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TOXICOLOGICAL EFFECTS OF TERTIARY MIXTURE OF METALS ON THE OXIDATIVE STRESS IN *CIRRHINA MRIGALA* AND *LABEO ROHITA*

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ABSTRACT

Present research work was conducted to measure the effects of tertiary metals mixture (Fe+Zn+Mn) on superoxide dismutase (SOD) activity in various tissues of two major carps, *Cirrhina mrigala* and *Labeo rohita* at controlled laboratory conditions. 90-day-old fingerlings of both fish species were exposed to 1/4th and 1/5th of their respective 96-hr LC_{50} value of Fe+Zn+Mn mixture, for 24 days. After 6, 12, 18, and 24-day exposure, fish from each treatment will be sampled, dissected and their tissues viz. brain, gills, kidney, and heart isolated for the SOD enzyme assay. The physical and chemical parameters of test media viz. pH, temperature, dissolved oxygen, total hardness, carbon dioxide, total ammonia, magnesium, and calcium were determined on a 12-hourly basis. It was observed that with an increase in metal concentration, the activity of enzymes increased significantly in both fish species which was maximum at 1/4th of LC_{50} with the mean value of 49.35±10.04 UmL^{-1} in *C. mrigala*. In *Labeo rohita*, SOD activity decreased with an increase in exposure duration. SOD activity was maximum on day 6 at 52.22±12.91 UmL^{-1}, and on day 24, it was minimum at 35.01±6.91 UmL^{-1}. Among the organs, the SOD activity followed the trend: gills > heart > kidney > brain. The various tissues of metals mixture treated fish *Cirrhina mrigala* showed significantly increased activity of SOD in comparison to *Labeo rohita*. All the physico-chemical parameters varied significantly at $p<0.05$ during this study period.

Keywords: *antioxidant enzymes, superoxide dismutase, Major carps*

INTRODUCTION

Freshwater has vital importance in human life, but this natural resource is contaminating rapidly due to the addition of inorganic and organic chemicals through leaching from agricultural land which has fertilizers and pesticides and in addition to it urban sewage contaminating the sediments of water bodies with metals and inorganic anions (Owa, 2013). Among these contaminants, heavy metals are the most deleterious pollutants that enter the food chain through aquatic organisms. The uptake of metals in the fish body varies in different fish species with their developmental stage, age, and physiological factors (Gagnaire *et al*., 2004). However, their tolerance limit varies from species to species and from metal to metal. Excessive exposure to metals replaces the cofactor of enzymes that inhibit their normal functioning (Khayatzadeh and Abbasi, 2010). Some metals cause toxicity by disturbing natural processes of transformation, and degradation and by amassing in various fish tissues, especially in the kidney and liver depending upon the affinity of metals with that organs (Oze *et al*., 2005).

In aquatic environments, metals usually affect fish in the mixture form. The effects of a metal mixture are different from that of individual metals due to the interaction of metals to produce synergistic or antagonistic effects within the organisms (Barata *et al*., 2006). Iron, zinc, and manganese are mostly used as trace elements in organisms (Rajkowska and Protasowicki, 2013). Iron (Fe) is mostly present in bound form with the enzymes, ferritin, transferrin, and hemoglobin (Valko *et al*., 2005). Increased level of Fe result in the formation of free radicals and produces reactive oxygen species (ROS) through the Fenton reaction which cause damage to DNA and oxidation of proteins and lipids (Emerit *et al*., 2004)

