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Original Research Article

Comparative Impact of Fungicides on Biomolecules of *Eisenia fetida*

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ABSTRACT:
Tebuconazole and copper oxychloride are two widely used fungicides in Haryana against fungi harming banana, apple, potato and cucurbits, chili, paddy, onions, etc. Both fungicides induce free radicle-induced oxidative stress by disturbing the antioxidant defense mechanism and other biochemical mechanisms. Even the copper from copper oxychloride accumulates in soil and poses a serious ecological threat to the environment and the non-target organisms, like farmer's friend earthworms. In the present study, the acute toxicity (LC_{50}) using probit analysis, antioxidant enzymes like Superoxide dismutase (SOD), Catalase (CAT), Peroxidase (POD), Ascorbate peroxidase (APX), Glutathione S-transferase (GST) and lipid peroxidation (MDA) activity in the whole-body extract while total antioxidant (TAC) and phenolic content (TPC) using the coelomic fluid of earthworm. All the objectives of *Eisenia fetida* were determined spectrophotometrically after exposure to 60 and 80% of the calculated LC_{50} after the 14^{th} and 28^{th} day of orientation. Results showed that both the fungicides are toxic to test organisms inducing oxidative stress with increased concentration. Here, fungicides-induced oxidative stress was determined by a noticeable change in the antioxidant enzyme activity. TAC and TPC value are irreversibly interconnected, and a decrease in these indicates a shift in earthworm biomarkers.

Keywords: Acute toxicology, Agrochemicals, *Eisenia fetida*, Oxidative stress, Antioxidant content, Phenolic content

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INTRODUCTION

Earthworms have been reported on the earth for over 20 million years. Earthworms are the most widely distributed soil invertebrate fauna, and they are regarded as soil biophysical engineers. Charles Darwin studied earthworms for 39 years and called them 'the unheralded soldiers of the soil.' He also wrote that, "No other creature on earth has done so much for mankind" (Sinha et al., 2012).

Earthworms may constitute up to 80% of the total biomass of the soil fauna.

Worms are essential in maintaining soil biophysical conditions like texture, supplement binging, microorganisms network structure, and assisting plant growth and development. Coelomic fluid released from dorsal pores of earthworms consists of watery fluid, plasma, and many coelomocytes such as amoebocytes, mucocytes, circular cells, and chloragogen cells which play a crucial role in both cellular and humoral immunity of earthworms in fighting against pathogenic micro-organisms and makes worms a potent therapeutic agent. The coelomic fluid possesses various bioactive compounds such as proteins, which show a variety of biological functions like antibacterial, anti-cancer, hemolytic, mitogenic, tumor static, cytotoxic, and proteolytic activities (Omar et al., 2012).

They can consume the pollutants by feeding and through the skin, affecting their immunocompetent coelomocytes (Ciğerci et al., 2018). They can be used as a sensitive biomarker to reveal the ecosystem health (Ciğerci et al., 2018; Homa et al., 2005). Pesticides are commonly used in agricultural practices to control pests to increase crop protection and productivity. Besides their effects on target organisms, agrochemicals also affect non targeted flora and fauna. Heavy soil treatment with pesticides can decline the populations of advantageous soil micro-organisms (Chen et al., 2018). Numerous studies have reported the effect of various pesticides. Using pesticides in agro-ecosystem, can harmfully affect earthworms, which play significant roles in soil functioning; for example, organic matter decomposition (Bart et al., 2019). Night crawlers are affected by pesticides at all organization levels. For instance, pesticides disturb enzymatic exercises, increment individual mortality, decline fertility and development, and change individuals' behavior, like feeding rate decline in the general community biomass and thickness. Fungicides are extensively employed in fields to check the growth of pests and in storage as well. Pesticides may adversely influence the development of advantageous fauna and its related bioprocessing in soil.

Acute toxicity is one of the many tools to assess the toxic level of any pesticides in ecological niches as it gives results very quick (Alves et al., 2013). Mortality is a close indicator of toxicity along with biomass and reproduction. Various enzymatic reactions and their products and by-products are indicators of xenobiotics toxicity. The cells acquire enzymes for antioxidant attributes by which they protect themselves from the negative impact of reactive oxygen species (ROS). Catalase (CAT), superoxide dismutase, peroxidase, and glutathione S-transferase are the antioxidant enzymatic part of the defense system of *Eisenia fetida* and are mainly confined in cytosol compartments (Saint-Denis et al., 1998; Song et al., 2009).

