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Research Article

Biochemical response of earthworm, *Eisenia fetida* to heavy metals toxicity

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Abstract
Soil heavy metal pollution is widespread and has severe adverse effects on soil organisms. Earthworms are the major soil organisms which perform several beneficial ecological functions butare vulnerable to damage from heavy metal pollution of soil. The present study was conducted to evaluate the potential toxicity of arsenic (As) and chromium (Cr) on the biochemical response of the earthworm, *Eisenia fetida*. Following exposure to various sub-lethal concentrations ofAs (34, 68, 102 and 136 mg/kg) and Cr(26, 51, 77 and 102 mg/kg) for 28 days, the levels of several biochemical markers, including superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), Glutathione-S-transferase (GST) and malondialdehyde (MDA) content were assessed. The results showed that both heavy metals significantly ($p<0.05$) impacted the antioxidant enzyme activities and MDA content during the entire exposure period. Compared with the control, SOD, CAT, POD and GST activities increased significantly ($p<0.05$) by (6.21-23.23, 6.32-18.6, 15.87-34.18 and 0.84-5.45% respectively) at14th day, but after prolonged exposure, these activities were significantly ($p<0.05$) decreased (9.58-38.13, 10.09-30.03, 19.05-53.16 and 2.26-9.36% respectively) at 28th day. The contents of MDA showed significant ($p<0.05$) increase (17.84-45.59%) in all exposure groups for entire exposure period. Therefore, it can be concluded that antioxidants play a direct role in the adaptive response of *E. fetida* for survival in heavy metal contaminated soil. This adaptive antioxidant response can be used as an important biomarker to assess the toxicity of heavy metals in the soil ecosystems.

Keywords: Antioxidant enzymes, Biomarker, Earthworms, *Eisenia fetida*, Heavy metals toxicity

INTRODUCTION

Soil heavy metal pollution is a major environmental problem due to the continuous input of heavy metals into the soils through different industrial and agricultural activities (Wu *et al.,* 2012). Because of their mobility, non-degradability and capability of bio-accumulation, heavy metals can pose serious environmental concerns when accumulated in soils in large quantities (Xu *et al.,* 2021). In addition to polluting the soil, higher concentrations of heavy metals alter the soil structure by influencing the soil organisms. Earthworms are important soil ecosystem engineers that influence soil functioning through their burrowing, feeding and casting activities (Wu *et al.,* 2015). They greatly aid in soil formation by decomposing organic debris and serve as an important source of food for many species (Datta *et al.,* 2016; Ma *et al.,* 2016). Soil contaminated with heavy

metals serves as a feeding ground for earthworms leading to bio-accumulation and bio-magnification (Basha and Latha, 2016).They act as important organisms in soil eco-toxicity assessment studies because of their high vulnerability and sensitivity to soil contaminants than other soil organisms (Zheng *et al.,* 2013; Chen *et al.,* 2018). Through their gut and permeable skin, they are regularly exposed to pollutants in soil and accumulate large amounts in their tissues.

In earthworms, heavy metals exposure results in cellular oxidative stress that induces several negative effects, including ionic leakage, membrane per-oxidation, DNA strand breaks and protein cleavage (Li *et al.,* 2015; Wang *et al.,* 2018). Due to their ionic nature, heavy metals can penetrate phospholipids of cell membranes and harm the cells by creating reactive oxygen species (ROS) such as hydroxyl radical (·OH) and perhydroxyl radical (HO_2) (Pratviel, 2012). Previous investigations have showed that earthworms exposed to environmental contaminants may produce ROS which cause oxidative damage to macromolecules such as lipids, nucleic acids and proteins resulting in cell damage (Liu *et al*., 2017; Yao *et al*., 2020; Wen *et al*., 2021). Several pollutants, including heavy metals and pesticides, are known to cause lipid peroxidation. The main oxidation product of cellular lipid membranes is malondialdehyde (MDA) and the amount of MDA can be utilized as a sensitive biomarker of cell damage (Zhang *et al.,* 2014). High concentrations of heavy metals in organisms can lead not only to lipid peroxidation, but also to inactivation of enzymes (Vlahogianni and Valavanidis, 2007). Earthworms can eliminate ROS and reduce lipid peroxidation and related MDA by antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and glutathione-S-transferase (GST) (Shi *et al.,* 2015). SOD and CAT are regarded as primary defense enzymes which protect the biological system from oxidative damage (Roubalova *et al.,* 2015). SOD converts superoxide anions into hydrogen peroxide (H_2O_2) - a less harmful product of the reaction and CAT is the peroxisomal enzyme which catalyzes the scavenging of H_2O_2 produced from SOD activity. Similarly, POD also decomposes H_2O_2 by oxidizing ascorbate and guaiacol (Sun *et al.,* 2007). GST is a phase II enzyme which is involved in xeno-metabolism and detoxification reactions by glutathionylation of xenobiotics (Saleeb *et al.,* 2020).

