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Assessment of anthracene on hepatic and antioxidant enzyme activities in *Labeo rohita* (Hamilton, 1822)

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are widespread pollutants across the global aquatic environment and demonstrate a wide variety of toxic effects in freshwater and marine organisms. The present study aim to estimate the risks of PAHs (Anthracene) to aquatic organisms' enzymatic biomarkers in Labeo *rohita* were assessed. Those include antioxidant enzymes (SOD, CAT, GSH), liver damaging enzymes (GOT, GPT,) and LDH, ALP in the liver, kidney, gills and muscle. Fish were grouped into six. Each group was exposed to any one of the different concentration (0(control), 0.5, 1.0, 2.0, 3.0 and 4.0mg/L) of anthracene. The exhibited induction of LDH activity suggesting an increase of anaerobic pathway of energy production. Antioxidant enzymes elevations suggest anthracene induced oxidative stress in aquatic organisms. The increased liver damaging enzymes suggest liver dysfunction due to anthracene in liver cells. The overall results suggest anthracene is a potent toxicant to aquatic environment.

Key-Words: *Labeo Rohita*, Anthracene, Anti-oxidant enzymes, PAHs, Hepatic Biomarkers.

Introduction

Polycyclic aromatic hydrocarbons (PAH) are a group of hydrophobic organic compounds that are ubiquitous pollutants derived from pathogenic sources. They are present, for example, in water bodies especially near densely populated and industrialized areas. PAHs are absorbed by fish via the gills and body surface. It also enters via ingestion of food or through contaminated sediment (Neff 1985). PAHs are rapidly transformed into more hydrophilic metabolites that are excreted; thus fish exposed to these compounds show only trace amounts of PAH in their tissues (Tuvikene 1995). PAH metabolites are usually determined in fish bile, where they are concentrated and stored prior to excretion. Biliary PAH metabolite analysis provides information about the actual exposure of fish to PAH compound and reveals the state and suitability of the aquatic environment for fish.

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The effects of pollutants such as polycyclic aromatic hydrocarbons (PAHs) have been extensively studied in species from sub-tropical to boreal regions and biomarkers have been developed for indicator species living specifically in the regions of concern. An increasing variety of foreign chemicals (xenobiotics) released by urban communities, industries and agricultures is entering the aquatic environment and bringing potential long-term adverse effects on aquatic organisms (Livingstone 1998). These xenobiotics include organic trace pollutants such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and dibenzo-p-dioxins (PCDDs), and many metals such as iron (Fe), cadmium (Cd) and lead (Pb). Such a diverse array of chemicals products can have many different mechanisms of toxicity, and numerous xenobiotics may also involve the common mechanisms of toxicity (Stohs and Bagchi 1995; Livingstone 2001). Once a PAH has been taken up by an organism it may be subjected to biotransformation reactions. In bivalve molluscs, PAH metabolism largely occurs through radical oxidation involving reactive oxygen species (ROS) (Stegeman and Lech 1991) which can be generated at various stages along the metabolic pathway (Livingstone 1991). ROS are produced

continually in living cells, and are essential in maintaining cell function in biological systems. However, an imbalance between formation and neutralisation of these reactive species (ROS) can induce oxidative damage (Valavanidis *et al*. 2006).

Crude oil spills, refinery activities and industrial and urban wastes are important sources of polycyclic aromatic hydrocarbons (PAH) in aquatic ecosystems. PAH are potentially carcinogenic (Shailaja and D'Silva 2003) and an important concern, because they are ubiquitous contaminants in coastal and freshwater zones (Akaishi *et al.* 2004). These compounds may induce hepatic lesions, physiological and biochemical disorders in fish**.** Contaminated fish may then be used to bio monitor the presence and importance of these pollutants. PAH contamination is especially important near industrialized areas (Aas *et al*.2000). Finding PAH metabolites in fish bile has proven to be a simple and sensitive method for screening fish for PAH contamination as well as a useful biomarker for assessing exposure to aromatic contaminants in aquatic environments (Leadly *et al*. 1999; Aas *et al*. 2000).