Reactive oxygenic species (ROS) act as a marker of the normal functioning of physiological processes within the cell of living organisms.TAC is directly associated with TPC as they possess a hydroxyl group, and hence, they play an essential role in neutralizing the free radicals (Balamurugan et al., 2007; Aldarraji et al., 2013). These phenolic compounds are present in the coelomic fluid of earthworms. Butoxidative stress starts appearing because of a broken balance between generation and neutralization of ROS. Excess production of ROSs can destroy the macromolecules like protein carbonylation, lipid peroxidation, and DNA. So, reactive oxygenic species can cause many severe diseases like cancer and bring ageing[5].

Tebuconazole is a triazole fungicide that is used to treat plant pathogenic fungi. Tebuconazole (TBZ) removes fungi by inhibiting their ability to spread spores. They effectively control most fungal pathogens but do not in preventing bacteria and viruses. It is mainly employed against the fungi that check the growth of grapes, cherries, almonds, and cereals.

Copper, a heavy metal prevalent in agricultural fields due to indiscriminate use as an agrochemical, is challenging to eliminate once it entereds oil or water and supposably damages the complete physiology of organisms like *E. fetida* (Di et al., 2020). Copper-based fungicides like Copper Oxychloride (COC) have been mainly employed to prevent and treat downy mildew in agriculture, specifically in vineyards.

Indiscriminate use of copper oxychloride causes the pilling of copper in cultivable soils. Copper-based fungicides affect non target flora and fauna like other agrochemicals and control fungi. Even a tiny concentration of $16 mgkg^{-1}$ of copper oxychloride can negatively impact *E. fetida* populations (Helling et al., 2000).

MATERIAL AND METHODS

Animal model

Earthworms are the most used model organisms for ecotoxicological studies (Farsani et al., 2021). As per OECD guidelines, 1984 and ISO, 1993, *Eisenia fetida* is the recommended species for studying the toxicological aspect of soil. Earthworms were procured from Bhoojeevan Organic Pvt Ltd, New Delhi. The species was confirmed using online taxonomical keys, and dissection of earthworms was done (Thakur and Yadav 2018)then were cultured in laboratory conditions at 20°C,80% humidity with 12 hours dark and 12 hours light cycle.

Acute Toxicity calculation: Fungicide – Tebuconazole, Copper oxychloride

The experiment was conducted as per OECD guidelines no. 207 (1984) of chemicals testing. Tropical artificial soil (Garcia 2004; Desilva et al., 2009) was prepared. 10% coconut peat as an alternative to sphagnum peat, 20% kaolin clay, and 70% fine industrial soil moistened up to 50% of its water holding capacity. For optimizing pH to 6 ± 0.5, calcium carbonate was employed.

Fungicides were added to soil on a dry weight basis. The doses were added in increasing geometric series. 4 replicates were prepared for each concentration. 10 adult well-clitellated earthworms from the culture beds, washed with deionized water, were accommodated under test conditions for 24 hours in tropical artificial soil. After acclimatization, worms were released in pots kept under laboratory conditions for 14 days. The mortality percentage was checked 14th day of the study. Regularly food was given, and moisture was maintained in the pots. Mortality was limited, and dead worms were removed on an urgent basis. The LC_{50} of fungicides on *Eisenia fetida* were calculated using the log dose/probit regression line method as per Kumar and Singh (2016) with slight modifications. Three samples were prepared

- 1st sample –Control (no fungicides added)
- 2nd sample- 60% of LC_{50}
- 3rd sample- 80% of LC_{50}

Determination of total phenolic content (TPC)

An extrusion fluid (Goven et al., 1994; Fourie et al., 2007) was prepared to collect coelomic fluid. A master solution was prepared by mixing 10 mg/ml guaiacol glycerol ether, 2.5 mg/ml EDTA, 95% saline, and 5% ethanol of pH 7.3. Earthworms and extrusion fluid were poured into the Petri plate to stimulate the spontaneous release of coelomic fluid.

Folin Ciocalteu's reagent method was employed to calculate TPC (Devatha et al., 2018). Samples were diluted with distilled water followed by 0.15 ml of FC reagent. Allow the reaction to proceed for 5 minutes, then mix (20% W/V) Na_2CO_3, and the content was thoroughly mixed, practicing a vortex shaker. The solutions were allowed to develop color in dark incubation of 30 minutes, and absorbance was recorded at 765 nm. The polyphenol content was evaluated as Ascorbic acid equivalents using the calibration curve.

