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Super oxide dismutase, Catalase and glutathione peroxidase activity in heavy metals contaminated earthworm, *Eisenia fetida*

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Abstract
The present study of 90 days was carried out to analyze the toxic effects of heavy metals *viz.* copper, tin and their combination on anti-oxidative enzymes *viz.* super-Oxide Dismutase, Catalase and Glutathione peroxidase in *Eisenia fetida.* The worms for this observation were exposed to different concentrations of heavy meals *viz.* Cu (0.06 ppm, 0.08 ppm, 0.10 ppm), Sn (0.06 ppm, 0.08 ppm, 0.10 ppm) and Cu+Sn (0.03+0.03 ppm, 0.04+0.04 ppm, 0.05+0.05 ppm). A dose and time dependent increase in activities of these enzymes was observed. Cu was found to be more toxic at its highest concentration followed by the highest concentration of combination of both metals Cu+Sn (0.05+0.05 ppm). Not much significant increase in activity of enzymes was observed with Sn.

Keywords: Anti-oxidative enzymes, Catalase, *Eisenia fetida*, Peroxidase, Super-Oxide Dismuatse

1. Introduction
Heavy metals constitute a group of elements which vary in their chemical as well as biological properties, as the name indicates these metals have specific gravity > 5 g-cm^{-3} (Holleman and Wiberg, 1985) [6]. Heavy metals kept under environment pollutant category due to their toxic effect on animals including human, plants and their food as well. Heavy metals have a property to persistent due to which they will get accumulate but not metabolized or breakdown into other intermediate compound. Hence these metals are accumulating in food chain through uptake at primary producer level and then through consumption at consumer level.
The earthworms have the property to bioaccumulate the contamination such as heavy metals, pesticides *etc.* These worms are present at the base of food chain hence the understanding and the detail study of the effect of these pollutants (heavy metals) on earthworms is very essential for predicting the potential food chain impacts of soil contamination (Roubalova *et al.*, 2014) [12]. Earthworms have many interactions with the soil and due to these interactions the earthworms are significantly affected by the pollution originated by the rigorous use of heavy metals and biocides in agriculture, industrial activities, and atmospheric deposition. Hence earthworms have been proved as valuable bio-indicators of soil pollution (Lanno *et al.*, 2004) [8]. The species, *E. fetida* is an ideal species for predicting the effects of heavy metals on them due to the limited difference between their sensitivity to metals and the ease with which they can easily be reared and handled in the laboratory (Edwards and Coulson, 1992) [4].
In earthworm, defense system is present against lipid peroxidation (under stress condition) which is 'antioxidant defense system'. There are many important enzymatic scavengers of superoxide ion ($O_2^{-\bullet}$) and hydroxide ion (OH^-) such as SOD (Super Oxide Dismutase), CAT (Catalase), GPx (Glutathione Peroxidase). These enzymes act as scavengers by preventing the generation of hydroxyl free radical and thus protect the cellular constituents from oxidative damage (Scott *et al.*, 1991) [13].
In the present investigation toxicity of Copper and Tin in earthworms was analyzed, as copper is a micronutrient (means toxic when taken in excess in requirements) (Monni et al., 2000; Blaylock and Huang, 2000) [10,1] and Tin is abundant in rural areas, hence the present investigation was carried out to study the toxic effects of copper and tin on the epigeic earthworm species *E. fetida* with the objective "To study the effect of heavy metals toxicity on antioxidative enzymes activity in *Eisenia fetida".*

2. Materials and Methods

2.1 Collection of earthworms

Fully clitellated *E. fetida* were procured from the stock culture maintained in Vermitechnology unit, Department of Zoology, Chaudhary Charan Singh Haryana Agricultural University, Hisar.

2.2 Experimental setup

Experiment was performed for 90 days in tubs of 30L capacity. Then further investigation was done after 90 days. The properly washed twenty earthworms after weighing inoculated in tubs having predigested and very well aerated cowdung provided by Department of Microbiology, CCS HAU, Hisar. Triplicates were maintained for each treatment. Treatments of different concentrations of heavy metals were given by direct spray in corresponding tubs according to Table 1.

