponin derived plant materials are known to cause hemolysis in fish (The Wealth of India, 1952; Chopra et al, 1958). The chemical analysis so far done on Z. armatum does not indicate the presence of any hemolytic factors like saponin (Simensen & Rao, 1929; Nazir & Handa, 1961; Deshpande & Shastri, 1977).

Thus, it can be concluded from the studies on hematological parameters of H. fossilis that (i) a clear understanding of normal levels of the various parameters under consideration in a particular species is necessary for toxicity studies as significant intraspecific variations exist relating to size and environment of the fish, and (ii) the piscicidal components present in the fruits of Z. armatum do not have any effect on the hematological parameters and the lethal effect could be due to the damage done to some other physiological processes.

CHAPTER III

OXYGEN CONSUMPTION STUDIES

INTRODUCTION

Respiration is an essential physiological process by which the living organisms obtain energy for carrying out their different functions. Respiration is classified as aerobic and anaerobic types depending on availability and utilization of oxygen. Aerobic respiration involves the use of oxygen for release of energy where as anaerobic respiration takes place in absence of oxygen. Respiration (aerobic) in multicellular organisms takes place in 3 stages.

(1) External respiration : It is usually called as 'breathing'. It refers to those mechanisms by which oxygen is taken into the body from the environment and CO_2 is expelled from the body into the environment. The exchange of gases takes place at the respiratory surfaces such as integument, gills, air-sacs or lungs.

(2) Transport of respiratory gases : This phase of respiration involves the transportation of oxygen from the respiratory surface to the tissues or cells and carbon-dioxide from the tissues to the respiratory surface.

(3) Internal or cellular respiration : This phase of respiration includes the oxidative processes by which oxygen is consumed by the cells to produce energy and carbon-dioxide. These are mostly enzymatic reactions.

In fishes the uptake of dissolved oxygen from water takes place mainly through gill and skin. However, in certain fishes specialised accessory respiratory organs have been developed to utilise atmospheric oxygen as an additional

adaptation. These fishes are called as amphibious or air-breathing fishes. The air-breathing fishes, in general, have a better survival capacity in less oxygenated water.

The gills and skin are the main routes for uptake of any toxicant in aquatic organisms besides their role in gaseous exchange as these two organs are in direct contact with the media. These organs could be damaged by the toxins thus affecting the process of respiration. The effect of a toxicant on respiratory system of an organism could be studied either at organismal level, by measuring the amount of O_2 taken by the fish from the medium or by measuring the rate of oxygen consumed by the tissues using manometric techniques in presence of the toxicant. The rate of oxygen consumed by the intact fish gives a measure of general body metabolism where as the rate of oxygen consumption by a tissue indicates the functioning of cellular oxidative pathways in that tissue.

Some reports are available on the effect of pollutants on gross respiration or oxygen uptake by the organism determined by direct or indirect means. However, studies on cellular respiration are very much wanting. Mount (1962) determined the chronic effect of endrin upon bluntnose minnows and guppies, and found that it had no effect either upon the oxygen consumption or ability of the fish to swim against current. Cairns and Scheier (1964) studied the chronic effect of dieldrin upon the oxygen consumption of Lepomis gibbosus and reported that there was significant increase in their oxygen

uptake. A near-linear relationship between uptake of C^{14}-labelled DDT and oxygen consumption in mosquitofish (Gambusia affinis) was reported By Murphy and Murphy (1971) at a DDT concentration of 40 mg/l. Waiwood and Johansen (1974) studied the effects of methoxychlor on survival, oxygen consumption and activity of white sucker (Catostomus commersoni). They found that in all fish before death at 0.10 ppm and 0.04 ppm of methoxychlor, the oxygen consumption rates were 2 to 3.5 times greater than those of controls. Luhn et al (1976) observed a gradual increase in the rate of respiration, coughing behaviour and heart rate of trout (Salmo gairdneri) with the increasing concentrations of pesticides. Reddy and Gomathy (1977) have shown that the lethal level of thiodon increased the aquatic respiration of Mystus vittatus, which is primarily a water breathing fish. Natarajan (1980) found no change in total oxygen uptake by Anabas scandens treated with sumithion. However, the aerial respiration increased and gill-respiration decreased on addition of the toxicant in the medium. The rate of oxygen consumption by stickle back (Gasterosteus aculeatus) exposed to zinc was studied by Brafield and Matthiessen (1976). They found that the oxygen consumption tended to rise and then become extremely erratic before declining as the death approached. Studies on the effect of toxicants on opercular rhythm have been measured by visual observations as an indirect evidence on the rate of respiration. Ellis (1937) and Jones (1947) have established relationship between pollution and respiratory activity in fish by observing the changes in the opercular rhythm.

The electronic measurement of opercular rhythm has been shown to be a practical system lending itself to automation and adoption for the biomonitoring of waste discharge (Morgan & Kuhn, 1974; Morgan, 1975). The distinct relationship between pollution and respiratory activity in fish has led in recent years to do routine investigations into the effect of pollution on breathing rate in fish (Spoor et al, 1971; Sparks et al, 1972). Some pesticides (Bergen, 1971; Kawatski & McDonald, 1974; Gupta & Balbir, 1974; Rao & Rao, 1979) and metal ions (Janssen, 1970; Hodson, 1976b; Gould & Maclnnes, 1977) have been shown to have adverse effect on the cellular respiration and thus cellular respiration could serve as an indicator system for pollution.

Rotenone, a plant derived toxicant has been only studied ~~to a greater~~ in some detail among the plant toxins. It is known to reduce the capacity of cells to withdraw oxygen from blood at tissue capillary bed (Perry & Conway, 1977). Lindahl and Oberg (1960) frist demonstrated in fish that gill filaments treated in vitro with rotenone reduced oxygen uptake. Horgan et al (1968) studied the action of rotenone at sub-cellular level and found that it acts principally by blocking the reduced nicotinamide adenine dinucleotide dehydrogenase segment in mitochondral respiratory chain. Fromm (1970) demonstrated that rotenone reduced perfusion flow rate in isolated gill arches. Nath et al (1980) have shown that the inhibitory action of different rotenoids on respiration in vitro depends on their structure. Drugs like antimycin A, oligomycin, dimercaprol and atractyloside are known either to inhibit oxidative phosphorylation or act as uncouplers (Harper et al, 1977).

The toxicity of various substances to aquatic organisms are influenced by different physical factors (Cairns, 1957). Some of them like dissolved oxygen (Kusins & Mangum, 1971), temperature (Arrington & Sellers, 1976), salinity (Nammalvar, 1977), pH, osmotic pressure and light (Igram & Wares, 1979) are known to influence the rate of oxygen consumption in fishes exposed to toxicants.

Plan of work

The present study on the effects of alcoholic extract of fruits of Z. armatum on oxygen consumption were done both at organismal level and cellular level in metabolically active tissues like liver and brain in fish. Studies at organismal level were done by subjecting the gill-breathing weed fish Tilapia mossambica to different concentrations of the extract and measuring the oxygen level in the medium to find out if the extract has got any effect on the rate of oxygen uptake from water with relation to the concentration and time of treatment. The cellular respiration experiments were done by treating in vitro fresh liver and brain tissue homogenates of Heteropneustes fossilis with different concentrations of the extract and measuring the rate of oxygen consumption by Warburg respirometer using glucose as the substrate.

MATERIALS AND METHODS

The gill-breathing fish Tilapia mossambica were obtained from Gauhati fish farm and Heteropneustes fossilis were purchased from the market at Gauhati, Assam. The fishes were

brought to Shillong and acclimatized to laboratory conditions at 20 ± 2°C at least for two weeks before using them for experiments. H. fossilis were given powdered dry fish and T. mossambica rice bran and oil cake as food, on alternate days. The gill breathing fish were aerated during acclimatization period.

The alcoholic extract of fruits of Z. armatum used in the experiments was prepared by CDRI as mentioned in Chapter I. Three different concentrations (15, 20, 30 ppm) of the extract and a control were run in triplicate for oxygen uptake study by the whole animal. The diluent water which was used in the experiments was unchlorinated tap water, having the same qualities as mentioned in bio-assay experiments. The experiments were carried out in closed jars containing 5 litre of tap water with 3 fish (T. mossambica) weighing approximately 30 g in each jar. The surface of water was layered with liquid paraffin to avoid contact with the atmosphere. The oxygen content of water both in control and experimental jars were estimated using Winkler's method (Welch, 1948) at 30 minutes intervals till 6 hrs. The water for oxygen estimation was taken out slowly through a stoppered out let. A few fish died in 20 and 30 ppm concentrations during the experimentation period and they were removed soon after their death. During these experiments other physico-chemical factors were kept constant. The oxygen content of the water was kept higher than the critical range. There was no death of fish in the control jars. The average rate of oxygen uptake for each 30 min. interval was calculated and expressed as mg oxygen consumed/g fish/30min.

The tissue respiration *in vitro* was carried out at two temperatures (20°C & 30°C) in the brain and liver tissues of *H. fossilis*. 10% tissue homogenates were used for measuring the rate of respiration. The oxygen consumption was measured by Warburg respirometers with glucose as the substrate (Dixon, 1943). The Warburg flask contained 3.2 ml reaction mixture as follows:

	Control	Experimental
Main chamber :	0.8 ml Sucrose (0.25M) 0.7 ml Phosphate buffer (1 M, pH. 7.4) 1.0 ml tissue homogenate (10%)	0.8 ml Sucrose (0.25M) 0.5 ml phosphate buffer (1 M pH, 7.4) 1.0 ml tissue homogenate (10%)
Central well :	0.2 ml of 10% KOH	0.2 ml of 10% KOH.
Side arm :	0.5 ml of glucose (1.0M)	0.5 ml of glucose(1.0M) 0.2 ml of *Z. armatum* fruit extract.

The TB (thermobarometer) was prepared with 3.2 ml of phosphate buffer in main chamber.

After allowing 10 minutes for equilibration at required temperature the contents of side arm were mixed with that in central chamber. The oxygen consumption was then measured in the barometer continuously for 30 minutes at 5 minutes interval. All chemicals used in the experiments were of analytical grade supplied by either Sigma Chemical Co., U.S.A. or Glaxo Laboratories, India.

RESULTS

Effect of the extract on oxygen uptake by fish (T. mossambica)

The oxygen uptake by Tilapia mossambica during initial stages of the experiments were at a higher level in all the three concentrations of the extract studied and also in the control (Table 12; Fig 12 & 13). In control fish, the oxygen consumption gradually reduced to a stable state by 4 hrs. At 15 ppm concentration of the extract there were prolonged fluctuations in the rate of oxygen uptake by fish which stabilized at a lower level ~~compared to that of~~ than control by 5 hrs. The patterns of oxygen consumption at 20 and 30 ppm were parallel. However, the initial stimulation of oxygen uptake was at an earlier time and to a comparatively higher level and the final stabilization point at a lower level in 30 ppm concentration ~~compared to~~ than that in 20 ppm. In general, it was seen that the rate of inhibition of oxygen consumption was dependent on the concentration of toxin and the stabilization points reached were lower as the concentration of toxin increased.

The maximum inhibition of the rate of oxygen consumption (-25%) at 15 ppm was observed between 5 and 5.5 hrs. In 20 and 30 ppm concentrations of toxicant after an initial fall, the rate of oxygen consumption reached maximum at 3 hrs and 2.5 hrs respectively and then gradually came down towards the end of the experiment.

It was observed that there was no death of fish in control and 15 ppm concentrations. At 20 and 30 ppm concentration few fishes died during experimentation. The rate of mortality was higher in 30 ppm concentration.

Effect of the extract on oxygen consumption by tissues

The results on the oxygen consumption by brain and liver homogenates of H. fossilis in presence of the extract are given in Table 13. There was no significant change in the cellular oxygen consumption rate in presence of the toxicant both at 20°C and 30°C temperatures However, at 30°C the rate of oxygen consumption was higher than at 20°C in both the tissues.

DISCUSSION

Effect of the extract on oxygen uptake by the fish.

The changes on environmental conditions cause stress to the animals and influence their metabolic state. All organisms, when subjected to stress, show different physiological responses. These are either short term or long term responses. The short term responses are meant for the immediate adjustment to the changed environment and occur spontaneously such as the changes in the rate of heart-beat, opercular movement, respiration etc. The long term responses occur gradually and in a comparatively slower rate to develop a stable changed physiological condition due to process of acclimatization to the changed environmental conditions.

Environmental stress like temperature (Bullock, 1955; Fry, 1958; Precht, 1958; Wares II & Igram, 1979), salinity (Job, 1959; Nammalwar, 1977), high oxygen level (Kewalramani & Pradhan, 1955; Wares II & Igram, 1979), pH, osmotic pressure and light (Igram & Wares II, 1979) have been shown to increase oxygen consumption in fish. In our experiments on oxygen consumption

by intact fish (Tilapia mossambica), it was seen that the rate of oxygen uptake was very high in the initial stage of the experiment. This might be due to the disturbances caused to the fish while transferring them from aquaria to experimental jars. This has been further supported by the fact that the fish which were introduced in different concentrations of the extract showed a higher level of oxygen consumption than control because of additional irritant stress acting upon them due to the presence of the toxin. In control fish, the higher rate of oxygen consumption decreased after a few hours of the beginning of the experiment. One reason for this decrease in oxygen consumption could be due to acclimation of the fish to the prevailing conditions. The fish which had become over active on transfer to the jar due to stress, gradually got acclimated and became quiescent and therefore, there might have been a decrease in its metabolic rate. The experiment being conducted in a static state there could have been a sudden lowering of dissolved oxygen during the initial few hours due to high rate of oxygen consumption by fish. It is known that lower dissolved oxygen level declines the rate of oxygen consumption in fish (Wares II & Igram, 1979), insects (Walshe, 1947) and snails (Kusins & Mangum, 1971). Hence, another reason for the decline in rate of oxygen consumption might be due to decrease in total dissolved oxygen concentration of the media (Table 13). Towards the end of the experiment it was observed that the rate of oxygen consumption of control fish again increased and stabilized at a little higher level. This may be due to the acclimatization

of fish to the jar condition or this could be due to the indegenous circadian rhythm. The fish showed a graded response in their rate of oxygen consumption to three different concentration of Z. armatum fruit extract tested. At 15 ppm, there was no definite alteration in the rate of oxygen consumption. The stabilization point of oxygen uptake was reached by the 4th hour. This was at a lower level to that found in control fish and at a higher level compared to 20 and 30 ppm. The response of fish at 15 ppm concentration of toxin could be considered as a sub-lethal response and could be related to absence of mortality during the test period. At 20 and 30 ppm of the extract the response of fish was parallel, both showing elevated rate of oxygen uptake between 2nd and 4th hour of the treatment following a gradual decrease. The stabilization points were observed after 4½ hours in both the concentrations. The increase in rate of oxygen consumption in 20 and 30 ppm can be related with their hyper active state requiring more metabolic energy. They also showed higher rate of opercular movement. Pesticides are shown to increase the rate of oxygen consumption of fish with the rise in activity (Woiwood & Johansen, 1974; Lingaraj & Venugopalan, 1975). Similar type of dose dependent graded response in rate of oxygen uptake has also been reported in zinc toxicity (Brafield & Matthiessen, 1976) and in pesticide toxicity (Luhn et al, 1976). Dechev and Matveeva (1978) observed that oil and detergents stimulated respiratory activity in fish during the initial stages. Later, there was a sharp drop in their respiratory activity. These results seems to be similar to that found in our studies at

higher concentrations (20 & 30 ppm).

It could be seen from these studies that the stimulation and inhibition percentage of the rate of oxygen uptake was also concentration dependent. The maximum stimulation were + 54%, +151% and + 156% and maximum final inhibition were - 25%, -39% and -56% at 15, 20 and 30 ppm of Z. armatum fruit extract respectively. The amount of oxygen consumed by fish in a given time is dependent on such factors as the amount of muscular activity displayed, with its consequent greater or lesser consumption of energy (Beamish, 1964).

The pattern of stimulation and inhibition of oxygen uptake by Tilapia mossambica observed with the increase in concentration of toxicant could be related to higher muscular activity exhibited by the fish due to irritant response produced by the toxicant. The stabilization points seen at different concentrations clearly show a lowering of metabolic activity at higher concentration.

The increase in oxygen uptake in terms of opercular frequencies after the threshold time, has been observed in some metal ions like copper (O'Hara, 1971), cadmium (Gardner & Yevich, 1970) and chemicals like phenol (Mitrovic et al, 1968), ammonia and cyanide (Jones, 1964). The decline in rate of oxygen uptake after a brief period of increased activity might be due either to the failure in the efficiency of the fish for detoxification or to the activity of the toxicant as a respiratory depressant acting on some other physiological or biochemical processess or both.

Effect of the extract on oxygen consumption by tissues

Tissue respiration gives a measure of oxidation process

occuring at sub-cellular level which involves enzymatic reactions to liberate energy. The inhibition of certain enzymatic steps due to hypoxic conditions by any type of stress will lead to decline in production of energy. This in turn will affect all other metabolic processes occuring in an animal, as most of them are energy dependent processes. It is known that many pesticides (Bergen, 1971; Kawatski & McDonald, 1974, Gupta & Balbir, 1974; Rao & Rao, 1979) and metal ions (Janssen, 1970; Hodson, 1976b; Gould & MacInnes, 1977) inhibit the tissue oxygen uptake creating a hypoxic condition and inhibiting oxidative enzymes of mitochandria. Rotenone treated gill filaments of fish are known to reduce oxygen uptake (Lidahl & Oberg, 1960). It has been reported to act by blocking the reduced nicotinamide adenine dinucleotide dehydrogenase segment in mitochondrial respiration. The results of the present study indicate that there was no change in oxygen consumption of both liver and brain tissue homogenates with glucose as substrate at all the three concentrations of Z.armatum fruit extract. The effect of toxicant on tissue respiration was studied at two temperatures (20°C & 30°C) because high temperature is known to enhance the rate of organismal and cellular respiration (Kanungo & Prosser, 1959a, 1959b; Parvatheswara Rao, 1972) and toxicity of many toxicants (Cairns & Scheier, 1959, 1962; Hodson, 1976b). Though there was a rise in rate of respiration at higher temperature both in brain and liver tissues, the Z. armatum fruit extract did not alter the rate at both the temperatures.

It can be,therefore, inferred from these studies on tissue oxygen uptake that the Z. armatum fruit extract does not have any direct inhibitory action on sub-cellular respiration process. The inhibition of oxygen uptake by intact fish could be due to paralysing effect of the toxicant on the different organs related to the oxygen uptake.

CHAPTER IV

ENZYMATIC STUDIES

INTRODUCTION

Enzymes are a group of functional proteins catalyzing different biochemical pathways in an organism. They control different metabolic functions in an animal. Many enzymes are regulatory in nature and respond promptly to various changes in the environment. The pattern of response of different enzymes varies depending on the nature and duration of exposure of the organism to a particular environmental factor. A large volume of literature is available on the effects of different xenobiotics on different enzymes in mammals. However, such studies in non-mammalian vertebrates are limited. The behaviour of a few enzymes to various xenobiotics like pesticides, fertilizers, industrial effluents and metal ions have been investigated in fish and other organisms (Shaw, 1954; Hewitt & Nicholas, 1963; Dixon & Webb, 1964; Christensen, 1971-72; McKim et al, 1973,1974. 1975, 1976; Brungs et al, 1977; Krishna Murthy, 1980). The activity of AchE and ATPase have been the main target of investigation with respect to the treatment of different toxicants.

Mixed function oxidase (MFO) in liver is known to metabolically degrade the pesticides. An increase in its level after the treatment with pesticides like aldrin, chloradane, malathion, parathion, pyrethrin and rotenone have been shown by various workers (Gibson, 1971; Ludke et al, 1972; Fabacher & Chambers, 1972a, 1972b; Chambers & Yarbrough, 1973; Stanton & Khan, 1973; Garretto & Khan, 1975; Mukhopadhyay & Dehadrai, 1978). Moffett and Yarbrough (1972) and McCorkle and Yarbrough (1974),

reported inhibition of succinic dehydrogenase (SDH) activity by DDT and mirex respectively in susceptible mosquito fish. The inhibition of gut invertase after phosphomidon exposure in cat-fish Clarias batrachus was shown by Ghosh and Konar (1973). A positive correlation between endrin toxicity with inhibition of α-amylase activity in hepatopancreas of C. batrachus was suggested by Bhattacharya et al (1975). Thomas and Murthy (1976) studied the effect of certain organic pesticides like phosphomidon, endrin and sevin on acid phosphatase activity in liver, kidney and intestine of fresh water fish Heteropneustes fossilis. A significant increase of these enzyme activities in the treated fish were seen with endrin. However, there was no significant change in the enzyme activities with other pesticides. Alterations in the activity of certain enzymes in liver and kidney of Channa punctatus exposed to endrin were studied by Sharma and Sastry (1979). Endrin inhibited acid-, alkaline-and Glucose-6-phophatase and lipase, and stimulated amylase activity in liver. In kidney, though there was slight elevation in phosphatase activity, the increase in acid- and alkaline phosphatase was insignificant. Sastry and Sharma (1979) found that the activity of acid- and alkaline-phosphatase and glucose-6-phosphatase in liver of C. punctatus were inhibited on exposure to endrin for 15 days, where as exposure for 30 days significantly elevated the enzyme level. The enzyme levels remained elevated till 30 days in kidney which has been attributed to the increase in transphosphorylation. Acute toxicity of endrin on the activities of some oxidases (Sharma et al, 1979) and Diazinon on the

activities of some brain enzymes (Sastry & Sharma, 1980a) have been studied in Ophiocephalus punctatus.

The effect of sublethal concentration of industrial effluents on kidney peroxidase have been studied by Mukherjee & Bhattacharya (1975). They showed a 70-100% inhibition of the enzyme in O. punctatus and C. batrachus. The inhibition of the activities of thyroid peroxidase and hepatopancreatic proteases respectively in O. punctatus treated with industrial pollutants have been reported by De & Bhattacharya (1976) and Bhattacharya and Mukherjee (1976) respectively. Verma et al (1979c) have reported the inhibitory effect of distillery waste on the activities of acid- and alkaline-phosphatases in liver and kidney of O. punctatus and Saccobranchus fossilis. Increase in exposure time decreased the enzyme activity significantly. Studies on mix function mono-oxygenase in aquatic organisms have shown that the activities of this enzyme could be an indicator of aquatic pollution by industrial effluents (Ahokas et al, 1976). The activity of MFO has been shown to be induced by polychlorinated biphenyls in trout liver (Addison et al, 1978; 1979). Petroleum and other compounds containing hydrocarbons are known to induce aryl hydrocarbon hydroxylase activity (Payne & Penrose, 1975). This enzyme has been suggested to be used as indicator of aquatic pollution by petroleum products. Alterations in the activities of many digestive, blood serum and metabolic enzymes have been studied with relation to the treatment of many metal ions like cadmium (Johanson & Larsson, 1978), copper (Lee et al, 1973),

lead (Hodson, 1976a; Sastry & Gupta, 1978; Sastry & Agarwal, 1979a), mercury (Kendall, 1975; Hinton & Koeing, 1975; Koller et al, 1977; Sastry & Agarwal, 1979b; Sastry et al, 1979; Sastry & Gupta, 1979; Sastry & Sharma 1980b), nickel (Whanger, 1973) and zinc (Watson & McKeown, 1976). Sublethal concentrations of phenol significantly elevated the activities of some blood plasma enzymes like LDH, GOT and GPT (Kristofferssen et al, 1974). The inhibitory activity of blood cell carbonic anhydrase in channel cat fish (Ictalurus punctatus) by inorganic cations, organometallic cations, some anions and pesticides were reported by Christensen and Tucker (1976).

Inhibition of LDH, kidney peroxidase and allontoinase by pesticides like aldrin, parathion, dieldrin and related compounds, kepone, endrin, DDT and PCB have been studied in vitro (Hendricksen and Joe, 1976; Anderson & Carter 1977; Passino & Contant, 1979). Chambers et al (1979) showed that the activities of AchE and alkaline-phosphatase were enhanced on chronic exposure to crude oil. Bogin and Abrams (1976) reported the inhibitory effect of garlic extract on hepatic and serum GOT, GPT, LDH and ChE in guinea pig.

The varied types of responses shown by different enzymes are reflected on the physiological activities of the organism leading to behavioural changes. Thus, the selection of an enzyme for a particular toxicant could be made from the behavioural response of the animal for that particular toxicant. The hyper activity of H. fossilis on treatment with Z. armatum

fruit extract and then a gradual reduction in its activity resulting in its inability to reach the surface of water for aerial respiration towards the lethal phase were assumed to be the impact of the toxin on either neural transmission or the lack of energy production in fish or both. Considering the behaviour of H. fossilis to Z. armatum fruit extract along with its effect on physiological parameters like blood parameters, oxygen uptake and respiration, studies on the key enzymes namely Acetylcholinesterase (AchE, E.C.3.1.1.7) responsible for neurotransmission and Adenosine triphosphatase (ATPase, E.C.3.6.1.3) for release of metabolic energy in treated fishes were undertaken.

Acetylcholinesterase

Nervous transmission is one of the suitable targets for any toxicant since this is the basis for coordination of different physiological functions. AchE helps in hydrolysis of acetylcholine (Dale, 1914), a neurotransmitter released during the transmission of nerve impulse in the cholinergic synapses (Nachmansohn, 1959). Besides cholinergic synapses, Ach and AchE are also present in neuromuscular junctions (Prosser, 1973; Rubin et al, 1979) and nerve free endplates (Weinberg & Hall, 1979). The level of AchE activity has been correlated with the general state of activity of animals (Vijayalakshmi et al, 1977). Therefore, alterations in AchE activity could be used as an indicator for any type of stress causing change in the activity of an animal. AchE activity has

been shown to be influenced by different physiological and physical factors like aestivation (Murali Mohan et al, 1977) , photoperiod, light intensity and motor activity (Wood & Rose, 1979), hydrostatic pressure (Millar et al, 1974), temperature, pH (Ngo & Laidler, 1978) and X-irradiation (Valcana et al, 1974).

Several investigators have reported the different molecular forms of AchE both in vertebrates and invertebrates. Principato et al (1978) reported three forms of AchE in Allolophora caliginosa. The enzymatic forms are shown to be monomeric, dimeric and tetrameric. The monomeric forms seems to be composed of two subunits. The AchE of Electrophorus electric organ is shown to contain six molecular forms, three globular and three asymmetric forms which are similar as that of Torpedo marmorata (Bon & Massolie, 1976a, 1976b; Bon et al, 1976; Rieger et al, 1976). In chick, three molecular forms of AchE as 11S, 6S and 4S components have been characterised. (Marchand et al, 1977). The 11S form represents most of AchE activity in adult brain. In fish there are six molecular forms of AchE like that of mammals (Vigny et al, 1979). Three are globular forms and other three asymmetric structures. The globular forms occur as monomer, dimer and tetramer where as the asymmetric forms are found to contain one, two and three tetramers. One of the asymmetric forms has been reported in the neuromuscular junction (Hall, 1973; Vigny et al, 1976). In brain of mammals 4S and 10S forms are found. The kinetic differences of AchE observed in different tissues of various animals (Mohan & Brink, 1970; Moudgil & Kanungo, 1973; GoodKin & Howard,

1974; Giri et al, 1977; Moss & Fahrney, 1978; Vanker & Mizukami, 1979) might be due to the tissue specific iso-enzyme variations.

Several AchE specific electrophoretically separable bands have been demonstrated on gels in various tissues of different animals. Three electrophoretically separable zones of eserine sensitive enzyme activity against acetylthiocholine in mammalian brain were identified in starch gel matrices by Bernsohn et al (1963) and Barron et al (1963). Maynard (1964a) has demonstrated three AchE specific bands in embryonic brain and skeletal muscle, as well as in adult chicken brain. Maynard (1964b) has described two isoenzymic forms of AchE by gel electrophoresis in three species of lobster nervous system. The rapidly migrating form was predominant in central ganglia and interganglionic connectives, while the slower moving enzyme was mostly present in peripheral nerves. Baldwin and Hochachka (1970) have shown two distinct bands of AchE in brain of trout, one being predominant in cold acclimatized and the other being predominant in warm adapted fish. Both forms were found in the fish acclimatized to intermediate temperature. Moudgil and Kanungo (1973) have reported only one band of AchE which was eserine sensitive in the cerebral hemisphere of rat of different ages. Vijayan and Brownson (1975) have shown three different bands in different parts of brain of rat. Gisiger et al (1978) separated five bands of AchE from rat sympathetic ganglion.

Many of the commercially available insecticides work by interfering with passage of nerve impulse (Corbett, 1974).

They are thought to act on (i) axonal transmission (ii) acetylcholine receptor or (iii) the enzyme acetylcholinesterase. The inhibition of acetylcholinesterase by toxicants stops the hydrolysis of acetylcholine leading to its accumulation and thus prevention of the smooth transmission of nervous impulses across the synaptic gap. This results in electrophysiological disturbances causing loss of muscular coordination and induction of convulsions leading to death.

Most of the work on specific anticholinesterase agents and other reaction-rate-influencing chemicals (*in vivo* and *in vitro*) are concerned with pesticides belonging to the group of carbamates, alkylphosphates, selenophosphates, fluorophosphates and organosulfates (Hayama & Kuwabara, 1962; Davis & Mulancy, 1967; O'Brien, 1967; Voss, 1969; Karczmar, 1970; Su *et al*, 1971; Gibson & Ludke, 1973; Corbett, 1974; Mukherjee & Bhattacharya, 1974; Thirugnanam & Forgash, 1975; Coppage, 1977; Olson & Christensen, 1980). Some metal ions like copper, mercury, lead and nickel (Abou-Donia & Menzel, 1967), alkyl lead (Galzigna *et al*, 1969; Heywood *et al*, 1978) and industrial effluents (Karczmar, 1970; Mukherjee & Bhattacharya, 1974) have also been shown to decrease the level of AchE. Carboxylesterases have been reported to be inhibited by organophosphate insecticides (Aldridge, 1953; Oppenoorth & Ven Asperen, 1960; Danford & Beardmore, 1978; Chin & Sudderuddin, 1979).

Several toxins from algal sources have been investigated for their effect on neuromuscular junction. *Gymnodinium veneficum* toxin stimulates transmission before blocking and acts as a depolarizing agent (Abbott & Ballentine, 1957). The toxin of

Gonyaulax catanella (Burke et al, 1960) and saxitoxin from Saxidomus giganteus block axonal and muscle conduction without depolarization and show very little effect if any on neuro-muscular junction (Kao & Nishiyama, 1965). Venoms from various sources, both from vertebrate and invertebrates are also known to possess neurotoxic action (Gardiner, 1972; Levinson et al, 1976; Tu et al, 1975; Sen et al, 1976).

The tissue specificity in terms of reaction to different toxicants could be explained by presence of different iso-enzyme patterns of AchE in various organs and tissues. Different isoenzymes of AchE have been shown to have marked differences in their inhibitory and recovery time on parathion treatment (Vijayan & Brownson, 1975). Ozretic and de Ligny (1978) have emphasised the need of isoenzyme studies on AchE for more specific monitoring of organophosphate pesticides. Some of the new aspects of target enzyme inhibition interaction includes the studies on the existence of isomers of AchE and their relative rates of inhibition by organophosphates, the likely identity between the receptor site for binding of acetylcholine and the multifaceted membrane bound AchE (KrishnaMurthy, 1980).

Most of the neurotoxic action usually lead to behavioural changes in their activity. The fish becomes excited at the initial stages of the treatment of the toxicant, then displays erratic convulsive movements with frequent tremors and finally shows a gradual cessation of activity leading to death.

Adenosinetriphosphatase (ATPase)

Most of the physical, physiological and biochemical processes in an organism depend on the availability of metabolic

energy supplemented by the breakdown of ATP molecules. ATPase is the enzyme which hydrolyses ATP molecule to liberate energy. Mainly three types of ATPase have been recognised in living systems depending on the requirement of different metal ions for their activity. They are Mg^{2+} ATPase, Na^{+}, K^{+} ATPase and Ca^{2+} ATPase. Mg^{2+} ATPase is mainly concerned with energy supply for general metabolism and Na^{+}, K^{+} ATPase primarily provides energy for active transport to maintain ionic equilibrium. Ca^{2+} ATPase is known to be responsible for active transport of Ca^{2+} ions on the axonal membrane. Therefore, the distribution of different ATPase depend on the functions carried out by them. In aquatic organisms Na^{+}, K^{+} ATPase plays an important role in osmoregulation which is an adaptation of an aquatic organism to its environment (Jampol & Epstein, 1970).

Horiuchi (1977) characterized Na^{+}, K^{+} ATPase from heavy microsomal fractions of gill of fresh water crayfish Procambarus clarki. He has reported the K_m of gill Na^{+}, K^{+} ATPase for ATP as 7.1×10^{-4}M and the K_m of Mg^{2+} ATPase as 8.7×10^{-4}M. The presence and properties of Mg^{2+} - HCO_3^{-} stimulated and SCN^{-} inhibited ATPase in mouse kidney was studied by Suzuki (1978). His studies indicate that SCN^{-} inhibited both Mg^{2+} - and Mg^{2+}-HCO_3^{-} ATPase activities at concentrations above 10^{-2}M. The K_m of Mg^{2+} ATPase in mitochondrial, microsome I, microsome II fractions for ATP were found to be 2.77 mM, 1.49mM and 1.47mM respectively.

The activity of the animals could also be related with ATPase activity as it is directly related with energy metabolism in the organism and also indirectly regulate the Ach levels (KrishnaMurthy, 1980). The endogenous rhythm of ATPase activity

have been studied in various organisms (Nishiitsutsuji-Uwo et al, 1967; Bakkerren et al, 1971; Reddy et al, 1977) and are discussed in relation to neuronal activity and activity of the animal as a whole.

Various factors like temperature (Shamoo et al, 1971; Kohenen et al, 1973; Adams et al, 1973; Zaugg & Mclain, 1976; Lagerspetz, 1977) photoperiod (Wanger, 1974), salt concentration (Epstein et al, 1967, 1969; Kamiya & Utida, 1968; Zaugg& Mclain, 1970; Thomson et al, 1976) and habitat (Maruyama, 1965; Srivastava et al, 1977) are known to influence ATPase activity in animals.