Anthracene ($C_6H_4.C_2H_2.C_6H_4$) is a solid polycyclic aromatic hydrocarbon with a low molecular weight, consisting of three fused benzene rings derived from coal-tar . Anthracene also referred to as Para-naphthalene, Green Oil which accompanies naphthalene in the last stages of the distillation of coal tar. Anthracene is used in the artificial production of the red dye alizarin. It is also used in wood preservatives, insecticides, and coating materials. Anthracene is colorless but exhibits a blue fluorescence under ultraviolet light. Anthracene is present in many estuarine systems at concentrations believed to cause sub lethal adverse effects, although its exact mode of toxicity remains uncertain.

If released to water, anthracene will be expected to adsorb very strongly to sediments and particulate matter. It was not hydrolyzed but may bio concentrated in aquatic organisms which lack microsomal oxidase (this enzyme enables the rapid metabolism of polyaromatic hydrocarbons). It undergoes direct photolysis near the surface of natural waters and may be subject to significant biodegradation based on laboratory tests.

Material and Methods

Chemicals

Anthracene technical grade was purchased from Loba chemie (pvt) Ltd and were used with 97%purity, respectively.AST, ALT, LDH and ALP kits were purchased from biosystem and the antioxidant substances were purchased from Himedia.

Test Species

One month old *L.rohita* fish with an average weight of 8 ±1.6gm and average length of 10 ±1.4cm were obtained from a local hatchery. Fish were kept in tanks with aerated tap water. Fish were fed on commercial feed and acclimatized to laboratory conditions at least one week before the experiments were performed.

Determination of Sub-Lethal Concentrations

Acute toxicity bioassays to determine the 96 h LC50 value of anthracene was conducted in a semi-static laboratory system according to the OECD guideline No 203 [1992] with the modifications indicated below. Anthracene was dissolved in filtered distilled water and added to the aquarium following the method of Vieira LR [2008]. The experiment was conducted in 100L aquarium and gentle aerated tap water. A set of 10 fish specimens were randomly exposed to each of the nominal anthracene concentrations (5.0, 10.0, 15.0, 20.0 and 25.0mg/L) and a control (0.00 mg/L) and the experiment was set in triplicate to obtain the LC50 values of the test anthracene for the species. The LC50 value of anthracene for *L.rohita* was determined as 10.0 mg/L. Based on the 96 hLC50 values, the fish were then exposed for 5 weeks to five sub-lethal concentrations of the anthracene (1/2.5 LC50 = ~4.00 mg/L, 1/3.3 LC50 = ~3.00 mg/L, 1/5 LC50 = ~2.00 mg/L, 1/10 LC50 = ~1.00 mg/L and 1/20 LC50 = ~0.5mg/L) and used for the enzyme assays. A set of 20 fish were also simultaneously maintained in tap water (0.00 mg/L) as the control. No mortalities occurred following the exposure to the five sub lethal concentrations of the anthracene during the experiment**.** Every week's two fish from each dose and the control group were sacrificed to take liver, kidney, Gills and muscles from each fish and weighed to the nearest milligram. Approximately 0.2gms of tissue was extracted and used for the determination of enzyme activities. The tissues were homogenized in a polytron-type homogenizer in ice cold condition at 4°C. The homogenates' strength was adjusted to 10% (w/v) with 0.25 M sucrose solution and was used as the source of enzymes.

Biochemical Analysis

Biosystem Kits were used for the determination of Glutamic Pyruvic Transaminase (ALT) and Glutamic Oxaloacetic Transaminase (AST), Lactate Dehydrogenase (LDH), Alkaline Phosphatase (ALP). All the enzymatic activities were measured in Autoanalyzer Biosystem A25 model Spain.

Total protein content was determined according to the Bradford (1951) method using bovine serum albumin (BSA) as a standard.

Antioxidant Enzyme Activities

CAT activity was assayed by the method of Caliborne (1985). Briefly, the assay mixture consisted of 1.95mL phosphate buffer (0.05 M, pH 7.0), 1mL hydrogen peroxide (0.019 M), and 0.05mL 10% PMS in a final volume of 3 ml. Change in absorbance was recorded at 240 nm. CAT activity was calculated in terms of nanomoles H_2O_2 consumed/min^{-1}/mg^{-1} protein.