Table 1: Description of treatments given to the earthworms along with control

Sr. No.	Treatments	Description
1.	Control	0.00 (ppm)
2.	T1	Copper (0.06 ppm)
3.	T2	Copper(0.08 ppm)
4.	T3	Copper (0.10 ppm)
5.	T4	Tin (0.06 ppm)
6.	T5	Tin(0.08 ppm)
7.	T6	Tin (0.10 ppm)
8.	T7	Copper+Tin (0.03+0.03 ppm)
9.	T8	Copper+Tin (0.04+0.04 ppm)
10.	T9	Copper+Tin (0.05+0.05 ppm)

2.3 Sample preparation

All procedures were carried out at 4 ºC then samples were frozen at -80 ºC until further use.

2.4 Preparation of Enzyme extract

After 90th day of treatment, earthworms were removed from each treated tub and placed on wet filter paper in petri-plates for a period of 24 h to allow depuration of their gut contents. 1 gm of gut cleared earthworm was homogenized in prechilled mortar and pestle in 50 mM phosphate buffer (pH 7.2) for SOD, 50 mM phosphate buffer (pH 7.4) for Catalase and in 100mM phosphate buffer (pH 6.0) for POD (Plate 2). The homogenate was centrifuged at 10,000 rpm for 10 min. at 4°C and the supernatant was used further for the assay of enzyme activity (Plate 3).

a. Assay of Super-Oxide Dismutase: The 3 ml assay mixture contained 2.5 ml of phosphate buffer, 0.1 ml EDTA, 0.1 ml methionine, 0.1 ml NBT, 0.1 ml riboflavin and 0.1 ml enzyme extract. Riboflavin was added last, and the tubes were shaken and illuminated with 4000 lx fluorescent tubes. The reaction was allowed to proceed for 20 min., then lights were switched off and the tubes were covered with black cloth. Then the absorbance of reaction mixture was read at 560 nm. One unit of SOD activity (U) was defined as the amount of enzyme required to cause 50% inhibition of NBT photo-reduction rate, and the results was expressed as U mg^{-1} protein.

$$\text{Percent inhibition} = \frac{V-v}{V} \times 100$$

Where,
V= Rate of assay reaction in absence of SOD
v= Rate of assay reaction in presence of SOD

b. Assay of Catalase: To 0.4 ml H_2O_2, 0.55 ml phosphate buffer was added along with 50 µl enzyme extract. This mixture was mixed thoroughly and incubated for 1 minute. Then after incubation 3.0 ml of potassium dichromate: acetic acid in 1:3 ratios was added to it. The control tube containing 0.6 ml buffer with 0.4 ml H_2O_2 without enzyme, all these tubes were kept in boiling water bath for 10 min, cool and the absorbance was recorded at 570 nm using dichromate: acetic acid as blank. After subtracting the absorbance of samples from that of control the amount of H_2O_2 can be calculated. One enzyme unit of CAT is defined as amount of enzyme required to consume one µmol of H_2O_2 U mg^{-1} protein.

c. Assay of glutathione Peroxidase: 2.15 ml phosphate buffer was pipetted in a cuvette. To it 0.6 ml of guaiacol (0.01%) and 0.1 ml enzymes extract was added to it. Thereafter, 25 µl of H_2O_2 was added to it. The solution was mixed well and 100% transmission at 470 nm was adjusted with it. Increase in absorbance was recorded for 3 min at 15 sec interval. One activity unit of POD was defined as the amount of enzyme required to oxidize one n-mole guaiacol, and the result was expressed as U mg^{-1} protein.

***Chemicals:** Phosphate buffer (50 mM for SOD [pH 7.2], CAT [pH 7.4] and 100mM for POD [pH 6.0]), Ethylene diamine Tetra Acetate (EDTA) (100 µM), Methionine (130 µM), Riboflavin (20 µM), Nitro Blue Tetrazolium (NBT) (750 µM), Peroxide (H_2O_2) (0.2 M for CAT and 30% for POD), Guaiacol (0.01%), 5% potassium dichromate, glacial acetic acid. Other chemicals of rigorously pure grade were taken from vermitechnology lab (CCS HAU, HISAR).

2.5 Statistical Analysis: The data is the Arithmetic mean of triplicates where means was compared by two-way ANNOVA, using software 'OPSTAT", developed at the Computer centre, College of Basic Sciences and Humanities, CCS Haryana Agricultural University, Hisar.

