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Catalase Enzyme Response to Chronic Pb+Cd Metal Mixture Exposure, Its Purification and Partial Characterization from the Kidney of Freshwater Fish, *Oreochromis niloticus*

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ABSTRACT

In the present study, kidney tissues were selected to measure the catalase (CAT) enzyme activity due to their responsibility in the elimination of the compounds generating reactive oxygen species (ROS). Variation in *Oreochromis niloticus* kidney CAT activity (114.33±0.33 to 159.5±0.03) was observed throughout the study period. During first week of experimental trial, higher CAT activity (159.5±0.03) was noted in Pb+Cd metal mixture stressed fish kidney in comparison to control fish (128.8±0.06). Similarly, lower kidney CAT activity (114.33±0.33) was recorded in second week of experimental trial in metal mixture exposed fish as compared to control group. Significant differences ($p<0.05$) were observed when compared kidney CAT activity among control and metal mixture stressed *O. niloticus*. Partial characterization of *O. niloticus* kidney CAT enzyme after its purification was also performed in this study. Purified kidney CAT showed specific activity 1011.84 and 1314.9 Umg^{-1} for metal stressed and control fish, respectively. Optimum pH, temperature and substrate concentration both for control and binary metal treated fish purified kidney CAT was measured 7.0, 25°C and 50 mM H_2O_2, respectively. Km values for control and metal mixture stressed fish were measured 7.59 and 1.17 mM H_2O_2 mL^{-1}, respectively and Vmax values for control and metal stressed fish were recorded 2.02 and 2.09 UmL^{-1}, respectively. This study will be helpful in understanding how fish oxidative stress biomarker CAT could be used in bio-monitoring studies of metal contamination.

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Authors' Contribution
SA conceived and designed the study. TA conducted the experimental work. TA, KA and MAZ analyzed the data. TA and KA wrote the article.

Key words
Renal antioxidant enzyme, Heavy metal stressed, Nile tilapia, Catalase

INTRODUCTION

Water is an essential component for all living organisms either terrestrial or aquatic. Today, the major problem of the world is the contamination of aquatic ecosystem (Osman and Kloas, 2010) with a wide range of pollutants (Vutukuru, 2005; Dirilgen, 2001; Voegborlo *et al*., 1999) that affect not only flora and fauna of the aquatic ecosystem but also terrestrial ecosystem (Dirilgen, 2001; Ambreen and Javed, 2015).

Extensive urbanization, industrialization, usage of chemical fertilizers and pesticides has increased the heavy metal concentration into the aquatic ecosystems during the past few decades (Faheem *et al*., 2012; Tariq *et al*., 2014). These pollutants has become a potential threat to aquatic organism due to the disturbance in the integrity of biochemical and physiological mechanisms in fish (Palace and Klaverkamp, 1993).

Antioxidant enzymatic systems may be used as a bio-indicator of metal contamination in fresh water aquatic ecosystems (Atli and Canli, 2007) due to their sensitivity to metals as their activities change significantly and could be helpful in environment monitoring studies (Atli and Canli, 2010).

Antioxidant enzymes are essential in coping oxidative stress caused by the metabolism itself and environmental factors. Catalase (CAT) is an antioxidant enzyme that acts as a first defensive line against oxidative stress (Mates *et al*., 1999; Sarwar *et al*., 2014) by break down of H_2O_2 to H_2O and O_2 molecule and help in redox regulation in various body tissues (Faheem *et al*., 2012).

Lead (Pb) and cadmium (Cd) are hazardous heavy metals that come naturally through erosion and manmade activities into aquatic ecosystems (Nanda, 1993) and began to accumulate in different body parts of an organism inhibited in this contaminated area especially fish (Elia *et al*., 2007) and results in distortion of aquatic ecosystem (Gilbertson and Carpenter, 2004) and ultimately threatened to human health (Tchounwou *et al*., 2003). These metals are known as pro-oxidants and become the reason of antioxidant system alteration (Almeida *et al*., 2009; Xie *et al*., 2009; Anushia *et al*.,

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2012; Souid *et al.*, 2013). CAT and superoxide dismutase (SOD) activities in different body tissues are affected in the presence of cadmium because it displace copper and iron from these enzymes (Romeo *et al*., 2000; Ercal *et al*., 2001).