Antioxidant enzyme activities and MDA are frequently used as biomarkers of heavy metals exposure in earthworms, *E. fetida* (Wang *et al.,* 2016). Studies on various oxidative stress indicators are crucial because they show changes in physiological health of earthworms caused by contaminants (Tiwari *et al.,* 2019). *E. fetida*, an epigeic earthworm species, is commonly employed as standard toxicology test organism because it is simple to cultivate in laboratory and is sensitive to a variety of toxicants (Organization for Economic Cooperation and Development, OECD, 1984). Therefore, the present study was conducted to measure the effect of heavy metals on oxidative damage, particularly on the production of the lipid peroxidation product MDA and antioxidant enzyme (SOD, CAT, POD and GST) activities in the earthworm,*E. fetida*.

MATERIALS AND METHODS

Earthworms and chemicals

The earthworm(*E. fetida*) was selected for the study owing to its high reproductive rate and ease of culture. Earthworms were obtained from the Vermicomposting Unit situated at Department of Zoology & Aquaculture, Chaudhary Charan Singh Haryana Agricultural University Hisar. They were maintained in culture tubs of 40L capacity with cattle dung as substrate. All tubs were covered with gunny bags and the moisture content was maintained constant between 40-45% by sprinkling water. In order to prevent the likelihood of pre-exposure to contaminants, healthy adult earthworms (400-500 mg) from third generation were used as test organisms (Alves *et al*., 2018). The heavy metals used in this experiment were sodium arsenate (AR/ACS) ($Na_2HAsO_47H_2O$) and potassium dichromate (GR) ($K_2Cr_2O_7$) purchased from Hi-Media. All other chemicals used in the study were of analytical grade. Research ethics committee approval was not required for this study because the experimental work was conducted with the lower invertebrate species (earthworms).

Experimental exposure

For achieving this experiment, earthworms were exposed to different concentrations of As and Cr that were designed according to their 14 d median lethal concentrations (14 d LC_{50}) obtained in an acute toxicity test for artificial soil following the OECD (Organisation for Economic Cooperation and Development, 1984)

Table 1. Description of treatments given to *E. fetida*.

Treatment	Description
Control	No heavy metals
T1	Arsenic (34 mg/kg)
T2	Arsenic (68 mg/kg)
T3	Arsenic (102 mg/kg)
T4	Arsenic (136 mg/kg)
T5	Chromium (26 mg/kg)
T6	Chromium (51 mg/kg)
T7	Chromium (77 mg/kg)
T8	Chromium (102 mg/kg)

guideline-207. The treatments (T1 to T8) given to earthworms are detailed in Table 1. Three replicates per doses were maintained. Exposures were performed for a period of 28 days.

Preparation of earthworm tissue homogenates

Earthworms were removed from each tub at 0, 14 and 28 days after exposure and placed in petri plates on wet filter paper for a period of 24 h to depurate their gut content. Then 1g of gut-cleaned earthworms were placed in a pre-chilled mortar with 10 ml of 0.1 M cold phosphate buffer (pH 7.2) and crushed under ice-cold conditions. The homogenate was centrifuged at 10,000 xg for 20 min at 4 °C. For further analysis, the supernatants were stored at -20 °C (Jeyanthi *et al.,* 2016).