Total antioxidant capacity

The antioxidant assay was evaluated employing phosphomolybdate reagent (28mM sodium phosphate, 4 mM ammonium molybdate, and 0.6M sulphuric acid) in acidic pH (Kumari and Shukla 2021). 0.1 ml of samples were treated with phosphomolybdate reagent followed by incubation at 95°C for 90 minutes, which formed a green color complex due to the reduction of Mo (+) to Mo (-). It then cooled down the samples to room temperature to read the absorbance at 695 nm. Here the control wa s methanolic phosphomolybdate solution, and agallic acid standard curve was employed for total antioxidant content determination.

Antioxidant enzymes assay

Earthworms were **3** homogenized using prechilled mortar and pestle after gut cleaning.50 mM potassium phosphate buffer (1:8 W/V; pH 7) was centrifuged with homogenized content for 10 minutes at four °C at 10,000 rpm. To study enzymatic activity and protein quantity, the supernatant was employed. The protein concentrations were measured using bovine serum albumin (BSA) as standard (Bradford 1976), and colorimetric measurement was detected at 595 nm to determine the light absorption value.

Peroxidase (POD) activity (EC 1.11.1.7) was measured by the guaiacol method (Kochba et al., 1977). Definition of one unit of POD is the amount of enzyme that oxidizes 1 micromole of guaiacol per minute per g fresh weight. A reaction mixture contained 100 mM potassium phosphate buffer (pH 6), 30 % H_2O_2, and guaiacol. After adding enzyme extract, an absorbance reading was taken at 470 nm. Ascorbate peroxidase (APX) activity (EC 1.11.1.1) reaction was started by adding H_2O_2 (Nakano and Asada 1981), and extinction of ascorbate was measured at 290 nm for 1 minute. Enzyme activity was expressed in µmol H_2O_2 min^{-1} g^{-1} DM. Catalase (CAT) activity (EC 1.11.1.6) was measured using the hydrogen peroxide method (Claiborne 1985), and the definition of one unit of CAT is the amount of enzyme that degraded 1 µmol of H_2O_2 per min per g fresh weight. A 0.1 ml sample was mixed with 100 mM phosphate buffer (pH 7.2, 1 mM EDTA). The addition of 10 mM H_2O_2 commenced the reaction. Glutathione-S-transferases (GST) activity (EC 2.5.1.18) was measured by measuring the rate of the combination of glutathione (GSH) and 1-chloro-2, 4-dinitro-benzene (CDNB). The appearance of reduced glutathione (GSH)-CDNB (1-chloro-2,4-dinitrobenzene) complex was monitored at 340 nm. The reaction mixture includes phosphate buffer saline, 200 mM glutathione reduced, 100 mM CDNB, and enzyme extract. The final GST activity is expressed as micromoles of the GSH-CDNB complex produced per minute per gram of protein.

Measurement of lipid peroxidation

MDA content was evaluated spectrophotometrically using Xiang and Wang (1990) method with slight alterations. Enzymatic fluid was mixed with a reaction mixture containing acetic acid (20%), thiobarbituric acid (TBA 1%), and deionized water (1 mL), then the contents were incubated for 1 hour in a water bath at 95°C. The MDA levels in the supernatant were determined at 532 nm and expressed as µmol/ mg protein.

RESULTS

Characterization of earthworm

They are small around 3-4 inches long with reddish-purple body color. The "rings" around worms are known as segments and closely paired setal arrangements. The head or anterior end of the earthworm has a prostomium, a projection covering the mouth which can constrain open splits in the soil into which they can crawl. *Eisenia fetida* is hermaphroditic with the position of clitellum in reproductively active worms from 26-32 segments. The cocoons are lemon-molded and are light yellow from the start, getting progressively brownish as the worms inside becoming adults. These cocoons are noticeable to naked eyes. Worms consist of developed nervous, circulatory, digestive, excretory, muscular, and reproductive systems. Lubricating mucous emitted by skin glands encourages worms to travel through the soil and balances out tunnels and castings.

Median lethal concentration (LC_{50})

The dose and duration relationship has been confirmed during the exposure period. Mortality has been increasing with the increasing concentration of both the fungicides. For analyzing the LC_{50} of both the fungicides, Probit analysis was done. Results were recorded after 14 days of exposure. The LC_{50} of Tebuconazole and Copper oxychloride were 294.9mg/Kg and 2511.88 mg/Kg, respectively.