Cadmium administration in fish increases or decreases the activities of CAT, glutathiones and SOD (Basha and Rani, 2003; Atli and Canli, 2007; Firat and Kargin, 2010; Souid *et al*., 2013). CAT activity in gastropods kidney is inhibited when exposed to cadmium and zinc (Chandran *et al*., 2005). Atli *et al*. (2006) observed enhanced CAT activity in liver of Cd exposed *Oreochromis niloticus*. Farombi *et al*. (2007) measured lower CAT activity in liver, gills, heart and kidney tissues of Cd and Cu stressed catfish.

In the field of ecotoxicology, oxidative stress has gained greater attention and it is considered that CAT activity act as a sensitive biomarker before harmful effects occur in fish (Gul *et al*., 2004; Sanchez *et al*., 2005). The present study data will provide an advantageous database for imminent research work about the effects of Pb+Cd metal mixture pollutants in aquatic organism's antioxidant enzyme system.

MATERIALS AND METHODS

Fish Sampling

Freshwater fish fingerlings of tilapia (*Oreochromis niloticus*) were chosen as an experimental animal and collected from the local Fish Seed Hatchery, Faisalabad, Pakistan and transferred live to Fisheries Research Farms at University of Agriculture, Faisalabad, Pakistan.

Acclimatization of fish fingerlings

Collected fish fingerlings were acclimatized to laboratory conditions for two weeks. During the period of acclimatization and experimental trial, 12h light and 12h dark photoperiod was maintained and the fish fingerlings were fed with standard fish feed twice a day.

Experimental trial

Two glass aquaria (one for control and one for Pb+Cd metal mixture stress fish group) were selected for experimental trial. After acclimatization, total 30 fish fingerlings were shifted randomly into selected glass aquaria (15 fingerlings in each aquarium). The duration of experimental trial was remained for two weeks.

Pb+Cd metal mixture toxicity to O. niloticus

Lead chloride and cadmium chloride pure compounds were dissolved in deionized water for making stock solution (1000 ppm) after measuring LC_{50} value of Pb+Cd for *O. niloticus* (55 mgL^{-1}).

At optimum water temperature, pH, dissolved oxygen and total hardness, chronic Pb+Cd metal mixture stress was given to *O. niloticus* fingerlings for two weeks. From stock solution, 183.4 mL Pb+Cd metal mixture solution was added in the aquarium having fingerlings of *O. niloticus* for metal stress. The total quantity of binary metal mixture solution was not added immediately so that fish fingerlings did not die (Naz *et al*., 2008).

Determination of water physico-chemical parameters

Various physico-chemical parameters including temperature, pH, dissolved oxygen, total hardness, total alkalinity, carbonates and bicarbonates were measured on daily basis throughout the experimental trial by following the standard methods described in A.P.H.A (1998).

Extraction of kidney and its homogenization

After completion of experimental trial, the fingerlings were dissected and kidneys were extracted from both the control and Pb+Cd metal mixture exposed *O. niloticus* and stored at -20°C for further analyses. The kidneys were weighed and then homogenized in phosphate buffer (10mM, pH 7.4) 4 times greater than the weight of organ for 15 min with the help of a homogenizer, filtered and centrifuged at 10, 000 rpm for 15 min. Both the pellets and supernatants were separated for further analyses.

CAT enzyme assay

The activity of CAT was determined by measuring its ability to decompose H_2O_2 at 240nm by following the methods of Chance and Mehaly (1977) with some modifications. A 50mM phosphate buffer (pH 7.0) and 10mM hydrogen peroxide (H_2O_2) were prepared to make buffer substrate solution. The reaction mixture (2mL) contained 1.95mL buffered substrate solution and 0.05mL enzymes extract. The buffered substrate solution was used as blank.