A level of Manganese (Mn) above its threshold value leads to neurotoxicity, a decrease in the level of RBC and internal hemorrhaging results in hematocrit formation (Chen *et al*., 2006). Manganese may be present in water in the environment from natural sources (rock and soil weathering) or as a result of human activities (such as mining, industrial discharges and landfill leaching). Zinc (Zn) accumulation leads to the production of reactive oxygen species (ROS) through extra-mitochondrial and mitochondrial pathways (Muyssen *et al*., 2006). In mitochondrial ROS production, Zn^{+2} cations interfere with the electron transport chain and inhibit

cellular respiration, while extra-mitochondrial pathways include the activation of protein kinase C which results in increased NADPH oxidase activity (Frazzini *et al*., 2006). To counteract the harmful effects of ROS various defense processes have evolved including the release of antioxidant enzymes to scavenge the highly reactive free radicals. These enzymes include superoxide dismutase (SOD) which removes superoxide radicals and converts it into hydrogen peroxide in mitochondria and peroxisomes (Parthiban and Muniyan, 2011).

Fish is used as a bioindicator to assess the level of aquatic pollution (Rashed, 2011). *Cirrhina mrigala* and *Labeo rohita* are omnivorous fish species so accumulate more metals than carnivorous fish species (Azmat *et al*., 2012). Therefore, this research work was conducted to measure the activity of SOD due to chronic exposure to a tertiary metals mixture (Fe+Zn+Mn) in various organs of *Cirrhina mrigala* and *Labeo rohita*.

MATERIALS AND METHODS

During the present study, juveniles of *Cirrhina mrigala* and *Labeo rohita* were procured from the Fish Seed Hatchery. These experiments were conducted in the laboratories of Fisheries Research Farms, Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad, Pakistan. These fish species were kept in cemented tanks for acclimatization to laboratory conditions. Stock solutions of metals were prepared, separately, by dissolving the chloride compounds of iron, zinc, and manganese in distilled water. To prepare tertiary metals mixture solution these individual stock solutions were mixed on an ion equivalence basis (1:1:1). After acclimatization, three groups (n=10) of 3 months old juveniles of both the fish species were taken and transferred to the 50 L water capacity aquaria separately, to determine the effect of Fe+Zn+Mn mixture on superoxide dismutase activity in the fish gills, kidney, heart and brain. During all the experiments, temperature (28ºC), pH (7.5), and total hardness (225mgL^{-1}) were kept constant. Both the fish species were exposed to $1/4^{th}$ and $1/5^{th}$ of their respective 96-hr LC_{50} of Fe+Zn+Mn mixture as determined by Naz (2013) for 24 days. The calculated sub-lethal concentrations were as follows

Fish species	Treatments	Sub-lethal concentrations (mgL^{-1})
Cirrhina mrigala	$1/4^{th}$	17.72±0.15
	$1/5^{th}$	14.17±0.12
Labeo rohita	$1/4^{th}$	20.98±0.12
	$1/5^{th}$	16.79±0.10

After 6, 12, 18, and 24 days of MM exposure, each fish species was sampled and dissected and their organs viz. gills, kidney, heart, and brain isolated and preserved at -4ºC for the analyses of enzyme activity. The physical and chemical parameters of test media viz. water temperature, pH, dissolved oxygen, carbon dioxide, total hardness, calcium, magnesium, and total ammonia were monitored after 12 hours by following the methods of A.P.H.A. (2012).

Enzyme Assay: Red blood cells were removed from the gills, kidney, heart, and brain by rinsing these organs with a phosphate buffer of pH 6.5 (0.2M) and homogenized in cold buffer (1:4w/v) with pestle and mortar. After homogenization, the organs homogenate were centrifuged for 15 minutes at 10,000rpm at 4ºC. After the centrifugation process, clear supernatant was preserved at -4ºC for enzyme assay while the residue was discarded. For the determination of superoxide dismutase activity, the sample was subjected to enzyme assay by following the methods described by Giannopolitis and Ries (1977).