Plate 1: *Eisenia fetida* (Dorsal Side)

Plate 2: Homogenate

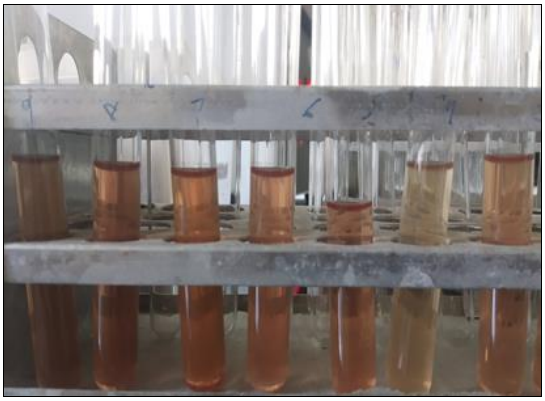

Plate 3: Samples after centrifugation

3. Results and Discussion

The activity of antioxidative enzymes (SOD, CAT & POD) increased after 90 days of exposure to heavy metals as compared to 1st day of treatment. Both metals did not exhibit synergistic effect *i.e.* only Copper is toxic for the earthworms at its highest concentration and also in combination with Tin.

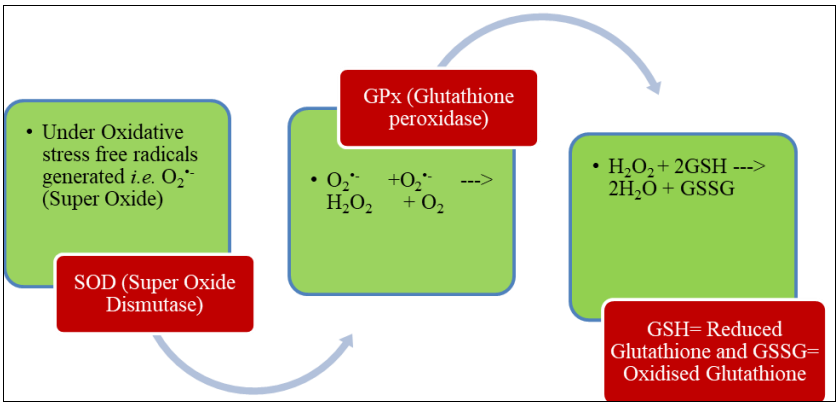

Plate 4: Action done by Antioxidative enzymes

3.1 Super Oxide Dismutase

SOD is an antioxidative enzyme which act first after the generation of Reactive Oxygen Species (ROS) and convert superoxide ions into peroxide ($O_2^{-\bullet}$ to H_2O_2). Dose and time dependent increase in SOD activity was observed in worms treated with various concentrations of copper and tin (Fig 1). The activity of SOD in the control group was observed to be 6.74 U-mg^{-1} protein and 6.96 U-mg^{-1} protein on 1st and 90th day of experiment, respectively. The activity of SOD increased in all treatments till the 90th day of experiment. Noticeably, the highest SOD activity was 8.38 U-mg^{-1} protein and 8.10 U-mg^{-1} protein in Cu (0.10 ppm) and Cu+Sn (0.005+0.05 ppm), respectively followed by 7.37 U-mg^{-1} protein in Sn (0.10 ppm) at 90th day of exposure.

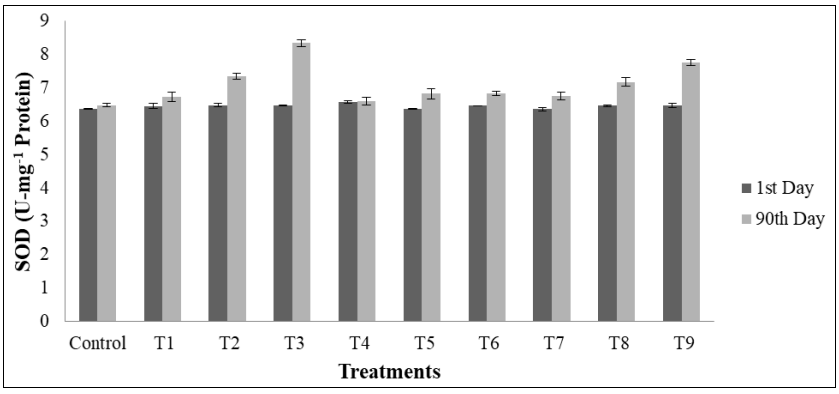

Fig 1: Effect of different concentrations of heavy metals on SOD activity of the earthworm, *E. fetida*

3.2 Catalase

Catalase enzyme deactivates H_2O_2 hence prevent oxidative damage at cellular level. As H_2O_2 concentration increased due to increased activity of SOD on heavy metal exposure, hence dose and time dependent increase in CAT activity was observed in present investigation (Fig 2). The activity in control group on 1st and 90th day of experiment was found to be 21.48 U-mg^{-1} protein and 21.56 U-mg^{-1} protein, respectively. The highest activity of CAT was 25.75 U-mg^{-1} protein in Cu (0.10 ppm) followed by 25.18 U-mg^{-1} protein in Cu+Sn (0.05+0.05 ppm). With highest concentration of Tin *i.e.* Sn (0.10 ppm) 21.26 U-mg^{-1} protein (CAT) was observed.

