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Responses of Biochemical Markers in Liver of Zebrafish, *Danio rerio*, Exposed to Two Different Heavy Metals, Lead and Cobalt

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Abstract | **Original Research Article**

The present study was aimed to investigate the changes due to two heavy metals exposure in the activity of the antioxidant enzyme, Catalase (CAT), Reduced glutathione (GSH) and Lipid peroxidation (LPO) in the liver of *Danio rerio* during 5, 10, 15 and 20 days of exposure period. Discharge of heavy metals into river or other aquatic body can change the environmental factor, species diversity and ecosystem, due to their toxicity and bio-accumulation. These heavy metals enter the aquatic ecosystem as a result of direct input of atmospheric deposition, leaching of minerals and soil erosion due to rain water. This cause the hazardous and weaken the mechanism concerned leading to physiological, biochemical and pathological changes. The lead is non essential elements while cobalt is an essential element for living organisms but their presences in fresh water in higher concentration are toxic to living organisms. Its effect on the oxidative enzymes in the liver is investigated in the present study.
Key-words: Zebrafish, Lead, Cobalt, Liver, CAT, LPO, GSH.

INTRODUCTION

Heavy metals have been recognized as strong biological poisons because of their persistent nature, tendency to accumulate in organisms and undergo food chain amplification [1], they also damage the aquatic fauna. The contamination of freshwaters with a wide range of pollutants has become a matter of great concern over the last few decades. The impact of metals, as well as other pollutants, on aquatic biota can be evaluated by toxicity test, which are used to detect and evaluate the potential toxicological effects of chemicals on aquatic organisms. However, little research has been done on the impact of contaminations on tropical ecosystems [2]. Fish are widely used to evaluate the health of aquatic ecosystems because pollutants build up in the food chain and are responsible for adverse effects and death in the aquatic systems. Fish can obtain their trace elements, either directly from the water through the gills or indirectly from food through the alimentary tract [3].

Heavy metals like Co, Zn, Ni, Cu, Se, Mn, Fe, and Cr are essential for the growth of organisms, while Pb, As, Hg and Cd are not only biologically non essential, but these heavy metals beyond optimum threshold levels found to be hazardous and toxic. After entering the water body these metals may participate, get absorbed on solid surface, remain soluble or suspended in water or taken up by fauna. The most important biological property of metal is their tendency to accumulate in the animal tissues.

Lead is a persistent metal which is commonly used in various industrial processes. It is toxic to living systems and may stay in the environment for a prolonged period of time, due to its persistency; it exists as a free metal in various compounds. Lead is a widespread environmental and occupational xenobiotic and is hazardous to humans and various ecosystems [4]. Its exposure to humans is mainly by ingestion through the mouth and inhalation from fumes and dust in the atmosphere [5]. Exposure to lead is mainly from anthropogenic sources due to its widespread usage. The form in which lead exists determines how toxic it is in the environment. Several studies link inorganic lead like lead acetate compounds to increased incidence of diseases in various organisms. Lead toxicity has been linked to incidence of neurological disorders, hypertension, cognitive impairments *et al*. [6]. Chen *et al*. 2012 reported that exposure of low doses of lead to the development of embryo of zebra fish resulted in embryonic toxicity, behavioral alteration, and adult learning/memory deficit in zebrafish [7]. It's accumulation in sediment is of significance for aquatic organisms. It is not a transition metal and cannot readily undergo valence changes, it can induce oxidative

damage through direct effects on the cell membrane, interactions between lead and haemoglobin, which increase the auto-oxidation of hemoglobin, auto-oxidized δ-aminolevulinic acid, interactions with glutathione reductase or through the formation of complexes with selenium, which decrease glutathione, peroxidise activity [8]. Lead deposits in various fish organs like liver, brain, kidneys, spleen, digestive tract and gills [9].