Effect on growth rate

Weight and length are the two parameters chosen to determine the impact of fungicides on the growth rate of *E. fetida* for 28 days. An increase and decrease in the weight and length of control and fungicides exposed worms were checked. Figures showed the changes caused by fungicides. The average body weight (figure 1) of worms showed a significant difference from control (347.36 ± 0.5 mg) to treated groups (308 ± 0.3 mg, 261.43 ± 0.2 mg, 238.47 ± 0.3 mg, and 261.1 ± 0.6 mg) after 28 days of exposure at different concentrations of fungicides while no significant difference in length (figure 2).

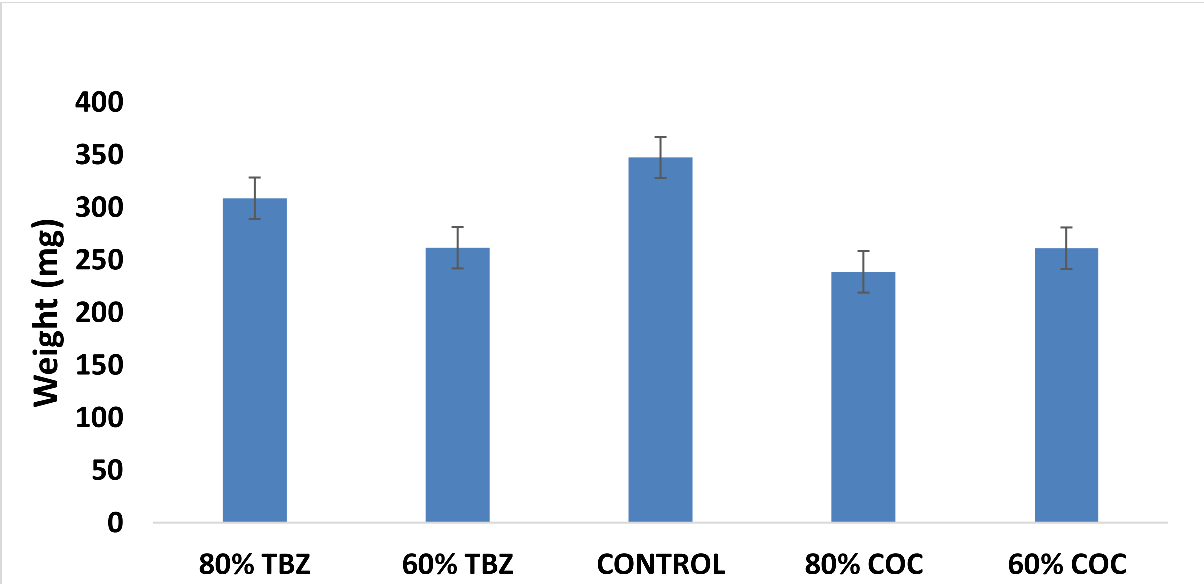

Figure 1: Graphical presentation of the average weight of worm in control and fungicides treated groups.