Many pesticides like DDT, DDE, aldrin, dieldrin and kepone (Desaiah et al, 1974, 1975a; Desaiah & Koch, 1975c; Price, 1976; Desaiah et al, 1977a; Banerjee et al, 1978; Cutkomp et al, 1976, 1980; Neufeld & Pritchard, 1979; Ghiasuddin & Matsmura, 1979; Srivastava et al, 1979), polychlorinated biphenyls (Koch et al, 1972), pyrethroids (Desaiah et al, 1975b), metal like chromium (Kuhnert et al,1976), synthetic detergents (Verma et al, 1979a, 1979b) are known to effect total ATPase, Mg^{2+} ATPase and Na^{+}, K^{+} ATPase activity in different tissues of vertebrates and invertebrates. Effect of Rubratoxin, a mycotoxin obtained from Pencillium rubrum, on Na^{+}, K^{+} and Mg^{2+} ATPase have been studied by Desaiah et al, (1977c). They have reported inhibition of the enzymes in brain, kidney and liver of mouse. These inhibitions were concentration dependent. There is no information regarding the inhibition of ATPases by plant toxins.

Certain chlorinated insecticides are known to cause repetitive discharge of nerve impulse (Matsumura & Narahashi, 1971). It also prolongs amplitude and duration of the negative afterpotential at synaptic junction which is caused by release of K^+ ions (Corbett, 1974). A nerve impulse passes down the axon as a result of sequential changes in the permeability of the axonic membrane to Na^+ and K^+ ioms. The maintenance of membrane potential is an active process and is dependent on the availability of metabolic energy by the activity of Na^+, K^+ ATPase. Therefore, inhibition of the activity of Na^+, K^+ ATPase will affect the smooth passage of nerve impulse. Studies on developing rat brain have revealed an increase in ATPase activity of nerve ending fractions from prenatal to ten days old animal (Abdel-Latif et al, 1967). They have also shown the correlation between appearance of electrical activity with the development of Na^+, K^+ stimulated ATPase in nerve ending fractions of developing rat brain.

ATP is known to be an antagonist of acetylcholine (Krishna Murthi, 1980). Hence, it has been suggested that the synthesis of ATP and ATPase mediated transport are involved in the regulation of acetylcholine level. The functions of neurons depend among other things on active Na^+ and K^+ transport as well as on passive permeability of the cell membrane. The passive permeability of cell membrane has been suggested by Duncan (1967) to be controlled by the activity of the membrane bound Mg^{2+} ATPase. Therefore, both Na^+, K^+ ATPase and Mg^{2+} ATPase activity in the nervous system have their role to play in neurotransmission.

Some works have been done on the direct action of the pesticides on the ATPase activity *in vitro*. Desaiah *et al* (1977[b]) have shown the non competetive inhibition of Mg^{2+} ATPase by kepone in rat liver. There was no change in K_m value. Kepone abolished the 2-4 Dinitrophenol (DNP) stimulated Mg^{2+} ATPase activity. DNP stimulated Mg^{2+} ATPase are known to be involved in oxidative phosphorylation that results in production of ATP (Lardy *et al*, 1958; Racker *et al*, 1975).

Plan of work :

This chapter deals with the effect of *Z. armatum* fruit extract on the activities of AchE, in brain and muscle and of total, Mg^{2+} and Na^+, K^+ ATPase in brain, muscle and gill of *H. fossilis*. The detailed experiments were as given below.

1. Effect of different concentrations of the extract on the activities of the enzymes *in vivo*.
2. Effect of LC_{50} concentration of the extract (20 ppm) on the activities of the enzymes *in vivo* with relation to the duration of treatment.
3. Effect of the extract on the activities of AchE and Mg^{2+} ATPase treated *in vitro*.
4. Effect of the extract on the kinetics and nature of inhibition of AchE and Mg^{2+} ATPase.
5. Isoenzyme pattern of AchE of brain and muscle by specific staining on polyacrylamide gels.

MATERIALS AND METHODS

The air-breathing fish H. fossilis was used for the enzymatic studies. The fish were collected and maintained in the laboratory as mentioned in the Chapter I. The weight of the fish used in the experiments ranged between 12-15 g indiscriminate of sex.

Treatments

1. Effect of different concentrations of the extract on the activities of the enzymes in the fish in vivo:

   A group of six fish were subjected to a range of concentrations of the extract (15, 20, 25, 35 and 45 ppm). The treatment procedure were the same as described in the Chapter I. The fish were sacrificed by decapitation at 6 hours post-treatment or at lethal phase whichever was earlier. The activities of AchE were estimated in brain and muscle, and ATPase in brain, muscle and gill tissues.

2. Effect of LC_{50} concentration of the extract (20 ppm) on the activities of the enzymes in the fish in vivo with relation to the duration of treatment :

   The fish were treated with 20 ppm (LC_{50}) concentration of the extract and the enzyme activities were estimated at different time intervals (1hr, 2hrs, 3hrs, 4hrs, 6hrs, 8hrs, 10hrs, 12hrs and 24hrs) in brain, muscle and gill tissues. At each time interval 6 fish were sacrificed for estimations. The treatment procedures were the same as described in Chapter I.

3. Effect of the extract on the activities of AchE and Mg^{2+} ATPase treated *in vitro* :

The effects of different concentrations of known inhibitors like eserine and KSCN for AchE and Mg^{2+} ATPase respectively were compared with inhibition by *Z. armatum* fruit extract *in vitro*. The inhibitors were added in the reaction mixture at different concentrations and the enzyme activities were estimated.

Tissue processing

The fish were killed by decapitation and different tissues were removed immediately. They were washed in ice cold 0.25M sucrose and blotted dry with filter paper. The tissues were kept deep frozen at -15°C till estimations were made. All estimations were completed within a week of experimentation. It was observed that deep freezing upto one week did not alter the activities of the enzymes studied. Each tissue was weighed in a single pan balance and 5-10% homogenate was prepared in 0.25M sucrose in an all glass Potter-Elvehjem type homogeniser. The homogenates were centrifuged at 14,000 x g at 0$\pm$ 2°C for 20 minutes to sediment mitochondrial and nuclear fractions. RThe supernatant thus obtained was used for estimations.

Enzyme assay :

(i) Acetylcholinesterase : The AchE activity was estimated spectrophotometrically following the method of Ellman *et al* (1961) using acetylthiocholine iodide as the substrate. The enzyme activity was measured at 412 nm in a double beam digital spectrophotometer (Beckman Model 26) using

silica cuvettes with 1.0 cm light path. The rate of increase in O.D. due to the yellow colour complex formed by the reaction of thiocholine, produced by the enzymatic activity, with di-thio-bis-nitrobenzoate (DTNB) was followed.

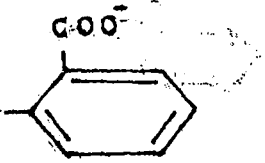
$$(CH_3)_3N^+ \cdot CH_2CH_2SCO\,CH_3 + H_2O \xrightarrow{AchE} (CH_3)_3N^+ \cdot CH_2CH_2S^-$$

Thiocholine

$$+ CH_3COO^- + 2H^+$$

$$(CH_3)_3N^+ \cdot CH_2CH_2S^- + RSSR \longrightarrow (CH_3)_3N^+ \cdot CH_2CH_2SSR + RS$$

(DTNB) 5 thio-2-Nitrobenzoic acid

where R = O_2N 

The final volume of the reaction mixture was 3.12 ml which consisted of

Phosphate buffer (pH 8.0)	.....	83.33 μ moles
DTNB	.....	0.32 μ moles
Substrates	.....	0.32 μ moles
Enzyme extract	.....	0.1 ml

The blank cuvettes contained all the reactants except the enzyme in 3. 12 ml. The increase in O.D. was noted at 30 seconds interval for 3 minutes when the rate of change was linear. The rate of increase in O.D. for the first 2 minutes was used for calculating the enzyme activity. The supernatant were suitably diluted so that the change in O.D. in one minute did not exceed 0.1. One unit of enzyme

activity was defined as μ moles of substrate hydrolysed/min/g tissue and was calculated as follows (Ellman et al, 1961).

$$R = \frac{A}{1.36 \times 10^4} \times \frac{1}{(400/3120)\ CO}$$

or

$$R = 5.74 \times 10^{-4} \times \frac{A}{CO}$$

where R = moles of substrate hydrolysed/min/g, A = change in absorbance/min. CO = original concentration of tissue (mg/ml).

(ii) Adenosine-triphosphatase : ATPase activity was estimated colorimetrically following the method of Swanson et al (1964). The inorganic phosphate liberated by enzyme activity was measured by the method of Fiske and Subba Row (1925)

$$ATP + H_2O \xrightarrow[K^+,\ Na^+,\ Mg^{2+}]{ATPase} ADP + P_i$$

The standard reaction mixture of 1 ml contained

tris-Hcl buffer, pH 7.5	.....	30 μ moles
KCl	.....	30 μ moles
$MgCl_2$	.....	3 μ moles
NaCl	.....	100 μ moles
Enzyme	.....	0.1 ml

The enzyme was added to start the reaction after preincubation of the mixture at 37°C for 5 minutes.

The reaction was stopped after 10 minutes by adding 1 ml of 10% ice-cold TCA (w/v) and was centrifuged. The liberated phosphate was estimated in the supernatant. The standard graph was prepared using different concentrations of K_2HPO_4. The Mg^{2+} ATPase activity was measured in presence of 1 mM oubain in reaction mixture, the latter being a specific inhibitor of Na^+, K^+ ATPase activity. One unit of enzyme activity was defined as μ moles of substrate hydrolysed or μ moles of P_i liberated/minute. The enzyme activities were expressed as total activity (units/g wet wt.) and specific activity (units/mg protein).

Protein estimation

The concentration of protein in the supernatant were estimated following Lowry et al; (1951) using Bovine Serum Albumin (BSA) as the standard. A linear standard graph was obtained using 10 to 70 μg concentrations of BSA.

Kinetic studies

(a) Determination of apparent K_m for the substrates :

The activity of AchE in brain and muscle and Mg^{2+} ATPase in brain, muscle and gill of H. fossilis was assayed at 0.01, 0.02, 0.03, 0.04, 0.05, 0.08, 0.1 and 0.2 mM of acetylthiocholine iodide and 0.25, 0.5, 1.0, 2.0, 2.5, 4.0 and 5.0 mM of ATP respectively. The apparent K_m both for AchE and Mg^{2+} ATPase were determined by Lineweaver-Burk plot of the data.

(b) Inhibition of AchE and Mg^{2+} ATPase :

The effect of eserine, KSCN and Z. armatum fruit extract on the activity of AchE and Mg^{2+} ATPase were studied. In brain AchE activity was assayed with acetylthiocholine iodide as substrate in presence of 5.1×10^{-6}M and 6.4×10^{-6}M eserine, and 1360 and 1600 ppm of the extract. However, in the muscle AchE activity was assayed in presence of 3.2×10^{-6} and 6.4×10^{-6}M eserine, and 1200 and 1600 ppm of the extract. Mg^{2+} ATPase activity was assayed with ATP as substrate in presence of 1.81×10^{-5}M and 5.45×10^{-5}M KSCN, and 80 and 240 ppm of the extract in the three tissues. The substrate concentrations were same as those used for the K_m studies for different tissues. The data were plotted on Lineweaver-Burk plot to determine the nature of binding of the inhibitors with the enzymes.

The K_i (Z. armatum fruit extract) for AchE in brain and muscle, and Mg^{2+} ATPase in brain, muscle and gill with respect to their respective substrates, acetylthiocholine iodide and ATP, were studied. The AchE activity was assayed at two concentrations of acetylthiocholine iodide, 0.1M and 0.05M and the extract concentrations of 1040, 1120, 1200, 1280, 1360, 1440, 1520 and 1600 ppm for brain and 560, 640, 720, 800, 960, 1040, 1120, 1200 ppm for muscle. The Mg^{2+} ATPase activity was assayed at two concentrations of ATP, 1 and 2 mM for brain and gill, and 2 and 4 mM for muscle, and the extract concentrations of 40, 80, 120, 200, 240, 320 and 400 ppm for the three tissues. TK_i was determined by Dixon plot of the data.

Isoenzyme pattern of AchE on polyacrylamide gel

Polyacrylamide gel electrophoresis was carried out to find the isoenzyme pattern of AchE in brain and muscle. 7.5% gel were prepared according to the method of Davis (1964). Electrophoresis was carried out inside a refrigerator at 2 ± 2°C using tris-citrate buffer 0.02M, pH 8.6 (Maynard, 1964) for about 2 hours with a constant current of 4 mA/gel. The gels were prerun for 30 minutes before loading the enzyme. About 18 units of AchE was loaded in 80% sucrose and bromophenol blue (0.001%) was used as the tracking dye. Following electrophoresis, the gels were specifically stained for AchE following the method of Koelle (1951). The gels were preincubated at 0°C in test tubes containing the following reaction mixture (Maynard, 1964):

0.6 ml of the solution containing 0.375% $CuSO_4$ 5 H_2O and 0.25% glycine,

0.6 ml of 9,5% Magnesium chloride,

0.5 ml of 0.2M Sodium acetate buffer (pH 6.1) and

0.4 ml of 0.1M Copper sulphate.

After 30 minutes the gels were transferred to another reaction mixture with the substrate that had all the above ingredients and in addition included freshly prepared acetylthiocholine iodide (0.3M). Incubation was carried out for 2 hours at room temperature (25°C). The gels were then washed with ice-cold water and preserved in distilled water. The white bands of copper thiocholine sulphate representing the AchE activity were observed, photographed and scanned in a double beam digital spectrophotometer (Beckman Model-26) at 425 nm

to find out the isoenzyme pattern.

All chemicals and biochemicals used were of analytical grade, purchased from either Sigma Chemical Co., USA; E. Merck, Germany; Glaxo Laboratories, Bombay; Centron Laboratories, Bombay or CSIR center for biochemicals, Delhi. The glass wares were of neutral glass purchased from Borosil Glass Co., Bombay. Double glass distilled water was used for all biochemical estimations.

RESULTS

Effect of different concentrations of Z. armatum fruit extract on the activities of AchE and ATPase in vivo (Table 15-18;Fig.15-17)

Z. armatum fruit extract inhibited the activities of AchE, Mg^{2+}- and Na^{+}, K^{+} ATPase in different tissues of H. fossilis. The activities of these enzymes showed a similar pattern of decrease with increase in concentration. The decrease in enzyme activities ~~were~~ may gradual with increasing concentration of the extract and reached a plateau after which the activities did not change. The specific activity of AchE was inhibited maximum upto about 50% in the brain and 40% in the muscle by 35 ppm concentration of the extract. The inhibition of total ATPase activity was maximum in gill (46%) followed by brain (38%) and muscle (35%). These maximum levels were reached by 20-25 ppm concentration of the extract. It is interesting to note that the maximum inhibition of the activity of ATPase was achieved at a much lower concentration of the extract than the maximum inhibition of AchE activity. It could also be seen that the percentage inhibition of the activities of total and Mg^{2+} ATPase was higher (35-45%), than

Na^+, K^+ ATPase (20-22%). Therefore, the Mg^{2+} ATPase which was more sensitive to Z. armatum was taken up lalong with AchE for kinetic studies.

Effect of LC_{50} concentration (20 ppm) of the extract on the activities of AchE and ATPase in vivo with relation to the duration of treatment (Tables 19-30; Figs. 18-21).

The activities of different enzymes (AchE, total ATPase, Mg^{2+} - and Na^+, K^+ ATPase) were inhibited gradually with time on treatment with LC_{50} concentration (20 ppm) of Z. armatum fruit extract. The pattern of inhibition of the activities of the enzymes in different tissues was similar. The inhibition was increased till 3-6 hrs of treatment and then it decreased gradually. The activities returned almost to the normal level by 24 hours. However, different enzymes showed different intensity and duration of inhibition in different tissues. The AchE activity was inhibited maximally upto about 40% in the three tissues studied. However, ATPase showed some tissue specific variations in the rate of maximum inhibition in the following order, (a) total ATPase - gill (50%) > muscle (35%) > brain (33%), (b) Mg^{2+} ATPase - gill (56%) > muscle (40%) > brain (33%) and (c) Na^+, K^+ ATPase-gill (30%) > muscle (22%) > brain (17%). The activities of AchE were inhibited earlier than the ATPases. However, the activities of Brain and gill ATPases were maximally inhibited by 3-4 hrs where as AchE and muscle ATPase were inhibited maximally by 6 hrs.

Effect of different concentration of a known inhibitor and *Z. armatum* fruit extract on the activities of AchE and Mg^{2+} ATPase *in vitro* (Table 31-35; Figs. 22 & 23)

The activities of muscle AchE showed inhibition much earlier than the brain AchE for both the inhibitors, eserine and the extract. Eserine completely inhibited both muscle and brain AchE at higher concentrations. The extract above 1600 ppm concentration inhibited muscle AchE activity maximally by 45% only. However, the brain AchE was inhibited maximally upto 29% with the extract concentration above 1600 ppm. The activities of brain Mg^{2+} ATPase showed maximum inhibition on *in vitro* treatment with the extract. There was no significant difference in the percentage inhibition of the activities of muscle and gill Mg^{2+} ATPase with the extract. In general, the patterns of inhibition of the activities *in vitro* of both the enzymes were similar to those *in vivo* reaching a plateau at higher concentrations. The concentration of the extract required for AchE inhibition was also much higher than that for inhibition of Mg^{2+} ATPase in all the tissues. However, the concentrations needed for maximum inhibition *in vitro* were much higher for both AchE (1600 ppm) and ATPase (400 ppm) than the maximum inhibition *in vivo*. KSCN inhibited partially brain, muscle and gill Mg^{2+} ATPase of *H. fossilis* upto 47%, 45%, and 43% respectively.

Kinetic studies (Tables 36-39; Figs. 24-27).

The apparent K_m (Michaelis constant) of brain and muscle AchE for acetylthiocholine iodide was found to be 7.27×10^{-4}M and 8.90×10^{-5}M respectively. They increased with eserine but did not change with Z. armatum fruit extract. The apparent K_m of brain, muscle and gill Mg^{2+} ATPase for ATP were found to be 9.72×10^{-4}M, 7.10×10^{-4}M and 8.71×10^{-4}M respectively. They decreased with KSCN but increased with Z. armatum fruit extract. The nature of inhibition of AchE with eserine was competitive and with Z. armatum non-competitive. The nature of inhibition of Mg^{2+} ATPase with KSCN and Z. armatum were non-competitive. The inhibition constant (K_i) of the extract for AchE with acetylthiocholine iodide as substrate was 700 and 500 ppm for brain and muscle respectively and K_i for ATPase with ATP as the substrate was 220, 240 and 250 ppm for brain, muscle and gill respectively as determined by Dixon's plot of the data.

Polyacrylamide gel electrophoresis of AchE (Fig - 28).

Specific staining of AchE on polyacrylamide gel showed one distinct band in brain tissue and two distinct bands in muscle of H. fossilis which were eserine sensitive. This result was supported by the absorption spectra of the gels at 425 nm.

DISCUSSION

Different behavioural and physiological manifestations in an organism treated with a toxicant are the result of the alterations which have taken place at cellular and sub-cellular level. The later changes are mainly the variations in the quantity and quality of different biomolecules controlling different biochemical pathways. The enzymes belong to an important group of biomolecules which control the synthesis and degradation of other biomolecules and also their own. The most significant character of the enzymes is to catylyze different reactions in the total net work of biochemical pathways operating in the cell to express its living characteristics. The enzymes are sensitive to various changes in their environment. Many enzymes show adaptive changes with the variations in the environment and there-by control the functioning of the organism. Studies on the effect of different toxicants on the regulations of the enzyme activities give proper insight into the mode of action of the toxicants at the cellular and sub-cellular level.

AchE and ATPase are the key enzymes of neural transmission and energy metabolism respectively in an organism. AchE is a hydrolase which catalyzes the hydrolysis of acetylcholine into choline and acetic acid. ATPase is also a hydrolase which is involved in liberation of energy by dephosphorylating ATP molecules.

Effect of different concentrations of Z. armatum fruit extract on the activities of AchE and ATPase in vivo :

The activities of both AchE and ATPase was inhibited in different tissues of H. fossilis with the treatment of different concentrations of the fruit extract of Z. armatum. However, the levels of inhibition were different in different tissues but the maximum inhibition in any case was not exceeding 50% of the normal level. The brain AchE was inhibited to a greater extent than the muscle AchE at higher concentrations (35 & 45 ppm). This indicates that the brain and muscle AchE have different regulatory mechanisms and the brain enzyme is more sensitive than that of muscle. However, at 20 ppm concentration there was not much difference in the percentage inhibition of the enzyme activity in brain and muscle. The inhibition of the enzyme activity increased and reached a plateau with increase in concentration in both the tissues. Among the three ATPase activities, Mg^{2+} ATPase was inhibited maximum and Na^{+}, K^{+} ATPase minimum in all the three tissues studied. The ATPase activities also showed a gradual increase in their percentage inhibition and then reached a constant level at higher concentrations.

The inhibition of AchE and ATPase by Z. armatum fruit extract was concentration dependent upto a certain limit and ran almost parallel. Similar concentration dependent inhibition of enzyme activity have been reported with different toxicants like pesticides (Desaiah et al, 1974; Cook et al, 1976;

Desaiah et al, 1977a; Chin & Sudderuddin, 1979), PCB (Koch et al, 1972) and detergents (Verma et al, 1979a). The maximum percentage inhibition of the activities of the enzymes in the present study was never above 50% of normal activity and reached a plateau at higher concentrations of the extract. This might be due to the fact that the inhibited levels of enzyme activity were enough to bring about physiological changes which were lethal to the fish. The inhibition of the activities of these enzymes might have brought about disturbances in neuronal transmission, ionic equilibrium and energy metabolism in the fish treated with the extract. This was seen in behavioural responses of fish to various concentrations of the extract. The activity of an enzyme in a tissue at a particular time is the outcome of the quantity and quality of the enzyme molecules existed at that time. Hence, the alterations in the activities of enzymes in vivo in relation to the treatment of the fish with any chemical in vitro could be the result of either the direct action of the chemical on the quality of the enzyme molecules or the indirect action of the chemical on the quantity of the enzyme molecules by regulating their rate of synthesis and degradation. It is not possible from these observations to say whether the inhibition of the activities of AchE and ATPase was the direct or indirect effect of the extract. However, it is true that the inhibition of the enzyme is at least a part of the physiological impact caused by the extract leading to the death of the fish at higher concentrations.

Effect of LC_{50} concentration (20 ppm) of the extract on the activities of AchE and ATPase *in vivo* with relation to the duration of treatment :

The studies on the activities of the enzymes with relation to the duration of the treatment of the toxin help in finding out the threshold time and pattern of physiological and metabolic changes occurring in the animal after the treatment. The changes in the activities of the enzymes (AchE, total ATPase, Mg^{2+} ATPase and Na^{+}, K^{+} ATPase) studied in these experiments showed similar pattern of inhibition with the time of treatment. However, the pattern of inhibitions ~~were~~ was different for different tissues. The maximum inhibition of the activities of all four enzymes ~~were~~ was found between 4-8 hours post-treatment. This coincided with the time of maximum mortality of treated fish. The time required for recovery of the activities of these enzymes was, however, much larger in comparison to that for inhibition. The threshold time to show inhibition of enzyme activity was smaller for AchE than the ATPases studied. However, the rate of inhibition was faster and the maximum inhibition was obtained earlier in case of ATPases than AchE. Brain and gill enzymes showing ~~comparatively~~ quicker inhibition indicate that either the enzymes in those tissues are more sensitive or the toxicant is promptly taken up by those tissues than the muscle. However, gills have been shown to be the major source for toxicant uptake to the body from the aquatic environment in the fish (Holden, 1973).

The recovery of animals from their affected state when treated with sublethal doses of toxicants has been known. The recovery in enzyme activities has been reported with the recovery of the animals in cases of pesticides such as chlorpyrifos (Thirugnanam & Forgesh, 1977), methomidophos (Chin & Sudderuddin, 1979) Rubratoxin B (Desaiah et al, 1977c). The recovery indicates the mechanism and level of tolerance of the animal for the toxin. In the present study, the recovery of the activities of AchE and ATPases were found to be a slower process taking about 24 hours when the maximum inhibition was seen by 6-8 hours.

These observations indicate that the initial irritant activity showing convulsive tremors in the fish immediately after the treatment of the extract might be due to the early effect of the toxicant on the nervous system inhibiting AchE and affecting neural integration. This follows the inhibition of Na^+, K^+ - and Mg^{2+} ATPase activities resulting in ionic imbalance and reduction in the availability of metabolic energy for active physiological functions. At sublethal concentrations recovery of these damages takes place gradually but in higher concentrations the cumulative action of all these and may be some others, both in terms of intensity and duration results in the death of the fish.

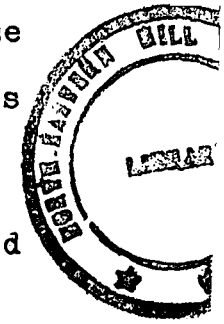
Effect of known inhibitors and Z.armatum fruit extract on the activities of AchE and Mg^{2+} ATPase in vitro:

Some chemicals inhibit enzyme activity by binding directly to the enzyme molecules either at the active site or at a regulator site. Most of the toxins act on the enzymes through one of the above processes and some of them are specific for specific enzymes. These chemicals which have similar structure like the substrate, bind at the active site and inhibit enzyme activity. They are called as competitive inhibitors. Prostigmine and phytostigmine (eserine) show structural resemblances with acetylcholine which enable them to become attached to the active site of acetylcholinesterase apparently by forces similar to those involved in normal reaction with acetylcholine. They, thus, compete with acetylcholine, so limiting its access to active site of the enzyme and its rate of breakdown (McIlwain & Bachelard, 1971). Therefore, eserine completely inhibits the AchE activity at higher concentrations. The effects of aquatic pollutants and other chemicals including eserine, on the AchE activity in vitro in muscle of fathead minnow (Pimephales promelas) were studied by Olson and Christensen (1980). They found eserine to be very powerful inhibitor, inhibiting AchE activity at concentrations as low as 3.2×10^{-7}M followed by carbamates, one-organo-oxy-phosphate, the arsenite ion and certain heavy metal cations. The inhibitory action of pesticides like dichlovos, temefos, chlorpyrifos on AchE activity in vitro has been studied by various workers (Thirugnanam & Forgash, 1975,

1977; Danford & Beardmore, 1978; Giri et al, 1977). In the present study eserine did show complete inhibition in vitro of brain and muscle AchE activity of H. fossilis. However, the extract of Z. armatum fruits could not inhibit completely the AchE activity in brain and muscle homogenates of H. fossilis even at very high concentrations. AchE activity was inhibited maximally upto 45% in muscle and 28% in brain at 1600 ppm of the extract and did not change upto 2400 ppm concentration of the extract. At lower concentrations of both eserine and the extract the AchE activity in muscle homogenates was inhibited at a ~~comparatively~~ higher rate than that in brain homogenates. These observations indicate that Z. armatum fruit extract inhibition is different from that of eserine and therefore, cannot be of a competitive nature. Besides, the responses of AchE to both the toxins in muscle and brain of H. fossilis were different indicating that this enzyme might exist in different isoenzyme forms in the two tissues studied.

The in vitro inhibition of Mg^{2+} ATPase by SCN^- (thiocynate) has been reported by various workers (Katz & Epstein, 1971; Tanisawa & Forte, 1971; Hansen et al, 1975; Suzuki, 1978; Miwa et al, 1980). Recently Komnick et al (1980) have shown the competitive nature of inhibition of HCO_3^- dependent Mg^{2+} ATPase by SCN^-. However, in the present study the activities of Mg^{2+} ATPase in vitro could not be inhibited completely by KSCN in the three tissue (brain, muscle and gill) homogenates of H. fossilis studied, indicating that the nature of inhibition was not of competitive type. The percent inhibition of Mg^{2+}

ATPase by KSCN in different tissues did not exceed 50%, even at higher concentrations, unlike that seen in AchE inhibition by eserine. The in vitro inhibition of ATPase by various toxicants like polychlorinated biphenyls (Koch et al, 1972), pesticides like aldrin, dieldrin and photodieldrin, DDT and TDE (Cutkomp et al, 1971; Desaiah & Koch, 1975c), kepone (Desaiah et al, 1977a, 1977b), pyrethroids (Desaiah et al, 1975b) have shown that both Na^+, K^+ = and Mg^{2+} ATPase are being inhibited by the toxicants. Riedel and Christensen (1979) have studied the effect of selected aquatic pollutants and other chemicals upon ATPase activity in vitro. The salts of silver, mercury, copper and cadmium were most effective in inhibiting ATPase activity followed by chlorinated hydrocarbon pesticide, dicofol. The pesticides such as DDT, dieldrin, lindane, carbaryl and malathion also inhibit ATPase activity, but to a lesser degree as the metal ions, methyl mercury, aluminium, fluoride, nickel and cobalt ions. In the present study with Z. armatum fruit extract, the maximum inhibition of Mg^{2+} ATPase obtained were 35%, 26%, and 26% in brain, muscle and gill respectively at 400 ppm. There was no further change with higher concentrations of the toxin. Thus, the extract could not inhibit Mg^{2+} ATPase completely, similar to that seen in AchE indicating that this inhibition also was not of competitive nature.



The concentration of Z. armatum fruit extract required for inhibition of AchE in vitro was found to be much higher than for the inhibition of Mg^{2+} ATPase activity. The inhibition of Mg^{2+} ATPase activity reached its maximum level around 400 ppm of Z. armatum fruit extract where as at that concentration

the inhibition of AchE activity started, reaching the maximum level at 1600 ppm concentration. This indicates that AchE inhibition follows Mg^{2+} ATPase inhibition on treatment with the extract. This is in conformity with the results of our time bound experiments on effect of Z. armatum fruit extract, where ATPase activities were seen to be inhibited before the inhibition of AchE activity. The inhibitor concentration for a particular enzyme depends on its molecular structure and nature of binding of the inhibitor molecule. The differences in inhibitor concentration for the two enzymes studied might be due to their structural differences and/or the nature of binding with the toxin.

Kinetic studies:

AchE - The kinetic studies on AchE from different laboratories indicate that there are variations in the K_m values of AchE in different tissues and in different animals. The AchE of the electric organ of Electrophorus electricus has shown the K_m value of 1.79×10^{-4}M for acetylthiocholine (Mohan & Brink, 1970). Moss and Fahrney (1978) reported K_m value of 2.5×10^{-4}M for fish brain AchE. The K_m values of rat brain AchE has been reported by several workers (Mohan & Brink, 1970; Beaver & Dobson, 1978; Moudgil & Kanungo, 1973; Moss & Fahrney, 1978). The values varied between 1.2×10^{-7} to 1.28×10^{-4}M. The lung AchE of rat was found to be 7.2×10^{-4}M (Giri et al, 1977) and that of synaptosomal AchE in Mongolian gerbil varied from 9.24×10^{-4}M (neonatal) to 3.02×10^{-4}M (adult) (Vanker &

Mizukami, 1979). Nistri et al (1978) have reported the K_m value of 1.41×10^{-4}M for AchE in central nervous system of leech. The apparent K_m values observed in the present study with acetylthiocholine as the substrate were 7.27×10^{-5}M and 8.9×10^{-5}M respectively, in brain and muscle of H. fossilis. These values are similar to those reported by the earlier workers. The difference in K_m values in brain and muscle may be due to the tissue variations observed in different animals by different workers. However, brain AchE has shown to have higher affinity for acetylthiocholine iodide than the AchE of muscle. Eserine showed a competitive nature of inhibition of AchE activity for the substrate acetylthiocholine iodide both in brain and muscle. This type of inhibition has already been reported in the literature. However, with Z. armatum fruit extract the nature of inhibition of AchE with acetylthiocholine iodide was of a noncompetitive type both in brain and muscle tissues. Therefore, the two possibilities by which AchE is being inhibited by the extract may be (1) by combining with some functional group of the free enzyme other than at active site or (2) by forming a comparatively inert enzyme substrate inhibitor complex combining with the enzyme substrate complex. The K_i of Z. armatum fruit extract for AchE in brain was higher compared to K_i for AchE in muscle showing that the inhibitor has got greater affinity to bind with muscle AchE rather than brain AchE. These differences in the kinetic properties of AchE among the brain and muscle tissues indicate that there might be tissue specific variations in the molecular structure of AchE for their functional adaptations.

Mg^{2+} ATPase - The apparent K_m value of Mg^{2+} ATPase in different tissues of H. fossilis were found to be different. Brain had the highest value (9.72×10^{-4}M) followed by gill (8.71×10^{-4}M) and muscle (7.1×10^{-4}M). It is difficult to say from these observations whether these differences are significant. However, these values show similarities with earlier findings. The K_m of gill Mg^{2+} ATPase in crayfish Procambrus clarki has been reported to be 8.7×10^{-4}M (Horiuchi, 1977). The mitochondral Mg^{2+} ATPase in liver of rat has been reported to possess a K_m value of 4×10^{-4}M for ATP. The values of K_m of ATPase in different tissues obtained in the present study seems to be consistent with the physiological state of the tissues. Komnick et al (1980) have reported competitive inhibition of Mg^{2+} ATPase by thiocynate. However, in the present study a non-competitive type of inhibition of Mg^{2+} ATPase by KSCN in brain, gill and muscle tissues of H. fossilis was observed using ATP as the substrate. More detailed kinetic studies with purified enzyme could resolve this conflict. The nature of inhibition of Mg^{2+} ATPase in brain, gill and muscle homogenates with Z. armatum fruit extract was also of a non-competitive type. Desaiah et al (1977b) have reported that the action of a non-competitive inhibitor like kepone to Mg^{2+} ATPase in perfused rat liver can be reversed by repeated washing of the tissue. Hence, the recovery of Mg^{2+} ATPase inhibition with time as observed in our in vivo studies may be due to the gradual removal of the non-competitive inhibitor by some biological process. The

inhibition constant (K_i) of Z. armatum fruit extract for Mg^{2+} ATPase was found to be 220, 240 and 250 ppm in brain muscle and gill respectively. This was lower than the K_i of the extract for AchE in brain and muscle. Therefore, it could be said that the Mg^{2+} ATPase was more sensitive to the inhibitory effect of the extract than AchE and was affected first.