SOD activities were measured using a spectrophotometer. About 0.3 g of fish liver was homogenized after addition of 3.0 ml of 10 mM Tris buffer (pH 7.5) and centrifuged. SOD activities were determined from their ability to inhibit the autoxidation of pyrogallol using a modification of the method of Wienterbourn (1975). The rate of pyrogallol auto-oxidation was measured at 325 nm. One unit of enzyme activity was defined as the amount of the enzyme, which gave 50% inhibition of the auto-oxidation rate of 0.1 mM pyrogallol in 1 ml of solution. Glutathione reductase (GSH) activity was determined by the method of Carlberg and Mannervik. (1985). The reaction mixture 2 ml containing 50mM - phosphate buffer, pH 8.2, 1 mM-EDTA, 0.2 mM-NADPH and 1 mM-GSSG. One unit of glutathione reductase was defined as the amount of enzyme catalysing oxidation of 1 umol of NADPH/min^{-1}/mg^{-1} protein. Statistical analysis the values are expressed as mean ± SE for five animals.

Results and Discussion

No difference was observed in the body weight gain of L.rohita during the whole period of anthracene exposure, but the food intake was decreased with increasing concentration. No mortality was encountered during to experimental period. Significance difference between the control and anthracene exposed fish were found for all the enzymes. Especially liver damaging enzymes such as ALT and AST concentrations were elevated (Fig 1-2) correspondingly with the increasing concentrations of the anthracene and also the activity noted was higher in liver sample when compared to kidney, gill and muscle in every week.

Another hydrolytic enzyme, ALP increases gradually starting from the 1st week to 5th week, the ALP enzyme level was higher than that control in all the concentration of anthracene. Further the LDH level was increased in the increasing concentration of anthracene. LDH level was higher in liver when compared to other tissue. (Fig. 1)

However, the antioxidant enzyme levels were significantly varies between the control and anthracene exposure fish. The specific activity of antioxidant enzymes such as catalase, SOD and GSH were significantly higher in the highest concentration of anthracene (4mg/L). The activity of antioxidant enzymes were increases gradually in increasing concentration of anthracene. The Lowest level of catalase activity in Liver, SOD and GSH activity in kidney was noted at the concentration of 0.5mg/L of anthracene on 1st week exposure (Fig3-4). The other tissue like Gill, Muscle and Kidney had slightly decreased in enzyme activity than liver.

Accumulation of PAH compounds like anthracene in the liver, kidney and other organs might have caused serious pathological damage due to the exposure (Braunsbeck 1994; Vutukuru *et al*. 2007). Burtis *et al*. (1996) and Vutukuru *et al*. (2007) reported that when the liver cell is damaged, tissue specific enzymes are released into the bloodstream, thus making the enzymes level in the blood to go up. Therefore, the significant increase of the enzymes' (AST, ALT and ALP) activities in the liver, kidney, gill and muscle of *Labeo rohita* exposed to anthracene-contaminated water could either be due to their possible leakage from the cytosol across damaged plasma membrane into the general blood circulation or increase in their synthesis as a result of the organ dysfunction. The elevated enzyme activity was concentration dependent could be considered to be manifestation of oxidative stress caused by anthracene. The level of AST, ALP and ALT were observed at the highest in tested concentration (4 mg/L) of anthracene.

LDH level was increased in the increasing concentration of anthracene treatment. These findings also suggest that animals are getting additional energy from the anaerobic pathway in an attempt to support the processes (e.g. detoxification mechanisms) needed to face chemical exposure. These results are in good agreement with findings from the literature reporting an increase of LDH in fish after exposure to anthracene (L.R.Vieira *et al*. 2008).

Anthracene were found to significantly induce all the anti-oxidant enzymes tested, namely SOD, CAT, and GSH, which are crucial in the detoxification of oxyradicals to non-reactive molecules (Van Der Oost et al. 2003). These results suggest that PAHs induce the production of $O^{\cdot}_2{}^{-}$ which is converted to hydrogen peroxide (H_2O_2) by action of SOD and then that H_2O_2 is converted into water by action of CAT. The pathway involving GPx also detoxify lipid peroxidase (Winston and Di Giulio 1991) and requires GR to catalyse the transformation of the oxidized disulfide form of glutathione (GSSG) to the reduced form (GSH), by making use of the oxidation of NADPH to NADP+, which is further recycled mainly by the pentose phosphate pathway. Since in the case of fish exposed to