Cobalt is an essential nutrient for man and is an integral part of vitamin $B_{12.}$ It performs important biochemical function but its higher concentration in aquatic ecosystems becomes toxic to fish as it interferes with the enzyme systems [10]. It is reported to be a potential carcinogenic compound and has been included recently in group 2A carcinogens *i.e.*, probably carcinogenic to humans. Cobalt can be absorbed from the surrounding water through the gills as well as from the diet. The uptake of waterborne cobalt increased with a rise in temperature and decrease in waterborne calcium. It exists in special fertilizers and waste water following the evolution of cobalt mines. Metals such as cobalt may cause environmental risk when occurring at raised levels [11] although cobalt is of relatively low abundance in the earth's crust and in natural waters.

Also, heavy metals are known to induce oxidative stress and carcinogenesis by mediating free radicals e.g. Reactive Oxygen Species (ROS) [12]. They deplete glutathione, resulting enhanced production of ROS such as catalase. ROS are considered as critical mediators for the metal-triggered tissue injuries and apoptosis. To prevent oxidation induced damage, there must be effective antioxidation system enzyme including free radical scavenging enzymes, such as Superoxide Dismutase (SOD) and Catalase (CAT) changes in the activity of enzymes and other biomarkers are the possible tool for aquatic toxicological research [13].

The zebrafish is selected for this experiment because it has great benefits, with regards to high fecundity, small size approx 2-5 cm long, easy to breed in laboratory, short generation time, rapid development, translucent embryos and easy to maintain under laboratory conditions. *Danio rerio* are considered to be a model organism for toxicological research and also recommended by the Organization for Economic Co-operation and Development [14].

MATERIALS AND METHODS

The present work was conducted in the Zebrafish laboratory, Department of Zoology, D.D.U. Gorakhpur University, Gorakhpur, India. Zebrafish, is also recommended by International Organization for Standardization [15] were collected and acclimatized for a month, stocked and reared under laboratory conditions. The aquariums were aerated continuously through stone diffusers connected to a mechanical air compressor and the water temperature was maintained at 25 ±2℃. The fishes were fed twice daily alternately with raw and chopped goat liver and shrimp powder. Tubifex worm, Tetrabit and spirulina granules purchased from pets shop were also supplemented.

For the present study, mature adult zebrafish approximately 3.5 cm in length and 1 g in weight were procured from stock aquarium and exposed to four different concentrations viz., 20, 30, 40 and 50 mg/l of cobalt and 05.00, 09.00, 13.00 and 17.00 mg/l of lead calculated from our previous toxicity test. The concentrations of heavy metals were decided for exposures were below the range of 80% 96-h LC_{50} as calculated earlier [16]. Low concentrations were selected since fish can survive the stress of the toxicant. Twenty fishes were exposed to each concentration. The water in the aquarium was replaced daily with fresh treatment of metals. Each experiment was accompanied by their respective control. After exposure periods of 5, 10, 15 and 20 days, required number of treated fish were removed from the experimental and control groups and their liver were removed and processed.

BIOCHEMICAL ASSAY

The activity of CAT was estimated according to procedures by Sinha [17]. This method is based on the fact that in acetic acid dichromate is reduced to chromic acetate when heated in the presence of H_2O_2 with the formation of perchromic acid as an unstable intermediate. The chromic acetate is measured colorimetrically at 620 nm. The catalase preparation is allowed to split H_2O_2 at different time intervals by the addition of a dichromic acetic acid mixture and the remaining H_2O_2 is determined colorimetrically. The results were expressed as µM H_2O_2 utilized/min/mg protein.