Specific staining of AchE on polyacrylamide gel:

Many enzymes have been shown to be present in different iso-enzyme forms with different biological activity. Several iso-enzymic forms of AchE have been reported in different mammalian and fish tissues (Bernsohn et al, 1963; Davis & Agranoff, 1968; Baldwin & Hochachka, 1970; Moudgil & Kanungo, 1973; Vijayan & Brownson, 1975; Gisinger et al, 1978). Different iso-enzymes show different enzyme activities and their kinetics also vary. The anticholinesterase activity of most of the toxins have been established by assaying the enzyme activity in presence of the toxin. Very little effort has been made towards the evaluation of the resources of different iso-enzymes of AchE to different toxins. Vijayan and Brownson (1975) have shown differential inhibition of iso-enzymic forms of AchE in vivo following parathion toxicity in different tissues of rat. They observed differential responses in the inhibition of the iso-enzymes of AchE in brain, serum and skeletal muscle of rat after parathion injection. The recovery of the brain iso-enzyme started after

2 hours, serum iso-enzyme by 24 hours when the skeletal muscle iso-enzymes were still inhibited. However, no report is seen on *in vitro* effect on iso-enzymes, may be due to the difficulty in getting the purified AchE iso-enzymes in measurable quantities. In the present study, it was not possible to purify the enzymes. However, due to the tissue specific variations in enzyme activities, kinetics and inhibition kinetics of AchE, it was decided to see if these results could be correlated to any iso-enzymic variations in AchE of brain and muscle of *H. fossilis*. On specific staining on polyacrylamide gel for AchE and recording the absorption spectra at 425 nm of the stained gels, it was seen that brain had one AchE band where as muscle had two. These bands were sensitive to eserine. These iso-enzymes might have different properties to account for the observed tissue specific variations in the response of AchE, However, this was a preliminary observation and a detailed study on the kinetics of the purified iso-enzymes is necessary to prove this proposition.

GENERAL DISCUSSION

The use of various types of biocides has become a common practice in modern fisheries management to control weed fishes and other aquatic organisms in different water bodies. Both natural and synthetic piscicides have been tried from time to time. Chemical piscicides like thanite, antimycin, ammonia, 3-trifluromethyl-4 nitro-phenol (TFM),copper sulfate and malachite green, and natural piscicides like rotenone, pyrethrin, nicotine, juglone and justicidin are commonly used. The chemical piscicides cause acute and chronic effects on the aquatic organisms even at very low concentrations. Their bio-degradability is slower and residual toxicity is higher compared to natural piscicides. Many of the natural piscicides also act as insecticides. Therefore, the natural piscicides have gained more significance in fish nursery management. Rotenone which is obtained mainly from Derris root and from some other groups of plants has been widely used as a piscicide throughout the world. Compounds commonly known as rotenoids and pyrethroids, similar to naturally obtained rotenone and pyrethrin are being synthesized and put into trial as piscicides and insecticides (Desaiah et al, 1973).

In our country, rotenone, which is the most commonly used piscicide for clearing ponds in nursery practices is at present imported. Therefore, attempts have been made to substitute various other plant products to develop indigenous natural piscicide. There are more than 150 plants in our country which have been reported to have piscicidal action (Chopra et al,

1949). But very few of them like Croton tiglium (Bhuyan, 1968), Milletia pachycarpa (Bhuyan, 1967), Randia dumetorum (Nandy & Chakraborty, 1976), Barringtonia acutangula (Chakraborty et al, 1972) and Madhuca indica (Choudhury, 1968) have been tested for their piscicidal action. In Japan, some plants like Marchantia polymorpha (Kanasaki & Ohta, 1976) and Justicia hyatai (Ohta et al, 1969) have been reported for their piscicidal action. Some of these natural piscicides are known to have useful side effects like fertilizing the pond by increasing the plankton productivity (Chakraborty et al, 1972).

The piscicidal action of most of those plant materials have been studied on the basis of their lethality alone. It is evident from the available literature that very little work has been done on the mode of action of these piscicides at physiological and biochemical level though some of them have been used in fish nursery management. Different toxins are known to produce their effects by combining with enzymes, cell membrane or other specialized functional components of cells. Such interactions between cell components and toxins induce different biochemical and physiological changes. The effects of toxins could be characterized on the basis of biochemical or physiological changes which may be produced at sublethal concentrations also. Therefore, the studies on physiological and biochemical effects of a toxin on any organism helps to understand the mode of its action which in turn helps in the better utilization of the toxin causing minimum hazard to the environment.

The present study was taken up with above ideas in view, to know the mode of action of piscicidal plants available in this region systematically and to find out if a suitable substitute for rotenone could be developed. Ten plants, commonly used in this region for fish catching, were collected, identified and toxicity tests were done using crude powder of these plants and their parts on a few fresh water fishes (Ramanujam & Ratha, 1980). The results of these bio-assays proved that the fruits of Z. armatum was most potent as a piscicide among the different plant parts studied. It also indicated the varied types of plants which have definite piscicidal properties and could be explored for their use in fish nursery management. The fishes which were treated with Z. armatum fruit powder could be recovered back to normalcy on transferring them to fresh water. Therefore, due to its stupefying action rather than lethal action, Z. armatum fruits could be used as an anaesthetising agent also. The studies on bio-assay have shown that both gill-breathing and air-breathing fishes were affected by the alcoholic extract of the fruit, though the concentrations of the extract required for air-breathing fish was comparatively higher than those for gill-breathing fish. Similar reports are available for other plants like Randia dumetorum (Nandy & Chakraborty, 1976) and Barringtonia acutagula (Chakraborty et al, 1972), and also with industrial pollutants (Das et al, 1980). This might be due to the physiological, behavioural and size differences of these two groups of fishes which are known to influence the effect of an toxicant (Sprague, 1973). Air-breathing fishes are more hardy

and less dependent on aquatic environment compared to gill-breathing fishes which are purely aquatic. Besides, the gill-breathing fishes are affected to greater extent by the changes in aquatic environment. Many toxins are known to drastically alter the aquatic environment making it harmful for the fish to live. However, our results on some major physico-chemical factors of water such as dissolved oxygen level, pH and conductivity showed no significant differences on addition of Z. armatum fruit extract.

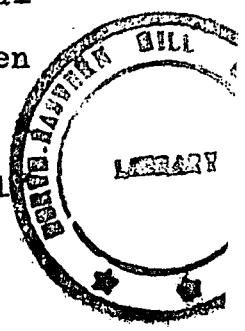
The behavioural response of fish mentioned by earlier workers during their experiments with different plant materials, though to some extent similar to that seen in our experiments, have not been utilised to know the mode of action of the toxicant. The actual changes in physiological and biochemical parameters are ultimately reflected as vbehavioural responses. Behavioural responses of an organism, therefore, give an approach for physiological and biochemical studies to be taken up. The fishes treated with Z. armatum fruit powder and extract showed rapid convulsive movement with occassional tremors, increase in opercular movement off gill-breathing fish, surfacing activity of air-breathing fish and then inability of the fish to reach the surface of water. These responses suggested broadly that the Z. armatum fruit extract could be affecting the nervous transmission or the availability of metabolic energy in the animals, thus affecting other physiological processes in the body. The availability of metabolic energy is dependent on the structure and function of different systems like blood

circulation, oxygen transport, cellular respiration and release of stored energy. Therefore, further studies were carried out on the functioning of these suspected systems in fish using Z. armatum fruit extract.

Conventional bio-assay experiments measure the strength of the toxicant using death as indicator. However, many toxicants at sub-lethal levels are known to cause serious physiological damages. Therefore, changes in physiological and biochemical parameters at sub-lethal concentrations help us to know the effect of the toxin before it becomes irreversible. The mode of action of many insecticides, herbicides and industrial effluents have been, therefore, studied at cellular and sub-cellular level. Chemical pesticides like sumithion and sevin (Koundinya & Ramamurthi, 1980) and natural toxicants like saponin (The Wealth of India, 1952) are known to have hemolytic and other adverse effects on hematological parameters. The hematological parameters like RBC count, Hb concentration, MCH and BOC studied in H. fossilis have indicated that there exists considerable intraspecific variations in hematological parameters with relation to the size of the fish. Similar intraspecific variations in hematological parameters have been reported by Dube and Munshi (1973) in Anabas testudineus, Joshi and Tandon (1977) in Clarias batrachus, and Pandey et al, (1976) in H. fossilis. In the present study H. fossilis showed a very high concentration of hemoglobin. There are reports indicating that the air-breathing fishes contain more hemoglobin than the purely aquatic fishes (Lenfant & Johansen, 1972). The fishes treated with four different concentrations of the extract of the fruit of Z. armatum did not alter the hematological

parameters studied (Ramanujam & Ratha, 1980a) and thus eliminated the possibility of the toxin's effect as a hemolytic agent. This has been further supported by chemical analysis of the fruits of Z. armatum done by Simensen and Rao (1929), Nazir and Handa (1961), Deshpande and Sastri (1977) showing the absence of known hemolytic factors like saponin. However, studies on oxygen binding capacity, and plasma characteristics of the treated fishes could not be conducted due to lack of facilities to completely rule out the possibility of any impact of the extract on blood.

The oxygen uptake by Tilapia mossambica from water treated with Z. armatum fruit extract have demonstrated a dose dependent graded response of the pattern of oxygen uptake showing an initial enhancement and ultimate inhibition both in terms of time and intensity. Similar type of dose dependent graded response in rate of oxygen uptake has been reported in zinc toxicity (Brafield & Matthiessen, 1976) and in pesticide toxicity (Luhn et al, 1976). In the present study, it was seen that at a sub-lethal concentration (15 ppm) there was no defined alteration in the rate of oxygen consumption, but at the end of 4 hours of treatment it stabilized at a lower level compared to control. However, at 20 ppm and 30 ppm the inhibition of oxygen uptake were more defined, and stabilized at a lower rate compared to control and 15 ppm concentrations. The commonly used natural piscicide, rotenone has been reported to decrease the oxygen uptake by gill filaments blocking the reduced nicotinamide adenine dinucleotide dehydrogenase segment in mitochondrial



respiration (Lindahl & Oberg, 1960). The results of the present study on tissue respiration showed no such change in oxygen uptake by brain and liver homogenates using glucose as the substrate. Thus, it could be said that the total oxygen consumption of fish was being inhibited indirectly and there was no direct effect of the extract on tissue respiration at sub-cellular level. The inhibition of oxygen uptake by fish could be due to the paralysing effect of the toxicant on different organs related to oxygen uptake. The total oxygen consumption of an organism is indicative of its general metabolism. Hence, the reduced oxygen consumption by the treated fish was indicative of the toxicant affecting the general metabolic activity of the animal. Further studies on the effect of the Z. armatum fruit extract on the activities of AchE, the key enzyme of neural transmission and ATPase, key enzymes of energy metabolism and ionic equilibrium, were taken up both in vivo and in vitro to find out the sites of damage caused by the toxin at sub-cellular level.

Studies on the effect of Z. armatum fruit extract on the enzymes of H. fossilis showed that the neural transmission, energy production and ionic equilibrium were being affected. The levels of AchE and ATPase activities in different tissues of the fish in vivo on treatment with different concentrations of the extract and with variation in duration of treatments have indicated that both the criteria are important in toxicity studies.

Many commercially available insecticides, industrial effluents and other toxins are known to interfer with the passage of nerve impulse inhibiting AchE activity, the key enzyme in neural transmission (Corbett, 1974; Mukherjee & Bhattacharya, 1974; Olson & Christensen, 1980). Various groups of toxins like pesticides, polychlorinated biphenyls, synthetic detergents and rubratoxin from Penicillium rubrum are known to inhibit Mg^{2+} ATPase and Na^{+}, K^{+} ATPase in different tissues of vertebrates and invertebrates (Koch et al, 1972; Desaiah et al, 1974, 1977c; Ghiasuddin & Matsumura, 1979; Verma et al, 1979b). The inhibition of Mg^{2+} ATPase results in short supply of energy from the stored ATP molecules and the inhibition of Na^{+}, K^{+} ATPase lead to the loss of ionic equilibrium in different membrane system. Inhibition of these enzyme activities are reflected in the behavioural changes of the animals.

In the present study, the behavioural changes like rapid convulsive movements with occassional tremors which is coinciding with the inhibition of AchE activity might be due to accumulation of AchE in the tissues. It is known that accumulation of AchE at synaptic junction will lead to loss of muscular co-ordination, induction of convulsions and ultimately death (Corbett, 1974). The activities of AchE in vivo were inhibited to a maximum level after 6-8 hours of the treatment of the extract. The inability of fish to reach the surface of water and gradual cessation of opercular movements of gill-breathing fish observed during the bio-assay studies might

be due to inhibition of Mg^{2+} ATPase activity as an added factor.

The levels of enzyme activities at different time intervals on treatment with the extract showed that the activities of AchE was inhibited earlier than Na^+, K^+ - and Mg^{2+} ATPase activities. Therefore, the synaptic transmission might have been affected at the early stage of effect. Besides, a nerve impulse passes down the nerve as a result of sequential changes in the permeability of the axonic membrane for Na^+ and K^+ ions. The maintenance of membrane potential is an active process and is dependent on the availability of metabolic energy. Therefore, inhibition of Mg^{2+} ATPase and Na^+, K^+ ATPase might have affected the passage of nerve impulse along the axons. It is implied from these studies that the fish was affected first by the blocking of synaptic transmission and then by short supply of metabolic energy due to the inhibition of Mg^{2+} ATPase followed by disturbances in ionic equilibrium due to inhibition of Na^+, K^+ ATPase.

It was observed that the enzyme activities were inhibited with the increasing concentration of the extract upto a certain maximum limit and then did not change at higher concentrations. The percentage inhibition of AchE activity in muscle was greater than that of brain at lower concentrations of the extract. The response was reversed at higher concentrations, though there was not much difference in percentage inhibition at 20 ppm concentration. Among the ATPase activities, Mg^{2+} ATPase was inhibited to greater extent than the Na^+, K^+ ATPase in all the three tissues studied. The maximum percentage inhibition of

enzyme activities, in any case, was not exceeding 50% and at higher concentration it reached a plateau. Similar concentration dependent inhibition by toxicants have been reported by different workers (Chin & Sudderuddin, 1979; Koch et al, 1972; Verma et al, 1979a). The recovery of fish at lower concentrations on transfer to freshwater might be due to lower inhibition of these enzyme activities which were reversible at sub-lethal level. At higher concentrations the inhibition might have persisted for a longer time, beyond the critical time for recovery, leading to the death of the fish. Those fishes which survived at higher concentrations might be having some extra tolerance capacity to the toxin by some unknown biological mechanism.

The in vitro studies on the effect of different concentrations of the fruit extract on the enzyme activities have shown the tissue specific inhibition of enzyme activities. It was seen that the AchE activity was inhibited at a comparatively higher rate in muscle homogenate than in brain homogenate. Similar tissue specific variations were found in Mg^{2+} ATPase activities also. The Mg^{2+} ATPase activity inhibition was higher in the brain homogenate than seen in muscle and gill. In general the concentration of the extract needed for the inhibition of enzyme activities in vitro were much higher than those in in vivo. This could be due to the fact that the in vivo inhibitions might not be the direct effect of the toxin on the enzyme at molecular level, rather the effect of the extract was amplified due to some unknown intermediary mechanism. The kinetics studies have also shown tissue specific differences in

these enzymes. The apparent Michelis constant (K_m) of AchE was fohnd to be different for brain and muscle of H. fossilis. However, the K_m for Mg^{2+} ATPase of brain, muscle and gill homogenates were in a very close range. The nature of inhibition of AchE and ATPase by Z. armatum fruit extract in brain, muscle and gill homogenates was found to be of non-competitive type. However, there were differences in the K_i values. Such tissue specific variations have been reported in literature for different enzymes and the reason for such variations have been attributed to their differences in molecular structures or different isoenzyme patterns. In the present study, the specific staining of AchE on polyacrylamide gels have shown two distinct bands in muscle and only one band in brain. The tissue specific inhibition of AchE by parathion has been shown by Vijayan and Brownson (1975), based on the isoenzymic differences of AchE seen in different tissues.

Finally, it can be concluded that the fruits of Z. armatum acts as a mild metabolic poison, the toxicity of which can be reversed at lower concentrations, but becomes lethal at higher concentrations. It does not have any significant effect on the hematological parameters studied nor on the cellular respiration process. The primary modes of action seems to be through the blockade of neural transmission and supply of metabolic energy by inhibiting the activities of AchE and ATPases. Thus, Z. armatum fruits showed a good promise to be used as an indigenous substitute for rotenone in fisheries

management, both as a stupefier at lower concentration to catch and transport the fish and also as a piscicide to remove the unwanted fish from ponds at higher concentrations. However, some more works on its secondary toxicity and the effects on plankton population are necessary before this could be applied in the field.

Table 1 : Piscicidal plants commonly used by the tribals of N.E. India in fishing.

Botanical name	Part(s) used	Local name
Croton tiglium L.	Seed and fruit	Jambola gota
Eupatorium odoratum L.	leaf and shoot	Assam-lata
Milletia pachycarpa Benth.	root	Bakoa-biri, Bokal bih, Bishloti
Myrica esculenta Buch-Ham.	bark	Soh-phi, Keifang, Naga-teng, Kai-phal
Polygonum hydropiper L. var. flaccidum steward	whole plant	Pani maricha
Polygonum hydropiper L. var. hydropiper	whole plant	Pani maricha
Potentilla fulgens wall. ex Lehm.	root	
Taxus baccata L.	leaf, shoot & seed.	Dingsableh
Xeromphis spinosa (Thumb.) Keay (= Randia dumetorum Poir.)	fruit	Dieng-makasing-Khlaw, Gurol, Behmona, Mainphal
Zanthoxylum armatum DC. (=Zanthoxylum alatum Roxb.)	root, fruit bark & leaf	Gaira, Tambul

Table 2 : Toxicity of some piscicidal plant parts (crude powder), on (a) *P. shalynius* (b) *D. dangila* and (c) *H. fossilis*

Botanical name	Part used	Minimum effective concentration (ppm)		Lethal time (hr)
Myrica esculenta Buch-Ham.	bark	(a)	80	12-15
		(b)	80	
		(c)	100	
Polygonum hydropiper L. var. *hydropiper*	leaf	(a)	100	10-12
		(b)	90	
		(c)	125	
Potentilla fulgens Wall.ex-Lehm.	root	(a)	150	8-10
		(b)	160	
		(c)	200	
Xeromphis spinosa (Thumb.) Keay (= *Randia dumetorum* Poir.)	Fruit	(a)	120	10-12
		(b)	120	
		(c)	140	
Zanthoxylum aramtum DCC. (= *Z. alatum* Roxb.)	fruit	(a)	50	8-9
		(b)	50	
		(c)	70	

Table 3 : The effect of different concentrations of *Z. armatum* fruit powder on quality of diluent water in open jars at different time intervals.

Concentration	Time (hr)	Oxygen content (ppm)	Conductivity (μ mohs/cm)	pH
0 ppm (Control)	0	8.00	44.10	6.08
	6	7.93	45.50	6.07
	12	7.86	44.80	5.97
	24	7.86	44.45	6.05
75 ppm	0	7.73	48.30	6.07
	6	7.86	47.95	5.96
	12	7.66	46.55	5.93
	24	7.66	45.50	6.12
100 ppm	0	7.73	45.50	5.98
	6	7.80	49.70	6.00
	12	7.66	48.30	5.98
	24	7.66	48.32	6.03
120 ppm	0	7.60	46.20	6.01
	6	7.86	51.10	5.98
	12	7.66	50.05	5.99
	24	7.60	50.05	6.03

Table 4. The rate of mortality of *P. shalynius* with different concentrations of *Z. armatum* fruit (crude powder) at different time intervals.

Concentration (ppm)	Percentage mortality			
	3 hrs	6 hrs	12 hrs	24 hrs
Control 0 (30)	0	0	0	0
50 (12)	0	0	0	0
60 (12)	0	0	0	25
70 (42)	0	12	55	98
80 (18)	0	28	95	100
90 (24)	0	42	96	100
100 (12)	10	92	100	100
150 (12)	100	100	100	100

Figures in parentheses represent the number of fishes used for each concentration.

Table 5 : The rate of mortality of P. shalynius with different concentrations of Z. armatum fruit (alcoholic extract) at different time intervals.

Concentration (ppm)	Percentage mortality			
	3 hrs	6 hrs	12 hrs	24 hrs
Control 0 (30)	0	0	0	0
5 (24)	0	0	0	4
6 (18)	0	22	22	22
7 (36)	42	86	100	100
8 (30)	54	93	100	100
9 (18)	66	100	100	100
10 (36)	72	100	100	100
12 (18)	100	100	100	100
15 (24)	100	100	100	100

Figures in parentheses represent the number of fishes used for each concentration.

Table 6 : The rate of mortality of H. fossilis (8-12 g. wt. group) with different concentrations of Z. armatum fruit (alcoholic extract) at different time intervals.

Concentration (ppm)	Percentage mortality			
	3 hrs	6 hrs	12 hrs	24 hrs
Control 0 (24)	0	0	0	0
12 (6)	0	0	0	0
15 (15)	20	40	67	67
20 (7)	71	85	100	100
25 (12)	75	91	100	100
30 (8)	88	100	100	100
35 (7)	100	100	100	100
40 (6)	100	100	100	100

Figures in parentheses represent the number of fishes used for each concentration.

Table 7 : The rate of mortality of H. fossilis (13-20 g. wt. group) with different concentrations of Z. armatum fruit (alcoholic extract) at different time intervals.

Concentration (ppm)	Percentage mortality			
	3 hrs	6 hrs	12 hrs	24 hrs
Control 0 (30)	0	0	0	0
12 (6)	0	0	0	0
15 (35)	3	20	37	37
20 (48)	37	60	75	84
25 (43)	42	72	88	96
30 (32)	50	100	100	100
35 (25)	69	92	100	100
40 (26)	69	100	100	100

Figures in parentheses represent the number of fishes used for each concentration.

Table 8 : LC_{50} values of Z. armatum for P. shalynius and two weight groups of H. fossilis calculated using methods (1) Drawing regression lines using the formula Y=a+bX, (2) Semi-log graphical interpolation and (3) Probit analysis.

Species of fish		Plant name	Z. armatum fruit							
		Nature of toxicant	Crude powder LC_{50} (ppm)				Alcoholic extract LC_{50}(ppm)			
		Time	3 hrs	6 hrs	12hrs	24 hrs	3 hrs	6 hrs	12 hrs	24 hrs
P. shalynius		Method								
		1	-	95	-	-	8.20	6.20	-	-
		2	-	92	69	-	7.70	6.40	5.90	5.90
		3	-	89	66	-	7.70	6.50	-	-
		Mean	-	92	67.5	-	7.87	6.37	5.90	5.90
H. fossilis	Gr.I (8-12g)	1	-	-	-	-	19.00	18.50	-	-
		2	-	-	-	-	17.73	16.50	14.00	14.00
		3	-	-	-	-	19.95	16.60	-	-
		Mean	-	-	-	-	18.90	17.20	14.00	14.00
H. fossilis	Gr.II(13-20g)	1	-	-	-	-	-	22.00	19.50	18.50
		2	-	-	-	-	35.00	18.50	16.25	16.13
		3	-	-	-	-	25.70	19.50	16.60	16.22
		Mean	-	-	-	-	30.35	20.00	17.45	16.95

Table 9 : The hematological parameters of H. fossilis in relation to its body size.

No fof fishes used	length in cm	RBC/mm^3 x 10^6	Hb g%	RBC mm^3/g.wt x 10^{-8}	Hb g%/g.wt.	(RBC/mm^3/cm) x 10^4	Hb g%/cm	MCH	Average BOC
8(4)	10.55 ± 1.22	1.72 ± 0.40	13.20 ± 0.86	21.52 ± 4.90	1.65 ± 0.10	16.25 ± 3.86	1.26 ± 0.07	7.88 ± 1.41	16.55 ± 1.08
10(6)	12.76 ± 0.60	2.19 ± 0.44	14.26 ± 1.56	21.96 ± 4.56	1.27 ± 0.15	17.17 ± 3.64	1.10 ± 0.03	6.05 ± 1.48	17.83 ± 0.58
12(7)	13.45 ± 0.86	2.38 ± 0.43	15.08 ± 0.94	19.79 ± 3.56	1.11 ± 0.05	17.57 ± 2.77	1.10 ± 0.08	5.74 ± 0.85	18.85 ± 1.10
13(4)	13.45 ± 0.44	2.66 ± 0.19	15.00 ± 1.35	20.47 ± 1.24	1.03 ± 0.03	20.00 ± 0.82	1.13 ± 0.08	5.03 ± 0.49	18.75 ± 1.69
14(4)	13.25 ± 0.25	2.39 ± 0.51	14.65 ± 1.23	17.10 ± 3.50	1.06 ± 0.13	18.25 ± 3.78	1.13 ± 0.08	6.21 ± 0.80	18.31 ± 1.81
15(4)	13.55 ± 0.41	2.13 ± 0.53	14.10 ± 1.25	14.24 ± 3.29	0.93 ± 0.09	16.00 ± 3.56	1.04 ± 0.09	6.69 ± 0.78	17.63 ± 1.74
16(3)	14.23 ± 0.45	3.10 ± 0.49	16.53 ± 0.46	19.37 ± 3.07	1.03 ± 0.02	21.67 ± 3.18	1.15 ± 0.03	5.42 ± 1.05	20.66 ± 0.35
17(2)	14.50 ± 0.42	3.01 ± 0.79	15.80 ± 0.84	17.70 ± 4.61	0.92 ± 0.14	20.50 ± 4.95	1.07 ± 0.15	5.36 ± 1.15	19.75 ± 1.06
18(4)	14.70 ± 0.34	2.64 ± 0.75	14.90 ± 1.32	14.66 ± 4.43	0.82 ± 0.10	18.25 ± 3.20	1.02 ± 0.05	5.85 ± 1.06	18.63 ± 1.57
20(3)	15.00 ± 0.20	3.10 ± 0.75	16.13 ± 1.39	15.51 ± 3.82	0.80 ± 0.14	20.30 ± 4.83	1.08 ± 0.08	5.36 ± 1.02	20.16 ± 1.60
22(3)	14.73 ± 0.28	2.83 ± 0.51	16.06 ± 1.33	12.86 ± 2.32	0.72 ± 0.06	19.33 ± 2.13	1.09 ± 0.05	5.75 ± 0.68	20.08 ± 1.57

Values are expressed as Mean ± S.D.

The figures in parenthesis indicate number of fish sampled for each weight group.

Table 10 : The effect of different concentrations of *Z. armatum* fruit extract on hematological parameters of *H. fossilis* (9-14g wt. group)

Parameters studied	Concentrations of fruit extract				
	0 ppm	15 ppm	20 ppm	30 ppm	35 ppm
Weight of the fish (g)	11.86 ±0.89 (22)	12.30 ±1.66 (11)	12.81 ±1.89 (11)	12.21 ±2.74 (14)	12.92 ±1.35 (14)
Length of the fish (cm)	13.09 ± 0.89	13.11 ± 0.64	12.93 ± 0.72	12.42 ± 1.2	12.69 ± 1.22
RBCx10^6/mm^3	2.29 ± 0.51	2.25 ± 0.35	2.22 ± 0.53	2.17 ± 0.43	2.14 ± 0.38
Hb g %	13.7 ± 1.06	13.73 ± 0.99	13.60 ± 1.00	13.58 ± 0.83	13.38 ± 0.61
MCH (g% Hb/RBC x 10^{-6})	6.61 ± 0.79	6.17 ± 0.70	6.10 ± 1.20	6.40 ± 0.93	6.35 ± 1.44
BOC	17.13 ± 1.33	17.16 ± 1.24	17.00 ± 1.25	16.97 ± 1.04	16.73 ± 0.76

Values are expressed as mean ± S.D. Figures in parentheses indicate the number of fishes studied in each concentration.

Table 11 : The effect of different concentrations of *Z. armatum* fruit extract on hematological parameters in *H. fossilis* (15-20g.wt. group).

Parameters studied	Concentrations of fruit extract				
	0 ppm	15 ppm	20 ppm	30 ppm	35 ppm
Weight of the fish (g)	17.12 ± 1.87 (16)	17.16 ± 1.9 (6)	16.88 ± 1.88 (9)	15.66 ± 1.31 (6)	16.66 ± 2.27 (9)
length of the fish (cm)	14.17 ± 1.42	13.55 ± 0.59	13.97 ± 0.66	13.76 ±0.81	14.03 ± 0.45
RBC/mm^3	2.74 ± 0.66	2.43 ± 0.53	2.44 ± 0.39	2.32 ± 0.70	2.43 ± 0.70
Hb g%	15.35 ± 1.40	14.03 ± 0.78	16.48 ± 1.33	14.80 ± 1.17	15.31 ± 1.17
MCH (g%Hb/RBC x 10^{-6})	5.85 ± 1.6	5.92 ± 0.97	6.96 ± 1.40	6.45 ± 0.91	6.29 ± 0.78
BOC	19.19 ± 1.75	17.54 ± 0.98	20.60 ± 1.67	18.50 ± 1.46	19.14 ± 1.46

Values are expressed as Mean ± S.D. Figures in parentheses indicate the number of fishes studied in each concentration.

Table 12 : Effect of different concentrations of *Z. armatum* fruit extract on oxygen uptake (mg O_2 consumed/30 min/ g fish) x 10^2 by *T. mossambica* till 6hrs post treatment

Time interval	0 ppm(control)	15 ppm	20 ppm	30 ppm
1st ½hr	8.26 ± 1.59	8.56 ± 0.67 (+3.56)	13.53 ± 1.29 (+63.77)**	9.49 ± 1.78 (+14.93)
2nd ½hr	6.33 ± 0.55	7.07 ± 0.81 (+11.67)	6.59 ± 0.63 (+9.59)	6.42 ± 0.58 (+1.52)
3rd ½hr	6.02 ± 1.23	3.32 ± 0.25 (+44.75)**	3.08 ± 0.31 (-48.75)**	6.88 ± 0.51 (+14.36)
4th ½hr	3.41 ± 0.42	5.27 ± 0.92 (+54.62)**	3.12 ± 0.30 (+8.31)	6.92 ± 1.25 (+103.22)**
5th ½hr	3.03 ± 0.46	3.78 ± 1.50 (+24.73)	7.37 ± 2.11 (+143.11)**	7.76 ± 0.56 (+156.07)**
6th ½hr	3.14 ± 0.50	3.94 ± 0.66 (+24.25)	7.91 ± 1.46 (+151.58)**	6.84 ± 1.42 (+117.53)**
7th ½hr	3.40 ± 0.70	3.02 ± 0.92 (-11.31)	6.78 ± 1.94 (+99.42)**	5.26 ± 0.75 (+54.73)**
8th ½hr	4.21 ± 1.25	5.40 ± 0.52 (+28.26)	7.76 ± 0.42 (+84.35)**	3.76 ± 0.74 (-10.68)
9th ½hr	5.12 ± 0.35	4.90 ± 0.56 (-9.04)	3.47 ± 0.44 (-12.70)**	3.36 ± 0.45 (-34.28)**
10th ½hr	4.97 ± 0.45	4.38 ± 0.11 (-11.78)	3.89 ± 0,56 (-21.75)*	2.39 ± 0.68 (-51.89)**
11th ½hr	5.07 ± 0.19	3.76 ± 0.45 (-25.86)**	3.36 ± 0.97 (-33.88)**	2.31 ± 0.65 (-54.48)**
12th ½hr	5.18 ± 0.95	4.24 ± 0.26 (-18.19)	3.16 ± 0.35 (-39.00)*	2.25 ± 0.60 (-56.61)**

Values are expressed as Mean ± S.D.

Figures in parentheses represent percentage change in oxygen uptake

* $p < 0.05$; ** $p < 0.01$.

Table 13 : The level of total oxygen (mg) in experimental jars with different concentrations of Z. armatum, during oxygen uptake experiment.

Time interval	Concentrations of Z. armatum fruit extract			
	0 ppm(control)	15 ppm	20 ppm	30 ppm
0 hr	34.03 ± 2.27	32.50 ± 0.45	36.08 ± 0.98	32.44 ± 0.63
1st ½hr	31.59 ± 2.14	29.97 ± 0.60	32.18 ± 0.98	29.71 ± 0.88
2nd ½hr	28.90 ± 2.08	27.11 ± 0.44	29.45 ± 0.95	27.11 ± 0.77
3rd ½hr	26.36 ± 1.98	25.41 ± 0.39	27.75 ± 0.92	24.42 ± 0.81
4th ½hr	24.64 ± 2.01	23.16 ± 0.21	26.10 ± 0.90	21.78 ± 0.65
5th ½hr	23.01 ± 2.00	21.35 ± 0.35	23.63 ± 0.88	18.96 ± 0.66
6th ½hr	21.42 ± 2.65	19.55 ± 0.34	20.97 ± 1.21	16.66 ± 0.34
7th ½hr	19.78 ± 2.09	18.15 ± 0.44	18.98 ± 1.68	15.13 ± 0.48
8th ½hr	18.00 ± 2.01	16.00 ± 0.32	17.07 ± 1.97	14.03 ± 0.61
9th ½hr	15.85 ± 2.29	14.05 ± 0.24	15.91 ± 2.20	13.23 ± 0.64
10th ½hr	13.86 ±2.19	12.30 ± 0.30	14.75 ± 2.29	12.55 ± 0.61
11th ½hr	11.85 ± 2.20	10.78 ± 0.19	13.78 ± 2.15	11.89 ± 0.58
12th ½hr	9.96 ± 2.03	9.15 ± 0.18	12.83 ± 2.28	11.25 ± 0.57

Table 14 : Effect of *Z. armatum* fruit extract on oxygen consumption by brain and liver homogenates of *H. fossilis* at two different temperatures.