Protein content estimation

Biuret method of Gornall *et al*. (1949) was used for the estimation of protein contents with the help of DC Protein Assay Kit (Bio-Rad Laboratories, USA) by using BSA (bovine serum albumin) as standard.

Purification of kidney CAT

Purification of kidney catalase was carried out by using methods of Nakamura *et al*. (2000) with some modifications. All purification steps were carried out at 4°C.

Ammonium sulfate precipitations

Crude extract of CAT was saturated with 25%

ammonium sulfate by dissolving 17.5g ammonium sulfate in 100mL. After 6 hours incubation, it was centrifuge at 13,000 rpm for 15 min at 4°C. The supernatant that was obtained from salting in procedure was subjected to salting out method by adjusting the saturation upto 50%. It was incubated at 4°C for overnight and then centrifuged at 13,000 rpm for 15 min at 4°C. Residues obtained from salting out were re-suspended and subjected to desalting with the help of dialysis bag in phosphate buffer (1.5mM; pH 7.4).

Ion exchange chromatography

The column of DEAE-cellulose (diethyl amino ethyl-cellulose) was prepared (1×20cm) for the purification of kidney CAT. Slurry was prepared and an amount of 250µL desalted sample was applied on column. The sample was eluted out with the help of 10mM phosphate buffer (pH 7.4) while the drop rate was kept constant (1 $mLmin^{-1}$). A total of 50 fractions with 2 mL of elution were collected. All the fractions optical density were noted at 280 nm against blank (buffer). Fractions showing higher absorbance were selected for protein content estimation and enzyme assay.

Gel filtration chromatography

Column (1×20cm) of sephadex G-150 was prepared in phosphate buffer (10mM; pH 7.0). An amount of 250µL of sample (with highest specific activity after ion exchange chromatography) was applied and 50 fractions with 2 mL were collected. Fractions showing higher absorbance at 280nm were selected for protein content estimation and enzyme assay.

Partial characterization of purified kidney CAT

Optimum pH, temperature and buffers concentration was determined by assaying the purified kidney CAT enzyme from both control and Pb+Cd metal mixture exposed Tilapia by following the methods of Nakamura *et al*. (2000) and Al-Bar (2012).

Statistical analysis

Data obtained in this study were analyzed by Mean Standard Deviation (Mean±SD). ANOVA was calculated to measure statistical difference in CAT activity among both metal stressed and control fish at *p<0.05* (Steel *et al.,* 1997). Multiple comparison test was also performed by applying LSD.

RESULTS

The present research work was performed to study the response of CAT against Pb+Cd metal mixture in the kidney tissues of *O. niloticus*. To purify and partially characterize the CAT from the kidney tissues of *O. niloticus* both from control and metal mixture stressed fish was also the objective of this study.

CAT activity in control and Pb+Cd metal mixture exposed O. niloticus

No fish mortality was observed in first week of experimental trial, however, at the end of second week, fish mortality was observed in metal mixture treated aquarium. After the administration of chronic Pb+Cd metal mixture concentration, CAT showed higher activity in kidney tissues of metal mixture stressed *O. niloticus* in comparison to control fish. However, during the second week of experimental trial, lower CAT activity was observed in metal mixture stressed fish as compared to its opponent i.e. control (Fig. 1). Significant differences were observed at *p<0.05* when both control and metal mixture treated fish kidney CAT activity was compared statistically. Multiple comparison test after analysis of variance revealed that all means were significantly different from each other.