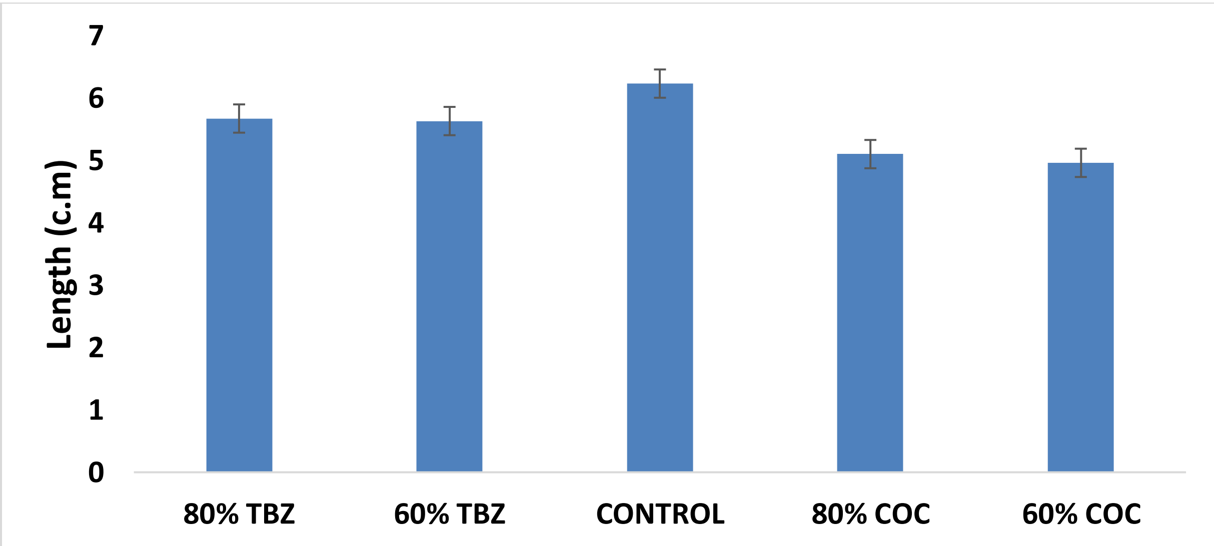

Figure 2: Graphical presentation of the average worm length in control and fungicides treated groups.

Total phenolic content &total antioxidant capacity analysis

Quantification of polyphenols showed a plentiful concentration that is believed to deliver an exceptional equivalence with antioxidant activity (Anwar et al., 2009b; Hussain et al., 2012). The polyphenols protect cells from ROS generated under stress and strengthen the antioxidant system (Sultana et al., 2007). The TPC/TAC can be withdrawn using a highly polar solvent, and water and methanol mixture is a potent candidate for extraction. All the total phenolic content and total antioxidant capacity of earthworms are cited in Tables 1 & 2.

Table 1: TPC of control and fungicide treated groups

Sample	TPC (14th day)	TPC (28th day)
80% TBZ	374.27± 2.12	373.26± 2.12
60% TBZ	371.6 ± 0.235	372.93± 3.06
CONTROL	376.93 ±1.17	375.1± 0.94
80% COC	375.1± 2.35	374.767± 0.94
60% COC	375.1± 0.47	374.767± 0.94

Table 2: TAC of control and fungicide treated groups

Sample	TAC (14th day)	TAC (28th day)
80% TBZ	389.27± 0.23	388.6± 0.23
60% TBZ	387.933± 0.23	388.1
CONTROL	388.93± 0.23	387.93± 0.23
80% COC	389.76± 0.47	388.6± 0.23
60% COC	388.6± 0.7	390.1± 0.94

Antioxidative enzyme assay

In the present work on *Eisenia fetida*, the toxicity of fungicides was determined by analyzing their effects on protein concentration and antioxidant enzymes like superoxide dismutase. Fungicide concentrations were 60% and 80% of LC_{50}.*E. fetida* activates antioxidant defense and detoxification system against excessive ROS by catalyzing superoxide radical to peroxides by SOD and maintaining homeostasis by scavenging it by CAT and POD (Gu et al., 2021). SOD maintains electron transport chain in living organisms are cited in Figures (POD 3; CAT 4; APX 5; GST 6; MDA 7) summarize all the antioxidant enzyme assay of treated and control groups