Temperature	Concentration of *Z. armatum*	µl O_2 consumed/hr/g tissue	
		Brain	Liver
30°C	0 ppm (Control)	307.6 ± 9.44	496.1 ± 14.31
	12.5×10^3 ppm	320.5 ± 11.03	512.6 ± 13.97
	25.0×10^3 ppm	300.3 ± 7.04	492.5 ± 7.64
	37.5×10^3 ppm	291.8 ± 9.13	492.2 ± 2.51
20°C	0 ppm (Control)	264.3 ± 13.20	438.3 ± 15.96
	12.5×10^3 ppm	275.4 ± 12.60	431.6 ± 13.70
	25.0×10^3 ppm	285.6 ± 14.54	458.4 ± 8.92
	37.5×10^3 ppm	275.8 ± 9.95	443.2 ± 14.87

Values are expressed as Mean ± S.D.

Table 15 : Specific activity (units/mg protein) x 10^3 of AchE in brain and muscle of H. fossilis treated with different concentrations of Z. armatum fruit extract.

Concentration	Brain	Muscle
0 ppm (Control)	41.00 ± 2.60	54.50 ± 3.40
15 ppm	34.60 ± 2.90 (-15.61)*	46.00 ± 5.20 (-14.81)*
20 ppm	31.80 ± 5.00 (-22.44)*	40.00 ± 4.80 (-25.93)**
25 ppm	22.70 ± 5.80 (-44.63)**	39.20 ± 3.50 (-28.07)**
35 ppm	20.10 ± 9.00 (-50.97)**	33.10 ± 2.70 (-39.44)**
45 ppm	20.42 ± 5.00 (-50.19)**	33.16 ± 1.80 (-39.16)**

Values are expressed as Mean ± S.D.

Figures in parentheses indicate percentage change.

* $p < 0.05$; ** $p < 0.01$.

Table 16 : Specific activity (units/mg protein) x 10^2 of total ATPase in brain, muscle and gill of H. fossilis treated with different concentrations of Z. armatum fruit extract.

Concentration	Brain	Muscle	Gill
0 ppm (Control)	63.45 ± 0.80	167.40 ± 8.68	147.53 ± 3.12
15 ppm	58.26 ± 0.93 (-8.18)**	151.03 ± 1.81 (-9.78)**	119.21 ± 5.72 (-19.20)**
20 ppm	48.35 ± 1.31 (-23.80)**	114.06 ± 2.98 (-31.86)**	87.71 ± 8.72 (-40.55)**
25 ppm	41.07 ± 0.59 (-35.27)**	112.79 ± 3.04 (-32.62)**	79.11 ± 1.38 (-46.38)**
35 ppm	39.47 ± 0.25 (-37.79)**	108.63 ± 3.68 (-35.11)**	79.53 ± 6.53 (-46.09)**
45 ppm	39.28 ± 0.82 (-38.09)**	108.80 ± 2.53 (-35.05)**	78.98 ± 3.45 (-46.46)**

Values are expressed as Mean ± S.D. Figures in parentheses indicate percentage change.
*$p<0.05$; ** $p<0.01$.

Table 17 : Specific activity (units/mg protein) x 10^2 of Mg^{2+} ATPase in brain, muscle and gill of *H. fossilis* treated with different concentrations of *Z. armatum* fruit extract.

Concentration	Brain	Muscle	Gill
0 ppm (Control)	49.65 ± 0.66	130.97 ± 7.81	133.87 ± 2.93
15 ppm	45.66 ± 1.25 (-8.04)**	118.22 ± 3.35 (-9.74)*	107.05 ± 4.58 (-20.03)**
20 ppm	36.03 ± 0.57 (-27.43)**	84.19 ± 3.58 (-35.72)**	76.38 ± 8.05 (-42.94)**
25 ppm	30.30 ± 0.24 (-38.97)**	83.04 ± 2.82 (-36.60)**	68.95 ± 1.23 (-48.49)**
35 ppm	28.62 ± 0.32 (-42.36)**	79.45 ± 4.10 (-39.34)**	68.86 ± 5.90 (-48.56)**
45 ppm	28.48 ± 0.30 (-42.64)**	78.58 ± 2.84 (-40.00)	68.51 ± 3.28 (-48.82)**

Values are expressed as Mean ± S.D. Figures in parentheses indicate percentage change.

* $p < 0.05$; ** $p < 0.01$.

Table 18 : Specific activity (units/mg protein) x 10^2 of Na^+, K^+ ATPase in brain, muscle, and gill of H. fossilis treated with different concentrations of Z. armatum fruit extract.

Concentration	Brain	Muscle	Gill
0 ppm (Control)	13.80 ± 0.54	36.43 ± 3.52	13.66 ± 1.69
15 ppm	12.59 ± 0.61 (-6.52)**	32.82 ± 2.92 (-9.91)*	12.32 ± 3.51 (-9.81)
20 ppm	12.31 ± 1.06 (-10.80)**	29.87 ± 2.61 (-18.00)*	10.80 ± 1.33 (-20.94)*
25 ppm	10.85 ± 0.48 (-21.38)**	29.76 ± 2.91 (-18.31)*	10.17 ± 0.50 (-25.55)**
35 ppm	10.85 ± 0.59 (-21.38)**	29.18 ± 2.50 (-19.90)**	10.43 ± 0.88 (-23.65)*
45 ppm	10.82 ± 0.36 (-21.59)**	29.14 ± 1.86 (-20.07)**	10.16 ± 0.43 (-25.62)**

Values are expressed as Mean ± S.D. Figures in parentheses indicate percentage change.

* $p < 0.05$; ** $p < 0.01$.

Table 19 : Tissue activity (units/g tissue) and specific activity (units/mg protein) x 10^3 of AchE in brain of H. fossilis at different time intervals on treatment with 20 ppm of Z. armatum fruit extract.

Time post treatment (hr)	Tissue activity		Specific activity	
	Control	Treated	Control	Treated
0	3.25 ± 0.15	-	43.2 ± 2.6	-
1	3.07 ± 0.20	2.36 ± 0.12 (-23.12)**	39.1 ± 2.8	29.7 ± 3.5 (-24.04)**
2	3.23 ± 0.24	2.20 ± 0.12 (-31.89)**	42.2 ± 3.4	28.7 ± 0.7 (-31.75)**
3	3.22 ± 0.18	2.16 ± 0.11 (-32.92)**	42.1 ± 2.6	28.1 ± 2.4 (-34.19)**
4	3.07 ± 0.26	2.01 ± 0.06 (-34.53)**	39.3 ± 3.1	25.7 ± 1.4 (-34.60)**
6	3.18 ± 0.09	2.05 ± 0.19 (-35.53)**	45.7 ± 3.8	27.1 ± 2.4 (-40.7)**
8	3.31 ± 0.14	1.99 ± 0.04 (-39.8)**	42.5 ± 2.6	25.4 ± 0.3 (-40.24)**
10	3.26 ± 0.17	2.34 ± 0.11 (-28.22)**	44.4 ± 1.9	31.1 ± 0.9 (-29.95)**
12	3.23 ± 0.09	2.65 ± 0.10 (-17.96)**	46.7 ± 0.7	38.5 ± 1.8 (-17.56)**
24	3.08 ± 0.12	2.78 ± 0.08 (-9.74)**	48.3 ± 1.9	45.0 ± 1.8 (-6.83)*

Values are expressed as Mean ± S.D.

Figures in parentheses indicate percentage change.

* $p < 0.05$; ** $p < 0.01$.

Table 20 : Tissue activity (units/g tissue) and specific activity (units/mg protein) x 10^3 AchE in muscle of H. fossilis at different time intervals on treatment with 20 ppm Z. armatum fruit extract.

Time post treatment (hr)	Tissue activity		Specific activity	
	Control	Treated	Control	Treated
0	1.64 ± 0.21	-	46.9 ± 4.3	-
1	1.62 ± 0.08	1.41 ± 0.15 (-12.96)*	50.6 ± 3.7	45.9 ± 4.6 (-9.29)
2	1.65 ± 0.09	1.32 ± 0.04 (-20.00)**	50.3 ± 2.6	40.7 ± 3.0 (-19.09)**
3	1.67 ± 0.07	1.00 ± 0.03 (-34.12)**	51.4 ± 3.3	31.4 ± 1.16 (-38.91)**
4	1.64 ± 0.08	1.08 ± 0.13 (-34.15)**	51.0 ± 4.1	30.9 ± 5.4 (-39.41)**
6	1.66 ± 0.06	0.98 ± 0.02 (-40.96)**	52.8 ± 2.8	31.0 ± 1.3 (-41.29)**
8	1.71 ± 0.06	1.00 ± 0.05 (-41.52)**	53.0 ± 2.3	31.1 ± 1.1 (-41.32)**
10	1.63 ± 0.08	1.13 ± 0.01 (-30.67)**	51.7 ± 2.4	37.3 ± 1.9 (-27.85)**
12	1.67 ± 0.11	1.42 ± 0.03 (-14.97)**	52.3 ± 1.7	45.4 ± 0.3 (-13.19)**
24	1.67 ± 0.05	1.62 ± 0.05 (-2.99)	52.5 ± 1.0	51.3 ± 1.0 (-1.33)

Values are expressed as Mean ± S.D.

Figures in parentheses indicate percentage change.

* $p < 0.05$; ** $p < 0.01$.

Table 21 : Tissue activity (units/g tissue) and specific activity (units/mg protein) x 10^3 of AchE in gill of H. fossilis at different time intervals on treatment with 20 ppm Z. armatum fruit extract.

Time post treatment (hr)	Tissue activity		Specific activity	
	Control	Treated	Control	Treated
0	1.94 ± 0.15	-	60.5 ± 1.4	-
1	1.85 ± 0.12	1.53 ± 0.24 (-17.30)*	58.7 ± 6.2	50.7 ± 7.4 (-13.63)
2	1.87 ± 0.14	1.44 ± 0.12 (-22.99)**	61.9 ± 5.7	47.4 ± 4.4 (-23.42)**
3	1.77 ± 0.20	1.26 ± 0.19 (-28.81)**	59.3 ± 1.2	41.4 ± 2.6 (-30.19)**
4	1.75 ± 0.19	1.14 ± 0.17 (-34.86)**	56.4 ± 4.0	36.6 ± 4.3 (-35.11)**
6	1.75 ± 0.08	1.02 ± 0.16 (-41.71)**	57.9 ± 3.2	34.0 ± 5.1 (-41.28)**
8	1.83 ± 0.36	1.24 ± 0.12 (-32.24)**	62.1 ± 3.9	41.6 ± 3.0 (-33.01)**
10	1.74 ± 0.23	1.32 ± 0.05 (-24.14)**	61.9 ± 7.7	47.5 ± 2.6 (-23.26)**
12	1.71 ± 0.10	1.50 ± 0.09 (-12.28)*	60.1 ± 1.0	54.6 ± 3.5 (-9.15)*
24	1.67 ± 0.07	1.59 ± 0.08 (-4.8)	56.4 ± 1.4	53.9 ± 1.5 (-4.43)*

Values are expressed as Mean ± S.D.

Figures in parentheses indicate percentage change.

* $p < 0.05$; ** $p < 0.01$.

Table 22 : Tissue activity (units/g tissue) and specific activity (units/mg protein) x 10^2 of total ATPase in brain of *H. fossilis* at different time intervals on treatment with 20 ppm *Z. armatum* fruit extract.

Time post treatment (hr)	Tissue activity		Specific activity	
	Control	Treated	Control	Treated.
0	45.44 ± 5.15	-	60.27 ± 7.04	-
1	44.94 ± 2.66	44.48 ± 2.97 (-1.02)	57.13 ± 4.24	55.78 ± 2.57 (-2.36)
2	48.29 ± 2.93	42.83 ± 2.83 (-11.28)*	62.98 ± 4.21	56.43 ± 4.51 (-10.4)
3	48.82 ± 2.55	36.15 ± 2.68 (-25.95)**	64.66 ± 3.34	46.42 ± 0.90 (-28.21)**
4	50.85 ± 2.40	34.73 ± 3.93 (-31.70)**	66.44 ± 3.88	44.39 ± 5.69 (-33.19)**
6	50.73 ± 2.42	35.24 ± 3.37 (-30.53)**	67.11 ± 2.88	50.40 ± 2.31 (-24.9)**
8	49.52 ± 3.32	37.83 ± 1.16 (-23.61)**	63.47 ± 3.13	48.47 ± 1.49 (-23.63)**
10	46.33 ± 2.51	42.10 ± 2.53 (-9.13)*	63.22 ± 4.75	55.75 ± 2.74 (-11.80)*
12	43.65 ± 2.01	40.09 ± 2.10 (-8.50)*	62.90 ± 1.64	58.20 ± 2.93 (-7.47)*
24	44.73 ± 0.51	41.45 ± 1.67 (-7.33)**	70.48 ± 3.38	67.21 ± 2.94 (-4.64)

Values are expressed as Mean ± S.D.

Figures in parentheses indicate percentage change.

* $p < 0.05$; ** $p < 0.01$.

Table 23 : Tissue activity (units/g tissue) and specific activity (units/mg protein) x 10^2 of total ATPase in muscle of H. fossilis at different time intervals on treatment with 20 ppm Z. armatum fruit extract.

Time post treatment (hr)	Tissue activity		Specific activity	
	Control	Treated	Control	Treated
0	54.19 ± 0.68	-	155.95 ± 7.92	-
1	56.76 ± 3.70	48.85 ± 3.41 (-13.94)*	170.80 ± 11.26	158.83 ± 8.92 (-10.16)
2	56.58 ± 1.27	45.82 ± 0.80 (-19.02)**	172.10 ± 5.30	142.13 ± 7.49 (-17.41)**
3	51.90 ± 1.39	39.26 ± 0.57 (-24.35)**	159.84 ± 5.50	117.72 ± 5.65 (-26.35)**
4	51.80 ± 2.80	37.00 ± 2.48 (-28.27)**	160.33 ± 14.65	117.13 ± 10.47 (-26.94)**
6	53.08 ± 1.86	34.23 ± 1.83 (-35.51)**	167.94 ± 2.46	107.77 ± 2.85 (-35.83)**
8	56.77 ± 1.70	40.77 ± 1.75 (-28.18)**	176.45 ± 5.60	126.60 ± 4.48 (-28.25)**
10	51.52 ± 2.51	41.44 ± 1.05 (-19.57)**	164.01 ± 8.65	134.25 ± 6.37 (-18.15)**
12	44.90 ± 0.90	38.82 ± 1.42 (-13.54)**	140.61 ± 4.26	124.15 ± 4.43 (-11.71)**
24	44.53 ± 1.68	41.85 ± 1.02 (-5.99)*	138.62 ± 3.65	133.90 ± 4.45 (-3.41)

Values are expressed as Mean ± S.D.

Figures in parentheses indicate percentage change.

* $p < 0.05$; ** $p < 0.01$.

Table 24 : Tissue activity (units/g tissue) and specific activity (units/mg protein) x 10^2 of total ATPase in gill of *H. fossilis* at different time intervals on treatment with 20 ppm *Z. armatum* fruit extract.

Time post treatment (hr)	Tissue activity		Specific activity	
	Control	Treated	Control	Treated
0	42.49 ± 3.62	-	136.51 ± 12.28	-
1	42.91 ± 4.16	36.52 ± 1.49 (-14.89)*	136.93 ± 19.00	120.36 ± 3.78 (-12.10)
2	41.58 ± 3.90	31.62 ± 1.06 (-23.95)**	138.72 ± 20.28	104.42 ± 4.51 (-24.73)*
3	43.81 ± 1.57	23.17 ± 2.47 (-35.70)**	145.85 ± 10.69	92.09 ± 8.90 (-36.86)**
4	44.58 ± 2.69	22.42 ± 1.28 (-49.71)**	143.06 ± 11.18	71.60 ± 5.04 (-49.95)**
6	43.19 ± 3.25	24.91 ± 2.04 (-42.32)**	143.18 ± 9.28	81.82 ± 2.11 (-42.86)**
8	35.58 ± 3.09	24.42 ± 2.25 (-31.37)**	120.85 ± 11.81	81.38 ± 6.57 (-32.66)**
10	38.36 ± 3.26	28.56 ± 2.93 (-25.55)**	135.28 ± 8.51	102.95 ± 9.62 (-23.90)**
12	38.66 ± 1.85	34.25 ± 1.71 (-11.41)**	136.72 ± 3.32	124.75 ± 5.29 (-8.76)**
24	39.77 ± 0.97	37.35 ± 0.66 (-6.08)**	134.21 ± 1.28	126.38 ± 0.39 (-5.83)**

Values are expressed as Mean ± S.D.

Figures in parentheses indicate percentage change.

* $p < 0.05$; ** $p < 0.01$.

Table 25 : Tissue activity (units/g tissue) and specific activity (units/mg protein) x 10^2 of Mg^{2+} ATPase in brain of H. fossilis at different time intervals on treatment 20 ppm Z. armatum fruit extract.

Time post treatment (hr)	Tissue activity		Specific activity	
	Control	Treated	Control	Treated
0	34.36 ± 2.65	-	45.50 ± 4.62	-
1	33.16 ± 3.91	33.13 ± 2.79 (-0.09)	42.13 ± 4.74	41.62 ± 4.19 (-1.21)
2	35.39 ± 2.57	32.16 ± 3.67 (-9.13)	46.21 ± 4.50	42.63 ± 3.78 (-7.75)
3	36.06 ± 1.27	25.85 ± 1.66 (-28.31)**	47.76 ± 1.40	33.49 ± 1.87 (-25.18)**
4	42.55 ± 2.29	27.36 ± 3.27 (-35.70)**	54.60 ± 2.95	34.96 ± 4.67 (-35.97)**
6	39.53 ± 1.29	26.12 ± 1.16 (-33.92)**	52.33 ± 2.38	37.51 ± 2.75 (-28.32)**
8	39.37 ± 1.50	28.59 ± 2.29 (-27.38)**	50.53 ± 2.79	36.48 ± 4.22 (-27.81)**
10	37.10 ± 2.71	33.62 ± 3.55 (-9.38)	50.61 ± 4.09	44.71 ± 3.02 (-11.65)
12	33.05 ± 1.69	30.16 ± 1.79 (-8.74)*	47.62 ± 2.42	43.78 ± 2.27 (-8.06)
24	35.70 ± 0.40	32.79 ± 1.26 (-8.15)**	56.05 ± 2.53	53.93 ± 2.24 (-3.78)

Values are expressed as Mean ± S.D.

Figures in parentheses indicate percentage change.

* $p < 0.05$; ** $p < 0.01$.

Table 26 : Tissue activity (units/g tissue) and specific activity (units/mg protein) x 10^2 of Mg^{2+} ATPase in muscle of *H. fossilis* at different time intervals on treatment with 20 ppm *Z. armatum* fruit extract.

Time post treatment (hr)	Tissue activity		Specific activity	
	Control	Treated	Control	Treated
0	45.27 ± 1.17	-	130.28 ± 6.72	-
1	47.06 ± 1.88	39.50 ± 2.74 (-16.06)**	147.37 ± 19.24	128.40 ± 10.49 (-12.87)
2	42.84 ± 2.33	33.65 ± 2.60 (-21.45)**	130.46 ± 9.27	104.30 ± 8.22 (-20.05)**
3	37.83 ± 0.63	26.51 ± 0.05 (-29.92)**	116.56 ± 4.39	79.45 ± 2.62 (-31.84)**
4	39.00 ± 1.34	25.73 ± 0.55 (-34.03)**	121.18 ± 8.73	81.37 ± 3.64 (-32.85)**
6	40.33 ± 1.99	24.27 ± 2.39 (-47.61)**	127.88 ± 4.46	76.69 ± 10.03 (-40.43)**
8	40.56 ± 2.78	26.36 ± 2.79 (-35.00)**	126.14 ± 10.19	82.01 ± 9.66 (-34.98)**
10	37.60 ± 3.01	29.09 ± 2.32 (-22.63)**	119.55 ± 7.66	93.78 ± 13.93 (-21.56)*
12	32.70 ± 1.56	27.60 ± 1.43 (-8.33)**	102.09 ± 6.71	88.28 ± 4.03 (-13.53)**
24	32.01 ± 0.85	29.82 ± 0.88 (-6.84)**	99.65 ± 2.92	95.41 ± 4.02 (-4.25)

Values are expressed as Mean ± S.D.

Figures in parentheses indicate percentage change.

* $p < 0.05$; ** $p < 0.01$.

Table 27 : Tissue activity (units/g tissue) and specific activity (units/mg protein) x 10^2 of Mg^{2+} ATPase in gill of H. fossilis at different time intervals on treatment with 20 ppm Z. armatum fruit extract.

Time post treatment (hr)	Tissue activity		Specific activity	
	Control	Treated	Control	Treated
0	34.75 ± 3.77	-	108.79 ± 14.52	-
1	34.69 ± 4.19	29.24 ± 0.41 (-15.71)*	110.71 ± 17.33	96.48 ± 9.83 (-12.85)
2	34.27 ± 4.03	25.31 ± 2.60 (-26.15)**	114.61 ± 21.12	83.43 ± 8.43 (-27.21)*
3	34.77 ± 2.39	21.99 ± 1.75 (-36.76)**	115.88 ± 12.58	72.11 ± 6.60 (-37.77)**
4	37.15 ± 1.53	16.50 ± 1.69 (-55.58)**	119.28 ± 3.06	52.77 ± 6.32 (-55.76)**
6	35.37 ± 3.58	18.48 ± 0.58 (-47.75)**	117.37 ± 12.16	60.92 ± 3.89 (-48.09)**
8	30.27 ± 1.91	19.97 ± 1.73 (-34.02)**	103.09 ± 8.30	66.57 ± 5.36 (-35.43)**
10	33.54 ±3.01	24.05 ± 2.31 (-28.29)**	118.62 ± 13.76	86.61 ± 5.78 (-26.99)**
12	33.71 ± 2.11	29.54 ± 1.45 (-12.37)**	119.21 ± 3.01	107.62 ± 5.43 (-9.72)**
24	35.19 ± 1.38	32.91 ± 0.31 (-6.48)*	118.72 ± 2.93	111.34 ± 0.94 (-6.22)**

Values are expressed as Mean ± S.D.

Figures in parentheses indicate percentage change.

* $p < 0.05$; ** $p < 0.01$.

Table 28 : Tissue activity (units/g tissue) and specific activity (units/mg protein) x 10^2 Na^+, K^+ ATPase in brain of *H. fossilis* at different time intervals on treatment with 20 ppm *Z. armatum* fruit extract.

Time post treatment (hr)	Tissue activity		Specific activity	
	Control	Treated	Control	Treated
0	11.08 ± 4.19	-	14.66 ± 3.94	-
1	11.82 ± 2.06	11.35 ± 4.40 (-3.98)	16.59 ± 1.70	14.15 ± 5.25 (-14.71)
2	12.88 ± 1.79	10.54 ± 2.44 (-18.17)	16.75 ± 1.97	13.91 ± 3.41 (-16.96)
3	12.75 ± 2.64	10.89 ± 2.81 (-14.59)	16.89 ± 3.38	14.04 ± 3.26 (-16.87)
4	8.30 ± 0.68	7.36 ± 1.61 (-11.32)	10.65 ± 0.96	9.41 ± 2.16 (-11.64)
6	11.20 ± 3.27	9.12 ± 3.04 (-18.57)	14.78 ± 4.13	12.87 ± 3.90 (-12.92)
8	10.15 ± 4.18	9.23 ± 1.97 (-9.06)	12.93 ± 5.00	11.82 ± 2.63 (-8.58)
10	9.21 ± 1.71	8.48 ± 1.70 (-7.93)	12.59 ± 2.53	11.29 ± 2.45 (-8.73)
12	10.61 ± 0.25	9,92 ± 0.39 (-6.50)	15.29 ± 0.14	14.41 ± 0.40 (-5.76)
24	9.03 ± 0.12	8.66 ± 0.42 (-4.10)	14.18 ± 0.68	14.05 ± 0.70 (-1.00)

Values are expressed as Mean ± S.D.

Figures in parentheses indicate percentage change.

Table 29 : Tissue activity (units/g tissue) and specific activity (units/mg protein) x 10^2 of Na^+, K^+ ATPase in muscle of *H. fossilis* at different time intervals on treatment with 20 ppm *Z. armatum* fruit extract.

Time post treatment (hr)	Tissue activity		Specific activity	
	Control	Treated	Control	Treated
0	8.91 ± 1.11	-	25.61 ± 3.38	-
1	9.70 ± 3.05	9.37 ± 1.75 (-3.40)	32.51 ± 9.04	30.39 ± 4.96 (-5.47)
2	13.69 ± 2.72	12.17 ± 2.38 (-11.10)	41.61 ± 7.82	37.78 ± 8.04 (-9.20)
3	14.05 ± 1.32	12.75 ± 0.94 (-9.25)	43.30 ± 3.37	38.29 ± 3.64 (-11.57)
4	13.07 ± 1.58	11.27 ± 2.06 (-13.77)	40.54 ± 5.56	35.76 ± 7.12 (-11.79)
6	12.74 ± 1.77	9.95 ± 1.57 (-21.89)*	40.28 ± 5.10	31.28 ± 4.43 (-22.34)*
8	16.19 ± 3.37	14.40 ± 1.39 (-11.06)	50.25 ± 9.79	44.69 ± 2.88 (11.06)
10	13.92 ± 3.86	12.35 ± 3.44 (-11.27)	44.45 ± 12.91	40.46 ± 14.12 (-8.98)
12	12.20 ± 0.79	11.23 ± 0.71 (-7.95)	38.17 ± 2.06	35.87 ± 1.44 (-6.03)
24	12.52 ± 0.61	12.03 ± 0.66 (-3.91)	38.97 ± 2.17	38.48 ± 1.16 (-1.26)

Values are expressed as Mean ± S.D.

Figures in parentheses indicate percentage change.

* $p < 0.05$.

Table 30 : Tissue activity (units/g tissue) and specific activity (units/mg protein) x 10^2 of Na^+, K^+ ATPase in gill of *H. fossilis* at different time intervals on treatment with 20 ppm *Z. armatum* fruit extract.

Time post treatment (hr)	Tissue activity		Specific activity	
	Control	Treated	Control	Treated
0	7.74 ± 0.75	-	23.66 ± 1.84	-
1	8.26 ± 1.25	7.27 ± 1.27 (-11.98)	26.36 ± 4.67	24.44 ± 3.35 (-7.28)
2	7.30 ± 1.29	6.31 ± 1.07 (-13.56)	23.97 ± 3.33	21.00 ± 9.08 (-12.39)
3	9.03 ± 1.49	6.16 ± 1.72 (-31.78)*	29.93 ± 7.00	20.77 ± 1.17 (-30.60)*
4	7.42 ± 1.23	5.90 ± 1.19 (-20.49)	23.78 ± 3.50	18.79 ± 3.41 (-20.98)
6	7.80 ± 1.79	6.43 ± 1.85 (-17.56)	25.77 ± 5.27	20.90 ± 5.02 (-18.90)
8	5.31 ± 1.24	4.45 ± 1.30 (-16.20)	18.03 ± 3.86	14.81 ± 4.18 (-17.85)
10	4.64 ± 1.13	3.95 ± 0.43 (-14.87)	16.23 ± 3.09	14.32 ± 2.57 (-11.77)
12	4.95 ± 0.25	4.70 ± 0.31 (-5.05)	17.50 ± 0.73	17.13 ± 0.36 (-2.11)
24	4.58 ± 0.43	4.45 ± 0.23 (-2.84)	15.84 ± 1.64	15.09 ± 0.8 (-2.52)

Values are expressed as Mean ± S.D.

Figures in parentheses indicate percentage change.

* $p < 0.05$.

Table 31 : Specific activity (units/mg protein) x 10^3 of AchE in brain of *H. fossilis* treated *in vitro* with Eserine and *Z. armatum* fruit extract.

Concentration of Eserine(M)	AchE Specific activity	Concentration of extract (ppm)	AchE specific activity
0.00 (Control)	33.76 ± 0.09	0.00 (control)	42.60 ± 0.10
1.28×10^{-6}	33.56 ± 0.48 (-0.59)	400	41.90 ± 0.10 (-1.64)**
2.56×10^{-6}	32.89 ± 0.28 (-2.58)*	800	40.05 ± 0.10 (-4.93)**
3.20×10^{-6}	32.45 ± 0.11 (-3.88)**	1200	38.50 ± 0.00 (-9.62)**
5.12×10^{-6}	31.00 ± 0.37 (-8.18)**	1600	30.40 ± 0.20 (-28.64)**
5.76×10^{-6}	22.77 ± 0.19 (-32.55)**	2000	30.50 ± 0.10 (-28.40)**
6.40×10^{-6}	11.66 ± 0.11 (-65.46)**	2400	29.70 ± 0.10 (-28.28)**
1.28×10^{-6}	4.40 ± 0.11 (-86.97)**	-	-
3.20×10^{-5}	0.77 ± 0.11 (-97.72)**	-	

Values are expressed as Mean ± S.D.
Figures in parentheses indicate percentage change.
* $p < 0.05$; ** $p < 0.01$.

Table 32 : Specific activity (units/mg protein) x 10^3 of AchE in muscle of *H. fossilis* treated *in vitro* with Eserine and *Z. armatum* fruit extract.

Concentration of Eserine	AchE specific activity	Concentration of extract (ppm)	AchE specific activity
0.00 (control)	49.03 ± 0.19	0.00 (control)	47.90 ± 0.06
3.20×10^{-7}	47.92 ± 0.32 (-2.26)*	400	43.60 ± 0.10 (-8.97)**
1.28×10^{-6}	40.42 ± 0.10 (-17.56)**	800	35.60 ± 0.10 (-25.68)**
3.20×10^{-6}	30.75 ± 0.39 (-37.29)**	1000	32.70 ± 0.13 (-31.73)**
5.12×10^{-6}	27.38 ± 0.12 (-44.16)**	1200	30.70 ± 1.10 (-35.91)**
6.40×10^{-6}	17.82 ± 0.57 (-63.65)**	1600	26.40 ± 0.30 (-44.89)**
1.28×10^{-5}	6.09 ± 0.10 (-87.58)**	2400	26.00 ± 0.10 (-45.72)**
3.20×10^{-5}	1.22 ± 0.13 (-97.51)**	-	-
6.40×10^{-5}	1.22 ± 0.13 (-97.51)**	-	

Values expressed as Mean ± S.D.

Figures in parentheses indicate percentage change.

* $p < 0.05$; ** $p < 0.01$.

Table 33 : Specific activity (units/mg protein) x 10^2 of Mg^{2+} ATPase in brain of H. fossilis treated in vitro with KSCN and Z. armatum fruit extract.

Concentration of KSCN (M)	Mg^{2+} ATPase specific activity	Concentration of Extract (ppm)	Mg^{2+} ATPase specific activity
0 (control)	46.67 ± 2.63	0 (control)	45.16 ± 0.34
1.81×10^{-5}	42.50 ± 0.17 (-8.94)	40	42.53 ± 1.73 (-5.82)
3.63×10^{-5}	35.37 ± 0.53 (-24.21)**	80	42.55 ± 0.98 (-5.78)*
6.60×10^{-5}	33.40 ± 0.17 (-28.43)**	160	36.17 ± 0.06 (-19.91)**
7.20×10^{-5}	27.37 ± 0.50 (-41.35)**	240	33.45 ± 0.62 (-25.93)**
9.09×10^{-5}	24.37 ± 1.04 (-47.78)**	320	30.62 ± 0.28 (-32.20)**
10.90×10^{-5}	24.50 ± 0.78 (-47.50)**	400	29.10 ± 0.55 (-35.56)**
-	-	480	29.49 ± 0.72 (-34.70)**

Values aree expressed as Mean ± S.D.

Figures in parentheses indicate percentage change.

* $p < 0.05$; ** $p < 0.01$.

Table 34 : Specific activity (iunits/mg protein) x 10^2 of Mg^{2+} ATPase in muscle of H. fossilis treated in vitro with KSCN and Z. armatum fruit extract.

Concentration of KSCN (M)	Mg^{2+} ATPase specific activity	Concentration of extract(ppm)	Mg^{2+} ATPase specific activity
0 (control)	139.37 ± 1.15	0 (Control)	132.91 ± 2.06
1.81×10^{-5}	120.08 ± 2.72 (-13.84)**	40	126.31 ± 1.24 (-4.97)*
3.63×10^{-5}	102.37 ± 0.68 (-26.55)**	80	120.59 ± 0.91 (-9.27)**
6.60×10^{-5}	96.19 ± 1.18 (-30.98)**	160	115.44 ± 1.07 (-13.14)**
7.20×10^{-5}	87.62 ± 0.73 (-37.13)**	240	101.26 ± 0.99 (-23.81)**
9×10^{-5}	76.58 ± 0.48 (-45.05)**	320	100.46 ± 0.99 (-24.42)**
10.9×10^{-5}	76.65 ± 0.53 (-45.00)**	400	98.12 ± 0.96 (-26.18)**
-	-	480	99.68 ± 0.92 (-25.00)**

Values are expressed as Mean ± S.D.

Figures in parentheses indicate percentage change.

* $p < 0.05$; ** $p < 0.01$.

Table 35 : Specific activity (units/mg protein) x 10^2 of Mg^{2+} ATPase in gill of H. fossilis treated in vitro with KSCN and Z. armatum fruit extract.

Concentration of KSCN (M)	Mg^{2+} ATPase specific activity	Concentration of extract(ppm)	Mg^{2+} ATPase specific activity
0 (control)	140.94 ± 1.21	0 (control)	124.28 ± 0.49
1.81×10^{-5}	135.69 ± 3.51 (-3.72)	40	110.79 ± 0.63 (-10.85)**
3.63×10^{-5}	118.26 ± 0.38 (-16.09)**	80	104.15 ± 0.25 (-16.20)**
6.60×10^{-5}	102.30 ± 1.14 (-27.42)**	160	98.60 ± 1.00 (-20.66)**
7.2×10^{-5}	85.24 ± 0.64 (-39.52)**	240	95.12 ± 0.90 (-23.46)**
9.0×10^{-5}	80.24 ± 1.02 (-43.07)**	320	92.98 ± 1.98 (-25.26)**
10.9×10^{-5}	81.04 ± 0.94 (-42.50)**	400	90.98 ± 1.98 (-26.80)**
-	-	480	91.65 ± 0.78 (-26.25)**

Values are expressed as Mean ± S.D.
Figures in parentheses indicate percentage change.
** $p < 0.01$.