Assay of antioxidant enzymes

Estimation of SOD activity

SOD activity was determined by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) using the method of Giannopolitis and Ries (1977). The reaction mixture (3.0 ml) contained 2.5 ml of 60 mM Tris-HCl (pH 7.8), 0.1 ml each of 420 mM L-methionine, 1.80 mM NBT, 90 µM riboflavin, 3.0 mM EDTA and enzyme extract. At the end riboflavin was added. The tubes were shaken properly and illuminated under fluorescent lamps (20W). The reaction was initiated by turning on the light. After 40 minutes of incubation, the reaction was stopped by turning off the light. The tubes were covered with black cloth to block light when the reaction was finished. The control was a non-irradiated reaction mixture that did not exhibit colour development. The reaction mixture without enzyme extract developed maximum colour and its absorbance decreased with the addition of enzyme. The absorbance was recorded at 560 nm. The amount of enzyme that prevents nitro blue tetrazolium photo-reduction by 50% is considered one enzyme unit. Percent inhibition was determined using the formula stated by Asada *et al.* (1974).

Per cent inhibition = V-v/V × 100 …….Eq. 1

Where,

V = Rate of assay reaction in absence of SOD.

v = Rate of assay reaction in presence of SOD.

Estimation of CAT activity

CAT activity was determined by slightly modified method of Xu *et al.* (1997). The assay mixture contained 1 ml of sodium potassium phosphate buffer (0.05M, pH 7.4), 1.25 ml of H_2O_2 (0.066M) and 250 µl of enzyme extract. The blank was prepared by adding 1ml assay buffer to 1.25 ml of H_2O_2 (0.066M) without enzyme extract along with the samples. One unit of enzyme activity was equal to the amount of enzyme consumed after the reduction of H_2O_2 by half over 100 seconds at 25° C. The absorbance of samples was subtracted from blank and the amount of H_2O_2 was calculated from the standard curve.

Estimation of POD activity

POD activity was determined by measuring the rate of guaiacol oxidation in the presence of H_2O_2 at 470 nm by adopting the method of Song *et al.* (2009). 2.15 ml of Potassium phosphate buffer (0.1M, pH 7.0) was pipetted in a cuvette. Then 0.6 ml of guaiacol (1%) and 0.1 ml of enzyme extract were added to it. Thereafter, 25 µl H_2O_2 (100mM) was added to it. The solution was thoroughly mixed and used to adjust 100% transmission at 470 nm. An increase in absorbance was recorded for 3 min at 15 sec. interval and change in O.D. was utilized for the calculation of enzyme activity using a molar extinction coefficient of 26.6 mM^{-1} cm^{-1} for guaiacol oxidation. The quantity of enzyme that resulted in a 0.01 absorbance unit per minute rise was considered one activity unit of POD and the results were expressed as Umg^{-1} protein.

Estimation of GST activity

GST activity was determined following method of Habig*et al.* (1974). The assay mixture contained 200µl CDNB (1 chloro, 2, 4 dinitrobenzene), 200µl GSH (reduced glutathione), 2500µl Na-phosphate buffer (0.1M, pH 6.5) and 100µl enzyme extract. The formation of the adduct of CDNB, *S*-2,4-dinitrophenyl glutathione (DNPG) was monitored by measuring the rate of increase in absorbance at 340 nm. Molar extinction coefficient (9.6 mM^{-1} cm^{-1}) was used to calculate enzyme activity. One unit of GST activity was defined as the amount of enzyme required for producing one nmol of DNPG min^{-1} mg^{-1} protein and the results were expressed as nmol/min/mg of protein.