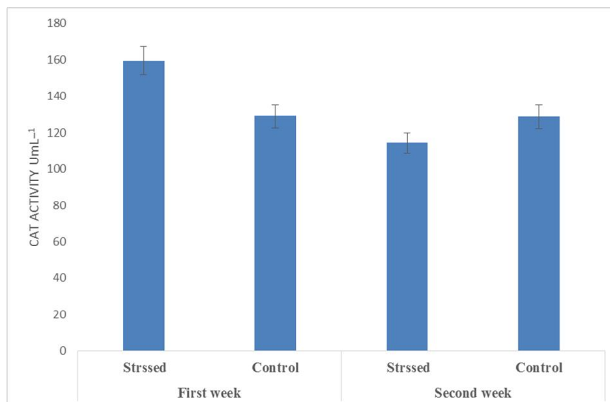
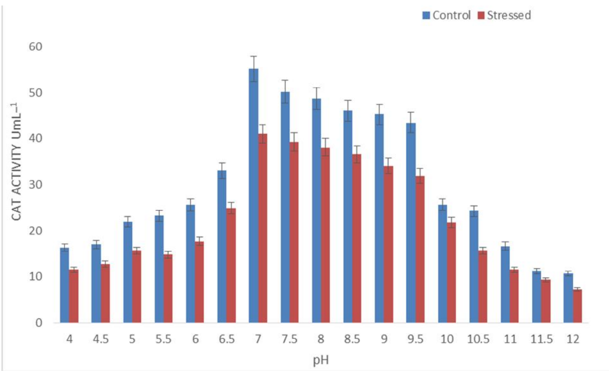


Fig. 1. Comparative CAT activity in kidney of *O. niloticus* during 1st and 2nd week of experimental trial

Significant statistical differences for purification inferences (from homogenate to Sephadex G-150 resins) were observed at *p<0.05* among both fishes. Highest specific activity (1314.9) was observed in control fish compared to metal stressed fish (1011.84). Fold purification was measured 18.95 and 15.66 in this study for control and metal mixture stressed fish, respectively (Table I).

Partial characterization of purified kidney CAT

CAT enzyme purified from control and metal stressed *O. niloticus* kidney was partially characterized. The effect of different pH on purified CAT both from control and metal stressed *O. niloticus* kidney was studied. From both control and metal mixture stressed fish, the pH at which purified CAT revealed maximum activity was measured 7 (Fig. 2A).



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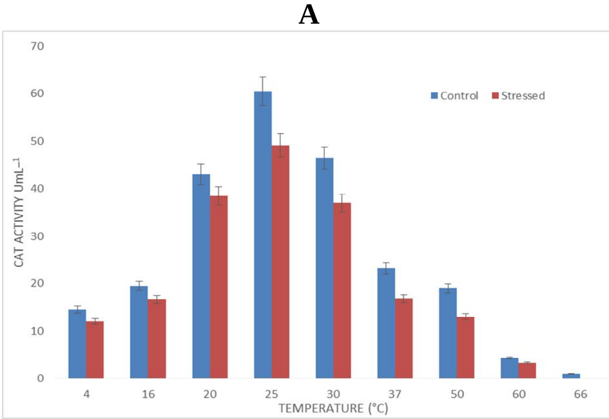

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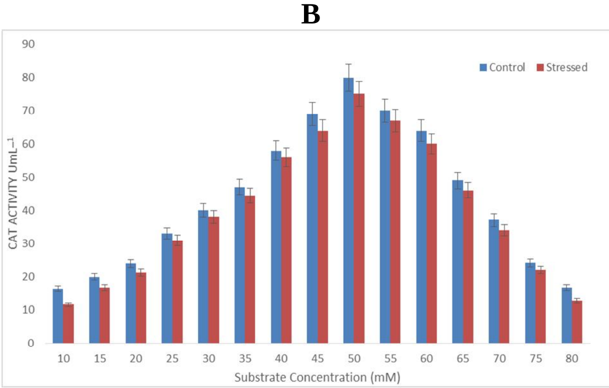


C

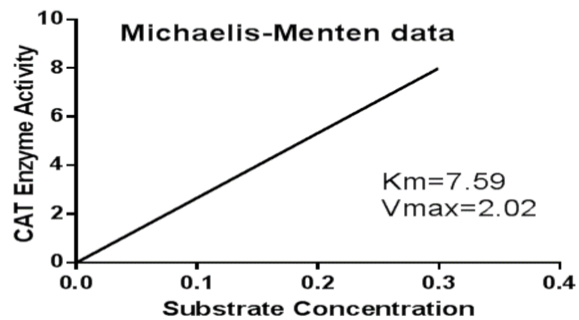
Fig. 2. Optimum pH (A), temperature (B) and substrate concentration (C) of purified kidney CAT control and Pb+Cd metal mixture stressed *O. niloticus.*