individual PAHs all the anti-oxidant enzymes measured were found to be induced, it is likely to conclude that a considerable amount of $O_2^{\cdot -}$ is produced originating abundant H_2O_2 that needs to be detoxified by both CAT and GPx pathways. GSH plays an important role in the detoxification of electrophilic substances and prevent cellular oxidative stress (Hasspieler *et al*. 1994). They can also bind, store and or transport a number of compounds that are not conjugates with GSH (Parkinson 2001). The GSH availability responds differently to distinct PAHs. GSH conjugation is involved in PAH removal, therefore it is in good agreement with the general detoxification. From these results, one can conclude that all the tested doses of anthracene have the capability of inducing oxidative stress on fish if the anti-oxidative stress defences of the cell are overtaken. Further this study shows that the accumulation of anthracene in the liver of *Labeo Rohita* led to the functional damage of these organs as reflected by the increased activities of the enzymes. This biochemical dysfunction may interfere with the homeostatic processes which may ultimately affect the survival and rational exploitation of this fish in their natural environment. The increase in the activities of the enzymes reinforces their important roles in the detoxification of toxicity of anthracene and further shows that AST, ALT and ALP are potential biomarkers that can be used for anthracene-induced liver toxicity in *Labeo rohita*.

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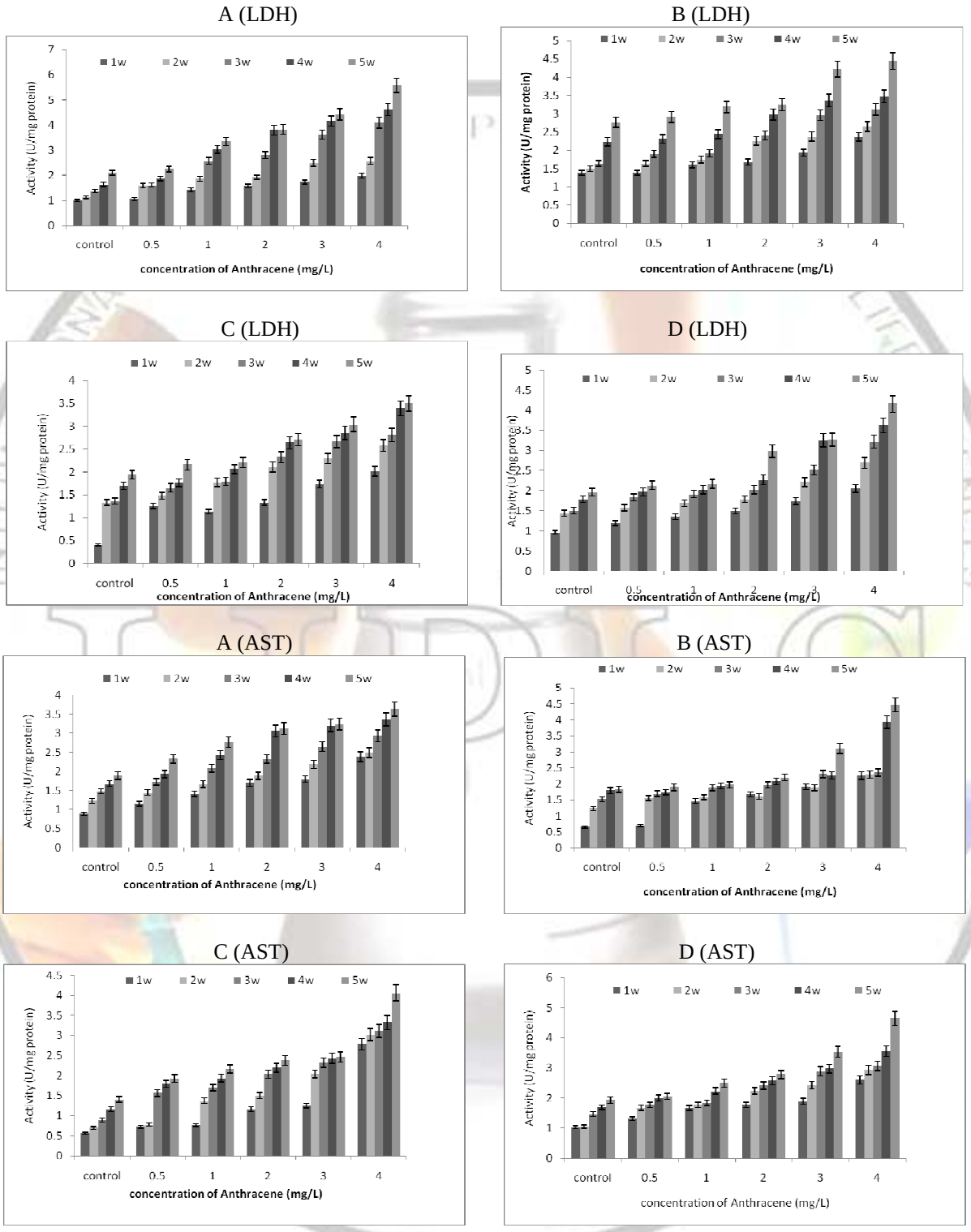