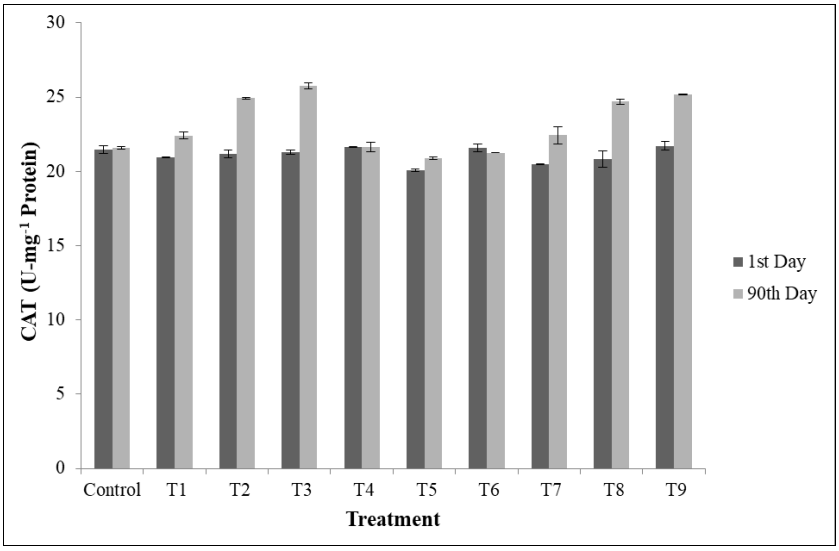

Fig 2: Effect of different concentrations of heavy metals on CAT activity of the earthworm, *E. fetida*

3.3 Glutathione Peroxidase

Peroxidase is a non-specific enzyme which is known to metabolize H_2O_2 into water, thus protect the cellular system under oxidative stress. The activity of this enzyme was observed to be increase in present investigation as the time of exposure and concentration of heavy metals increased (Fig 3). The activity of POD in the control group was observed to be 0.51 U-mg^{-1} protein and 0.50 U-mg^{-1} protein on 1st and 90th day of experiment, respectively. The highest POD activity was 0.78 U-mg^{-1} protein in Cu (0.10 ppm) followed by 0.72 U-mg^{-1} protein in Cu+Sn (0.05+0.05 ppm) on 90th day of experiment. In Sn (0.10 ppm) the POD activity was found to be 0.63 U-mg^{-1} protein.

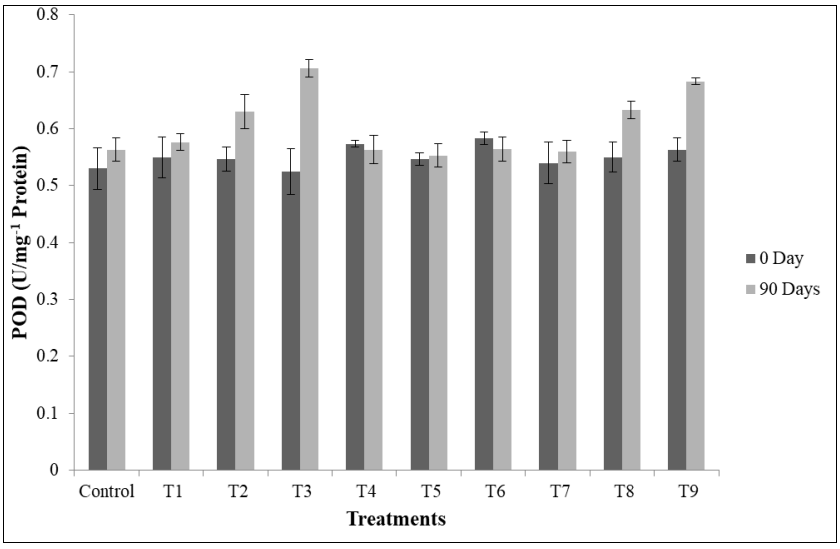

Fig 3: Effect of different concentrations of heavy metals on POD activity of the earthworm, *E. fetida*