Procedure: One ml buffer was taken in the cuvette as blank and inserted into the spectrophotometer to note the readings of blank, after taking the reading spectrophotometer was adjusted at zero at A_{560} nm. Then 5-6 cuvettes were taken and set in a lightbox with an internally mounted light bulb of 30 watts. Firstly 1 ml of buffer was added to each cuvette, then 0.05 ml of enzyme extract and 0.016 ml of riboflavin were added in each cuvette. All the cuvettes were incubated in a light box for 12 minutes. The cuvettes were transferred to the spectrophotometer, where 0.067 ml of EDTA/NaCN solution and 0.033 ml of NBT were added to the illuminated reaction mixture. The absorbance was noted after 20 s of reaction. The activity of superoxide dismutase was determined by measuring the % age inhibition of NBT.

Calculation:

$$\%\text{age inhibition} = \frac{\text{Blank (Abs)} - \text{Sample (Abs)} \times 100}{\text{Blank (Abs)}}$$

RESULTS

The antioxidant enzyme, superoxide dismutase (SOD) activity in the tissues viz. brain, gills, kidney, and heart of *Cirrhina mrigala* and *Labeo rohita* was determined after exposure to sub-lethal concentrations of Fe+Zn+Mn mixture. The physical and chemical characteristics of test media viz. water temperature, pH, dissolved oxygen, carbon dioxide, total hardness, calcium, total ammonia, and magnesium contents were monitored on a 12-hourly basis.

Superoxide Dismutase activity *Cirrhina mrigala* :Table 1 shows the variations in SOD activity in the organs of *Cirrhina mrigala* after exposure to Fe+Zn+Mn mixture for 24 days. Analysis of variance shows statistically significant differences among treatments, duration of exposure, and organs for SOD activity in *Cirrhina mrigala* (Table 2). Among the

treatments (control, 1/4th and 1/5th of LC_{50}), significantly higher activity of SOD was recorded as 49.35±10.04 UmL^{-1} in the 1/4th of LC_{50} exposed *Cirrhina mrigala*, whereas the significantly lower activity of SOD was measured in control fish as 38.70± 1.09 UmL^{-1} (Figure 1). During different durations of metal mixture exposure, the SOD activity decreased with increasing exposure durations up to 24 days. On the 6th day of exposure, the activity of SOD was maximum (51.91±4.99 UmL^{-1}), which decreased to 36.99±3.30 UmL^{-1} on the 24th day of metals exposure (Figure 2). In the organs of tertiary metals mixture exposed *Cirrhina mrigala*, significantly higher SOD activity was recorded in the gills with the mean value of 62.98±12.71 UmL^{-1} at day 6 while at day 18, maximum activity was measured in the heart as 48.03±8.98 UmL^{-1}. The order followed by the SOD activity in the organs in all exposure treatments was as follows: 6 > 12 > 18 > 24 days (Figure 3). In the control fish group, significantly higher SOD activity was observed in the gills at 39.30±6.84 UmL^{-1} followed by the heart, kidney, and brain (Figure 4). However, in 1/4th of LC_{50} exposure, maximum activity was observed in the gills with the mean value of 59.62±11.92 UmL^{-1} (Figure 5), and minimum in the brain was 36.04±8.52 UmL^{-1}, while at 1/5th of LC_{50} of Fe+Zn+Mn exposure, the SOD activity followed the order: gills > heart > brain > kidney (Figure 6).

Labeo rohita: The SOD activity in the tissues (gills, brain, heart, and kidney) of *Labeo rohita*, under different treatments of Fe+Zn+Mn mixture, is presented in Table 3. Analysis of variance shows that statistically highly significant differences existed among all the treatments, organs, and in different duration for the induction of SOD activity in fish exposed to tertiary metals mixture (Table 4). The activity of SOD altered significantly ($p<0.05$) in the tissues of fish at three different treatments, showing maximum mean SOD activity in fish treated with 1/4th of LC_{50} of Fe+Zn+Mn mixture.