Fig. 1 Effect of Anthracene on LDH & AST activity in A-liver ,B-gill,C-kidney,D-muscle of *L. rohita*

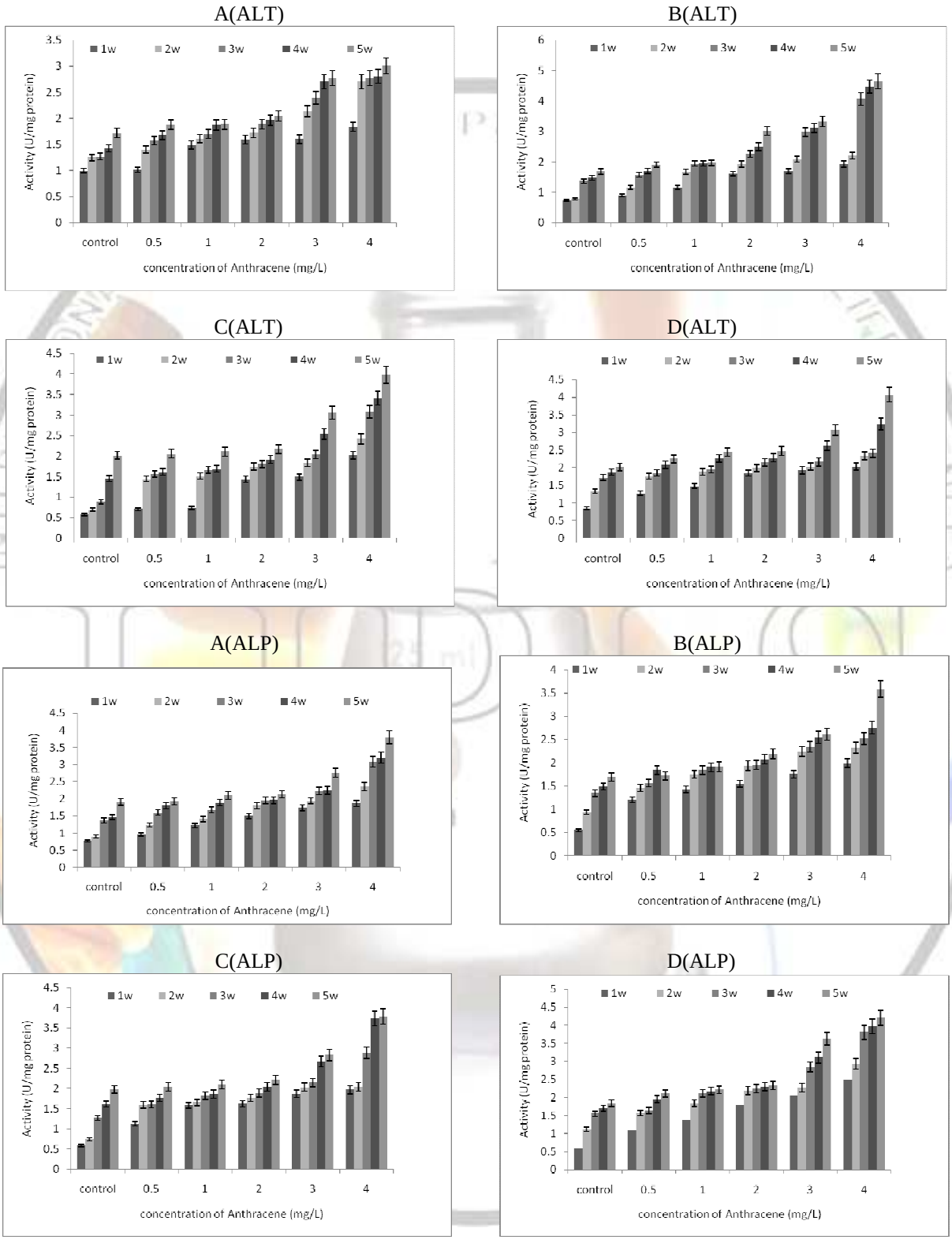

Fig.2 Effect of Anthracene on ALT & ALP activity in A-liver ,B-gill,C-kidney,D-muscle of *L. rohita*