Table 36 : Effect of Eserine and *Z. armatum* fruit extract on the apparent K_m and V_{max} of AchE in brain and muscle of *H. fossilis* using Acetylthiocholine iodide as substrate.

	Tissue	Control	Z. armatum fruit extract (ppm)				Eserine concentration (M)		
			1200	600	1600	1360	6.4×10^{-6}	3.2×10^{-6}	5.1×10^{-6} M
$K_m \times 10^{-5}$ M	Brain	7.27	-	-	7.27	7.27	32.00	-	24.24
	Muscle	8.90	8.90	8.90	-	-	25.89	13.60	-
$V_{max} \times 10^3$	Brain	44.4	-	-	30.3	37.7	44.4	-	44.4
	Muscle	58.8	40.0	50.0	-	-	58.8	58.8	-

Table 37 : Effect of KSCN and *Z. armatum* fruit extract on the apparent K_m and V_{max} of Mg^{2+} ATPase in brain, muscle and gill of *H. fossilis* using ATP as substrate.

	Tissue	Control	*Z. armatum* fruit extract (ppm)		KSCN concentration (M)	
			240	80	1.18×10^{-5}	5.45×10^{-5}
$K_m \times 10^{-4}$ M	Brain	9.72	13.51	11.11	7.14	6.84
	Muscle	7.10	10.00	8.33	5.95	4.59
	Gill	8.71	11.11	10.00	7.58	6.76
$V_{max} \times 10$	Brain	6.16	4.94	5.71	4.17	3.28
	Muscle	15.39	12.82	14.29	12.20	8.85
	Gill	13.25	11.29	12.00	11.11	9.52

Table 38 : The nature of inhibition of (A) AchE with Eserine and *Z. armatum* and (B) Mg^{2+} ATPase with KSCN and *Z. armatum* fruit extract in brain, muscle and gill of *H. fossilis*.

(A) Acetylcholinesterase

Substrate	Inhibitor	Nature of inhibition		
		Brain	Muscle	Gill
Acetyl thiocholine -iodide	Eserine	Competitive	Competitive	
	Z. armatum	Noncompetitive	Noncompetitive	

(B) Mg^{2+} ATPase

ATP	KSCN	Noncompetitive	Noncompetitive	Noncompetitive
	Z. armatum	Noncompetitive	Noncompetitive	Noncompetitive

Table 39 : Inhibitor constants (K_i) of *Z. armatum* fruit extract for AchE and Mg^{2+} ATPase in brain, muscle and gill for *H. fossilis*

Tissue	(K_i)	
	AchE	Mg^{2+} ATPase
Brain	700 ppm	220 ppm
Muscle	500 ppm	240 ppm
Gill		250 ppm

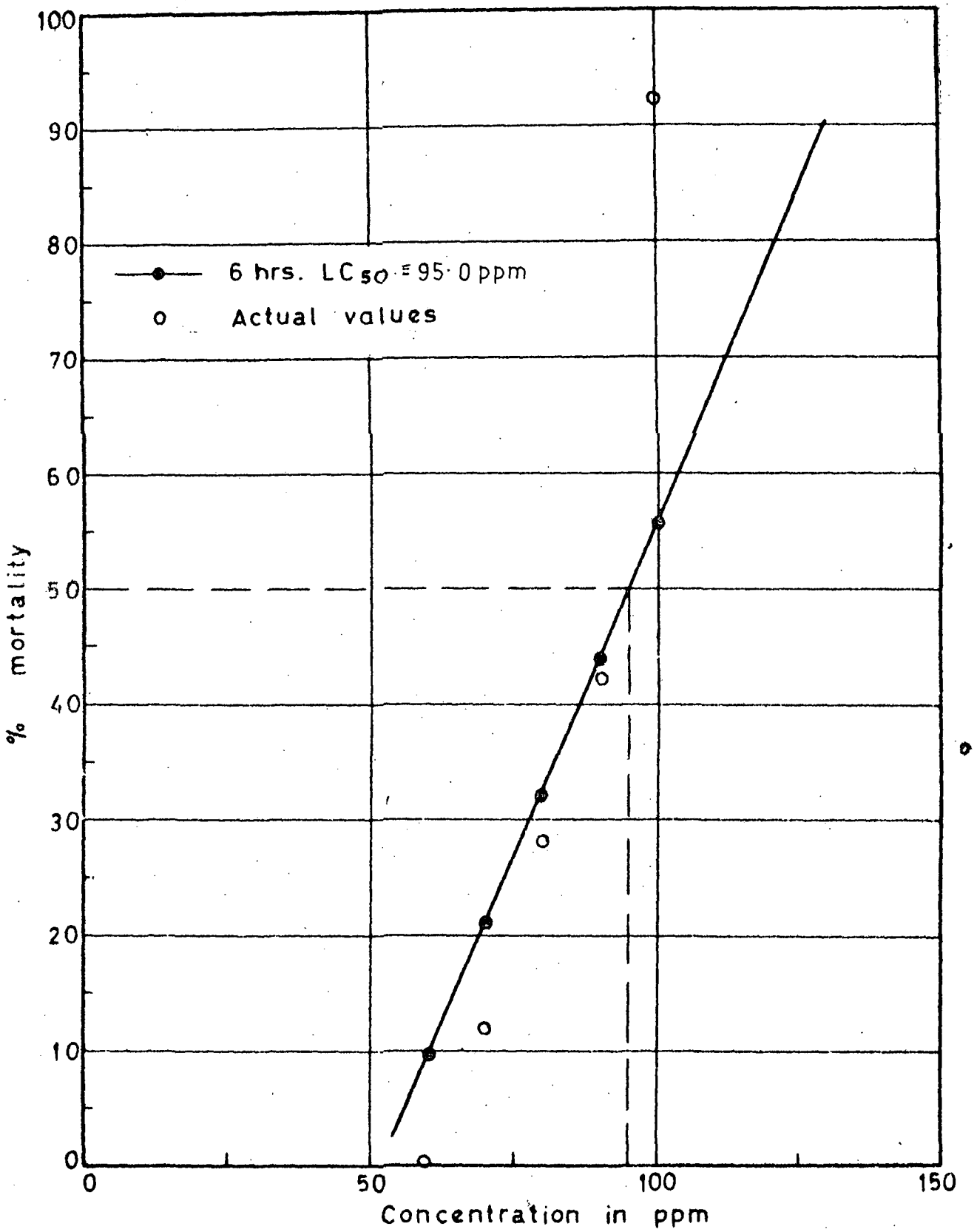


FIG. 1. -GRAPH FOR DERIVATION OF 6 hrs LC_{50} VALUE OF Z·ARMATUM FRUIT CRUDE POWDER FOR P·SHALYNIUS USING THE EQUATION $y = a + bX$ (Actual values are also plotted)

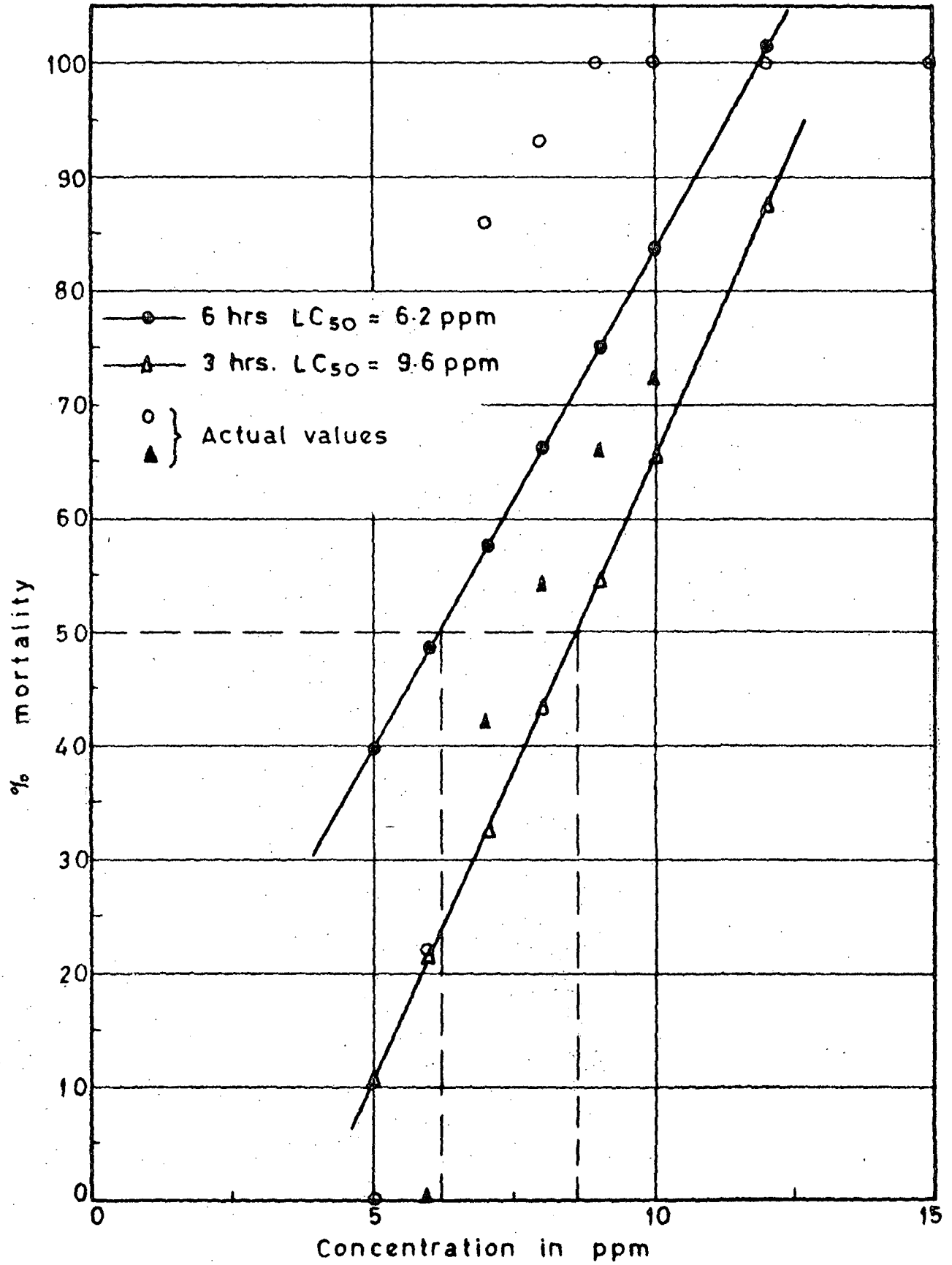


FIG. 2. -GRAPH FOR DERIVATIONS OF 3 hrs AND 6 hrs LC_{50} VALUES OF Z. ARMATUM FRUIT ALCOHOLIC EXTRACT FOR P. SHALYNIUS USING THE REGRESSION EQUATION $y = a + bX$ (Actual values are also plotted).

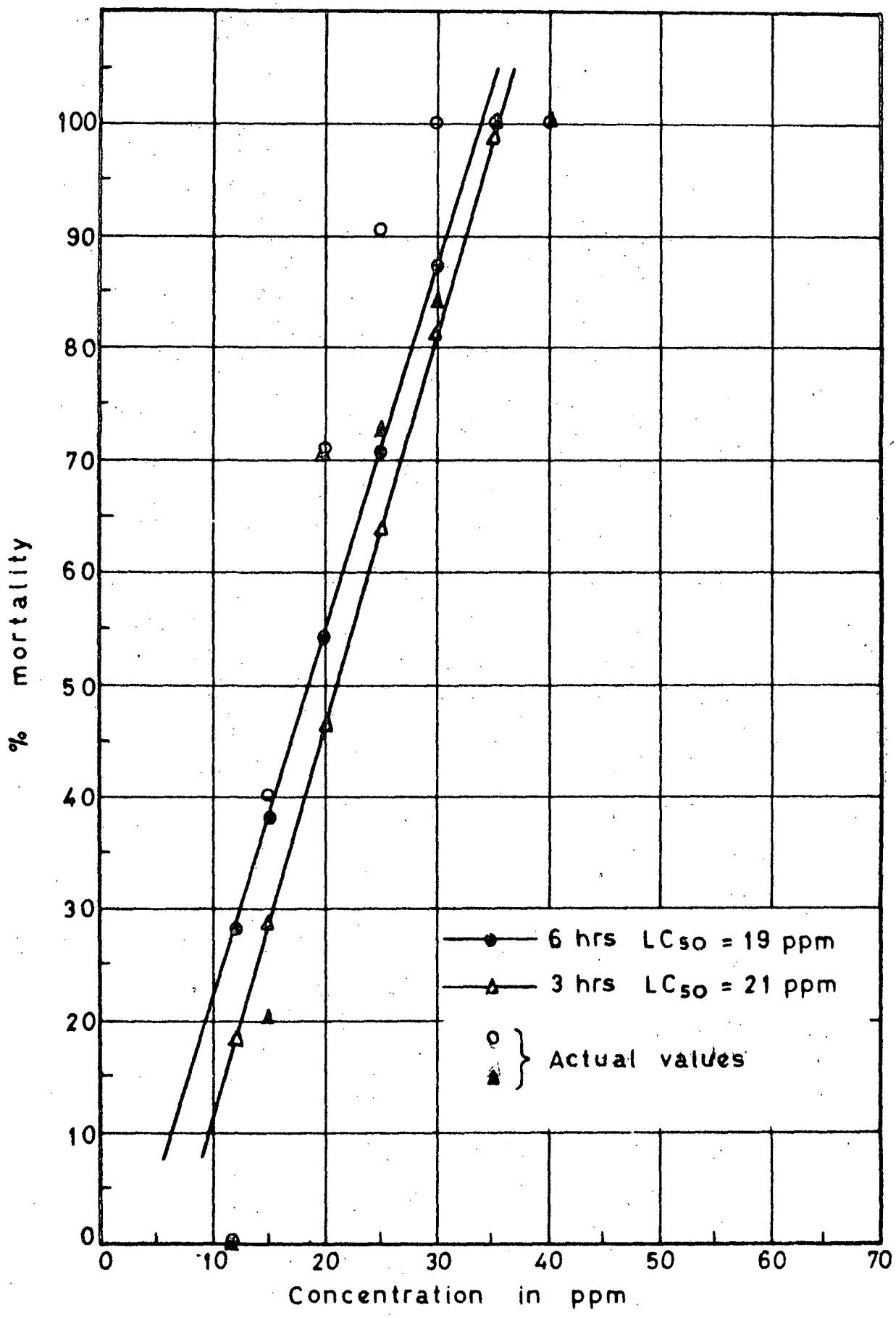


FIG. 3. -GRAPH FOR DERIVATION OF 3 hrs AND 6 hrs LC_{50} VALUES OF Z. ARMATUM FRUIT ALCOHOLIC EXTRACT FOR H. FOSSILIS (8-12 g. wt. group) USING THE REGRESSION EQUATION $y = a + bX$ (Actual values are also plotted).

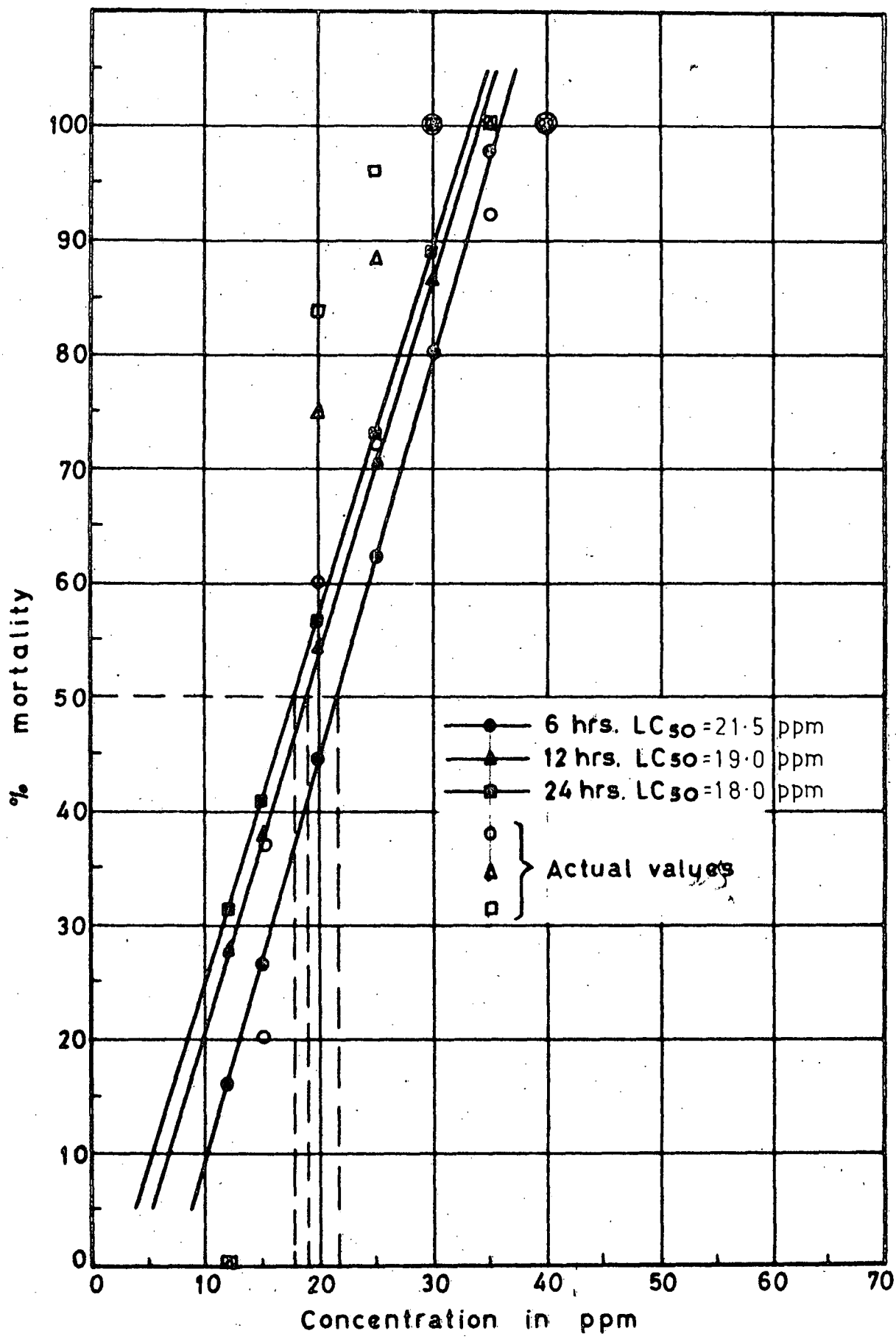


FIG. 4. -GRAPH FOR DERIVATION OF 6 hrs, 12 hrs AND 24 hrs LC_{50} VALUES OF Z. ARMATUM FRUIT ALCOHOLIC EXTRACT FOR H. FOSSILIS (13-20 g. wt. group) USING THE REGRESSION EQUATION $y = a + bX$ (Actual values are also plotted).

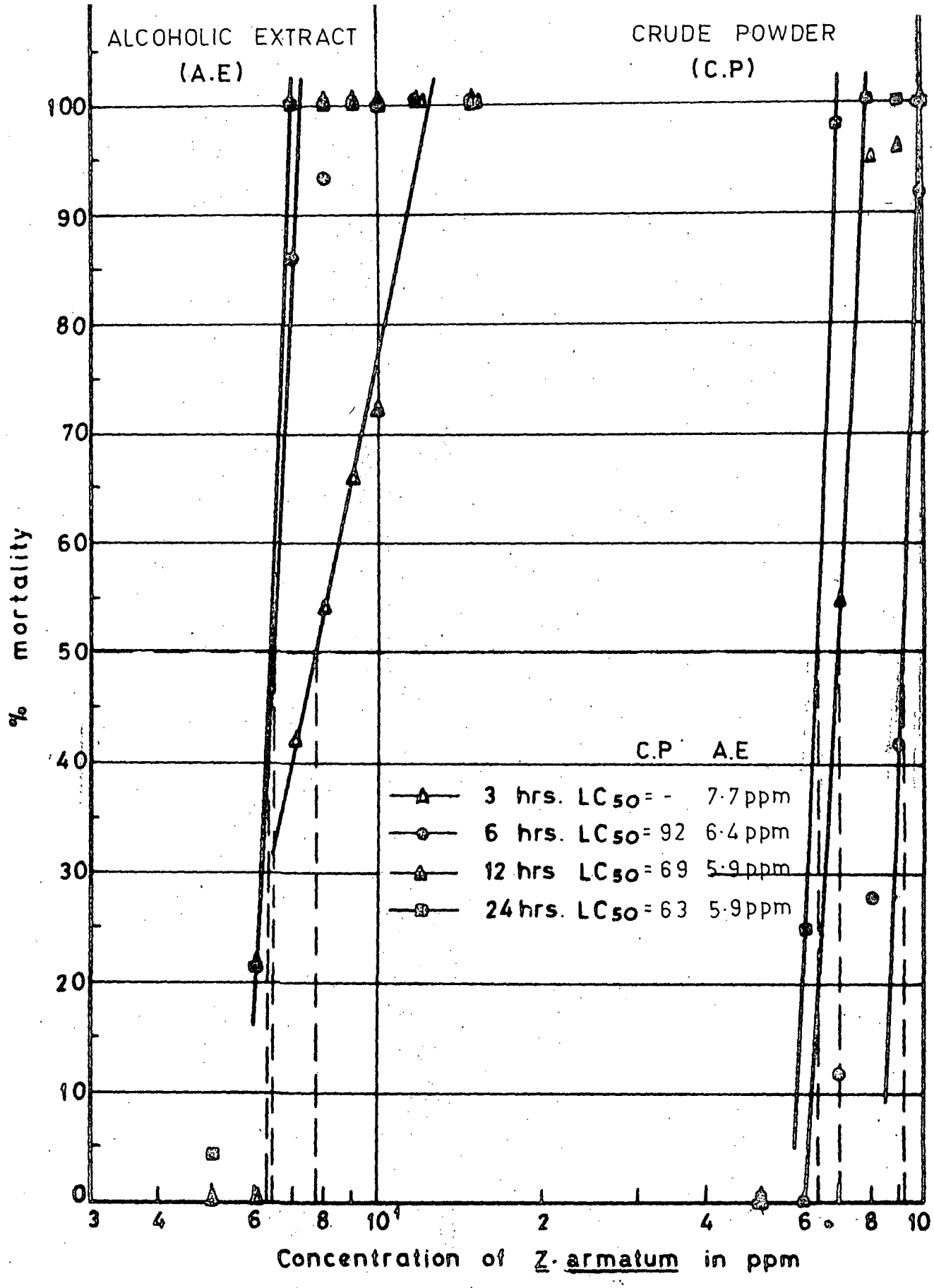


FIG. 5. -SEMILOG GRAPH FOR DERIVATION OF 6 hrs, 12 hrs AND 24 hrs LC_{50} VALUES OF Z. ARMATUM FRUIT CRUDE POWDER AND 3 hrs, 6 hrs, 12 hrs AND 24 hrs LC_{50} VALUES Z. ARMATUM FRUIT ALCOHOLIC EXTRACT FOR P. SHALYNIUS FOLLOWING DUDOROFF et al. (1951) METHOD

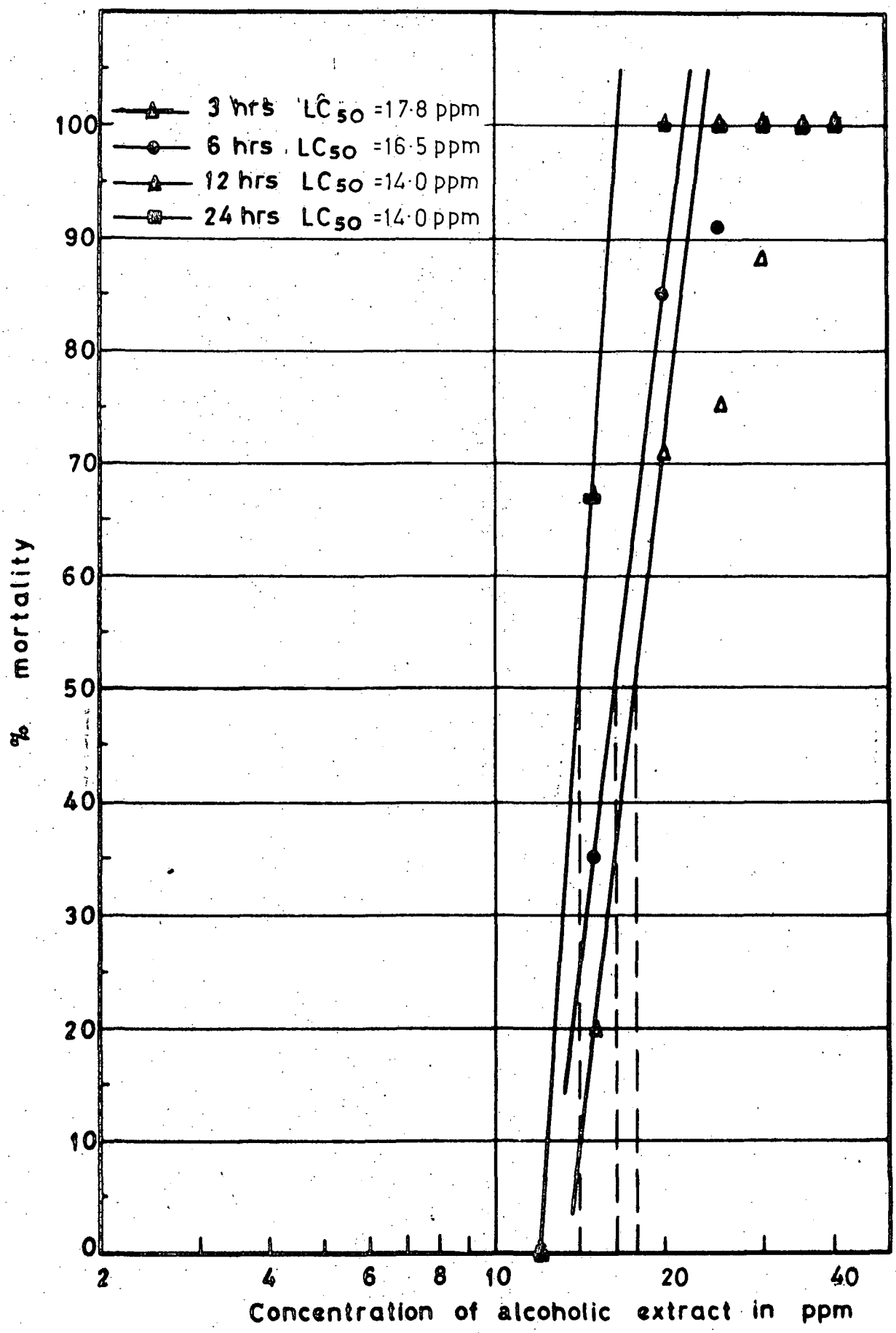


FIG. 6. -SEMILOG GRAPH FOR DERIVATION OF 3 hrs, 6hrs, 12 hrs AND 24 hrs LC_{50} VALUES OF Z. ARMATUM FRUIT ALCOHOLIC EXTRACT FOR H. FOSSILIS (8-12 g. wt. group) FOLLOWING DUDOROFF et al.(1951) METHOD

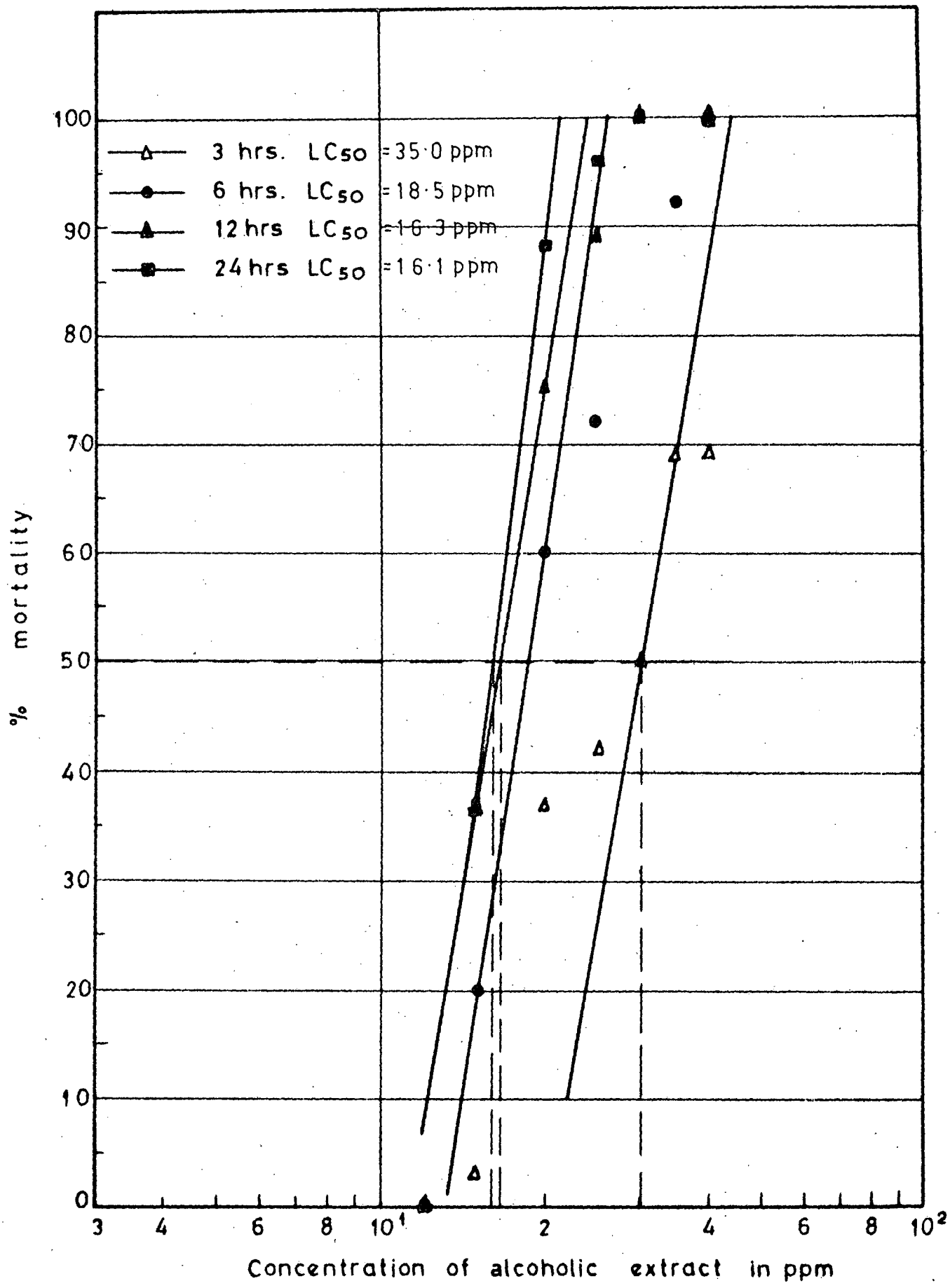


FIG. 7. -SEMILOG GRAPH FOR DERIVATION OF 3hrs, 6hrs, 12hrs AND 24hrs LC_{50} VALUES OF Z. ARMATUM FRUIT ALCOHOLIC EXTRACT FOR H. FOSSILIS (13 - 20 g. wt. group) FOLLOWING DUDOROFF et al. (1951) METHOD .