Fig 4 shows the percent changes in antioxidative enzymes, such as SOD, CAT and POD, activity after the 90 days of heavy metals exposure. All these enzymes activity increased at the 90th day and the maximum percent increment was observed with Cu+Sn (0.05+0.05 ppm) and Cu (0.10 ppm) in case of SOD where 31.92% and 23.78% increment was observed, respectively. Activity of CAT (%) also increased as 21.10% and 18.70% in Cu (0.10 ppm) and Cu+Sn (0.05+0.05 ppm) respectively. Similarly increment in POD activity (%) was observed with Cu (0.10 ppm) and Cu+Sn (0.05+0.05 ppm) also *i.e.* 44.44% and 28.57%, respectively. With tin at maximum concentration *i.e.* (0.10 ppm) 9.61% increment was observed in POD activity whereas SOD and CAT activity decreased by 1.21% and 1.48% respectively.

Similar trend of results was also observed by many scientists, according to Gaete *et al.* (2010) [5] decrease in GST level was observed at highest concentration of mercury and the organisms which possess lowest oxidative damage was found due to increased protein content. At the same time activity of catalase also provide evidence against oxidative stress. The activity of GST increased in *E. fetida* when exposed to the higher concentrations of copper. Zhang *et al.* (2014) [14] found that Imidacloprid could significantly stimulate the activity of CAT and SOD in earthworms. The Guadipyr could also enhance the activity of CAT, SOD and AChE in *Daphnia magna* (Qi *et al.*, 2013) [11]. Cao *et al.* (2017) [2], reported oxidative stress induced by microcystins in *E. fetida* which led to lipid peroxidation and disruption of the antioxidant system. The increase in activity of SOD had a better protection against oxidative stress which might be due to increase in expression level of mRNA or the post-transcriptional activation (Costa *et al.*, 1997) [3]. Increased

concentration of heavy metals reduced the antioxidative enzymes activities in plants (Hou *et al*., 2007) [7] whereas in fish, activities of SOD and CAT increased after Cd exposure (Messaoudi *et al*., 2009) [9].

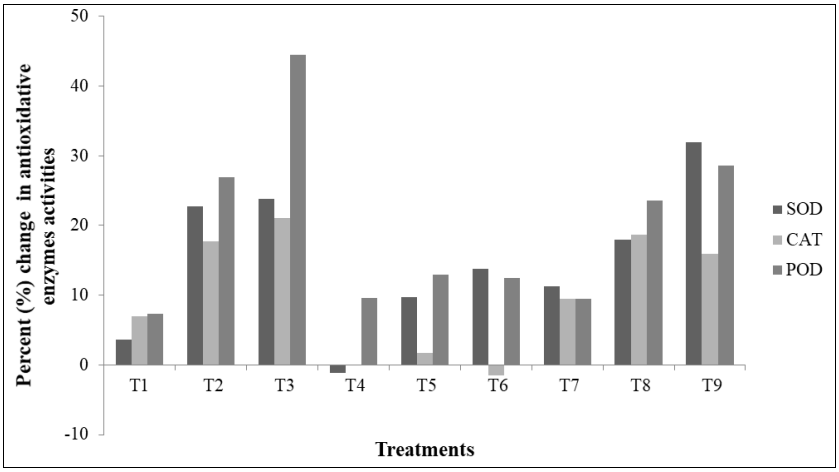

Fig 4: Percent change (%) in antioxidative enzymes activity earthworm, *E. fetida*

4. Conclusion

Activity of different antioxidative enzymes was assayed and the result revealed that exposure of earthworms (*E. fetida*) to heavy metals individually as well in combination led to increase in activities of antioxidative enzymes (SOD, CAT & POD). The increment in activities of enzymes was dose and time dependent. Maximum increment in SOD activity was found to be 23.78% and 31.92% in Cu (0.10 ppm) and Cu+Sn (0.05+0.05 ppm), respectively and the activity of CAT was increased by 21.10% and 18.70% in Cu (0.10 ppm) and Cu+Sn (0.04+0.04 ppm), respectively. The increment in POD activity was found to be 44.44% and 28.57% in Cu (0.10 ppm) and Cu+Sn (0.05+0.05 ppm), respectively. The decrement in activity of SOD was 1.21% with Sn (0.06 ppm) and that of CAT was 0.04% in Sn (0.06 ppm) and 1.48% in Sn (0.10 ppm).

5. Acknowledgement

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