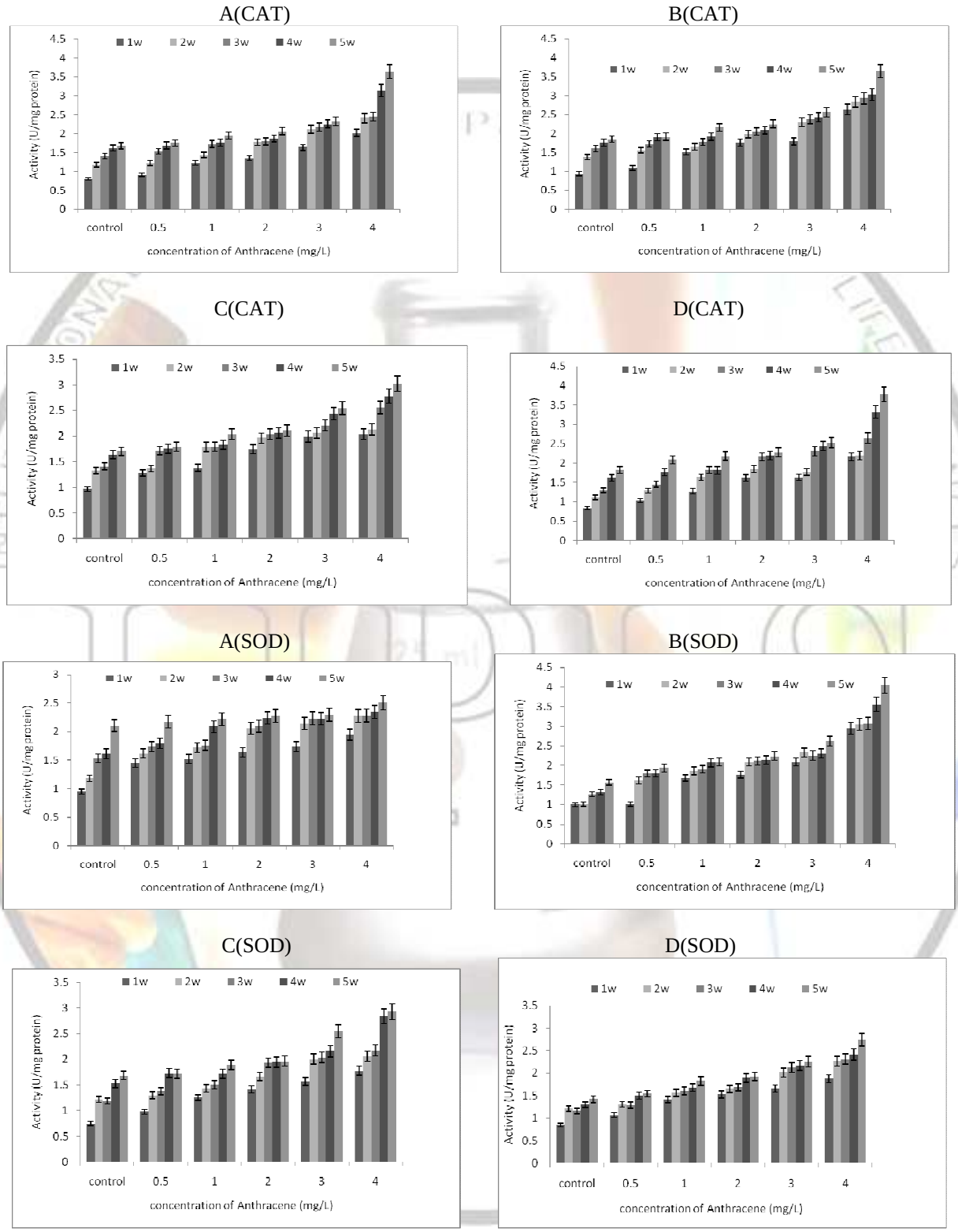

Fig.3 Effect of Anthracene on CAT & SOD activity in A-liver ,B-gill,C-kidney,D-muscle of *L. rohita*