Estimation of MDA content

For estimation of MDA, gut-cleaned earthworms from each treatment were homogenized in 5 ml TCA (0.1%) and centrifuged at 8000 xg for 15 minutes. The supernatants were used to determine MDA content by following the method of Heath and Packer (1968). In 1 ml supernatant, 4 ml trichloroacetic acid (20%) containing 5% thiobarbituric acid (TBA) was added. The reaction mixture was heated at 95 °C for 30 min. with constant stirring. Then it is cooled quickly on an ice bath and centrifuged at 8000 xg for 10 minutes. The absorbance of the supernatant was recorded at 532 nm against distilled water and the value of non-specific absorption at 600 nm was subtracted from it. The concentration of MDA was calculated by using molar extinction coefficient (155 $Mm^{-1}cm^{-1}$) and the results were expressed as nmol g^{-1}f.wt.

Statistical analysis

All the data, which are presented as the mean ± stand-

ard deviation (SD), were analyzed by one-way analysis of variance (ANOVA) using the SPSS 23.0 software. Post-hoc comparisons (Duncan test) were performed to identify the significant differences between the treatment and control groups. Significant differences from the control were identified as $p<0.05$.

RESULTS AND DISCUSSION

Effect of heavy metals on antioxidant enzyme activities of *E. fetida*

The levels of antioxidant enzymes (SOD, CAT, POD and GST) were actively altered due to heavy metal-induced stress.

SOD activity in response to heavy metals

As shown in Table 2, SOD activity significantly ($p<0.05$) increased in all treatment groups upto 14 days, with the maximum increase (23.23 and 21.65%) recorded at the highest Cr and As concentrations T8 (102 mg/kg Cr) and T4 (136 mg/kg As) respectively. After that, a significant decline ($p<0.05$) in activity was recorded on day 28, with the maximum decline i.e. 38.13% and 34.26% being noted in earthworms treated with T8 and T4 respectively. According to ANOVA results, heavy metal exposure duration, dosage and their interactions had a significant ($p<0.05$) impact on SOD activity.

As the first line of defense against ROS, SOD is crucial in catalyzing superoxide anions into H_2O_2 (Ighodaro and Akinloye, 2018). SOD activity is a reflection of changes in oxidative stress in living cells. Under normal physiological conditions, SOD keeps a dynamic equilibrium and eliminates the excessive O^{2-} from organisms. But, stress can easily disrupt the equilibrium between the formation and removal of O^{2-} by affecting SOD activity (Liu *et al.,* 2011). The increased SOD activity suggests that the exposure of earthworms (*Eudrilus eugeniae*) to high concentrations of heavy metals leads to over production of ROS (Tiwari *et al.,* 2019). This increase may be ascribed to the de novo synthesis of the enzyme protein by activation of the SOD gene by superoxide-mediated signal transduction (Liu *et al.,* 2011). According to (El-Demerdash *et al.,* 2009), the decrease in SOD activity in organisms (rat) after prolonged exposure to arsenic may be due to the accumulation of superoxide radicals which interfere with SOD activity at high concentration of heavy metals.

CAT activity in response to heavy metals

Changes in CAT activity in earthworms exposed to As and Cr are shown in Table 3. CAT activity of the control worms did not show any significant ($p<0.05$) variation during the experimental duration. However, the activity in earthworms increased significantly ($p<0.05$) up to 14 days of heavy metals exposure and decreased as the heavy metals concentration increased on day 28. In the present study, the changes in the CAT activity were in line with those in the SOD activity following 28 daysof exposure to heavy metals. The increase in CAT activity was highest in T8 (102 mg/kg Cr) (18.6%) followed by T4 (136 mg/kg As) (17.35%). However, compared to exposure of 14 days, CAT activities were significantly ($p<0.05$) reduced after 28 days.

CAT is present in peroxisomes and mitochondria and plays an important role in cellular antioxidant defense mechanism due to its ability to decompose H_2O_2 to water and oxygen (Wu *et al.,* 2012).The increased CAT activity in heavy metal exposed worms can be attributed to the increased substrate concentration (Liu *et al.,* 2011). While the inhibition of CAT activity could be explained by an accumulation of H_2O_2 and other ROS that exceed the scavenging capacity and become inhibitors of CAT activity (Liu *et al*., 2018).The present results agree with the findings of Lin *et al.* (2010), who reported