By keeping pH at optimum level *i.e.* 7, effect of different temperatures on purified kidney CAT was studied for measuring the optimum temperature. The temperature at which purified kidney CAT showed maximum activity was observed 25°C both for control and metal stressed fish (Fig. 2B).

By keeping the pH and temperature at optimum level *i.e*. 7.0 and 25°C, optimum substrate concentration was determined both for control and metal stressed fish purified kidney CAT. The substrate concentration at which purified kidney CAT showed maximum activity was observed 50 mM both for control and metal stressed fish (Fig. 2C).

Vmax value was measured 2.02 UmL^{-1} for control fish while, 2.09 UmL^{-1} for Pb+Cd metal mixture stressed fish kidney purified CAT. Low value of Vmax indicates CAT stronger ability to bind with H_2O_2 (Fig. 3A).

Km value was noted 7.59 mM $H_2O_2mL^{-1}$ and 1.17 mM $H_2O_2mL^{-1}$for control and Pb+Cd metal mixture stressed *O. niloticus*, respectively (Fig. 3B).



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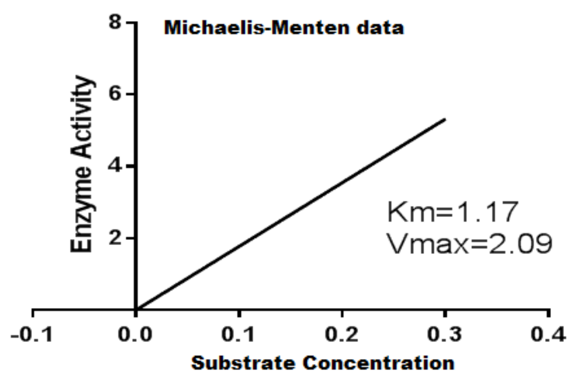

B

Fig. 3. Km and Vmax graph for purified kidney CAT from control (A) and metal stressed (B) *O. niloticus.*

Partial characterization of purified kidney CAT from control and metal stressed O. niloticus

In Table II, all the characterization parameters measured in this study are compared between both control and metal stressed *O. niloticus.* Although the values of optimum pH, temperature and substrate

Table I.- Comparative kidney CAT purification results from control and metal stressed *O. niloticus*.

	Control *O. niloticus* kidney			Metal stressed *O. niloticus* kidney		
Steps	Specific Activity (U/mg)	Yield (%)	Enrichment (fold)	Specific Activity (U/mg)	Yield (%)	Enrichment (fold)
Crude enzyme	69.29	100	1.0	64.59	100	1.0
$(NH_4)_2SO_4$ precipitation	86.42	65.23	1.24	72.43	65.88	1.12
Desalted	89.42	57.14	1.29	74.35	60.64	1.15
DEAE-Cellulose	524.24	46.75	7.56	413.33	54.22	6.40
Sephadex G-150	1314.9	44.67	18.95	1011.84	53.05	15.66

Table II.- Comparative partial characterization of purified kidney CAT from control and metal stressed *O. niloticus.*

Parameters	Control kidney CAT	Metal stressed kidney CAT
Specific activity (U/mg)	1314.9	1011.84
Fold Purification	18.95	15.66
Optimum pH	7.0	7.0
Optimum temperature (°C)	25	25
Optimum phosphate buffer (mM)	50	50
Km (mM $H_2O_2mL^{-1}$)	7.59	1.17
Vmax (mM $H_2O_2mL^{-1}$)	2.02	2.09

concentration are same for both control and metal stressed fish, but the specific activity was recorded lower on the average basis in metal mixture stressed fish as compared to control one. When both control and metal mixture stressed fish kidney CAT activities were compared after measuring its optimum pH, temperature and substrate concentration, significant differences ($p<0.05$) were observed statistically.