Glutathione (GSH) content in the liver was estimated according to the method of Paglia *et al*. [18]. Tissue (liver) was lyses with 2.0 ml of 1g/l EDTA (ethylene diamine tetraacetic acid) solution and 1.5 ml of precipitating reagent (1.67 g glacial metaphosphoric acid, 0.2 g EDTA, 30 g sodium chloride, distilled water to 100 ml) was added. After mixing, the solution was allowed to stand for five minutes then centrifuged at 3000 rpm for 15 min. 0.50 ml of filtrate was added to 2 ml of disodium hydrogen phosphate (Na_2HPO_4) (0.1M, pH 7.4) and 0.25 ml of DTNB reagent (40 mg) was dissolved in 100 ml of 10 g/l (1%) sodium citrate. A blank was prepared from 1.5 ml of precipitating reagent, 1 ml of distilled water, 2 ml of disodium hydrogen phosphate and 0.25 ml of DTNB reagent. The absorbance of yellow color was read at 412 nm within a minute after adding DTNB. The results were expressed as GSH mg/mg protein.

LPO levels were estimated with thiobarbituric acid reacting substances (TBARS) and colour reaction

for malondialdehyde (MDA) according to procedures in Placer *et al*, (1966) [19]. Tissues were homogenized in chilled 0.15 M KCl using a Teflon pestle to obtain 10% w/v homogenate. One ml of homogenate was incubated at 37 ± 0.5℃ for two hours. To each sample, 1 ml of 10% w/v trichloro acetic acid (TCA) was added. After thorough mixing, the reaction mixture was centrifuged at 2000 rpm for 10 minutes. 1 ml of supernatant was then taken with an equal volume of 0.67% w/v TBA (thio-barbituric acid) and kept in a boiling water bath for 10 minutes, cooled and diluted with 1 ml of distilled water. The absorption of the pink colour obtained which measured at 535 nm against a blank. The concentration of MDA was read from a standard calibration curve plotted using 1,1,3,3' tetra-methoxypropane and the results were expressed as µmol of MDA formed/ min/ mg protein.

The protein contents of tissues were assayed using the method of Lowry *et al*. with bovine serum albumin as the standard [20]. Two way analysis of variance (ANOVA) was applied to test the significance of the data. All the data are expressed as means (n=6) ± standard deviation (SD) and difference were considered significant at $P<0.05$.

RESULTS AND DISCUSSION

Changes in liver exposed with both heavy metals (lead and cobalt): Catalase activity levels were significantly reduced in 5, 10, 15 and 20 days during exposure on zebrafish liver. In 5 days of exposure period minimum changes observed in cat activities at each concentrations of lead was 140.55±1.63 (93%), 135.60±1.25 (90%), 129.25±1.65 (85%) and 120.65±1.90 (80%) µm h_2o_2 utilized/min/mg protein as compared to control 151.35±1.86 (100%). But after 20 days of treatment period at all concentrations the maximum changes was78.95±1.25 (53%) at 17.00 mg/l in CAT activity were observed which showed a concentration and time-dependent action of lead (Table-1). However in case of cobalt after 5, 10, 15 and 20 days of treatment period, maximum changes in CAT activities were observed after 20 days exposure period at each concentrations which was 128.25±1.65 (85%), 118.38±1.36 (78%), 108.85±1.25 (71%) and 90.32±1.63 (60%) µM H_2O_2 utilized/min/mg protein as compared to control 151.63±2.25 (100%), but after 5 days of treatment period at all concentrations the minimum change was 122.61±1.15 (82%) as compared to control 149.60±2.10 (100%) at 50.00 mg/l in CAT activity were observed which showed a concentration and time dependent action of cobalt (Table - 2). In this experiment the results were shown that lead was more toxic as compared to cobalt.

Alteration in GSH level after 5, 10, 15 and 20 days treatment of both heavy metals are presented in table 3 and table 4. The reduction in GSH level was maximum after 20 days of treatment of 17.00 mg/l of lead and it was found to be only 1.63±0.18 (46%) GSH mg/mg protein as compared to control 3.55±0.21 (100%),(Table- 3). While in case of cobalt, reduction was maximum after 20 days treatment period 2.28±0.17 (67%) GSH mg/mg protein as compared to control 3.38±0.16 (100%) at 20.00 mg/l concentration (Table-4).