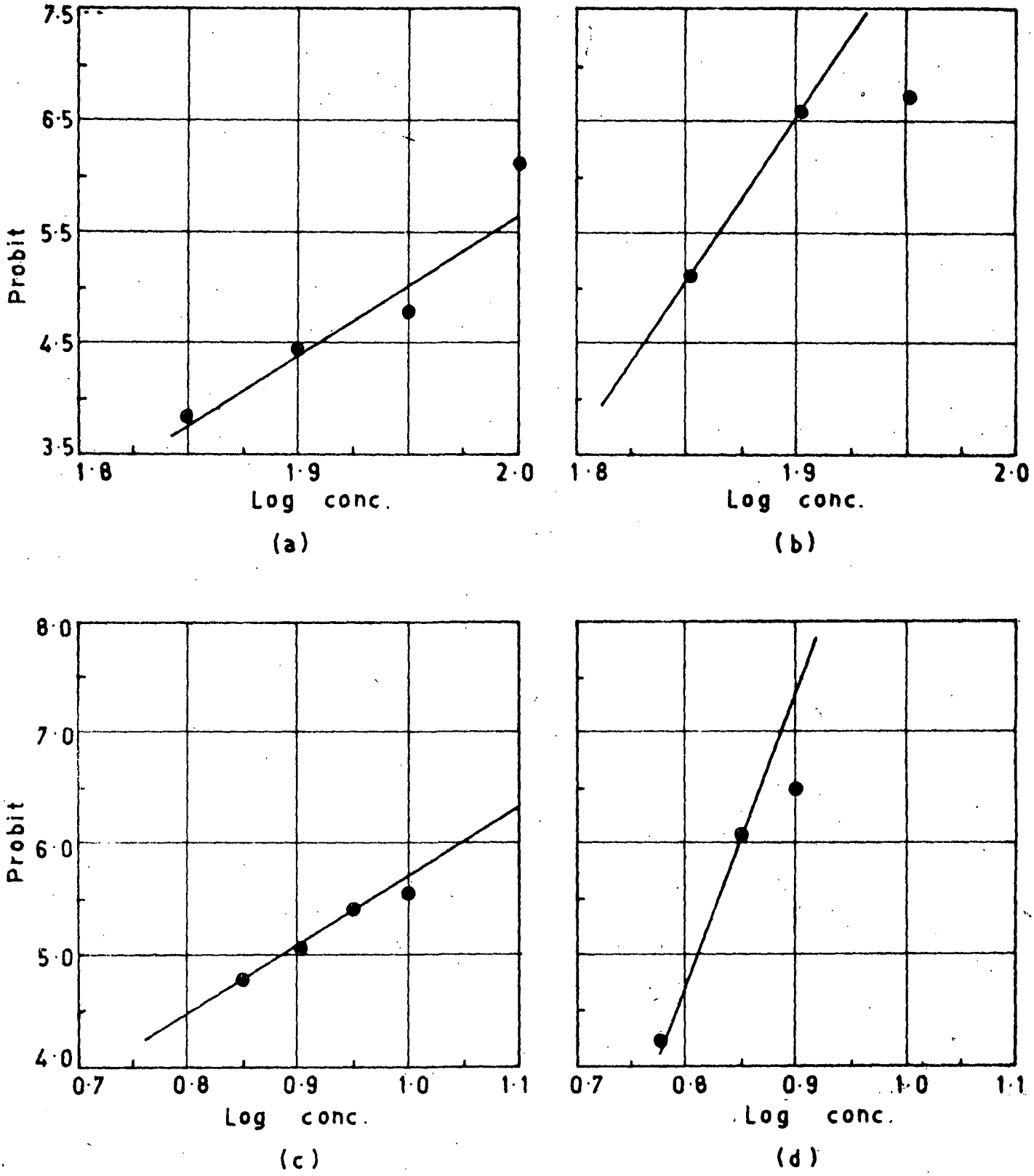


FIG. 8 - GRAPH FOR DERIVATION OF (a) 6 hrs AND (b) 12 hrs LC_{50} OF Z. ARMATUM FRUIT CRUDE POWDER AND (c) 3 hrs AND (d) 6 hrs LC_{50} OF Z. ARMATUM FRUIT ALCOHOLIC EXTRACT FOR P. SHALYNIUS FOLLOWING PROBIT ANALYSIS METHOD

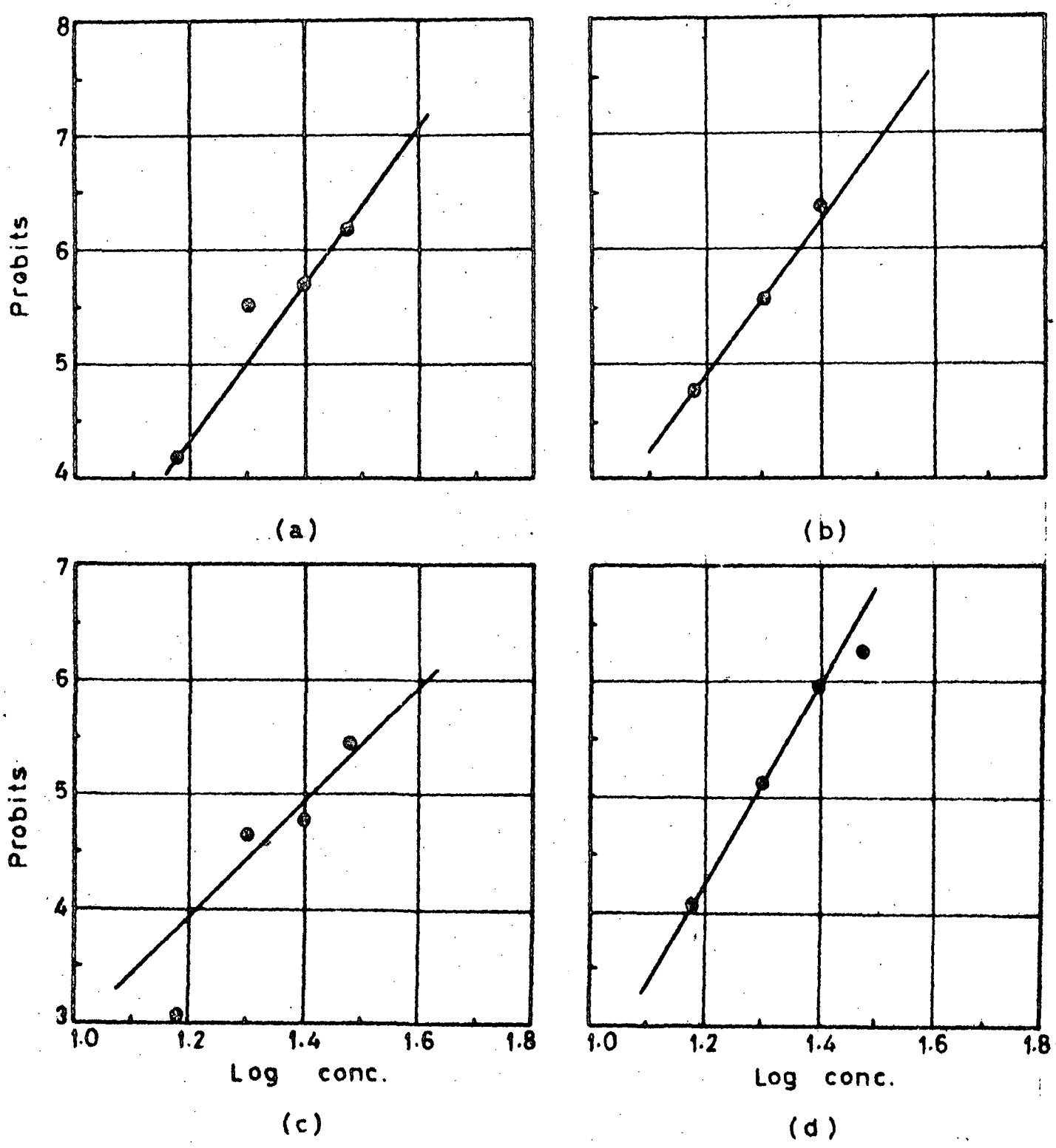


FIG. 9. -GRAPH FOR DERIVATION OF (a) 3 hrs AND (b) 6 hrs LC_{50} OF Z. ARMATUM FRUIT ALCOHOLIC EXTRACT FOR H. FOSSILIS (8-12 g. wt. group) AND (c) 3 hrs AND (d) 6 hrs LC_{50} OF Z. ARMATUM FRUIT ALCOHOLIC EXTRACT FOR H. FOSSILIS (13-20 g. wt. group) FOLLOWING PROBIT ANALYSIS METHOD

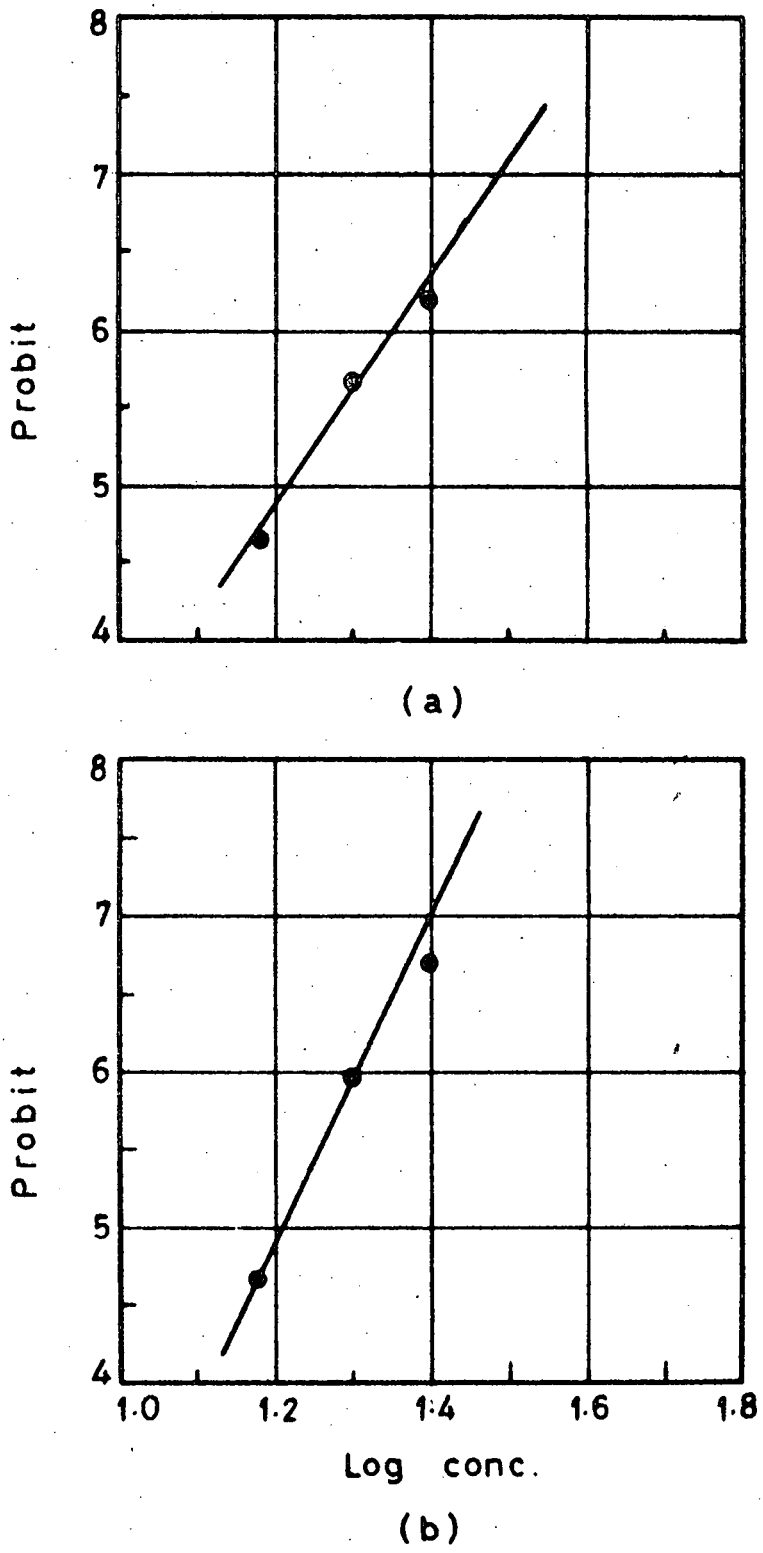


FIG. 10. -GRAPH FOR DERIVATION OF (a) 12 hrs AND (b) 24 hrs LC_{50} OF Z·ARMATUM FRUIT ALCOHOLIC EXTRACT FOR H·FOSSILIS (13-20 g. wt. group) FOLLOWING PROBIT ANALYSIS METHOD

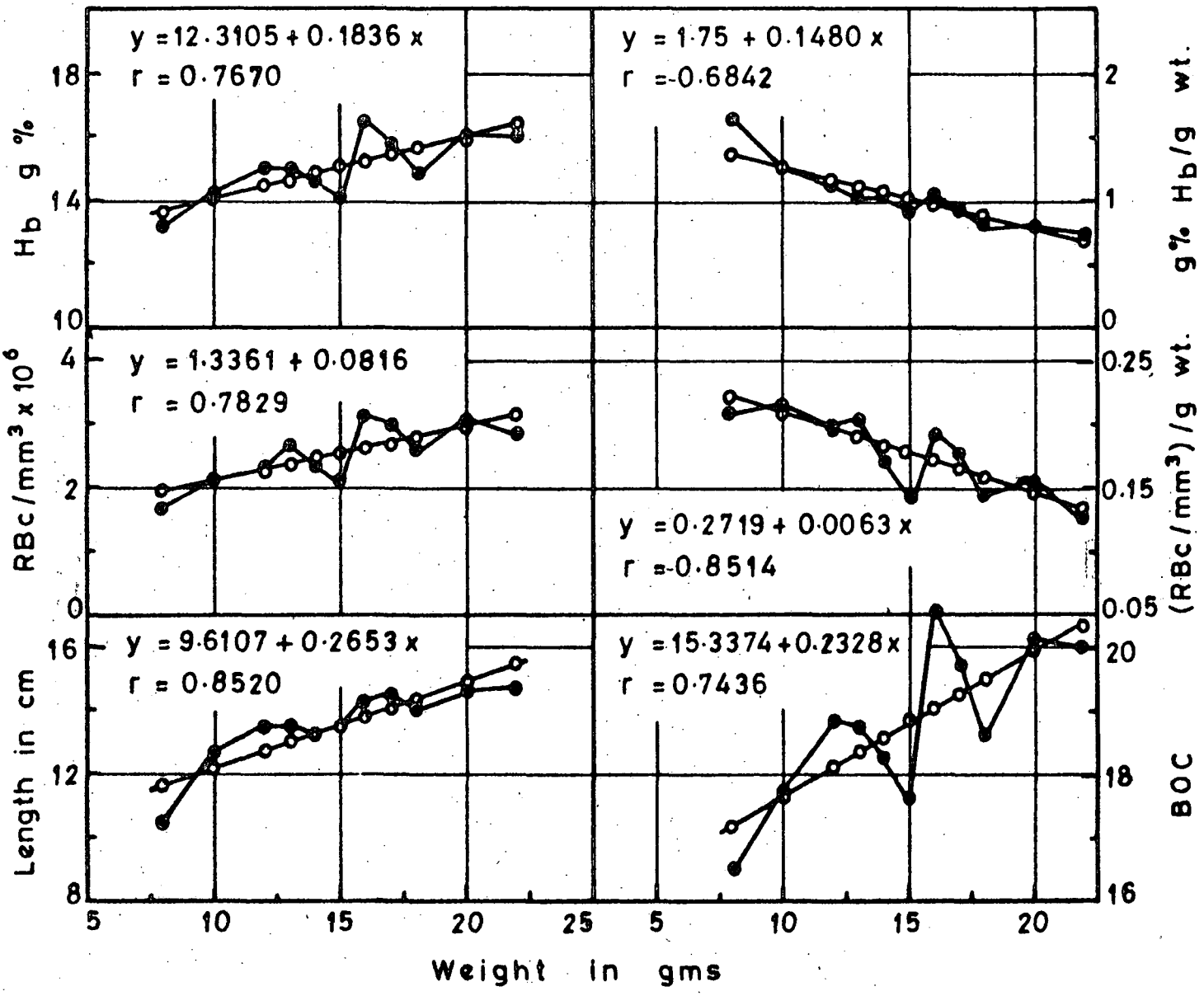


FIG.11. -THE HAEMATOLOGICAL PARAMETERS OF H. FOSSILIS IN RELATION TO ITS BODY WEIGHT (—o— Observed values —o— Calculated values using the regression equation y = a + bX).

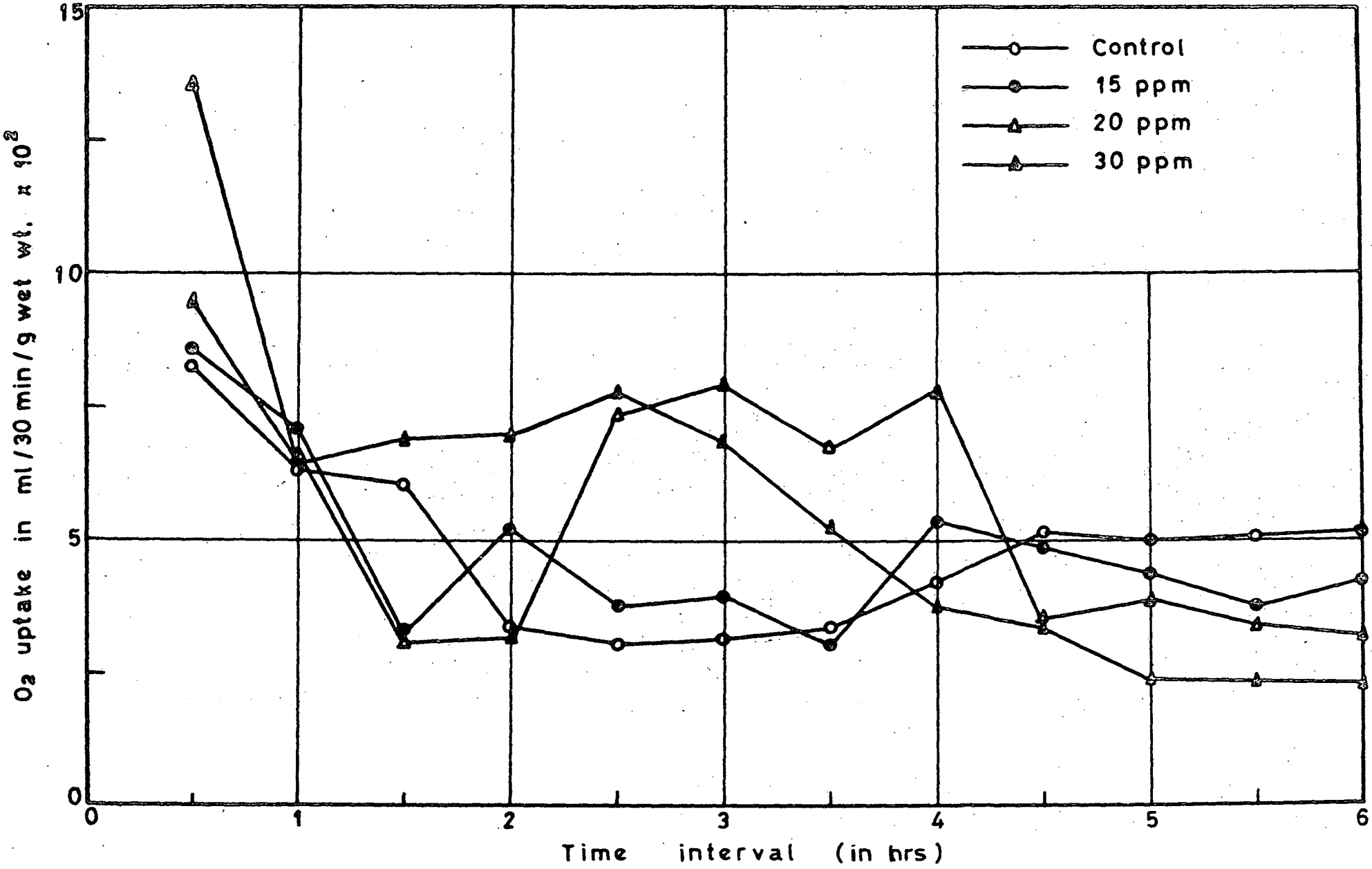


FIG.12. -THE PATTERN OF OXYGEN UPTAKE BY TILAPIA MOSSAMBICA TREATED WITH DIFFERENT CONCENTRATIONS OF ALCOHOLIC EXTRACT OF Z. ARMATUM FRUIT (Each point represent percentage change in oxygen uptake for 30 minutes).

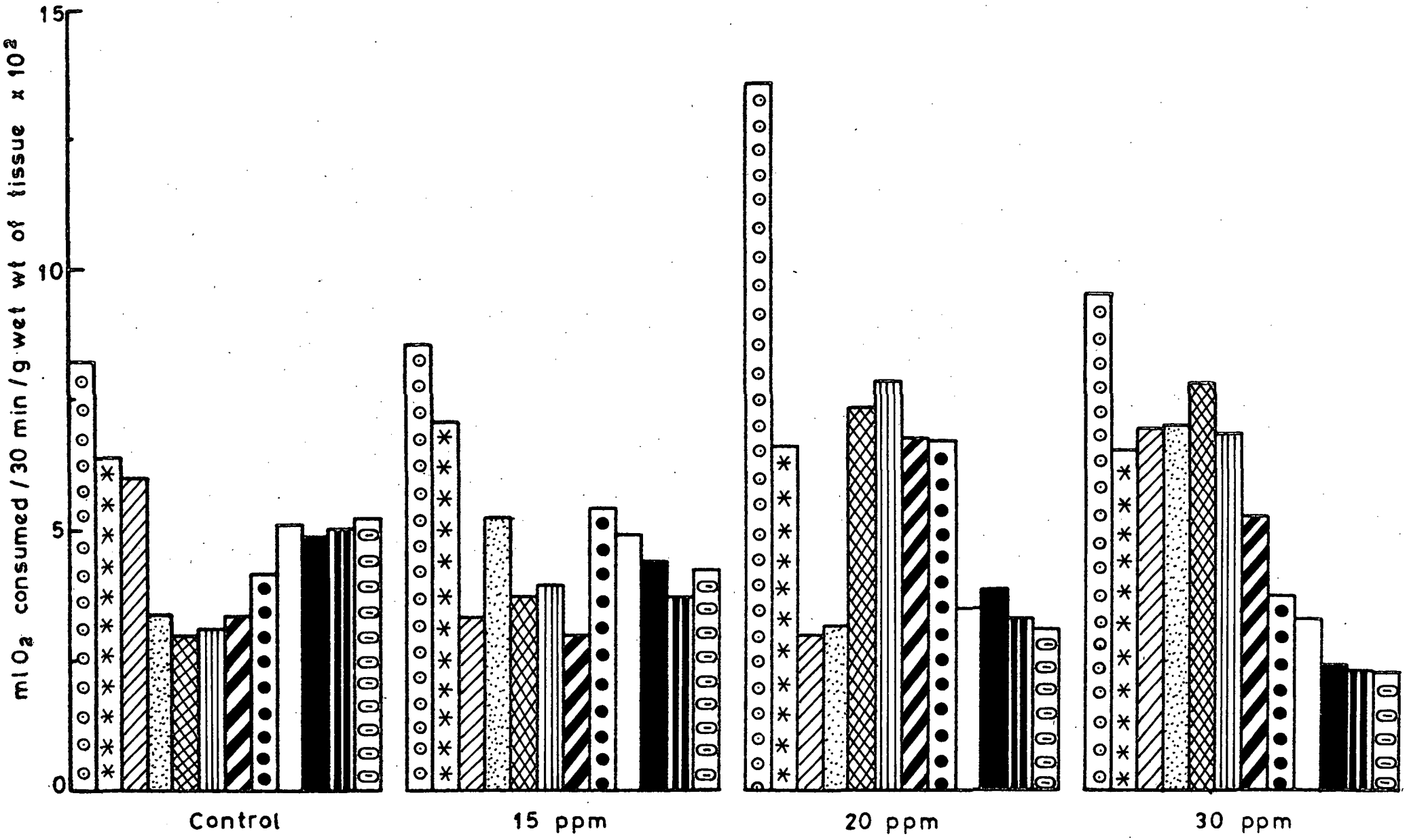


FIG.13. -THE PATTERN OF OXYGEN UPTAKE BY TILAPIA MOSSAMBICA TREATED WITH DIFFERENT CONCENTRATIONS OF ALCOHOLIC EXTRACT OF Z. ARMATUM FRUIT (Each point represent percentage change in oxygen uptake for 30 minutes).

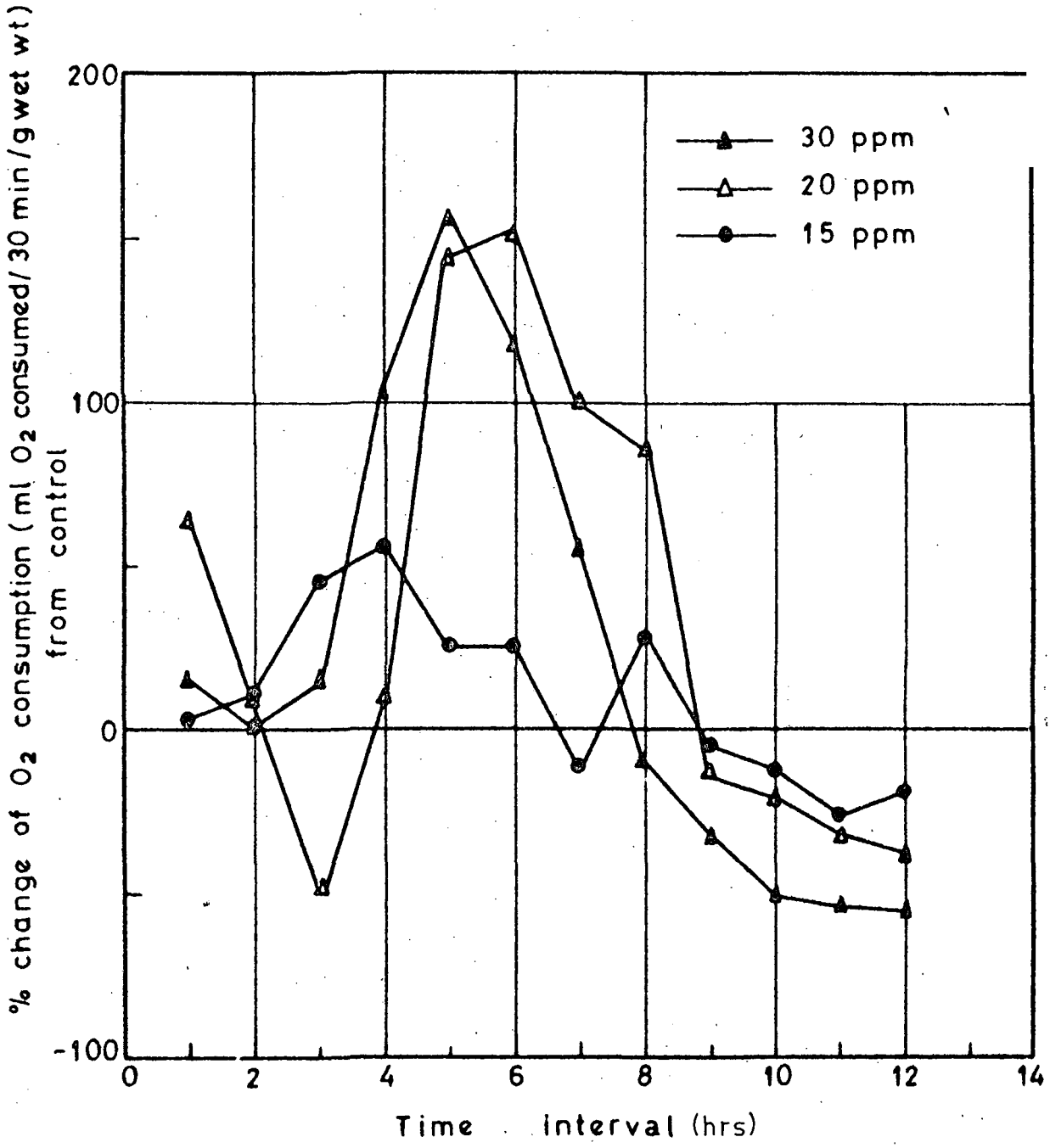


FIG. 14. -THE PERCENTAGE CHANGE IN OXYGEN UPTAKE OF TILAPIA MOSSAMBICA TREATED WITH DIFFERENT CONCENTRATIONS OF ALCOHOLIC EXTRACT OF Z. ARMATUM FRUIT (Each point represent change in oxygen uptake for 30 minutes).

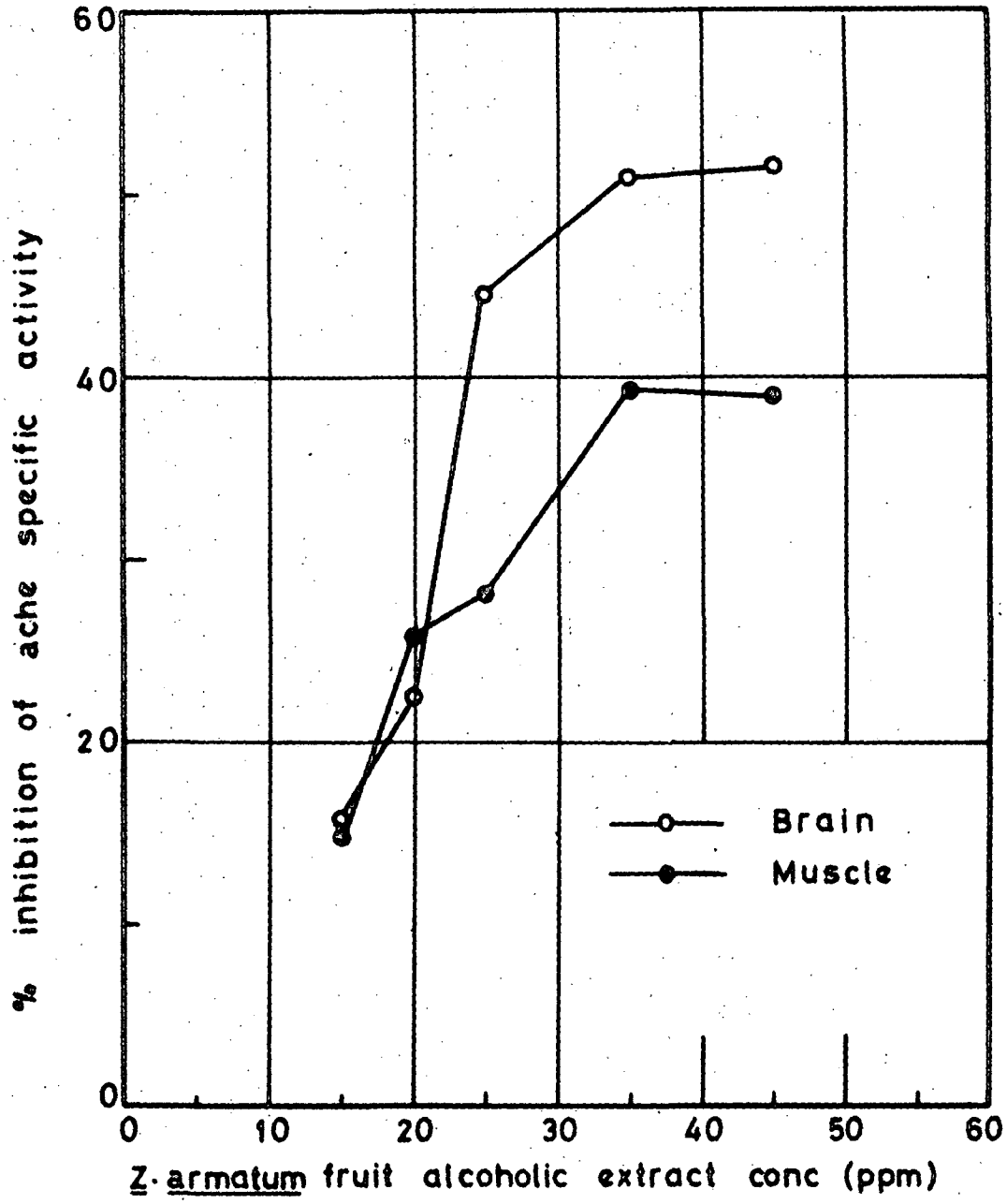


FIG.15. -PERCENT INHIBITION OF ACHE SPECIFIC ACTIVITY (units/mg protein) IN BRAIN AND MUSCLE OF H. FOSSILIS TREATED WITH DIFFERENT CONCENTRATIONS OF Z. ARMATUM FRUIT EXTRACT

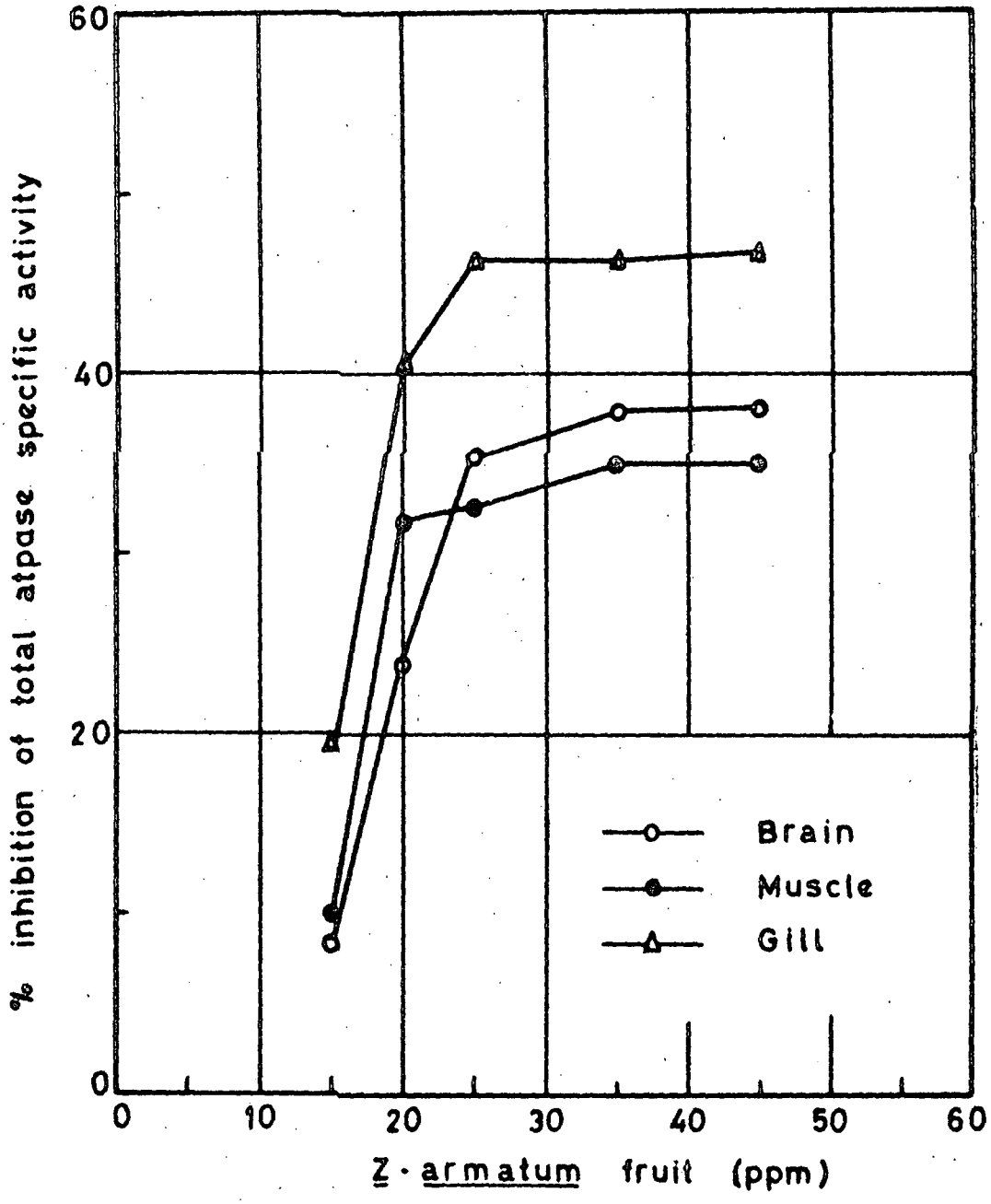


FIG.16. -PERCENT INHIBITION OF TOTAL ATPASE SPECIFIC ACTIVITY (units/mg protein) IN BRAIN, MUSCLE AND GILL OF H·FOSSILIS TREATED WITH DIFFERENT CONCENTRATIONS OF Z·ARMATUM FRUIT ALCOHOLIC EXTRACT