DISCUSSION

The present study was conducted to assess the impact of chronic metal mixture on the CAT activity in the *O. niloticus* kidney tissues due to their responsibility in the elimination of the compounds generating reactive oxygen species (ROS). Partial characterization of purified kidney CAT both from Pb+Cd metal mixture stressed and control *O. niloticus* was also performed in this study.

A wealth of information is present in literature in which individual effect of different heavy metals on fish antioxidant systems are studied. However, very little literature was found in which metal mixture effect on fish antioxidant systems studied although in aquatic ecosystem different metals disturb fish physiology jointly not individually.

Aquatic environments are the ultimate destination for most of the metals released from natural and man-made sources. Fish liver and kidney tissues are highly endowed with antioxidant enzymes including catalase (CAT), glutathione peroxidase (GPX), superoxide dismutase (SOD), glutathione S-transferase (GST) and glutathione reductase (GR) to protect them from oxidative stress.

Variation in *O. niloticus* kidney CAT activity was recorded in this study period. During the first week of experimental trial, higher CAT activity was noted in metal mixture stressed fish kidney compared to control fish group. Lower kidney CAT activity was recorded in second week of experimental trial in metal treated fish compared to control group of fish. Significant differences were observed at $p<0.05$ when compared kidney CAT activity among control and metal mixture stressed *O. niloticus*. Variation in responses of the antioxidant enzymes to metal exposures, depending upon body tissues, metals and exposure types (lethal or sub-lethal).

Elevated renal CAT activity in first week of experimental trial in this study is associated with increased production of ROS or oxidative stress by metal mixture. Further, redox active (Cu, Cr and Fe) and redox-inactive metals (Pb, Cd and Hg) can cause significant increases in rate of ROS production and followed by a situation known as oxidative stress that becomes the reason of several dysfunctions in DNA, proteins and lipids (Ercal *et al*., 2001; Pinto *et al*., 2003). Enhance kidney CAT activity in first week of experimental trial are according to the findings of Palace and Klaverkamp (1993) in the liver tissues of rainbow trout (*Oncorhyncus mykiss*), Avci *et al*. (2005) in muscle and hepatic tissues of *Silurus glanis*, Hansen *et al*. (2006) in brown trout (*Salmo trutta*), Atli *et al.* (2006) in brain, gills, liver, kidney and skin of *O. niloticus*, Atli and Canli (2008) in gills, liver and muscle tissues of *O. niloticus*, Lin *et al*. (2011) in gills and liver tissue of genetically improved farmed tilapia (*O. niloticus*).

At the end of experimental trial, decrease in renal CAT activity was observed in metal mixture stressed fish in comparison to control fish which might be associated with direct binding of Pb+Cd to the CAT thiol (-SH) group that transferred active CAT to inactive. Lower CAT activity in various tissues of fish is associated with the direct effect of different metal exposures and increased generation of ROS (Radi and Matkovics, 1988; Basha and Rani, 2003; Dautremepuits *et al*., 2004; Ahmad *et al*., 2005; Atli *et al*., 2006). In the killifish, *Fundulus heteroclitus*, inhibited hepatic CAT activity was found by Pruell and Engelhardt (1980) both in vivo and in vitro exposure to dissolved Cd^{2+}.

Lower kidney CAT activity at the end of experimental trial are according to the findings of Palace *et al.* (1992) who exposed rainbow trout to cadmium and reported lower CAT activity in the hepatic tissues and concluded that reduction in CAT level is due to direct binding of metals that alter its structure. Bainy *et al*. (1996) observed lower CAT activity in erythrocytes, gills, kidney and liver tissues of *O. niloticus* collected from metal polluted areas and are according to the findings of present study inferences. Similarly, Romeo *et al*. (2000) measured lower CAT activity in the kidney tissues of the sea bass, *Dicentrarchus labrax* kept under Cd stress compared to control fish. Lower CAT and glutathione peroxidase activity in hepatic tissues of *Cyprinus carpio* captured from polluted areas compared to non-polluted areas of Karakaya Dam Lake was also observed by Yilmazi *et al.* (2006).