Table 2. Effect of heavy metals on SOD activity of *E. fetida*

Sr. No.	Treatments	SOD activity (U mg^{-1} protein)		
		0 Days	14 Days	28 Days
1.	Control	16.39 ± 0.63^{a}	16.83 ± 0.84^{a}	16.45 ± 0.03^{h}
2.	T1	16.45 ± 0.03^{a}	17.54 ± 0.18^{c}	15.86 ± 0.03^{g}
3.	T2	16.34 ± 0.31^{a}	18.32 ± 0.11^{d}	15.23 ± 0.05^{e}
4.	T3	16.91 ± 0.35^{a}	19.15 ± 0.13^{f}	14.65 ± 0.03^{d}
5.	T4	16.65 ± 0.10^{a}	21.25 ± 0.84^{h}	13.97 ± 0.09^{b}
6.	T5	16.48 ± 0.06^{a}	17.36 ± 0.10^{b}	15.48 ± 0.01^{f}
7.	T6	16.35 ± 0.31^{a}	18.73 ± 0.06^{e}	15.16 ± 0.02^{e}
8.	T7	16.77 ± 0.32^{a}	19.56 ± 0.04^{g}	14.53 ± 0.01^{c}
9.	T8	16.49 ± 0.42^{a}	21.48 ± 0.01^{i}	13.29 ± 0.03^{a}

Mean±SD; Values not sharing the same letter are significantly different (Duncan, $P<0.05$).

that the CAT activity followed a trend of increase and decrease as the duration of exposure of earthworms (*E. fetida*) to triclosan increased. The present results also corroborate with the studies of Tiwari *et al.* (2019) who found that exposure to pesticides at low and high concentrations significantly altered the activities of antioxidant enzymes in earthworm, *E. eugeniae*.

POD activity in response to heavy metals

The POD enzyme activity in *E. fetida* exposed to As and Cr over 28 days is shown in Table 4. A concentration-dependent significant increase ($p<0.05$) until day 14 was observed, with the elevated POD enzyme activities most marked (29.87 and 34.18%) in the earthworms exposed to the highest concentration of As and Cr i.e. T4 (136 mg/kg As) and T8 (102 mg/kg Cr) respectively. However, on day 28, the POD activities of these treatments were significantly ($p<0.05$) lower than that of the controls, and at this time point, the POD activity decreased with an increase in the heavy metals concentration.

POD can remove H_2O_2 by oxidizing co-substrates like ascorbate or guaiacol (Zhang *et al.,* 2013). POD activity can be reduced either due to a decrease in protein synthesis or irreversible inactivation of enzymes by the accumulation of free radicals produced by the metabolism of pollutants (Liu *et al.,* 2011). Similar results were obtained by Chao *et al.* (2016) who reported that POD activity in earthworms (*E. fetida*) decreased when exposed to different concentrations of lead compared to control.

GST activityin response to heavy metals

As shown in Table 5, after treatment with heavy metals, significant enhancement in GST activity was observed until day 14in a dose and time-dependent manner.

Table 3. Effect of heavy metals on CAT activity of *E. fetida*

Sr. No.	Treatments	CAT activity (U mg^{-1} protein)		
		0 Days	14 Days	28 Days
1.	Control	21.45 ± 0.16^{a}	21.65 ± 0.50^{a}	21.38 ± 0.36^{d}
2.	T1	$21.63\pm0.06^{a,b}$	23.09 ± 0.58^{b}	20.76 ± 0.62^{d}
3.	T2	21.17 ± 0.88^{a}	$24.12\pm0.52^{c,d}$	$19.64\pm0.34^{b,c}$
4.	T3	22.21 ± 0.14^{b}	$25.53\pm0.11^{f,g}$	$19.43\pm0.72^{a,b}$
5.	T4	$21.63\pm0.31^{a,b}$	$26.17\pm0.78^{f,g}$	$18.71\pm0.66^{a,b}$
6.	T5	22.25 ± 0.04^{b}	$23.43\pm0.08^{b,c}$	$20.49\pm0.39^{c,d}$
7.	T6	$21.76\pm0.27^{a,b}$	$24.52\pm0.88^{d,e}$	$19.37\pm0.70^{a,b}$
8.	T7	21.23 ± 0.13^{a}	$25.31\pm0.59^{e,f}$	$18.97\pm0.24^{a,b}$
9.	T8	21.44 ± 0.31^{a}	26.34 ± 0.33^{g}	18.43 ± 0.76^{a}

Mean±SD; Values not sharing the same letter are significantly different (Duncan, $P<0.05$).