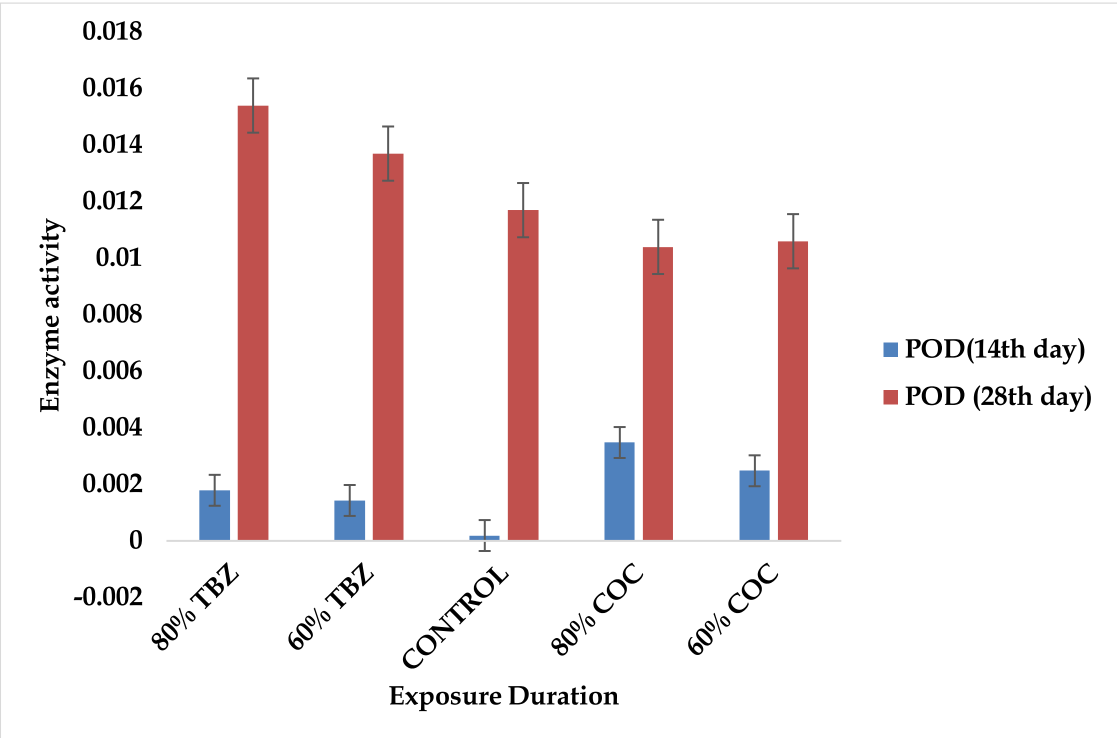

Figure 3: Graphical presentation of POD in control and fungicides treated groups. Significant difference was observed between the control and treated groups p << 0.05

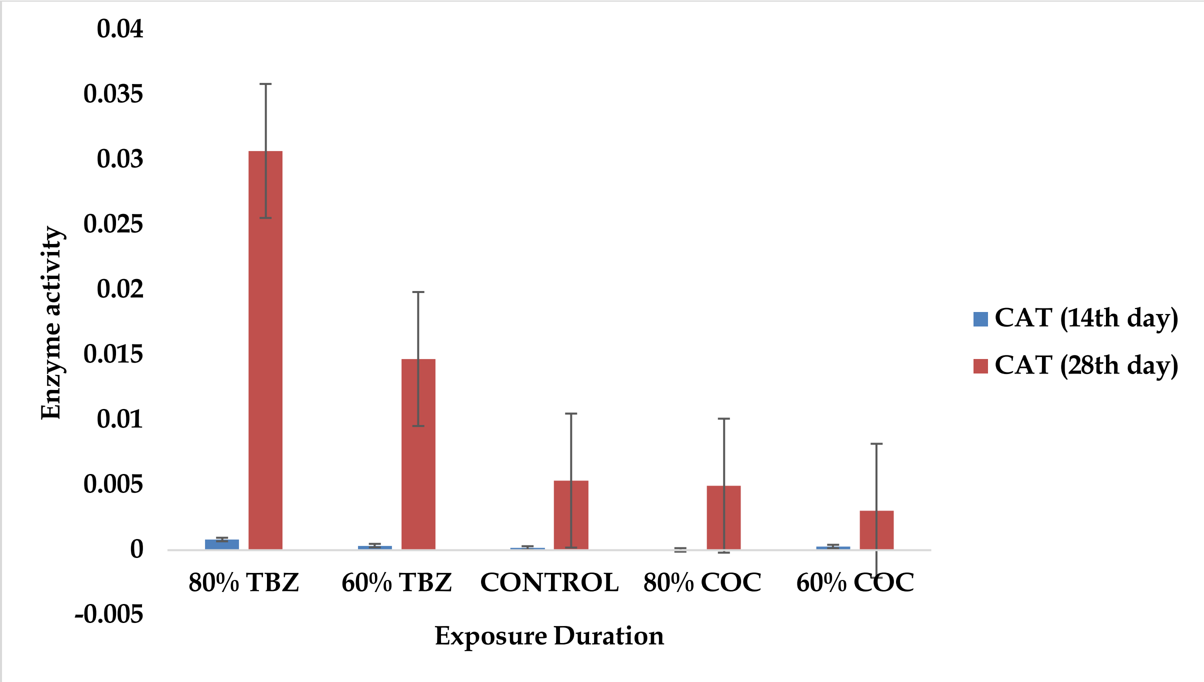

Figure 4: Graphical presentation of CAT in control and fungicides treated groups. Significant difference was observed between the control and treated groups $p < 0.05$

At 60%and 80% LC_{50} POD and APX isozyme, CAT activity shows a cycle of increaseas the time passes. Compared with the controls, the higher doses of fungicides increased and stimulated POD, APX isozyme, and CAT activity in the earthworm, and significant differences were found on days 14 and 28th. Peroxidase activity is a potent biomarker for the sublethal toxicity of xenobiotics to worms for oxidative stress conditions (Song et al., 2009).