Among different treatments, SOD activity was higher in 1/4th of LC_{50} as 51.04±5.05 UmL^{-1} followed by 1/5th (45.19±4.89 UmL^{-1}) and control (31.67±1.59 UmL^{-1}). Among the organs, a comparison of means shows that SOD activity followed the trend: gills (45.13±12.48 UmL^{-1}) > kidney (43.15±13.07 UmL^{-1}) > heart (42.10±6.99 UmL^{-1}) > brain (40.15±8.83 UmL^{-1}) (Figure 7). In *Labeo rohita*, SOD activity decreased with an increase in exposure duration. SOD activity was maximum on day 6 at 52.22±12.91 UmL^{-1}, and on day 24, it was minimum at 35.01±6.91 UmL^{-1} (Figure 8). During all the exposure durations significantly higher activity was observed in the gills 45.13±12.82 UmL^{-1}, kidney (43.15±5.03 UmL^{-1}), heart (42.10±3.38 UmL^{-1}), and brain (40.15±12.48 UmL^{-1}). In the control group, maximum activity was observed in the heart as 34.04±4.19 UmL^{-1}, while the kidney and gills showed maximum enzyme SOD activity at both 1/4th and 1/5th of LC_{50} concentrations of the mixture.

Figure 13 reveals a treatment-wise comparison of SOD activity in two species of fish, *Cirrhina mrigala* and *Labeo rohita*, treated with sub-lethal concentrations viz. 1/4th and 1/5th of LC_{50} of Fe+Zn+Mn mixture for different exposure durations. The various tissues of metals mixture treated fish *Cirrhina mrigala* showed significantly increased activity of SOD in comparison to *Labeo rohita*. A similar pattern was recorded for different durations viz. 6, 12, 18, and 24 days after exposure to different concentrations of Fe+Zn+Mn mixture

Table 1. Change in superoxide dismutase activity in the tissues of Fe+Zn+Mn exposed C. mrigala

Treatment	**Duration (Days)**	**Organs**				**Mean±SD**
		Brain	**Kidney**	**Heart**	**Gills**	
Control	**6**	44.47±2.42	45.76±2.51	46.24±2.62	48.34±2.87	**46.20±1.61**
	12	40.73±2.07	39.45±1.93	41.56±2.15	40.57±2.03	**40.58±0.87**
	18	36.87±1.70	31.89±1.18	37.67±1.80	32.74±1.31	**34.79±2.90**
	24	30.73±1.05	32.67±1.29	33.99±1.42	35.54±1.51	**33.23±2.04**
Mean±SD		**38.20±5.87**	**37.44±6.50**	**39.86±5.26**	**39.30±6.84**	
1/4th	**6**	40.67±3.84	51.92±3.59	58.17±3.67	71.18±3.89	**55.48±12.72**
	12	43.75±2.27	64.05±3.15	58.04±3.15	70.13±3.25	**58.99±11.30**
	18	36.46±1.45	47.10±2.35	53.47±3.29	41.32±2.10	**44.59±7.35**
	24	23.26±0.87	29.43±0.99	44.79±2.48	55.83±2.52	**38.33±14.77**
Mean±SD		**36.04±8.52**	**48.12±14.15**	**53.62±5.55**	**59.62±11.92**	
1/5th	**6**	42.68±3.97	48.41±3.49	55.66±3.45	69.42±3.75	**54.04±11.55**
	12	30.21±1.09	40.31±2.95	51.04±3.15	63.47±3.49	**46.25±14.28**
	18	39.63±2.07	31.72±1.34	52.96±2.89	45.01±2.22	**42.33±8.95**
	24	39.93±1.92	20.14±0.67	46.88±2.65	50.69±3.09	**39.41±13.60**
Mean±SD		**38.11±5.44**	**35.14±12.10**	**51.64±3.69**	**57.14±11.25**	

Table 2. Change in superoxide dismutase activity in the tissues of Fe+Zn+Mn exposed *L. rohita.*