The effect of both metals on LPO also showed a significant change that is ($p<0.05$) at different concentrations and exposure periods. At 17.00 mg/l of lead treatment for 20 days there was drastic increase in the MDA level 17.66±0.49 (134%), as compared to 13.15±0.17 (100%), (Table - 5) and in the case of cobalt concentration at 50.00 mg/l for 20 days, increment in MDA level was 16.14±0.54 (125%), (Table 6). However, it was observed that the changes were more profound with the lead exposure as compared to cobalt.

Heavy metal pollution is one of the most important environmental problems today. Fish are exposed to unnaturally high levels of these metals including lead. Lead (Pb) and its products are harmful pollutants in the environment as well as being produced by manufacturing and mining actions [21]. Several researchers have reported that toxic and non-biodegradable heavy metals such as lead accumulate in many fish species, causing toxicological effects [22]. Pb has been recognized as strong biological poisons because of their persistent nature, toxicity, tendency to accumulate in organisms and undergo food chain amplification [23]. As fish are constantly to exposed pollutants in contaminated water, they could be used as excellent biological markers of heavy metals in aquatic ecosystem [24].

In present study, the zebrafish was exposed to cobalt chloride ($CoCl_2.6H_2O$) and lead acetate ($C_4H_6O_4Pb.3H_2O$) for a period of 5, 10, 15 and 20 days at suitable concentrations i.e. for the cobalt 20 mg/l, 30 mg/l, 40 mg/l and 50 mg/l and for the lead 05.00 mg/l, 09.00 mg/l, 13.00 mg/l and 17.00 mg/l and recorded a significant reduction in CAT (catalase) and GSH (reduced glutathion) but in the LPO we observed significant enhancement in the liver of zebrafish. Maximum reduction was recorded in GSH and CAT at the higher concentration for the lead at 17.00 mg/l and maximum increase in LPO was recorded at same concentration. Same pattern was recorded into cobalt a significant reduction in CAT (catalase) and GSH (reduced glutathion) but in the LPO, we were observed significant enhancement in the liver of zebrafish. Maximum reduction was recorded in GSH and CAT at the higher concentration was at 50.00 mg/l as compared to the lower concentration of 20 mg/l and maximum increase in LPO was recorded at the 50.00 mg/l as

compared to 20.00 mg/l. These observations revealed that the decline in CAT, GSH and upgrade LPO levels in liver was directly proportional to concentration of cobalt and lead. In this investigation it is clear that cobalt is less toxic than lead. The heavy metals cause free radicals mediated cellular damage which leads to metabolic alterations such as the enzymatic activities and membrane transport mechanism and injuries of biological system at different levels.

CAT is the primary enzyme responsible for eliminating the ROS formed during bio-activation of xenobiotics in hepatic tissues and the induction of CAT system provides the first-line of defense against ROS. CAT activity, however, gradually decreased after 5, 10, 15 and 20 days of exposure to heavy metals and the values obtained were significantly ($p<0.05$) lower than those of the control. Decreased CAT activity decreases in reaction rates resulting from the excess production of H_2O_2. This could have been because of the flux of superoxide radicals, which has been shown to inhibit CAT activity [25]. Tripathi and Singh [26], observed a decrease in CAT activity in the livers, gills, and brains skeletal muscles of *Channa punctatus* (Bloch). The increase or decrease of enzyme activity is related to the intensity of cellular damage. Thomas and Murthy [27], described the Monocrotophos treatment resulted in the decrease of CAT activity in the liver of Asian stinging catfish (*Heteropneustes fossilis*). A decrease in the activity of CAT has been previously reported in *Cyprinidae* fish living in Seyhan dam Lake of Turkey and in starlet (*Acipenser ruthenus* L.) from the Danube river of Serbia [28]. Similar observation was found in the liver, brain, gills and kidney of *Labio rohita after* lethal and sub lethal concentrations of Malathion [29].