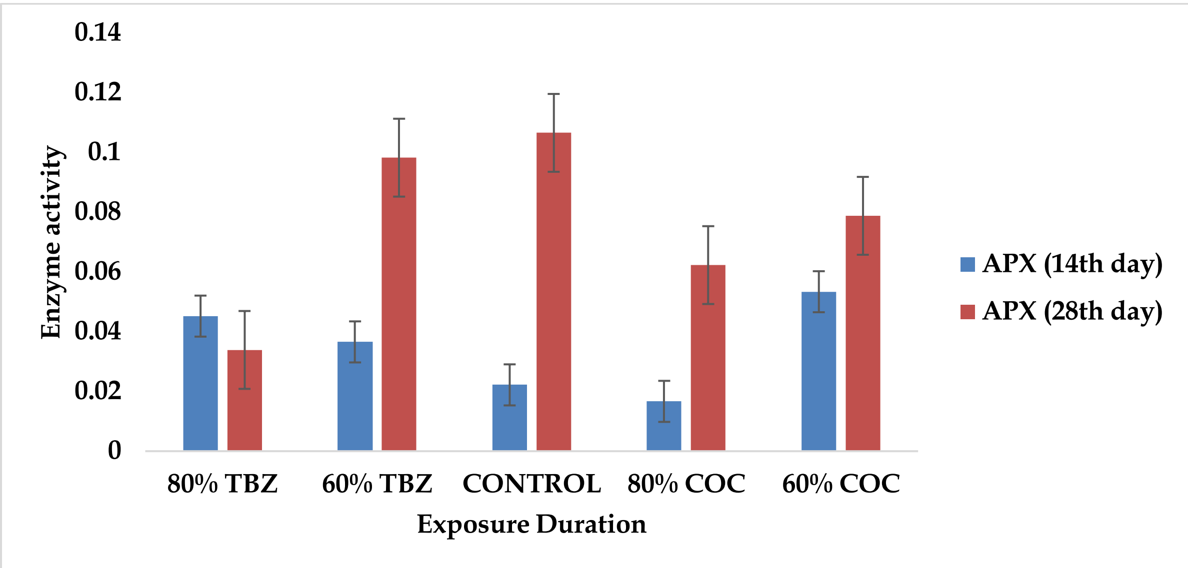

Figure 5: Graphical presentation of APX in control and fungicides treated groups. Significant difference was observed between the control and treated groups $p << 0.05$

GST is involved in the cellular detoxification ofawide range of xenobiotics (Ferguson and Bridge 2019). GSH, as long as three amino acids long peptide and a free radical scavenger, convert harmful poisons into harmless products to be excreted out of the body. After treatment, a significant change in GSH content was observed, indicating that tebuconazole and copper oxychloride could up-regulate GSH content in the earthworm.