Treatment	Duration (Days)	Organs				Mean±SD
		Brain	Kidney	Heart	Gills	
Control	**6**	35.18±1.44	37.16±1.73	38.21±1.84	39.48±1.95	**37.51±1.82**
	12	31.23±1.09	32.47±1.26	33.97±1.40	31.59±1.15	**32.32±1.22**
	18	29.45±0.97	25.63±0.87	35.65±1.54	28.45±0.95	**29.80±4.23**
	24	27.16±0.90	29.23±0.96	28.33±0.93	23.46±0.73	**27.05±2.54**
Mean±SD		**30.76±3.39**	**31.12±4.90**	**34.04±4.19**	**30.75±6.72**	
1/4th	**6**	71.43±3.94	57.15±3.42	48.65±3.34	69.42±3.75	**61.66±10.73**
	12	44.38±1.28	53.18±2.55	39.83±0.82	69.35±3.11	**51.69±13.02**
	18	45.79±2.48	68.61±2.85	53.53±2.59	37.38±1.28	**51.33±13.28**
	24	31.50±1.17	49.31±2.95	40.79±2.48	36.28±2.31	**39.47±7.58**
Mean±SD		**48.28±7.50**	**57.06±8.34**	**45.70±6.26**	**53.11±15.58**	
1/5th	**6**	67.18±3.89	54.24±3.52	46.67±3.80	61.92±3.84	**57.50±8.97**
	12	30.42±1.37	47.67±2.18	52.33±2.70	65.08±3.20	**48.88±14.34**
	18	39.83±2.50	28.15±1.57	44.31±2.95	31.19±1.68	**35.87±7.49**
	24	28.24±1.77	35.01±2.22	42.88±2.65	47.92±2.78	**38.51±8.67**
Mean±SD		**41.42±17.90**	**41.27±11.84**	**46.55±4.16**	**51.53±15.47**	

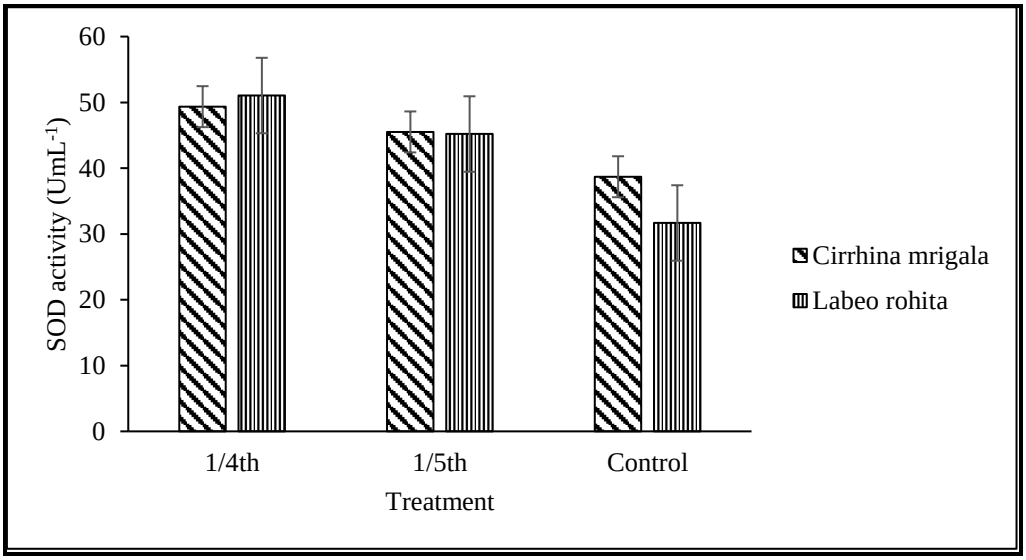

Figure 1. Comparison of SOD activity among C. mrigala and L. rohita exposed to different concentrations of Fe+Zn+Mn