The reduced glutathione (GSH) antioxidant system is the principal protective mechanism of cells and is a key factor in the development of immune response by immune cells. Reduced glutathione reduction might increase the risk of the oxidative stress [30]. However, oxidative stress can induce GSH rising by protective role in the organisms exposed to heavy metals. Reduced GSH and its metabolizing enzymes provide the major defense against ROS induced cellular damage [31]. Doyotte *et al.* [32] pointed out that a decreased enzyme activity response may accompany a first exposure to pollutants, which can be followed by an enhancement of antioxidant system. Thus, the existence of an inducible antioxidant system may reflect an adaptation of organisms. While Dimitrova *et al*. [33] Suggested that the superoxide radicals by themselves or after their transformation to H_2O_2 (Hydrogen peroxide) cause an oxidation of the cysteine in the enzyme and decrease superoxide dismutase activity. Consequently, the decreased and increased superoxide dismutase activities might have reflected a cellular oxidative stress due to heavy metal exposure. On the other-hand, the enzymatic antioxidants such as superoxide dismutase, GSH have been shown to be sensitive indicators of increased oxidative stress in *Mugil sp.* obtained from polluted area containing high concentration of pollutants [34]. Cysteine is the limiting factor of GSH synthesis in cells and reduced glutathione has sulfide functional groups that can capture unpaired electrons and thus is capable of removing harmful free radicals [35]. Farmobi *et al.* [36] reported an increase in activity of GST and GSH in liver of catfish from Ogun River as compared to adaptive and protective role of the bimolecule against oxidative stress induced by heavy metals. Joseph *et al*. [37] were found that GSH level was higher ($p<0.05$) in control with a value of 3.05±0.01, when compared to groups exposed to concentrations of Pb(NO3)2 (2.53±0.29, 0.94±0.14 and 0.82±0.10), at 28 days. For test organisms exposed to concentrations of ZnCl2, GSH level was lower (0.10±0.05) though not significant at ($p<0.05$) in control at 28 days.

Malondialdehyde (MDA) is one of the LPO products deriving from oxidative attack on cell membrane phospholipids and circulating lipids, and its level directly reflects the degree of oxidative damage induced by contaminants [38]. The measurement of MDA content provides a relative measure of potential for pollutants to cause oxidative injury [39]. The elevated MDA level was considered as result of oxidative stress from xenobiotics. MDA, a major oxidation product of peroxidized polyunsaturated fatty acids, has been considered as an important indicator of lipid peroxidation.

The enhanced levels of LPO in the liver of *Danio rerio* in response to 20 days of exposures to lead and cobalt were observed during the present study suggest that production of ROS is increased which could be associated with the metabolism of the heavy metals leading to the peroxidation of membrane lipids in liver tissues. Saida Mohammed *et al.* [40] were detected increase of Malondialdehyde (MDA) in liver tissue of rats in treated groups with cobalt and copper. Lipid peroxidation is indicated by the presence of MDA in tissues.

Table-1: Effect of Lead on CAT activity (µM H_2O_2 utilised/min/mg protein) in the liver of zebrafish

Concentrations(mg/l)	Treatment period (days)			
	5	10	15	20
Control (0.00)	151.35±1.69 (100%)	150.65±1.35 (100%)	149.70±1.60 (100%)	150.25±1.30 (100%)
05.00	140.55±1.63 (93%)	134.73 (89%)	129.30±1.75 (86%)	120.15±1.65 (80%)
09.00	135.60±1.25 (90%)	128.54±1.63 (85%)	120.25±1.37 (80%)	108.96±1.14 (73%)
13.00	129.25±1.65 (85%)	121.35±1.35 (81%)	103.75±1.30 (69%)	91.35±1.28 (61%)
17.00	120.65±1.90 (80%)	106.30±1.25 (71%)	89.34±1.69 (60%)	78.95±1.25 (53%)

Summary of computation for ANOVA

Source of variations	Degree of freedom	Sum of squares	Variance	F-values	Sign. level
Variation due to Operations	3	1876.45	625.48	11.73	P<0.05
Variation due to Concentrations	4	6167.01	1541.75	28.92	P<0.05
Total interaction	12	639.59	53.29		
Total	19				

*Dose selected was below 80% of 96-h LC_{50}.