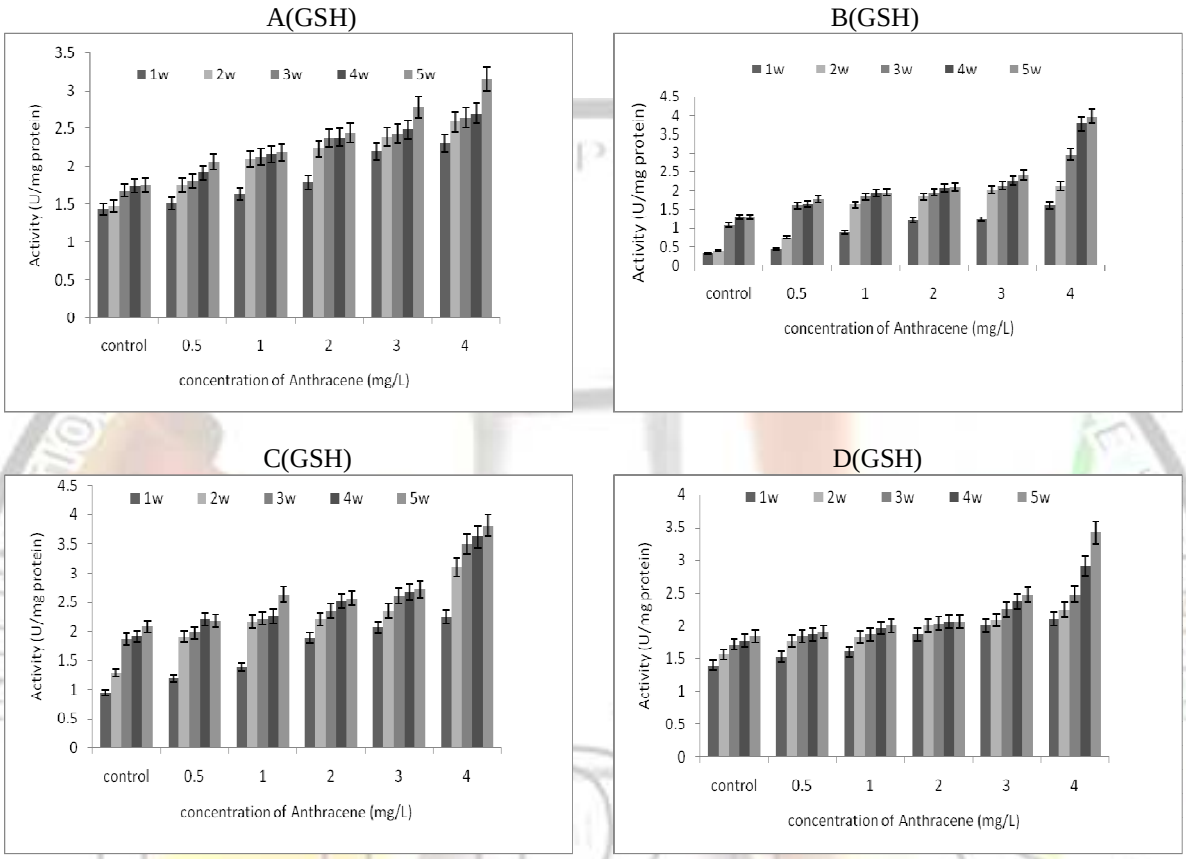

Fig.4 Effect of Anthracene on GSH activity in A-liver ,B-gill,C-kidney,D-muscle of *L.rohita*