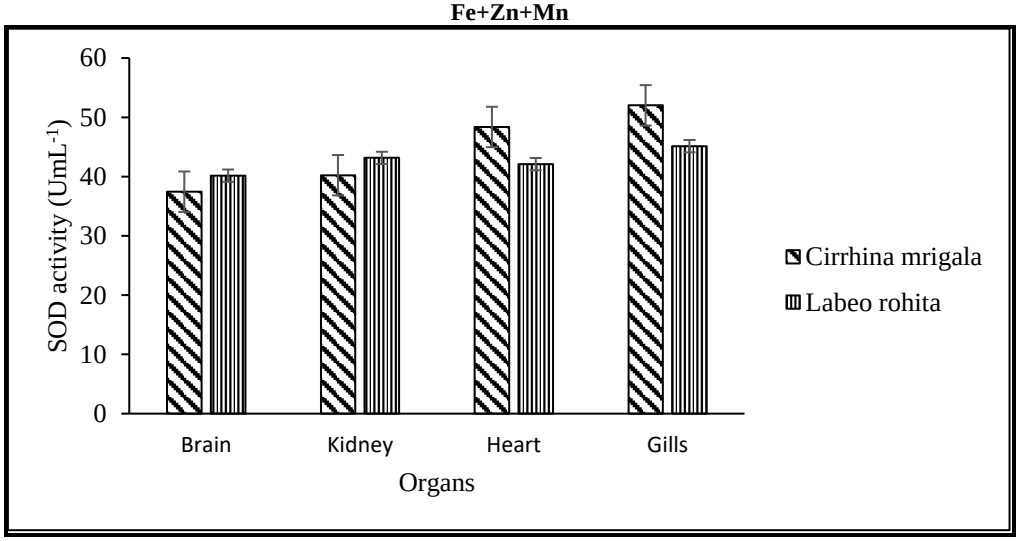

Figure 2. Comparison of SOD activity in different tissues among *C. mrigala* and *L. rohita* exposed to Fe+Zn+Mn.

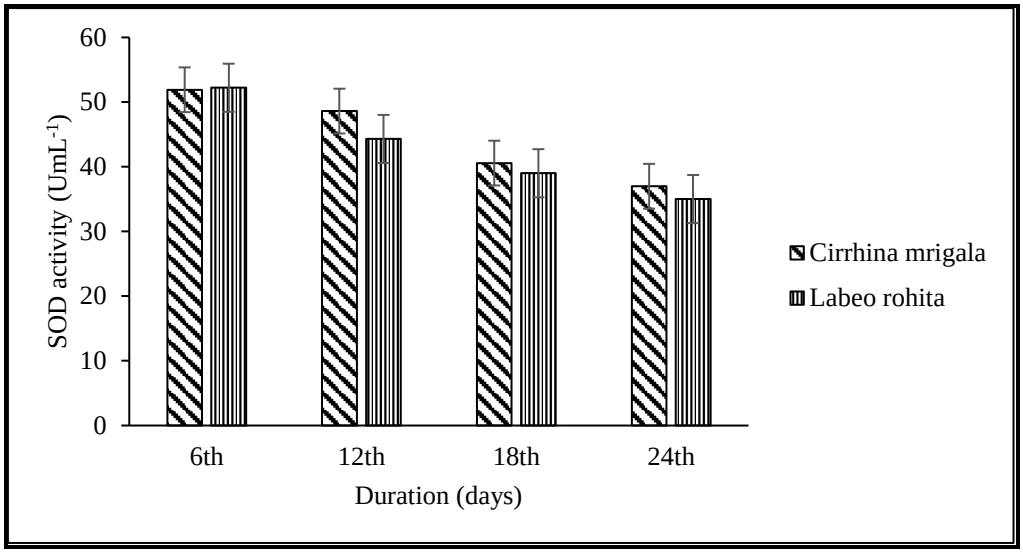

Figure 3. Comparison of SOD activity among *C. mrigala* and *L. rohita* exposed to Fe+Zn+Mn for different durations.