* Values are mean ± SD of six individual observations and significant at p<0.05 (two-way ANOVA).

Table-2: Effect of Cobalt on CAT activity (µM H_2O_2 utilised/min/mg protein) in the liver of zebrafish

Concentrations(mg/l)	Treatment period (days)			
	5	10	15	20
Control (0.00)	149.60±2.10 (100%)	151.35±1.90 (100%)	150.40±1.36 (100)	151.63±2.25 (100%)
20.00	145.32±1.30 (97%)	141.63±1025 (93%)	135.35±1.73 (90%)	128.25±1.65 (85%)
30.00	139.65±1.67 (93%)	132.26±1.17 (87)	128.62±1.25 (86%)	118.38±1.36 (78%)
40.00	130.90±1.25 (87%)	126.35±1.65 (83%)	120.32±1.15 (80%)	108.85±1.25 (71%)
50.00	122.61±1.15 (82%)	118.73±1.90 (78)	109.21±1.63 (73%)	90.32±1.63 (60%)

Summary of computation for ANOVA

Source of variations	Degree of freedom	Sum of squares	Variance	F-values	Sign. level
Variation due to Operations	3	932.759	310.91	10.10	P<0.05
Variation due to Concentrations	4	3802.33	950.58	30.89	P<0.05
Total interaction	12	369.21	30.76		
Total	19				

*Dose selected were below 80% of 96-h $LC_{50.}$, * Values are mean ± SD of six individual observations and significant at p<0.05 (two-way ANOVA).

Table-3: Effect of Lead on GSH activity (GSH mg/mg protein) in the Liver of zebrafish

Concentrations(mg/l)	Treatment period (days)			
	5	10	15	20
Control (0.00)	3.50±0.16 (100%)	3.54±0.19 (100%)	3.49±0.18 (100%)	3.55±0.21 (100%)
05.00	3.13±0.18 (89%)	3.05±0.26 (86%)	2.96±0.25 (85%)	2.93±0.18 (83%)
09.00	2.98±0.15 (85%)	2.65±0.32 (75%)	2.13±0.15 (61%)	2.01±0.15 (57%)
13.00	2.73±0.20 (78%)	2.17±0.22 (61%)	2.02±0.31 (58%)	1.88±0.22 (53%)
17.00	2.65±0.13 (76%)	2.08±0.16 (59%)	1.96±0.18 (56%)	1.63±0.18 (46%)

Summary of computation for ANOVA

Source of variations	Degree of freedom	Sum of squares	Variance	F-values	Sign. level
Variation due to Operations	3	0.98	0.32	7.31	P<0.05
Variation due to Concentrations	4	5.78	1.44	32.35	P<0.05
Total interaction	12	0.53	0.04		
Total	19				

*Dose selected was below 80% of 96-h LC_{50}.

* Values are mean ± SD of six individual observations and significant at p<0.05 (two-way ANOVA).

Table4: Effect of Cobalt on GSH activity (GSH mg/mg protein) in the Liver of zebrafish

Concentrations(mg/l)	Treatment period (days)			
	5	10	15	20
Control (0.00)	3.55±0.16 (100%)	3.55±0.13 (100%)	3.40±0.33 (100%)	3.38±0.16 (100%)
20.00	3.15±0.13 (94%)	3.12±0.11 (88%)	3.08±0.09 (91%)	3.01±0.15 (89%)
30.00	3.10±0.14 (93%)	3.03±0.13 (85%)	2.95±0.12 (87%)	2.90±0.18 (86%)
40.00	3.02±0.10 (90%)	2.98±0.14 (84%)	2.83±0.20 (83%)	2.70±0.13 (80%)
50.00	2.95±0.16 (88%)	2.88±0.12 (81%)	2.78±0.18 (82%)	2.28±0.17 (67%)

Summary of computation for ANOVA

Source of variations	Degree of freedom	Sum of squares	Variance	F-values	Sign. level
Variation due to Operations	3	0.26	0.08	8.08	P<0.05
Variation due to Concentrations	4	1.25	0.31	28.49	P<0.05
Total interaction	12	0.13	0.01		
Total	19				

*Dose selected was below 80% of 96-h LC_{50}.