Table 4. Effect of heavy metals on POD activity of *E. fetida*

Sr. No.	Treatments	POD activity (U mg^{-1} protein)		
		0 Days	14 Days	28 Days
1.	Control	0.59 ± 0.02^{c}	0.61 ± 0.00^{a}	0.60 ± 0.01^{f}
2.	T1	$0.53\pm0.02^{a,b}$	0.63 ± 0.00^{a}	0.51 ± 0.00^{e}
3.	T2	$0.56\pm0.01^{b,c}$	0.68 ± 0.01^{b}	0.47 ± 0.00^{d}
4.	T3	0.58 ± 0.02^{c}	0.73 ± 0.01^{c}	0.43 ± 0.01^{c}
5.	T4	$0.54\pm0.02^{a,b}$	$0.77\pm0.03^{d,e}$	0.39 ± 0.01^{b}
6.	T5	0.52 ± 0.01^{a}	0.62 ± 0.01^{a}	0.50 ± 0.00^{e}
7.	T6	0.51 ± 0.01^{a}	0.70 ± 0.01^{b}	0.46 ± 0.01^{d}
8.	T7	$0.53\pm0.01^{a,b}$	$0.75\pm0.01^{c,d}$	0.39 ± 0.00^{b}
9.	T8	$0.52\pm0.01^{a,b}$	0.79 ± 0.03^{e}	0.37 ± 0.01^{a}

Mean±SD; Values not sharing the same letter are significantly different (Duncan, $P<0.05$).

Maximum increases i.e. 5.12 and 5.45% were observed in T4 (136 mg/kg As) and T8 (102 mg/kg Cr) respectively. However, the increase was less intense in T1 (34 mg/kg As), T2 (68 mg/kg As), T5 (26 mg/kg Cr) and T6 (51 mg/kg Cr) with no significant ($p<0.05$) differences in GST activity. After 14th day, GST activity showed a tendency of decrease but only became significant ($p<0.05$) with concentrations T4 and T8 compared to the control.

GST is an important phase II enzyme in earthworms that plays a key role in the cellular detoxification of various xenobiotic compounds (Ezemonye and Tongo, 2010). It is primarily involved in the chemical disposition of toxins and has the catalytic capacity to conjugate glutathioneto different hazardous electrophiles and inactive aromatic compounds via non-catalytic binding (Ray *et al.,* 2019).The present investigation clearly showed that the GST activity was severely affected by the high concentrations (T3, T4, T7 and T8) of heavy metals. Maity *et al.* (2008) also found that in *Lampito-mauritii*, GST activities were increased initially when exposed to different concentrations of Pb (75, 150 and 300 mg/kg) in contaminated soil.

According to Xu *et al.* (2013), slight oxidative stress triggers the expression of antioxidant enzymes that enable organisms to partially or completely overcome stress caused by hazardous chemicals. However, severe oxidative stress can suppress the activity of antioxidant enzymes because of excessive ROS. Wang and Xie (2014) reported that the combined pollution of Cu, Pb and Cd had a significant induction effect on the activities of GST and SOD in earthworms (*E. fetida*) and these enzyme activities were closely related to the concentration of heavy metals as well as the duration of exposure. The presentfindings on antioxidant enzymes