DISCUSSION

Contamination of natural aquatic environments is increasing day by day due to the dumping of untreated industrial and urban wastes that are affecting aquatic fauna adversely (Rauf *et al*., 2009). Metals are present in the effluents in the form of a metals mixture because of different sources of discharge (Lange *et al*., 2002). The mechanism of metal mixture toxicity is different from that of individual metals as metals in mixture form react collectively and produce more lethal effects on the organisms (Jezierska and Witeska, 2001). Iron, zinc, and manganese are the trace metals needed for proper functioning in the fish body (Rajkowska and Protasowicki, 2013). However, excess of these metals can produce various disorders in the body of fish. Iron above its permissible limit may cause neurodegenerative abnormalities and immune system disorders (Fraga and Oteiza, 2002). Disturbance in ionic balance, oxidative stress, and hypoxia can be caused by increased levels of zinc while manganese above its certain level may cause oxidative stress and disturb the neurotransmitters (Avci *et al*., 2005). Therefore, these metals can increase oxidative stress in the body by enhancing the production of reactive oxygen species (Chen *et al*., 2006). To neutralize the effects of these reactive oxygen species, the fish presents a defense mechanism that includes various antioxidant enzymes and SOD is one of them (Pandey *et al*., 2010).

During the present investigation, the activity of SOD increased significantly with an increase in the exposure concentration of MM from $1/5^{th}$ to $1/4^{th}$ of 96- hr LC_{50} values while it was significantly lower in control fish of both species. Therefore, with an increase in exposure concentration of MM, the defense system of the fish would have activated to release more enzymes to counteract oxidative stress caused by the exposure to metals. The activity of enzymes was significantly less in the control group of both fish species. After 10 days exposure of to $1/10^{th}$ and $1/20^{th}$ of LC_{50} values of arsenic to *Clarias batrachus* by Bhattacharya and Bhattacharya (2007) the activity of SOD increased with an increase in the concentration of arsenic which also support the present findings. In another study by Soundararajan *et al*. (2009), 0.1 ppm and 0.05 ppm arsenic were given to two fish groups of *Tilapia mossambica* for 5 and 10 days, respectively. They found that the activity of SOD increased at a higher concentration of exposure to the fish till the 15^{th} day then it decreased significantly with the increase in duration of arsenic exposure. This shows the damage in the defense mechanism of fish due to prolonged exposure to metals. Li *et al*. (2009) exposed the *Oryzias laptipes* toiron. The SOD activity firstly increased with an increase in the concentration of iron while it decreased with the duration of exposure further. Khalid *et al*. (2015) reported that during acute exposure of Cr, Cu, and Cd to the *Labeo rohita*, the level of SOD activity decreased with the increase in metallic ion concentration after 96 hrs of exposure while after chronic exposure of 1 week, the activity was decreased in the liver of *Labeo rohita*. The present results are in conformity with the findings of Mohanty *et al*. (2013) who observed concentration and duration-dependent increase in SOD activity in $CdCl_2$ exposed *Labeo rohita*. Liu *et al*. (2006) exposed the fish, *Carassius auratus* with different concentrations of copper viz. 0.0023, 0.005, 0.01, 0.05 and 0.25 mgL^{-1}, and found that the SOD activity decreased upto 0.05 mgL^{-1} while it increased upto 0.25 mgL^{-1}. The increase in SOD activity with increasing copper concentration was because Cu acts as a co-factor for SOD. In various exposure concentrations of Cd and Cr to the *Channa marulius* (20-140 mgL^{-1}) and *Wallago attu* (10-80 mgL^{-1}) it was found by Batool *et al*. (2014) that increase in the activity of SOD was more significant in *C. marulius* as compared to the *W. attu* that increased with the increasing concentration of Cd and Cr.