Lower CAT activity was observed in hepatic, gills, cardiac and renal tissues of African catfish kept under cadmium (Cd) and copper (Cu) stressed by Farombi *et al.* (2007). Firat and Kargin (2010) noted lower CAT activity in red blood cells of *O. niloticus* kept under Zn+Cd metal mixture compared to individual metal effect in which higher CAT activity was measured.

CAT activity show variation in different aquatic animals when exposed to metals which depends upon exposed duration, environmental factors, divergence and compounds of heavy metals used for stress (Atli and Canli, 2010). As a result of oxidative stress, fish adapted to either increase or decrease antioxidants level (Firat and Kargin, 2010).

A great improvements on purification and characterization of CAT have been realized in superior organism principally in mammals but less in fish species. From both control and metal mixture stressed fish, the pH at which purified CAT revealed maximum activity was measured 7 and are according to the findings of Peterson and Salin (1995) in *Halobacterium halobium*; Nakamura *et al*. (2000) in beagle dog; Aydemir and Kuru (2003) in chicken erythrocytes; Yasseen and Jadallah (2009) in bovine liver; Zeng *et al*. (2010) in *Serratia marcescens*; Arabaci and Usluoglu (2012) in *Malva sylvestris*; Al-Bar (2012) in liver of *Camelus dromedaries*; Tariq *et al.* (2014) in *Cirrhinus mrigala* liver and Sarwar *et al.* (2014) in liver of *Ctenopharyngodon idella*.

Similarly, temperature at which purified kidney CAT shown highest activity was recorded 25°C and are according to the inferences of Yasseen and Jadallah (2009) in bovine liver, Arabaci and Usluoglu (2012) in *Malva sylvestris*, Al-Bar (2012) in liver of *Camelus dromedaries*, Sarwar *et al.* (2014) in liver of grass carp (*Ctenopharyngodon idella*) and Tariq *et al.* (2014) in liver of *Cirrhinus mrigala*.

Optimum substrate concentration was measured 50mM for purified kidney CAT and are according to findings of Peterson and Salin (1995) in *Halobacterium halobium*, Aydemir and Kuru (2003) in chicken erythrocytes, Arabaci and Usluoglu (2012) in *Malva sylvestris*, Tariq *et al.* (2014) in liver of *Cirrhinus mrigala* and Sarwar *et al.* (2014) in liver of grass carp (*Ctenopharyngodon idella*).

For control and metal mixture stressed fish, Km values were measured 7.59 and 1.17 mM H_2O_2 mL^{-1}, respectively while, Vmax values were recorded 2.02 and 2.09 $UmL^{-1}$for control and stressed fish, respectively in this study. However, no data about Km and Vmax values are available in literature about fish for comparison. Al-Bar (2012) measured Km value for purified CAT 22.7 mM H_2O_2 mL^{-1} and Vmax value for purified catalase was found 7.9 UmL^{-1} in the liver of *Camelus dromedaries.* The K_m value for purified liver catalase was noted 6 mM H_2O_2 in *Ctenopharyngodon idella* by Sarwar *et al.* (2014).

The present study suggested that the enzymes which are antioxidant in function are highly sensitive to metal pollution as their activities change significantly, suggesting they could be helpful in predicting sub-lethal metal toxicity and useful as an early warning tool in bio-monitoring studies.

CONCLUSION

On the basis of this study and previous studies, it is concluded that antioxidant enzymes are helpful in preventing the harmful effects of metals. Moreover, they are cautionary indicators for severe damage to organisms living in aquatic environment. Consequences of existing research work further reveals that CAT is a susceptible bio-indicator of an organism antioxidant defense system. However, it is still essential to study further antioxidant system enzymes in different aquatic animal models to understand better.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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