Table 5. Effect of heavy metals on GST activity of *E. fetida*

Sr. No.	Treatments	GST activity (nmol min^{-1} mg^{-1} protein)		
		0 Days	14 Days	28 Days
1.	Control	102.34±0.01^{a,b}	102.93±1.07^{a,b}	103.23±0.30^{c}
2.	T1	103.28±1.86^{b}	104.16±1.41^{b,c}	101.81±2.75^{c}
3.	T2	103.56±1.59^{b}	105.29±1.61^{b,c,d}	101.25±1.37^{b,c}
4.	T3	101.63±3.11^{a,b}	105.75±0.57^{c,d,e}	99.45±1.07^{a,b}
5.	T4	102.45±1.11^{a,b}	107.98±0.59^{d,e}	98.39±1.41^{a}
6.	T5	99.96±1.08^{a}	100.28±2.80^{a}	98.11±0.44^{a}
7.	T6	100.73±0.18^{a,b}	102.53±0.37^{a,b}	98.93±0.27^{a}
8.	T7	101.79±0.55^{a,b}	106.18±1.91^{c,d,e}	98.42±0.35^{a}
9.	T8	102.37±1.11^{a,b}	108.27±1.46^{e}	98.13±0.53^{a}

Mean±SD; Values not sharing the same letter are significantly different (Duncan, $P<0.05$).

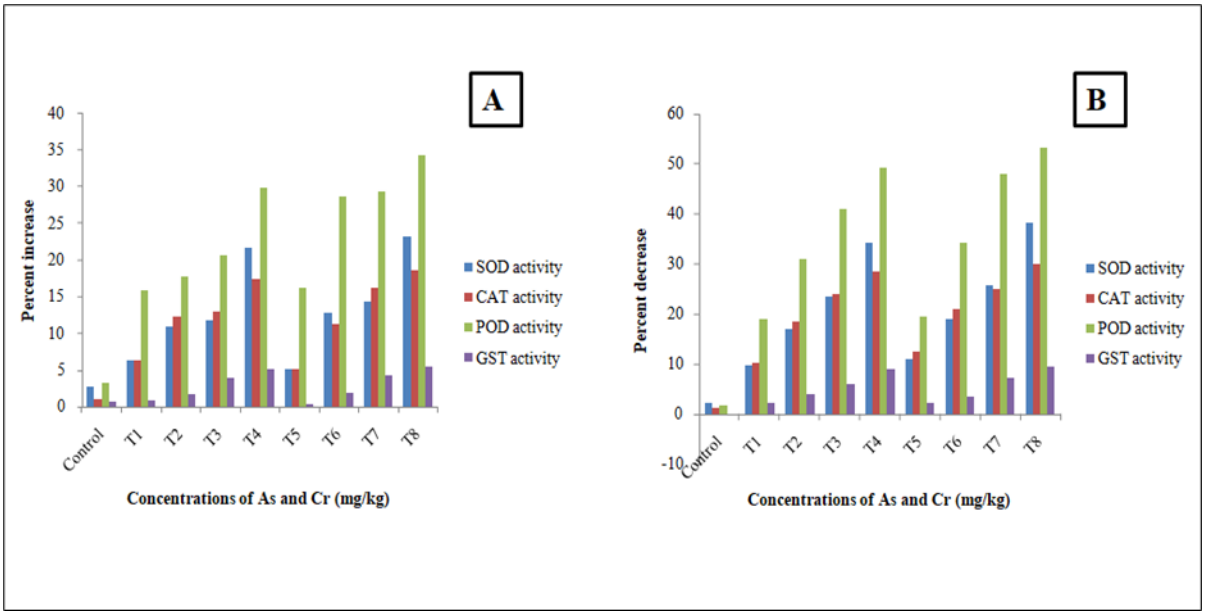

Fig. 1. *(A) Percent increase in antioxidant enzymes from 0 to 14 days of As and Cr exposure (B) Percent decrease in antioxidant enzymes from 14 to 28 days of As and Cr exposure*