Present results also revealed that when the duration of exposure increases from 6 to 24 days the

activity of SOD in both the fish species decreased significantly. In *Labeo rohita*, the activity decreased suddenly while it changed gradually in *Cirrhina mrigala*. The work of Vinodhini (2009) shows in conformity with the present findings in which *Cyprinus carpio* was exposed to Cd, Pb, and Ni for 32 days that caused a significant decrease in the activity of SOD at the 32nd day of exposure in the liver and kidney tissues of the fish. *Channa punctata* was exposed to the metals mixture for 30 days by Pandey *et al*. (2010). After 15 and 30 days of exposure, the activity of SOD significantly decreased in the gills.

Among the organs, it was observed that the activity of SOD was significantly maximum in the gills while it was minimum in the brain in both of the fish species after exposure to the tertiary mixture of metals (Fe+Zn+Mn). Fish gills are the organs of multiple functions as exogenous toxicants have direct contact with the gill lamellae, so MM would attach to the surface of gills to imbalance the physiological functions. Our results conform with the findings of Loro *et al*. (2012) who reported that when *Fundulus heteroclitus* was given exposure to a sublethal concentration of water-borne zinc 500mgL^{-1}, the activity of SOD was significantly increased in the gills than muscles and intestine. In another study by Basha and Rani (2003), the fish tilapia was exposed to water-borne Cd^{+2} that caused an increase in the activity of SOD in both the liver and kidney but this increase was higher in the liver (86.61%) as compared to the kidney (86.32%). The renal activity of SOD was not much increased probably due to its less role in detoxification as compared to the liver. The same findings were observed in the results of Hassan *et al*. (2015) who exposed the fish, *Cirrhina mrigala* to Cd and Cu for 90 days. Statistical variations were seen in the SOD activity of gills and kidneys. Gills showed the highest activity while the kidney exhibited the lowest activity. The activity of SOD was also lowest in the brain of medaka fish than in the liver when it was exposed to iron after 14 days of exposure by Li *et al*. (2009) which confirms present results of low activity of SOD in the brain of both the fish species. Low activity of SOD in the brain shows less penetration of metals in the brain due to blood-brain barrier. When catfish were exposed to chromium for 28 days, the SOD activity was the same in both gills and kidneys, showing the oxidative stress that occurred in the kidney due to chromium exposure (Madhavan and Elumalai, 2016). Contrary to present findings when goldfish were exposed to 20mgL^{-1} of Cd by Zikic *et al*. (2001) the SOD activity decreased after 1 day of exposure while it increased after the 7th and 15th days of exposure.

All the physico-chemical parameters of the test media varied significantly throughout the study period. Temperature, pH, and total hardness were kept constant while total ammonia, carbon dioxide, and calcium level increased significantly with an increase in tertiary metals mixture concentration. The dissolved oxygen and magnesium levels decreased significantly. with the increased level of stress in the metals exposure media. In a study by Hansen *et al*. (2007), it was observed that calcium contents of the test media increased with the increase in exposure concentration of Cd and Zn to the *Salmo trutta*. In another study by Abdullah *et al*. (2007), it was observed that increasing concentration of different metals viz. Zn, Fe, Pb, Ni, and Mn caused a significant decrease in dissolved oxygen contents of the test media while total ammonia and carbon dioxide increased concomitantly with increasing concentrations of metals.

CONCLUSION

In the organs of tertiary metals mixture exposed *Cirrhina mrigala*, significantly higher SOD activity was recorded in the gills with the mean value of 62.98±12.71 UmL^{-1} at day 6 while at day 18, maximum activity was measured in the heart as 48.03±8.98 UmL^{-1}. The order followed by the SOD activity in the organs in all exposure treatments was as follows: 6 > 12 > 18 > 24 days. The various tissues of metals mixture treated fish *Cirrhina mrigala* showed significantly increased activity of SOD in comparison to *Labeo rohita*.

CONFLICT OF INTEREST

The authors have no conflict of interest.

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