* Values are mean ± SD of six individual observations and significant at p<0.05 (two-way ANOVA).

Table 5: Effect of lead on LPO activity (µM of MDA formed/30 min/mg protein) in the liver of zebrafish

Concentrations(mg/l)	Treatment period (days)			
	5	10	15	20
Control (0.00)	13.18±0.65 (100%)	13.57±0.56 (100%)	12.85±0.39 (100%)	13.15±0.73 (100%)
05.00	13.96±0.39 (106%)	14.25±0.83 (105%)	14.59±0.46 (114%)	15.23±0.65 (116%)
09.00	14.15±0.48 (107%)	14.98±0.66 (110%)	15.38±0.73 (120%)	15.96±0.39 (121%)
13.00	14.88±0.67 (113%)	15.59±0.78 (115%)	15.97±0.64 (124%)	16.73±0.68 (127%)
17.00	15.35±0.45 (116%)	16.24±0.45 (120%)	16.38±0.45 (127%)	17.66±0.49 (134%)

Summary of computation for ANOVA					
Source of variations	**Degree of freedom**	**Sum of squares**	**Variance**	**F-values**	**Sign. level**
Variation due to Operations	3	5.23	1.74	9.73	P<0.05
Variation due to Concentrations	4	24.60	6.15	34.28	P<0.05
Total interaction	12	2.15	0.17		
Total	19				

*Dose selected was below 80% of 96-h LC_{50}.

* Values are mean ± SD of six individual observations and significant at p<0.05 (two-way ANOVA).

Table-6: Effect of cobalt on LPO activity (µM of MDA formed/30 min/mg protein) in the liver of zebrafish.

Concentrations(mg/l)	Treatment period (days)			
	5	10	15	20
Control (0.00)	12.88±0.46 (100%)	13.25±0.35(100%)	13.45±0.67 (100%)	12.90±0.43 (100%)
20.00	12.96±0.40 (101%)	13.68±0.43 (103%)	14.13±0.54 (105%)	14.58±0.16 (113%)
30.00	13.08±0.30 (102%)	13.95±0.38 (105%)	14.86±0.39 (110%)	15.04±0.38 (117%)
40.00	13.46±0.62 (105%)	14.15±0.76 (107%)	15.14±0.76 (113%)	15.88±0.43 (123%)
50.00	13.94±0.35 (108%)	14.97±0.54 (113%)	15.93±0.65 (118%)	16.14±0.54 (125%)

Summary of computation for ANOVA					
Source of variations	**Degree of freedom**	**Sum of squares**	**Variance**	**F-values**	**Sign. level**
Variation due to Operations	3	8.33	2.77	14.85	P<0.05
Variation due to Concentrations	4	10.39	2.59	13.88	P<0.05
Total interaction	12	2.24	0.18		
Total	19				

*Dose selected was below 80% of 96-h LC_{50}.

* Values are mean ± SD of six individual observations and significant at p<0.05 (two-way ANOVA).

CONCLUSIONS

The biochemical investigations can be used to study the mode of action of heavy metals and cause for death of aquatic organisms. Thus biochemical alterations in zebrafish may be considered as biomarkers to access the health status of the fishes as well as aquatic bodies polluted by heavy metals. Further research should be done in order to have a clear picture of heavy metal mediated oxidative stress and their effects on the environment and the risk they pose on it.

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