Table 6. Effect of heavy metals on MDA content of *E. fetida*

Sr. No.	Treatments	MDA (nmol g^{-1}f.wt.)		
		0 Days	14 Days	28 Days
1.	Control	$3.59\pm0.13^{c,d,e}$	$3.76\pm0.15^{a,b}$	3.94 ± 0.04^{a}
2.	T1	$3.27\pm0.12^{a,b}$	3.61 ± 0.06^{a}	3.98 ± 0.08^{a}
3.	T2	3.11 ± 0.07^{a}	3.93 ± 0.08^{b}	4.23 ± 0.03^{c}
4.	T3	$3.50\pm0.13^{c,d}$	5.24 ± 0.09^{c}	6.11 ± 0.15^{d}
5.	T4	$3.68\pm0.15^{d,e}$	5.53 ± 0.18^{d}	6.54 ± 0.07^{f}
6.	T5	$3.62\pm0.14^{c,d,e}$	3.88 ± 0.12^{b}	$4.03\pm0.12^{a,b}$
7.	T6	$3.41\pm0.13^{b,c}$	$3.83\pm0.12^{a,b}$	$4.17\pm0.11^{b,c}$
8.	T7	$3.57\pm0.15^{c,d,e}$	5.31 ± 0.17^{c}	6.26 ± 0.08^{e}
9.	T8	3.76 ± 0.06^{e}	5.74 ± 0.13^{e}	6.91 ± 0.01^{g}

Mean±SD; Values not sharing the same letter are significantly different (Duncan, P<0.05).

are also in agreement with the previous studies on heavy metals exposure (Liu *et al.,* 2015; Maity *et al.,* 2018; Otmani *et al.,* 2018). Liang *et al.* (2022) also observed that in earthworms (*E. fetida*), Cd exposure enhanced the activities of SOD, POD, CAT and GST.

Effect of heavy metals on MDA content of *E. fetida*

In comparison with the control, enhancement in MDA content was observed in all treatment groups after 14 and 28 days of exposure (Table 6). But there was no clear change in the MDA content of the earthworms exposed to T1(34 mg/kg As), T2 (68 mg/kg As), T5 (26 mg/kg Cr) and T6 (51 mg/kg Cr) for the entire exposure period. However, the MDA contents of the T3 (102 mg/kg As), T4 (136 mg/kg As), T7 (77 mg/kg Cr) and T8 (102 mg/kg) were significantly greater ($p<0.05$) than the control level on days 14 and 28, with increases of 42.72, 43.73, 42.97 and 45.59% respectively. The ANOVA results indicated that there was significant ($p<0.05$) influence of the dose of heavy metals, exposure duration, and interaction of dose and duration on the MDA content.

Increased MDA levels represent the extent of intracellular oxidative damage caused by excessive ROS levels. Lipid peroxidation is therefore frequently utilized as a sign of increased oxidative damage (Xue *et al.,* 2009; Lin *et al.,* 2010). It is one of the most important products of the body's membrane lipid peroxidation. The non-significant changes in T1, T2, T5 and T6 exposure groups may be explained by the possibility that excessive ROS can be scavenged by activated antioxidant enzymes that reduced the oxidative stress and prevented the accumulation of MDA in earthworms. However, the observed increase in the MDA content under the high concentrations can be attributed to an increase in H_2O_2 and excessive ROS, which resulted in peroxidation of lipid membranes (Liu *et al.,* 2012; Zhang *et al.,* 2013). Increased lipid peroxidation indicated by the elevated MDA content with heavy metals concentration is consistent with the results of (Xue *et al.,* 2009; Markad *et al.,* 2015; Wang *et al.,* 2016 and Chao *et al.,* 2016).

Conclusion

The study of biochemical response including antioxidant enzymes (SOD, CAT, POD and GST) and MDA content indicated ROS production and oxidative stress in *E. fetida* after exposure to different concentrations of As and Cr. The results suggested that the low concentrations of heavy metals may increase antioxidant enzyme activity in the earthworm *E. fetida*, but that high concentrations and prolonged exposure inhibited the activity of these enzymes. Therefore, it can be concluded that antioxidants play a direct role in the adaptive response of *E. fetida* for survival in heavy metal contaminated soil and these changes in the levels of antioxidant enzymes (SOD, CAT, POD and GST) and MDA content can be used as important biomarkers for monitoring toxicity of pollutants in the soil ecosystem.

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Conflict of interest

The authors declare that they have no conflict of interest.

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