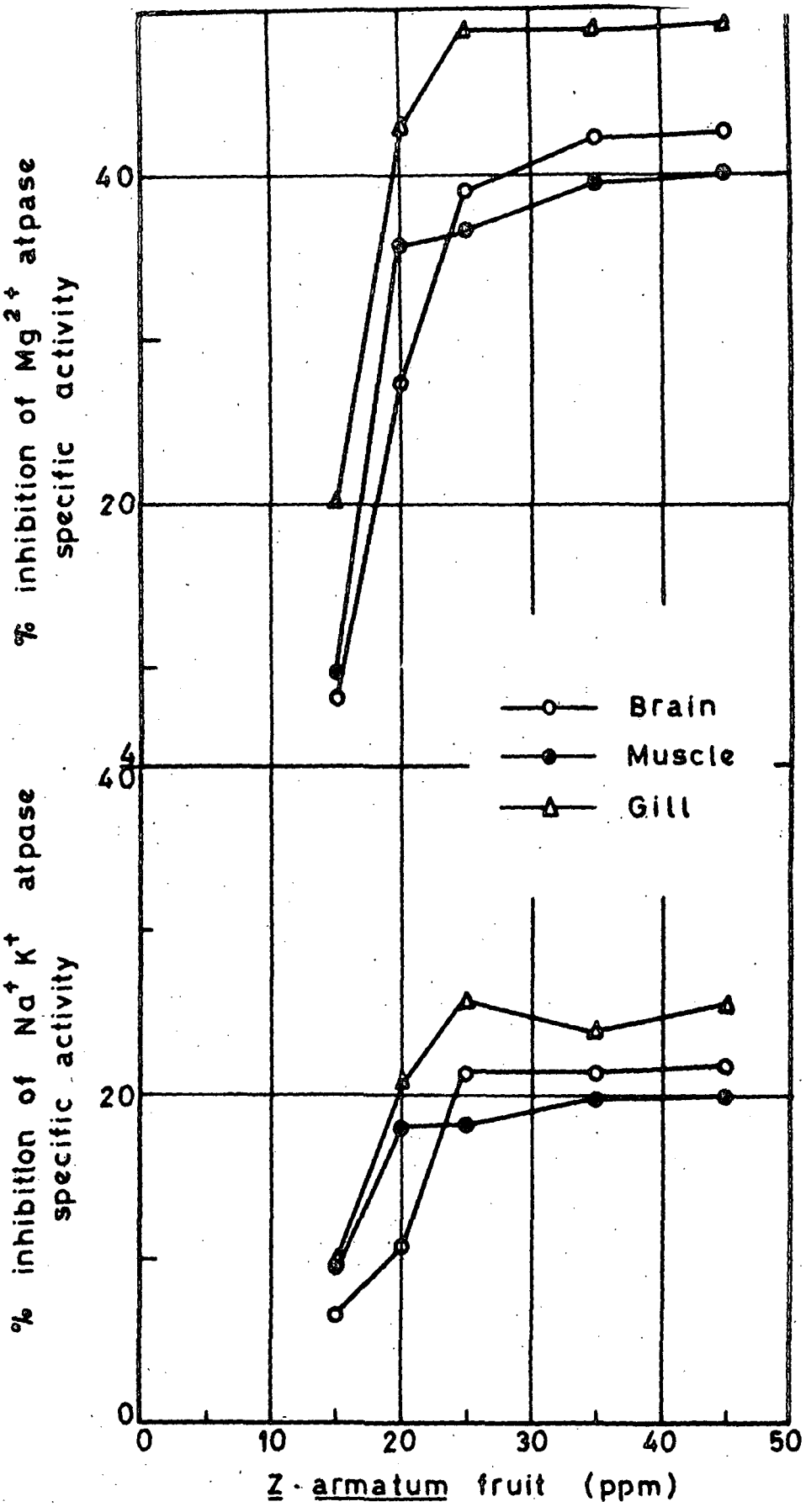


FIG. 17. -PERCENT INHIBITION OF Mg^{2+} ATPASE SPECIFIC ACTIVITY AND Na^{+}, K^{+} ATPASE SPECIFIC ACTIVITY (units/mg protein) IN BRAIN, MUSCLE AND GILL OF H. FOSSILIS TREATED WITH DIFFERENT CONCENTRATIONS OF Z. ARMATUM FRUIT ALCOHOLIC EXTRACT

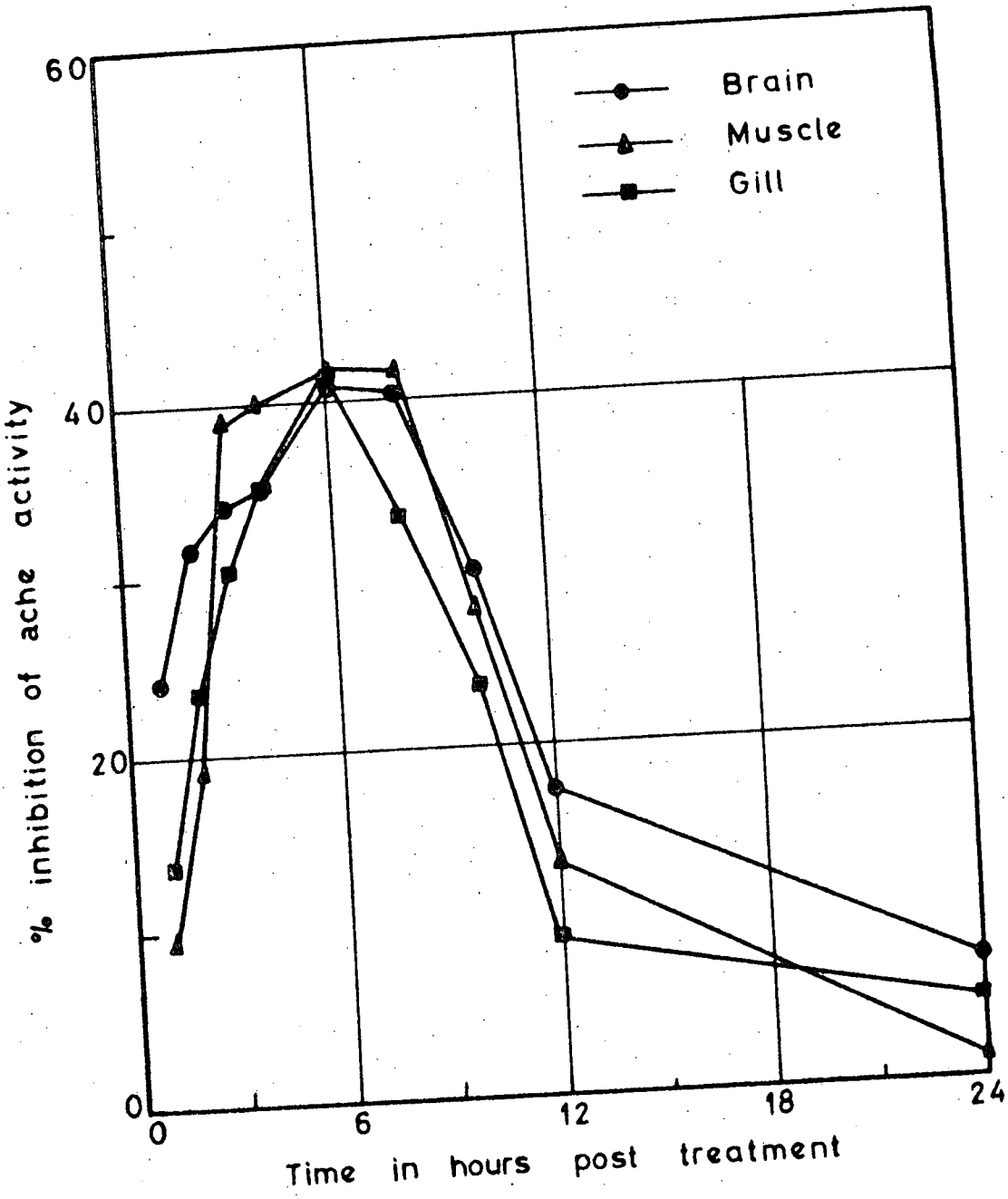


FIG. 18. -PERCENT INHIBITION OF ACHE SPECIFIC ACTIVITY (units/mg protein) IN BRAIN, MUSCLE AND GILL OF H. FOSSILIS TREATED WITH 20 ppm (LC_{50}) Z. ARMATUM FRUIT EXTRACT AT DIFFERENT TIME INTERVALS TILL 24 HOURS

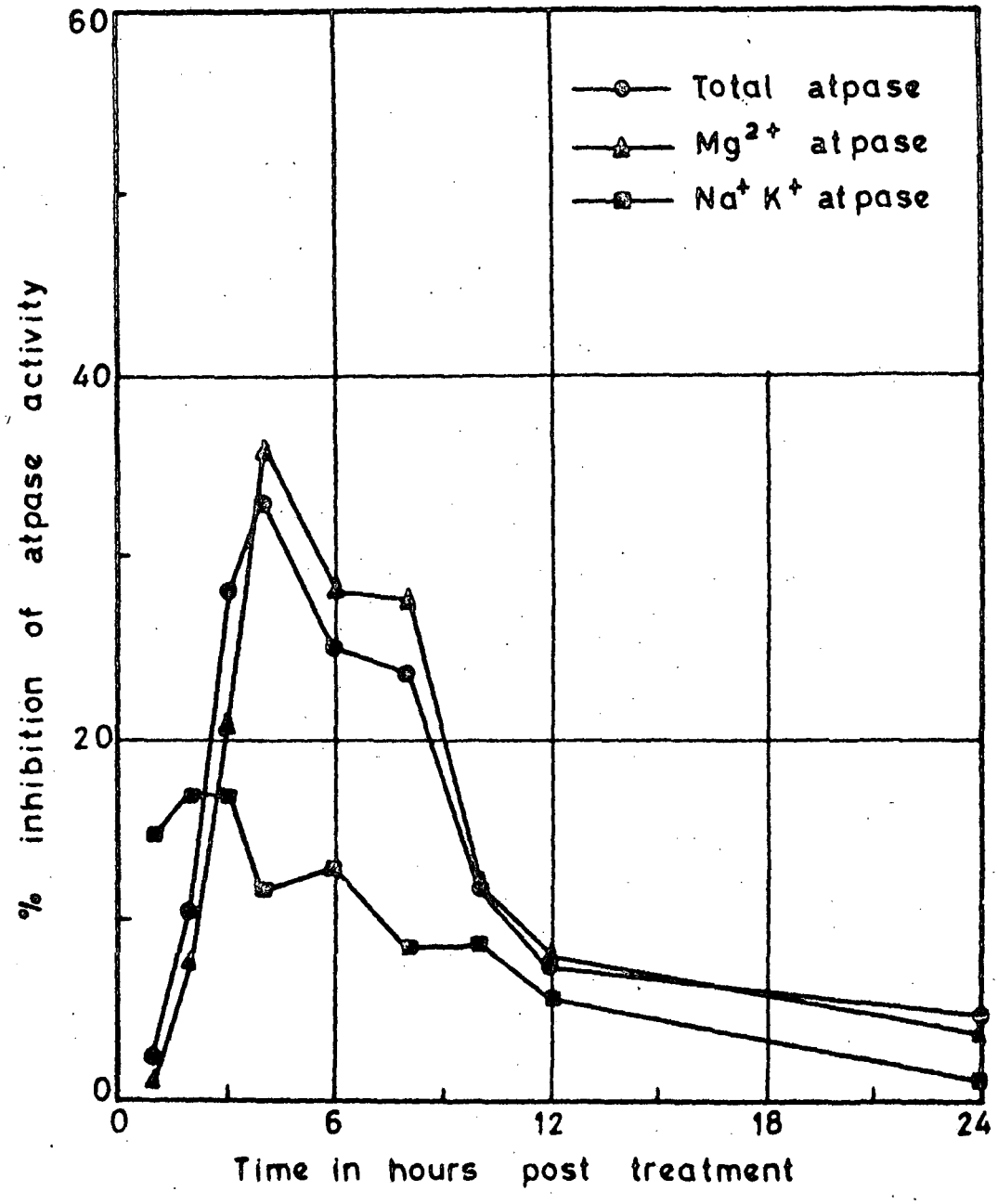


FIG.19. -PERCENT INHIBITION OF (a) TOTAL ATPASE (b) Mg^{2+} ATPASE AND (c) Na^{+} K^{+} ATPASE SPECIFIC ACTIVITIES (units/mg protein) IN BRAIN OF H·FOSSILIS TREATED WITH 20 ppm (LC_{50}) Z·ARMATUM FRUIT EXTRACT AT DIFFERENT TIME INTERVALS TILL 24 HOURS

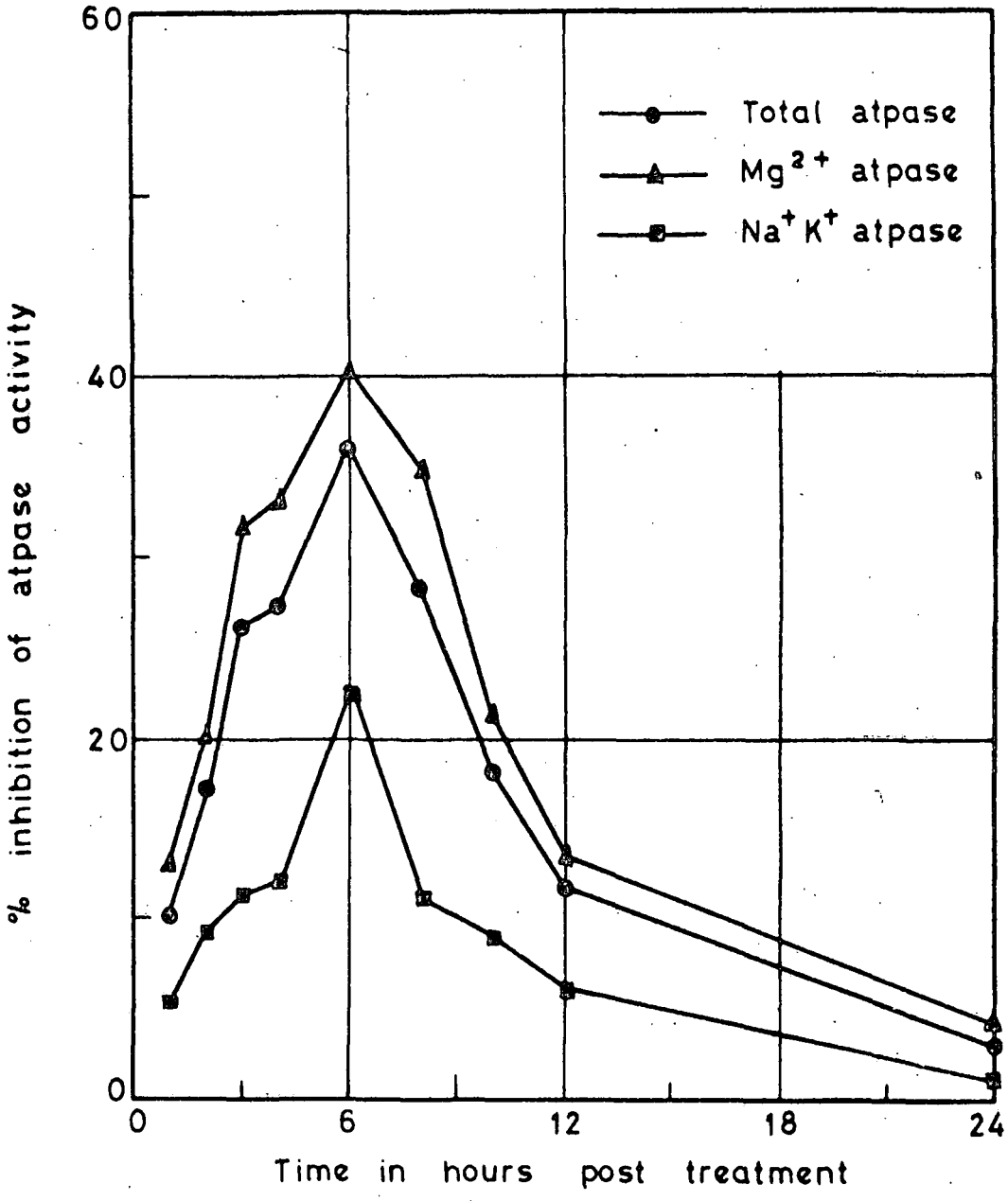


FIG. 20. -PERCENT INHIBITION OF (a) TOTAL ATPASE (b) Mg^{2+} ATPASE AND (c) Na^{+} K^{+} ATPASE SPECIFIC ACTIVITIES (units/mg protein) IN MUSCLE OF H. FOSSILIS TREATED WITH 20 ppm (LC_{50}) Z. ARMATUM FRUIT EXTRACT AT DIFFERENT TIME INTERVALS TILL 24 HOURS

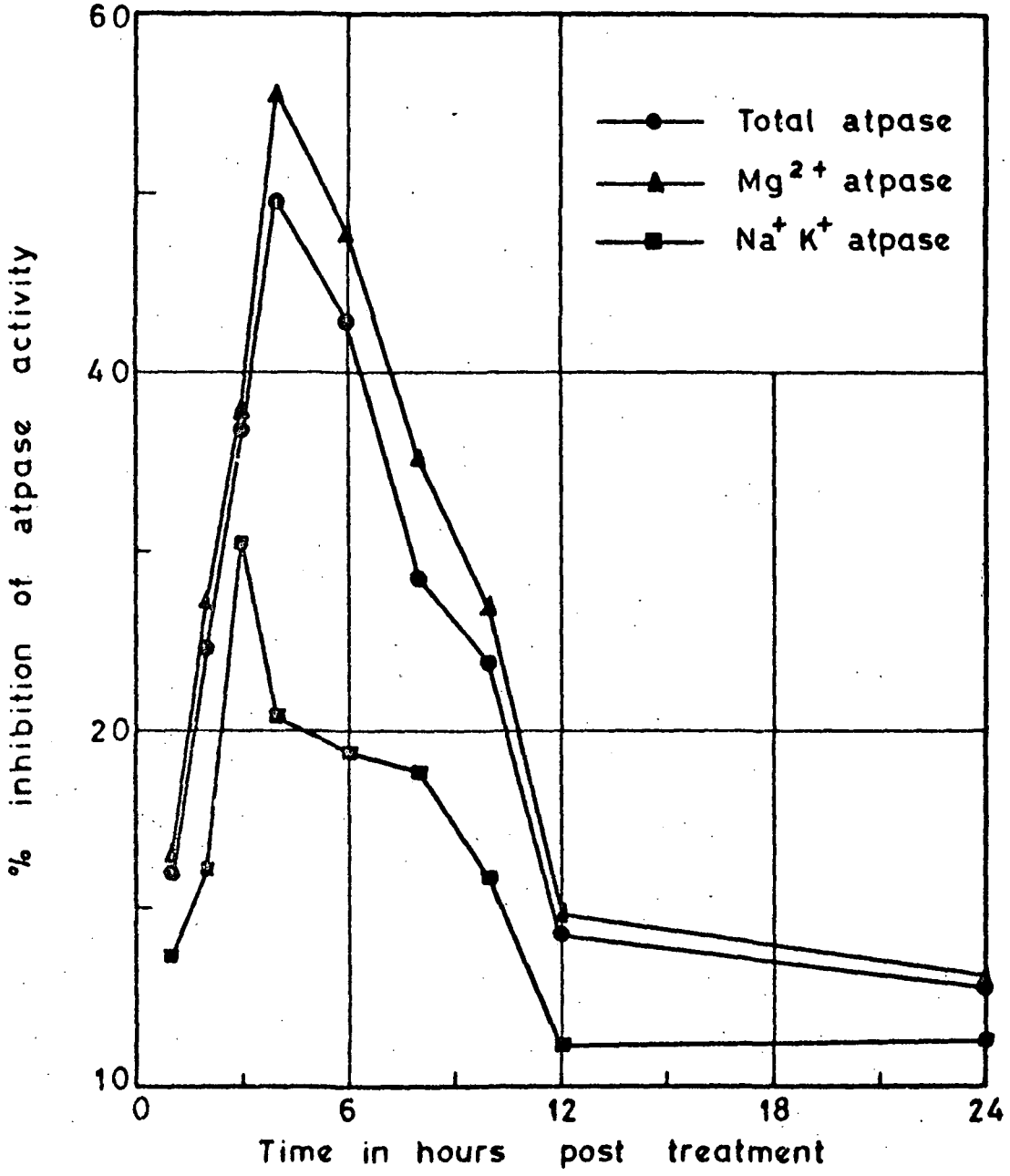


FIG. 21. -PERCENT INHIBITION OF (a) TOTAL ATPASE (b) Mg^{2+} ATPASE AND (c) Na^{+} K^{+} ATPASE SPECIFIC ACTIVITIES (units/mg protein) IN GILL OF H. FOSSILIS TREATED WITH 20 ppm (LC_{50}) Z. ARMATUM FRUIT EXTRACT AT DIFFERENT TIME INTERVALS TILL 24 HOURS.

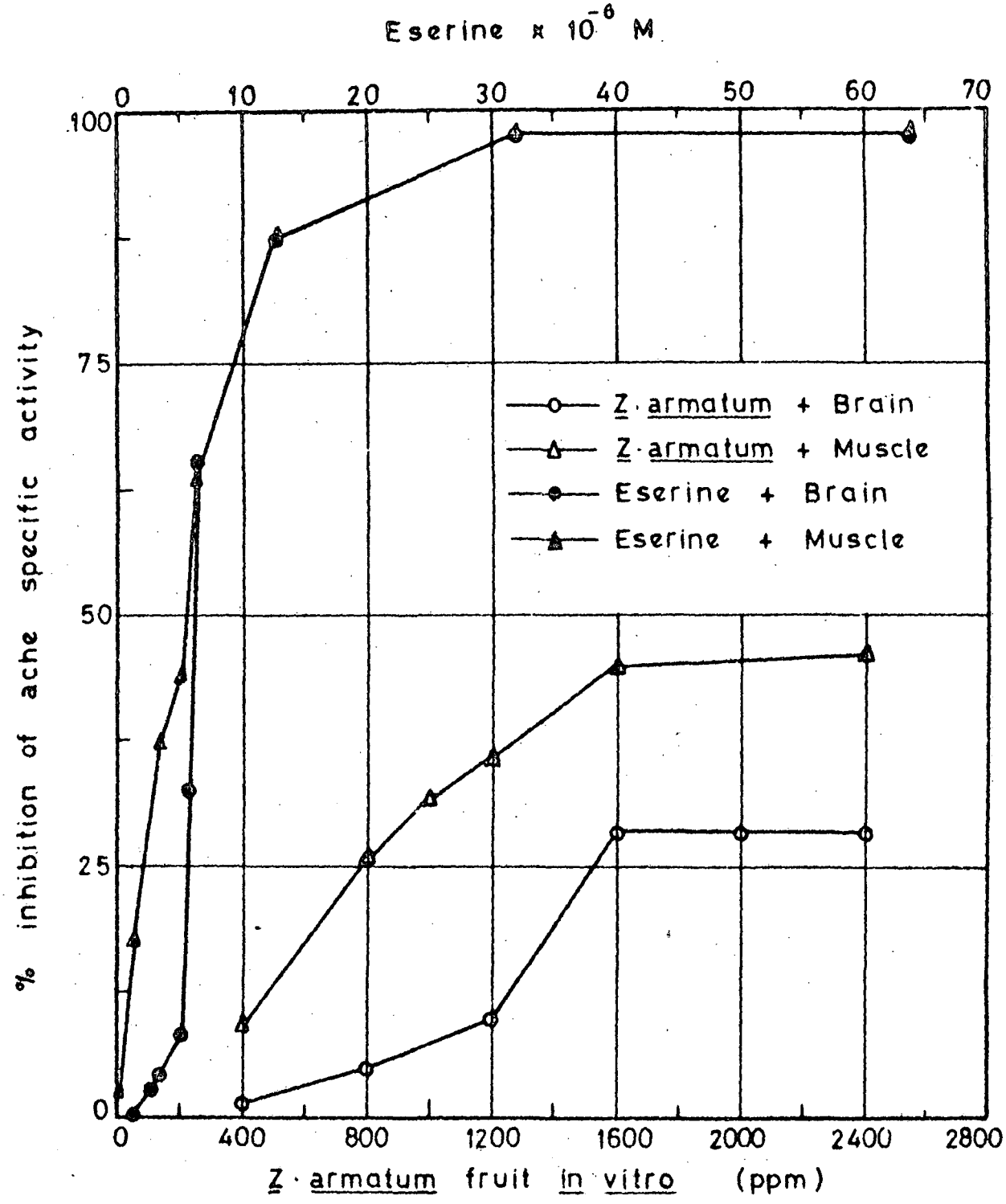


FIG.22. -EFFECT OF DIFFERENT CONCENTRATIONS OF ESERINE AND Z. ARMATUM FRUIT EXTRACT ON ACHE SPECIFIC ACTIVITY (units/mg protein) IN BRAIN, MUSCLE AND GILL OF H. FOSSILIS

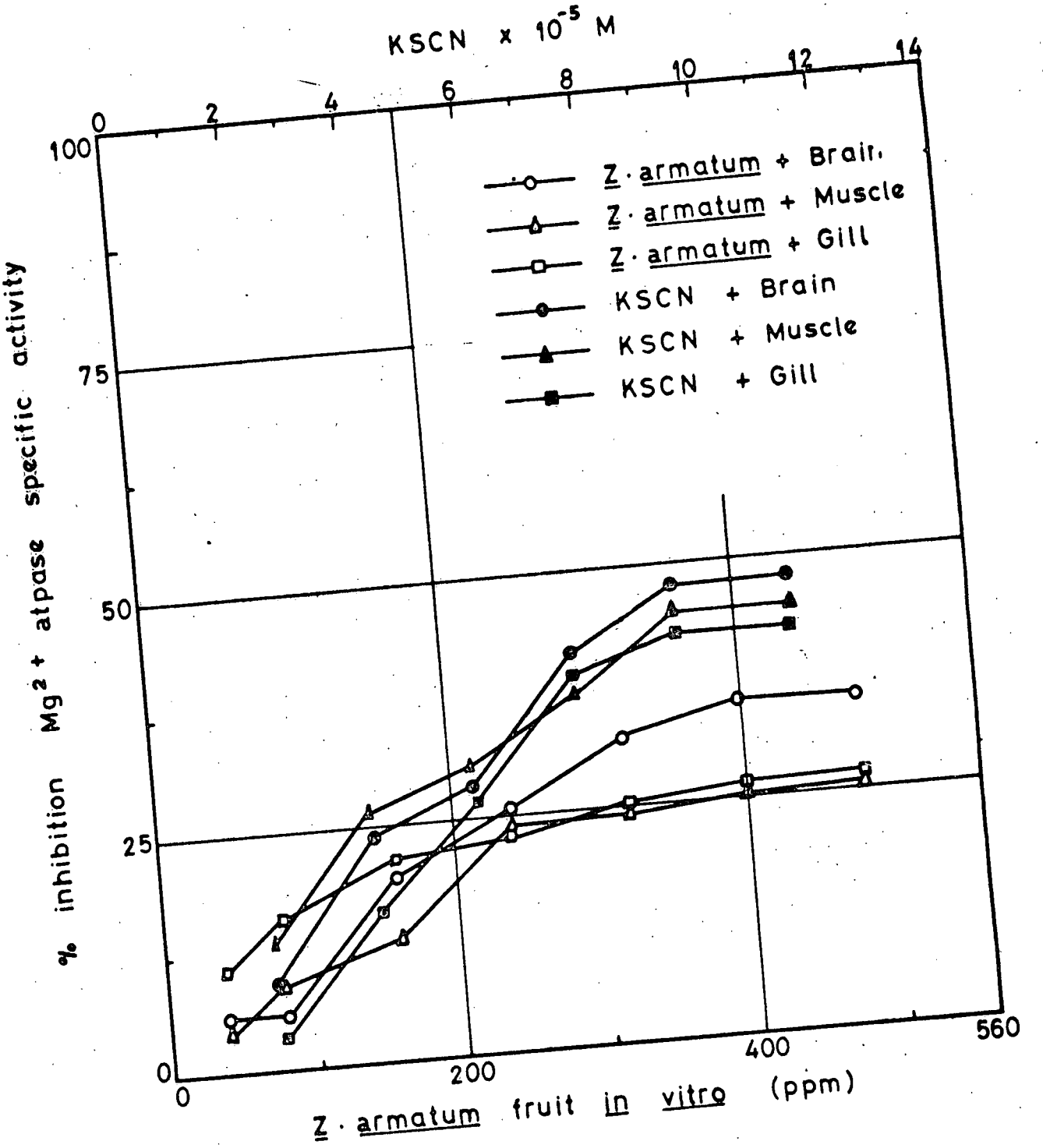


FIG. 23. -EFFECT OF DIFFERENT CONCENTRATIONS OF KSCN AND Z. ARMATUM FRUIT EXTRACT ON THE Mg^{2+} ATPASE SPECIFIC ACTIVITY (units/mg protein) IN BRAIN, MUSCLE AND GILL OF H. FOSSILIS

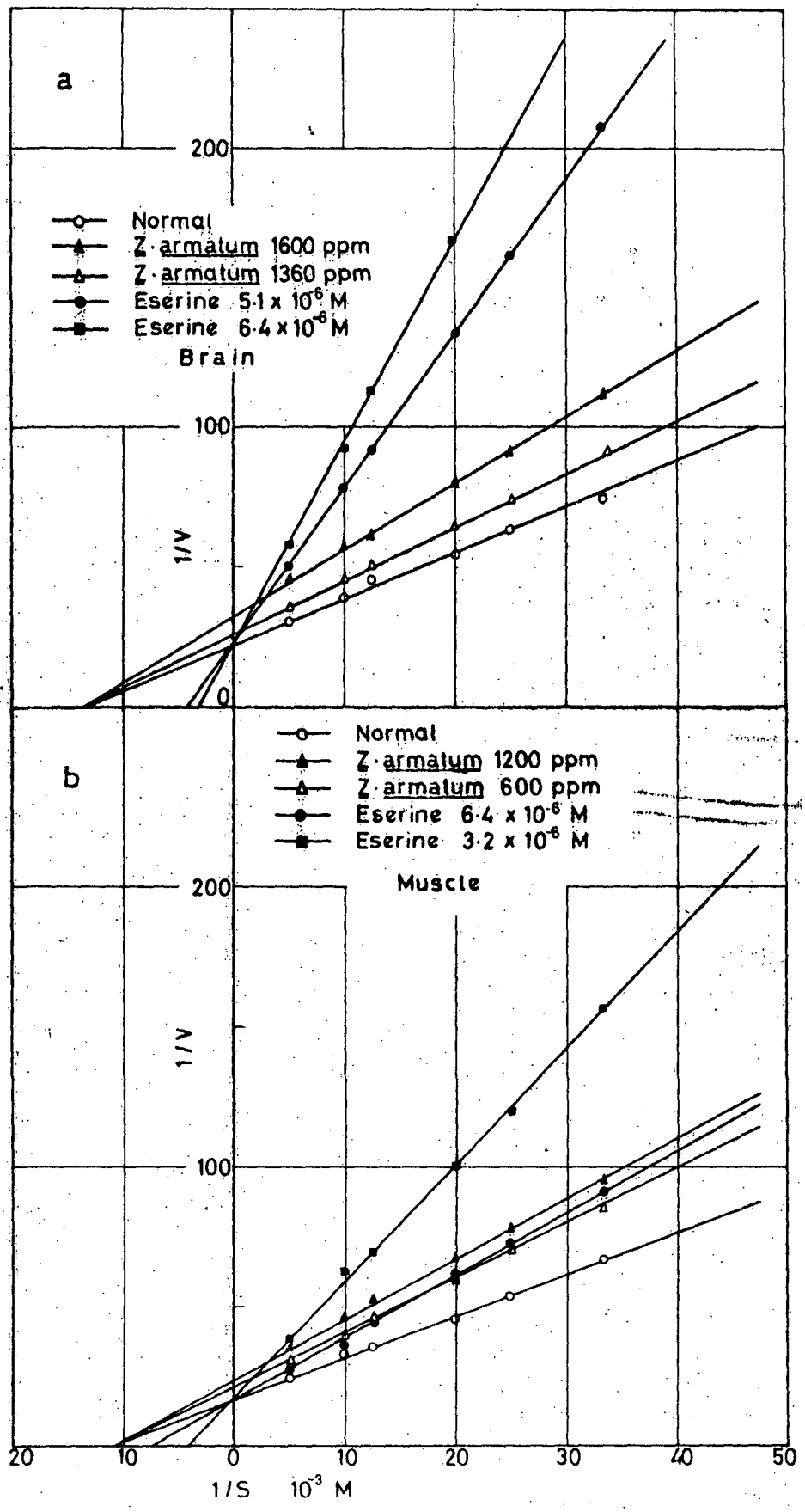


FIG. 24. -LINEWEAVER BURK PLOT TO DETERMINE THE APPARENT K_m FOR (a) BRAIN AND (b) MUSCLE ACHE AND NATURE OF INHIBITION BY Z·ARMATUM FRUIT EXTRACT AND ESERINE (a known inhibitor)

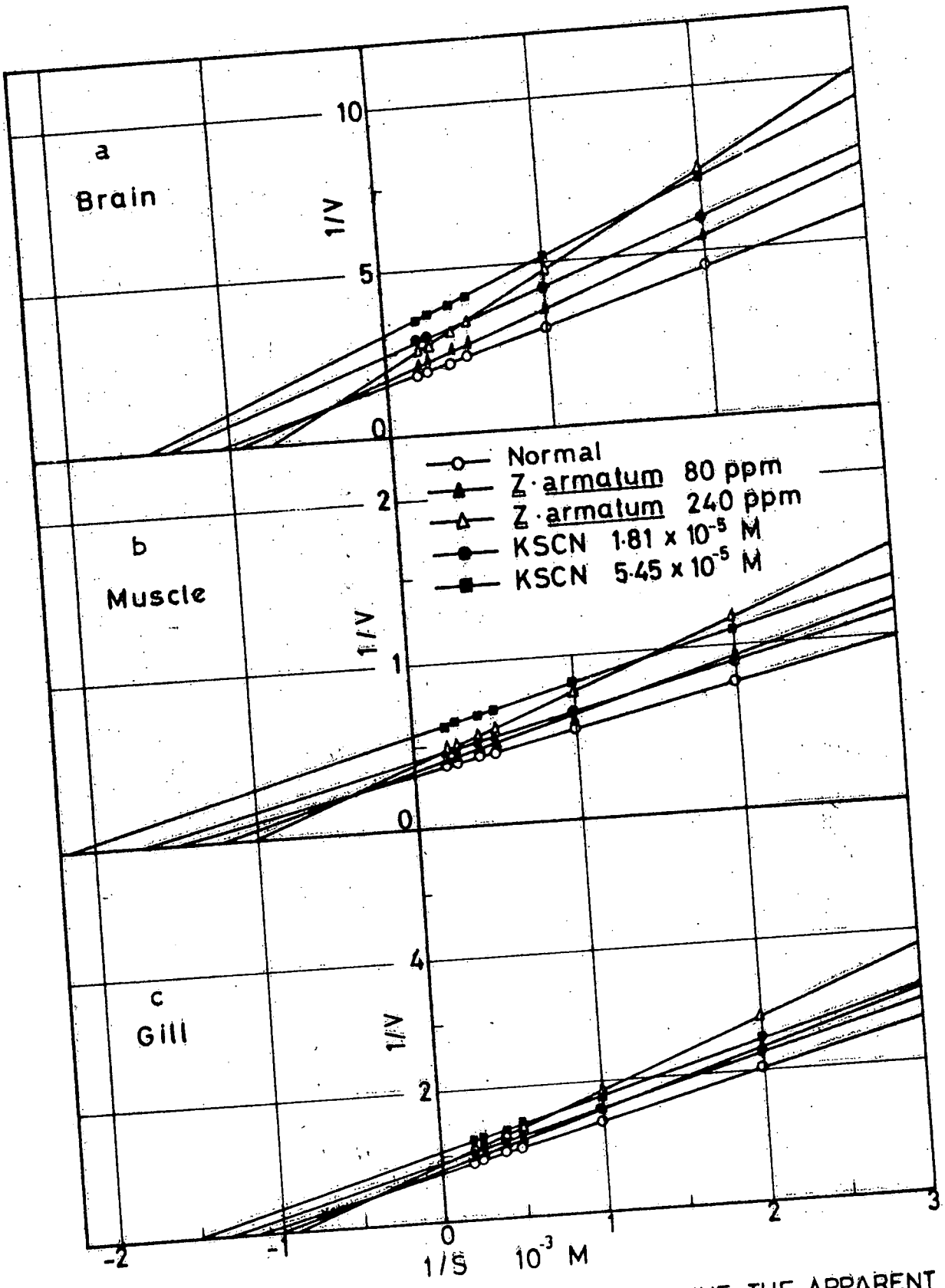


FIG. 25. -LINEWEAVER BURK PLOT TO DETERMINE THE APPARENT Km FOR (a) BRAIN (b) MUSCLE AND (c) GILL Mg^{2+} ATPASE FOR ATP AND NATURE OF INHIBITION BY Z·ARMATUM FRUIT EXTRACT AND KSCN (a known inhibitor)

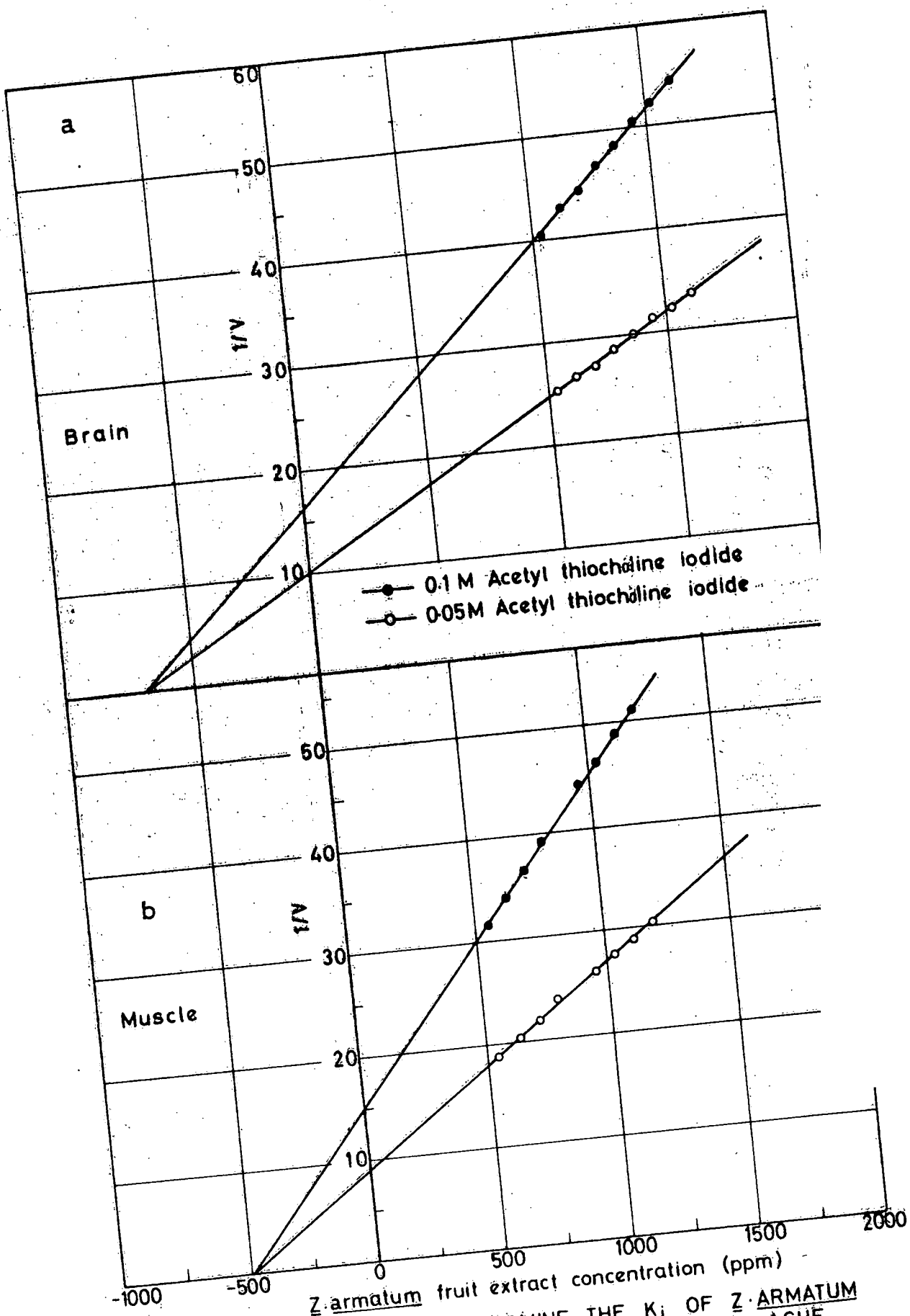


FIG. 26. -DIXON PLOT TO DETERMINE THE K_i OF Z. ARMATUM FRUIT EXTRACT FOR (a) BRAIN (b) MUSCLE ACHE

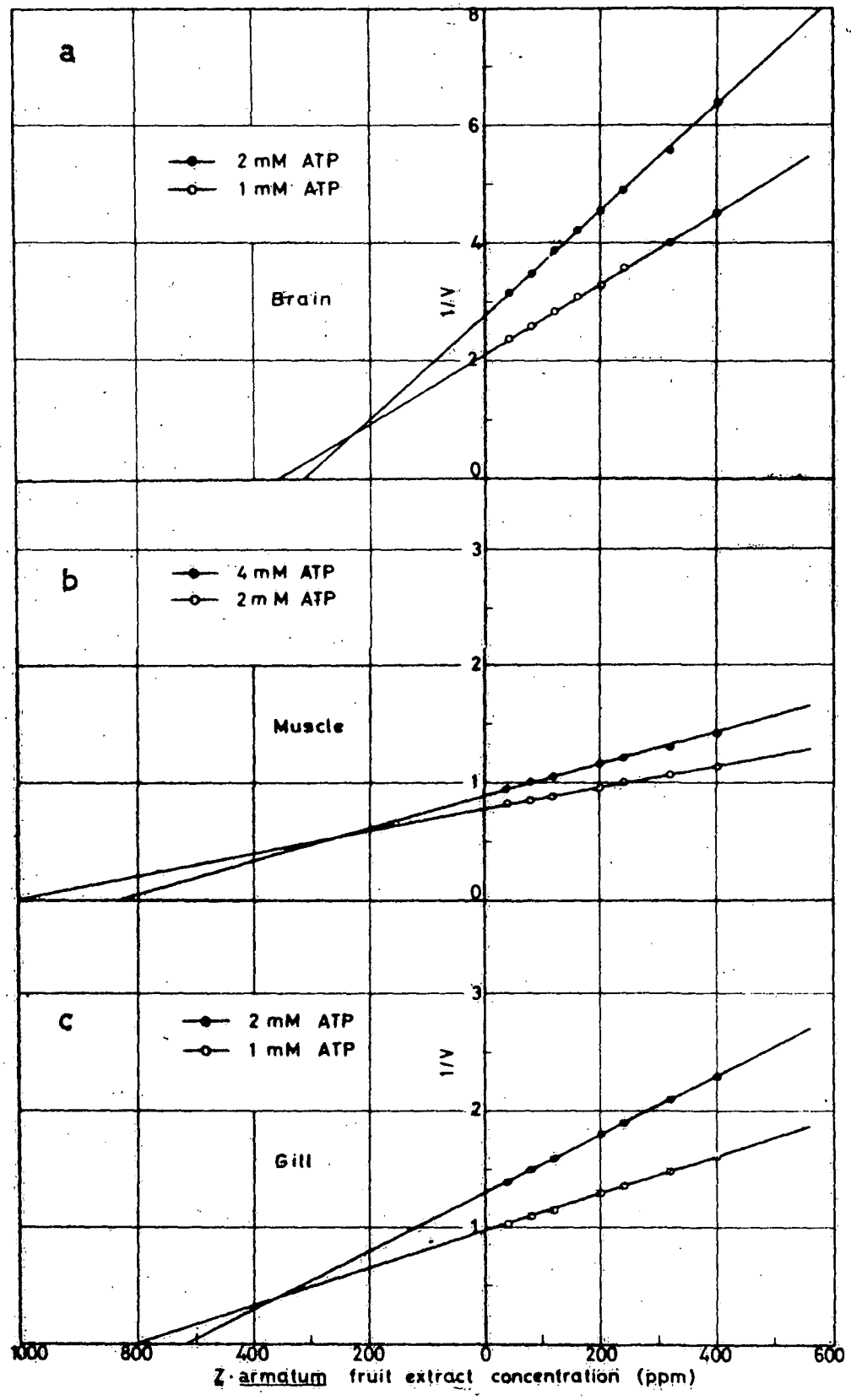


FIG. 27 -DIXON PLOT TO DETERMINE THE K_i OF Z. ARMATUM FRUIT EXTRACT FOR (a) BRAIN (b) MUSCLE AND (c) GILL Mg^{2+} ATPASE

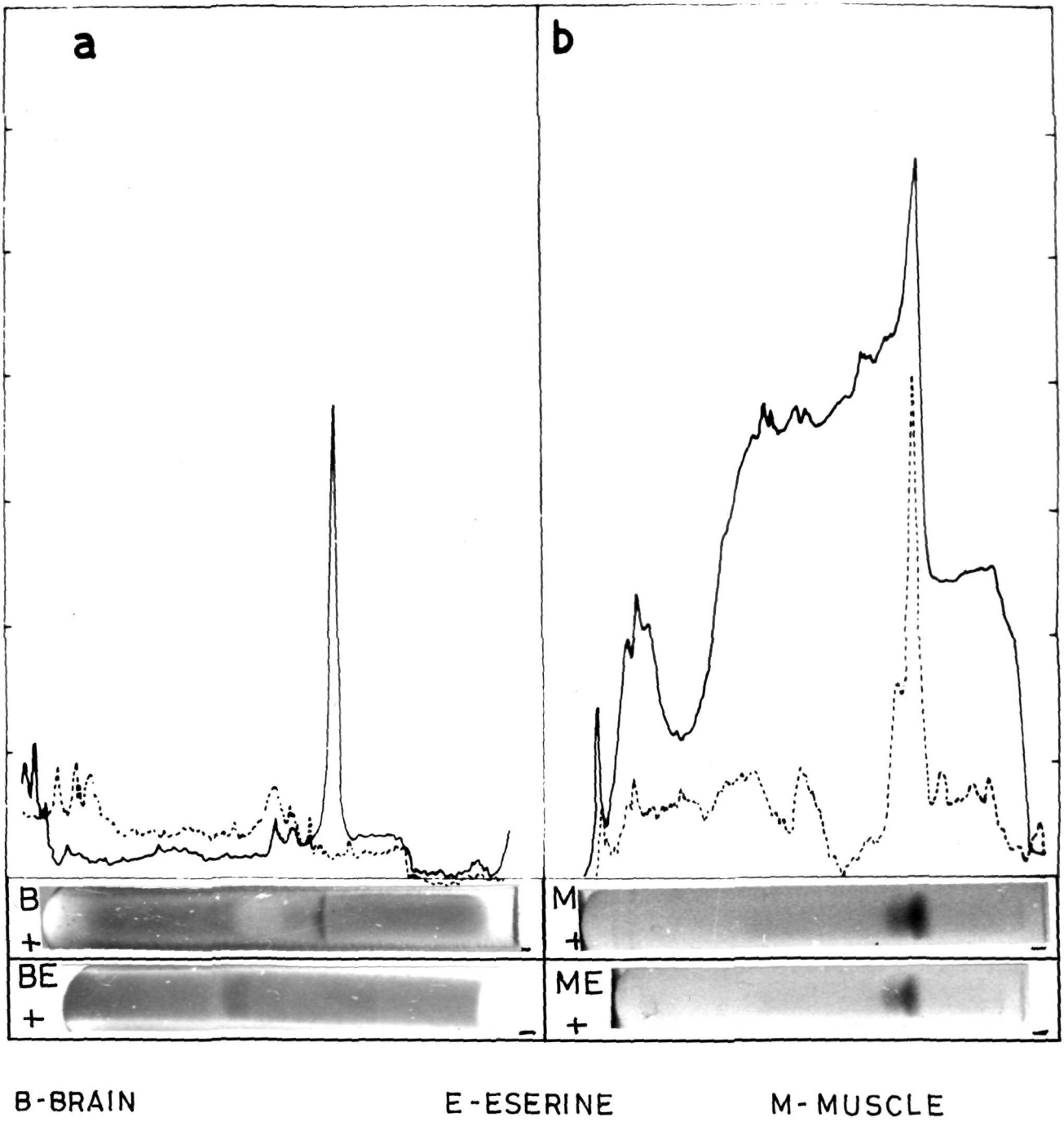


Fig. 28 The Specific staining on polyacrylamide gel and the absorption spectra at 425 nm of the gels stained for AchE with acetylthiocholine iodide (——) and eserine + acetylthiocholine iodide (----) in (a) brain and (b) muscle of H fossilis

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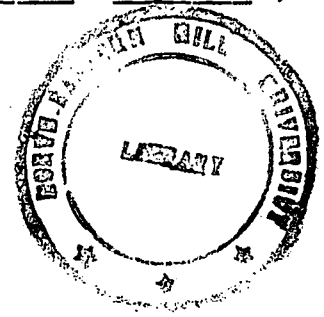

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APPENDIX

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EFFECT OF THE ALCOHOLIC EXTRACT OF *ZANTHOXYLUM ARMATUM* FRUITS ON CERTAIN HAEMATOLOGIC PARAMETERS OF *HETEROPNEUSTES FOSSILIS*

It has been reported earlier[1] that there are about ten plants and their parts which are used as piscicidal agents in fish capture by the people in North-Eastern India. Among these, we found the extract of the fruits of *Zanthoxylum armatum* DC (=*Z. alatum* Roxb.) to be the most potent in its piscicidal activity. However, the mode of action of its toxicity is not yet fully understood. The detailed mechanism of action of the only piscicidal plant product, Rotenone, derived from Derris root has been investigated[2–4]. At the sub-cellular level, Rotenone is known to block the nicotinamide adenine dinucleotide dehydrogenase segment of the mitochondrial respiratory chain, resulting in reduced oxygen uptake by fishes[5,6]. Many toxins are known to have a direct effect on the haematologic parameters[7–9]. The present study, was, therefore aimed to find out if the alcoholic extract of the fruits of *Z. armatum* had any similar effect on some of the haematologic parameters of the cat fish *H. fossilis*.

The fishes were collected from nature and were acclimatized to the laboratory conditions at 20 ± 2° C in aquaria for three weeks, and were fed on alternate days. The weight of the fishes ranged from 8–22 g and the length 10–15 cm. The fishes were weight-sorted into two groups (9–14 g and 15–20 g) in order to compare the effect of the toxin. About 500 g of shade dried powdered fruits of *Z. armatum* were extracted with alcohol following Indian Pharmacopeia and concentrated to 39 g. A 10 l glass jar containing 6 l of tap water was used with 6 fishes for each set of experiment. Four different concentrations of the fruit extract (15, 20, 30 and 35 ppm) were used. The blood samples were taken from fishes treated with 15 ppm after 12 hrs, in 20 and 30 ppm treated fish after 6 hrs. and with 35 ppm treatment after 3 hrs, since the lethal time was different for different concentrations. The blood samples were collected through the caudal vein and the RBC number/mm³ and Hb g% were estimated using haemocytometer and haemoglobinometer respectively[11]. The haematologic values like RBC number, Hb concentration and Hb/RBC ratio of control and treated fishes of the two weight groups were calculated (Table I). It could be seen from the data that the

TABLE I

Effect of different concentrations of Z. armatum (=Z. alatum) *fruits on the haematologic parameters of* H. fossilis

		Average wt. of fish (g)	Average length of fish (cm)	RBC/mm³* X 10^6	g% Hb*	g%Hb/RBC* × 10^{-6}
9–14 g. Wt. group.	0 ppm. (control) (22)	11·9	13–1	2·3±0·1	15·7±0·2	6·6±0·2
	15 ppm. (13)	12·3	13·1	2·2±0·1	13·7±0·3	6·2±0·
	20 ppm. (11)	12·2	12·9	2·2±0·1	13·6±0·3	6·1±0·
	30 ppm. (14)	12·2	12·4	2·2±0·4	13·6±0·2	6·4±0·
	35 ppm.	12·3	12·7	2·4±0·1	13·4±0·2	6·3±0·
15–20 g. Wt. group.	0 ppm. (control) (16)	17·1	14·2	2·7±0·2	15·3±0·3	5·8±0·
	15 ppm. (6)	17·2	13·6	2·4±0·2	14·0±0·3	5·9±0·
	20 ppm. (9)	16·9	14·0	2·4±0·1	16·5±0·4	6·9±0·
	30 ppm. (6)	15·7	13·8	2·3±0·3	14·8–0·5	6·4±0·
	35 ppm. (9)	16·7	14·0	2·4±0·1	15·3±0·4	6·2±0·

* The values expressed as Mean ± S.E.M.

Numbers in parentheses indicate the number of fishes used.

four different concentrations apparently had no significant effect on the two weight groups of fishes.

Certain pesticides, insecticides and piscicides have been reported to affect the different haematologic parameters like erythrocyte number, haemoglobin concentration, packed cell volume, mean corpuscular volume, erythrocyte haemoglobin, mean corpuscular haemoglobin and haematocrit[7-9]. Saponin is one of the plant derived piscicides known to cause haemolysis in fishes[12,13]. Beside toxins, the lower oxygen tension of water has been shown to change the haematologic parameters in fish[14]. However, in the present study, the authors did not observe any change in the oxygen levels of the experimental medium with change of time. Further, the experimental fish, *H. fossilis* being an air-breather would not be much influenced by the changes in oxygen concentration of waters The chemical analysis so far done on *Z. armatum* does not indicate the presence of the any of the known haemolytic factors such as Saponin or Rotenone[15]. Thus, it is evident from the foregoing account that piscicidal components present in the fruits of *Z. armatum* do not have haemolytic properties and the lethal effect could be due to interference in other physiological processes.

The authors are thankful to Professor R. George Michael, Head of the Zoology Department, for his interest in this study and to Dr. Nitya Nand, Director, CDRI, Lucknow, for helping in the preparation of the alcoholic extract of the fruits. The award of JRF from CSIR (to SNR) and financial assistance from NEHU and UGC (to BKR) are gratefully acknowledged.

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STUDIES ON PISCICIDAL PLANTS OF NORTH-EASTERN INDIA: HOPE FOR AN INDIGENOUS PLANT POISON FOR FISH NURSERY MANAGEMENT

IN modern fisheries plant products[1-2] like rotenone[3], jugalone[4], pyrethrin and pyrethroids[5] are used to remove predatory and weed fishes from rearing ponds. The toxic action of these plant products have been observed to be of short duration depending on the concentration of the toxin. In many cases, the fishes which have been poisoned can be revived back to normalcy by transferring them to fresh water. Chemical poisons are sometimes used in fishery management However, due to their acute toxicity on fishes, sustained residual toxicity and their side effects on other aquatic organisms, they are not much acceptable. Among the plant products, the most important one is rotenone which is obtained from Derris root (*Derris trifoliata* Lour.) and is at present imported to India. Hence, there is an urgent need to find out a suitable substitute for rotenone from indigenous piscicidal plants.

Chopra *et al.*[6] have described 112 plants in India reported to have piscicidal action. Out of these, more than 40 plants occur in N.E. India[7]. The authors have collected and identified 10 plants reported to have piscicidal effect from N.E. India (Table I). Toxicity studies were carried out on 5 of them on two species of fresh water fishes commonly found in hill streams, *Puntius shalynius* (Yazdani and Talukdar), *Danio dangila* (Hamilton) and on one species of brackish water air-breathing fish, *Heteropneustes fossilis* (Bloch.). The toxicity experiments were carried out in 10 l. glass jars with a minimum of 10 fishes. The plant parts were first air-dried in shade and finely powdered. The results are provided in Table II. These results indicate that the fruits of *Zanthoxylum armatum* DC (=*Z. alatum* Roxb.) have more acute toxic effect among the different plants screened. *Zanthoxylum armatum* occurs commonly in the hilly tracts of N.E. India and the fruits are extensively used for catching fishes locally.

Some of the investigated plants for piscicidal action are *Barringtonia acutangula* (L.) Gaertn., *Croton tiglium* L., *Derris trifoliata* Lour. and *Milletia piscidia* Wt.[8-11]. Many of the piscicidal plants contain saponin, alkaloids, glycosides and essential oils[7]. They mostly act on the nervous system causing paralysis or on the blood resulting in haemolysis or directly act on the muscle activity[12].

The behavioural responses of the fishes in relation to the toxins studied by the authors were similar to earlier workers made on different plants[8-11]. In the initial phase of treatment of the toxin, the fish was more active, then showed erratic movements, turned upside down and finally collapsed at the bottom of

TABLE I

Details of piscicidal plants commonly used by the N.E. Indian tribals in fishing

Batanical name	Part/s used	Local name
Croton tiglium L.	seed and fruit	Jambola gota
Eupatorium odoratium L.	leaf and shoot	Assam-lota
Milletia pachycarpa Benth.	root	Bakoa-biri; Bokol-bih; Bishloti.
Myrica esculenta Buch.-Ham.	bark	Soh-phi; Keifang; Naga-teng; Kaiphal
Polygonum hydropiper L. var. *flaccidum* Steward	whole plant	Pani maricha
Polygonum hydropiper L. var. *hydropiper*	whole plant	..
Potentilla fulgens Wall. *ex* Lehm.	root	..
Taxus baccata L.	leaf, shoot and seed	Dingsableh
Xeromphis spinosa (Thunb.) Keay (=*Randia dumetorum* Poir.)	fruit	Dieng-makasing-khlaw; Gurol, Bel.mona; Mainphal
Zanthoxylum armatum DC. (=*Z. alatum* Roxb.)	root, fruit, bark and leaf	Gaira, Tambul

TABLE II

Comparative lethal dosage of different piscicidal plants on P. shalynius, D. dangila *and* H. fossilis

Botanical name	Part used	Minimum effective concentration (ppm)	Lethal time (hr)
Myrica esculenta Buch.-Ham.	bark	80–100	12–15
Polygonum hydropiper L. var. *hydropiper*	leaf	100–125	10–12
Potentilla fulgens Wal *ex* Lehm.	root	150–200	8–10
Xeromphis spinosa (Thunb.) Keay (=*Randia dumetorum* Poir.)	fruit	120–140	10–12
Zanthoxylum armatum DC. (=*Z. alatum* Roxb.)	fruit	50–70	8–9

the jar. The gill movement increased at the initial phase and gradually decreased towards the lethal phase. It was also observed that the dosage required for air-breathing fishes was more compared to that of gill-breathers. Further studies on the purification of the toxins, their physiological mode of action and residual toxicity are in progress.

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Glucose-6-Phosphate Dehydrogenase (G6PDH) and Lactate Dehydrogenase (LDH) Activities in two Air-breathing and two Gill-breathing Species of Fish—A Comparative Study

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The activities of G6PDH and LDH were studied in the brain, gill and muscle tissues of two species each of the air-breathing and the gill-breathing fishes. In all cases LDH activities were significantly higher than those of G6PDH. G6PDH activites between the two groups of fishes indicate that the primary gill-breathers had higher activities in comparison to the air-breathing species. The highest activity of G6PDH was seen in the gills and lowest in the muscle whereas the LDH levels were in the reverse order. These enzymatic variations between the tissues and species are possibly due to the physiological, metabolic and behavioural differences.

Key Words: G6PDH, LDH, Adaptation, Glucose metabolism

Introduction

The main route of glucose metabolism in most of the tissues is by Embden-Meyerhof-Parnas (EMP) pathway, when glucose is converted to pyruvate. The reversible reduction of pyruvate to lactate is the terminal step that characterizes glycolysis in vertebrates. A secondary pathway of glucose metabolism is the Hexose monophosphate (HMP) shunt which diverges from glycolysis at the level of glucose-6-phosphate. It has been shown earlier that the presence of glucose-6-phosphate dehydrogenase (G6PDH, E.C. 1.1.1.49) is a strong evidence for the HMP shunt (Hellman 1964, Kauffman et al. 1969) and the presence of lactate dehydrogenase (LDH, E.C. 1.1.1.27) indicates the capacity for anaerobic glycolysis (Hochachka 1973). Hochachka (1961) reported that during aerobic metabolism both EMP and HMP pathways were operative whereas exclusive EMP participation for glucose metabolism is seen during anoxic condition.

A shortage of dissolved oxygen has been shown to be the primary environmental factor which stimulated the development of air-breathing mechanism in fishes (Carter & Beadle 1931). Purely gill-breathing fishes live

in well aerated water and are more active whereas the air-breathing fishes usually live in oxygen-deficient water and less active. This habitat difference is expected to have some impact on their metabolism.

Therefore, a comparative work has been carried out on the two key enzymes, LDH and G6PDH in gill, brain and muscle tissues of two species of surface-dwelling, active and purely gill-breathing fishes (*Puntius shalynius* and *Danio dangila*) and two species of bottom dwelling, sluggish and air-breathing fishes (*Heteropneustes fossilis* and *Channa orientalis*) to find out, if there exists, any difference in their glucose metabolism.

Materials and Methods

The fishes were collected from natural sources and were acclimatized to laboratory conditions for three weeks. They were kept in aquaria at 20±2°C with 12 hours' dark and light conditions. Food was given on alternate days and aeration was done only for the gill-breathing fishes. The weight range of different species of fishes used was : 1.2-2.0 g (*P. shalynius*), 1.0-1.6 g (*D. dangila*), 4.0-6.0 g (*C. orientalis*) and 8.0-12 g (*H. fossilis*). Eight matured fishes from each species were decapitated at a fixed time of the day and tissues (brain, gill and muscle) were removed, washed in ice-cold sucrose 0.25 M, blotted dry and were immediately deep-frozen at −15°C until estimations were carried out. All estimations were completed within a week of sacrifice.

Enzyme assays: A known percentage of homogenate for each tissue (10% for muscle and gill and 5% for brain) was prepared in 0.25 M ice-cold sucrose and centrifuged at 14,000×g for 20 min at 0±2°C. The supernatant collected was used for enzyme assays. Both G6PDH and LDH activities were measured spectrophotometrically at 340 nm according to the methods of Langdon (1966) and Kornberg (1955) respectively. The protein contents were estimated according to Lowry et al. (1951). The enzyme activity is expressed both in terms of total activity (units/g wet wt.) of tissues and specific activity (units/mg protein). All biochemicals used were purchased from Sigma Chem. Co., USA and other chemicals were of analytical grade from BDH.

Results and Discussion

The levels of G6PDH activity in the different tissues studied showed a general pattern of gill>brain>muscle in all the four species of fishes. The level of G6PDH was extremely low in the muscle (table 1).

Table 1 (*A*) *Total activities (units/g wet wt.)×10³ and* (*B*) *Specific activities (units/mg protein×10³) of G6PDH and LDH in brain, gill and muscle of four different species of fishes (Data has been expressed as mean±S.D. calculated from 8 experiments)*

	Species of fish	Brain		Gill		Muscle	
		G6PDH	LDH	G6PDH	LDH	G6PDH	LDH
A	*H. fossilis*	13.3±4.3	77.8±7.9	18.9±4.4	31.3±7.4	0.9±0.2	146.4±60.3
	C. orientalis	11.8±5.6	102.8±5.8	18.2±6.8	62.9±23.4	0.8±0.3	66.3±22.2
	P. shalynius	18.7±9.2	54.8±17.9	25.9±4.8	44.1±9.8	0.8±0.3	66.7±17.1
	D. dangila	27.2±4.9	76.6±16.5	27.7±6.0	36.9±5.6	2.9±0.9	116.9±38.5
B	*H. fossilis*	37.9±6.7	230.1±48.7	116.3±20.2	154.6±27.9	4.9±1.3	584.2±98.0
	C. orientalis	27.2±4.7	291.4±60.2	93.2±45.6	312.2±98.9	7.0±3.4	391.5±25.9
	P. shalynius	51.6±7.1	150.3±30.2	103.6±11.6	175.7±33.7	3.4±0.3	270.5±83.2
	D. dangila	48.6±12.1	131.7±13.8	93.4±21.1	144.2±9.7	15.3±4.6	593.8±75.6

The specific activity of LDH is significantly high in muscle in comparison to brain and gill in all the species. However, the total activity of LDH is significantly high only in the muscle of *H. fossilis*. This higher activity of LDH in muscle may be due to the higher rate of anaerobic glycolysis taking place in muscle. Hochachka (1969) has shown that the energy requirement in the muscle tissue usually arises suddenly and is supplied by rapid glycolysis resulting in increased formation of pyruvate. In an anaerobic tissue like muscle, the higher activity of LDH is necessary to convert the accumulated pyruvate into lactate utilizing the NADH produced during pyruvate formation and allow the anaerobic glycolysis to continue. On the other hand, the brain and gill do not face such sudden energy requirements. Besides, the oxygen is more readily available to those two tissues enabling aerobic metabolism.

The higher activity of G6PDH in gill and brain in comparison to muscle indicate the existence of efficient HMP shunt in addition to that of glycolytic pathway. It is known that the HMP pathway is predominantly found in red blood corpuscles (Harper et al. 1977). The gill tissue having high network of blood circulation and circulating red blood corpuscles for oxygen uptake should show a correspondingly higher G6PDH activity.

The higher level of G6PDH activity observed in the brain may be due to the increased rate of pentose production for nucleic acid synthesis and a higher rate of phospholipid synthesis. These results are similar to those reported by Farnararo et al. (1977) in two other species, *Anguilla anguila* and *Ameiurus rebulosus*. The negligible amount of G6PDH in muscle does not necessarily mean that the muscle cannot synthesize pentoses or nucleotides. Ribose could be synthesized in the muscle by a reversal of the HMP shunt utilizing fructose-6-phosphate, glyceraldehyde-3-phosphate and the enzymes transketolase and transaldolase (Harper et al. 1977). Mayers (1977) has proposed that in insect larvae the HMP shunt serves as a by-pass from glucose-6-phosphate to produce triose phosphates which again enter glycolytic pathway. This process could save the ATP which is needed for converting fructose-6-phosphate to fructose-1-6-diphosphate if ATP is in short supply. The negligible amount of G6PDH activity indicating a near absence of HMP shunt in the anaerobic tissues like muscle and the presence of HMP shunt and glycolytic pathway in the aerobic tissues like brain and gill of these four species of fishes are in keeping with the findings of Hochachka (1961).

Our studies show that the G6PDH activities in all cases are much lower in comparison to that of LDH. Perhaps the HMP shunt is a secondary pathway of glucose metabolism in fishes, unlike that of some snail hepatopancreas where the activity of G6PDH has been shown to be much higher than that of mammalian liver (Mark et al. 1977). However, our studies indicate that the anaerobic glycolysis perhaps operates at a much higher rate that than of HMP shunt for glucose metabolism in the four species of fishes investigated. Further studies using radioactive substrates can confirm this hypothesis.

It is also found that the G6PDH activities in all the tissues of the primary gill-breathers, are generally higher in comparison to those of the amphibious species. G6PDH activity were reported to increase in cold acclimated brook trouts (Yamaguchi et al. 1975, Hochachka & Hochachka 1973) and the phenomenon was probably associated with increase in lipogenesis during cold acclimation (Fried & Levin 1973). The purely gill-breathing species of fishes like *D. dangila* and *P. shalynius* used in our experiments are basically cold-water species and normally available at high altitudes (Yazdani & Talukdar 1975), compared to those of the sluggish air-breathing fishes. Thus, the tissue specific and

species specific differences observed in these two enzyme activities in the two groups of fishes are mainly in keeping with the physiological status of the tissues and in turn reflects their adaptation and behaviour to their respective habitat.

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