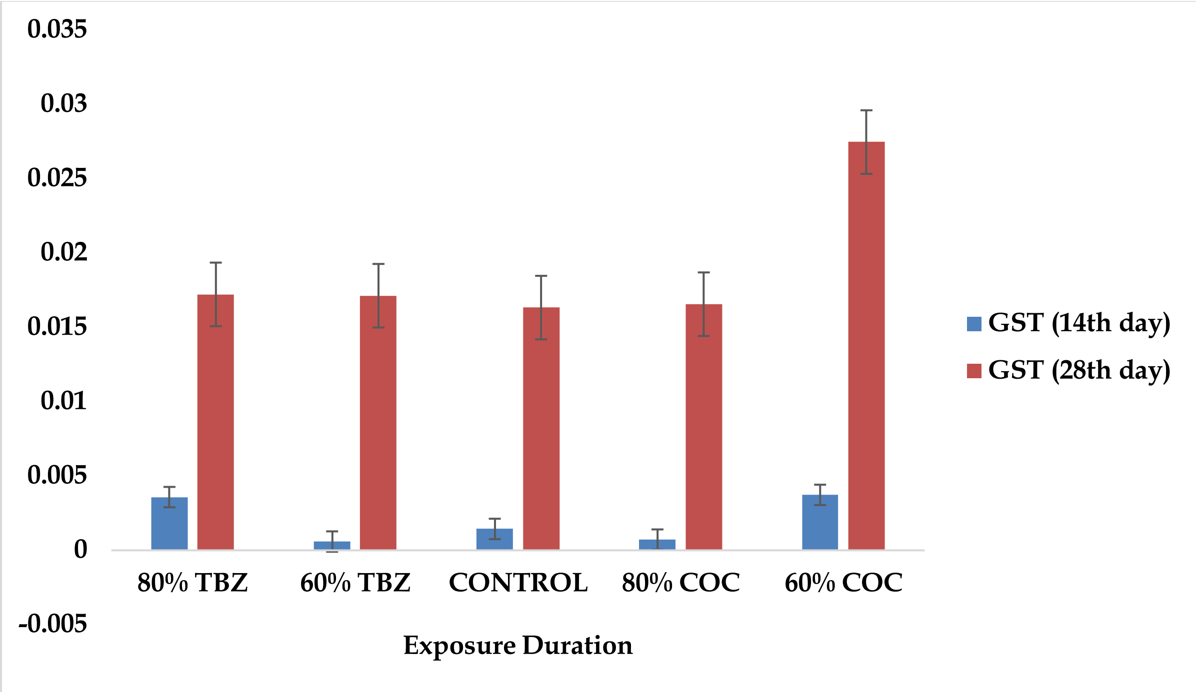

Figure 6: Graphical presentation of GST in control and fungicides treated groups. Significant difference was observed between the control and treated groups $p << 0.05$

MDA acts as an index senescence physiology, a byproduct of lipid peroxidation of the cell membrane. MDA levels aggravate lipid damage and explain the degree of lipid peroxidation. After tebuconazole and copper oxychloride treatment, a significant change in MDA content was observed, indicating that they could up-regulate MDA content in the earthworm (Sinha and Saxena 2006).

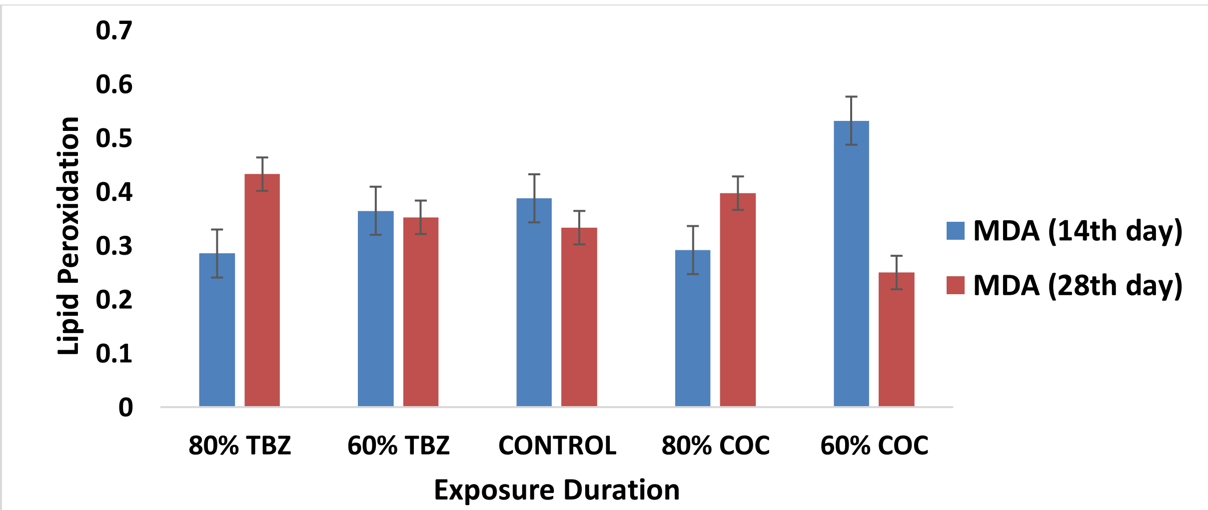

Figure 7: graphical presentation of MDA in control and fungicides treated groups. No significant difference was observed between the control and treated groups $p > 0.05$

The total protein concentration in *Eisenia fetida* was determined after 14 and 28days (figure 8). It was observed that compared to control, in which protein concentration increaseson the 14th day but then decreases slightly, in the fungicides exposed sample, there is a significant decrease in protein concentration on the 28th day. The protein synthesis rate shows a dip under stressful conditions (Hertwig et al., 1992).

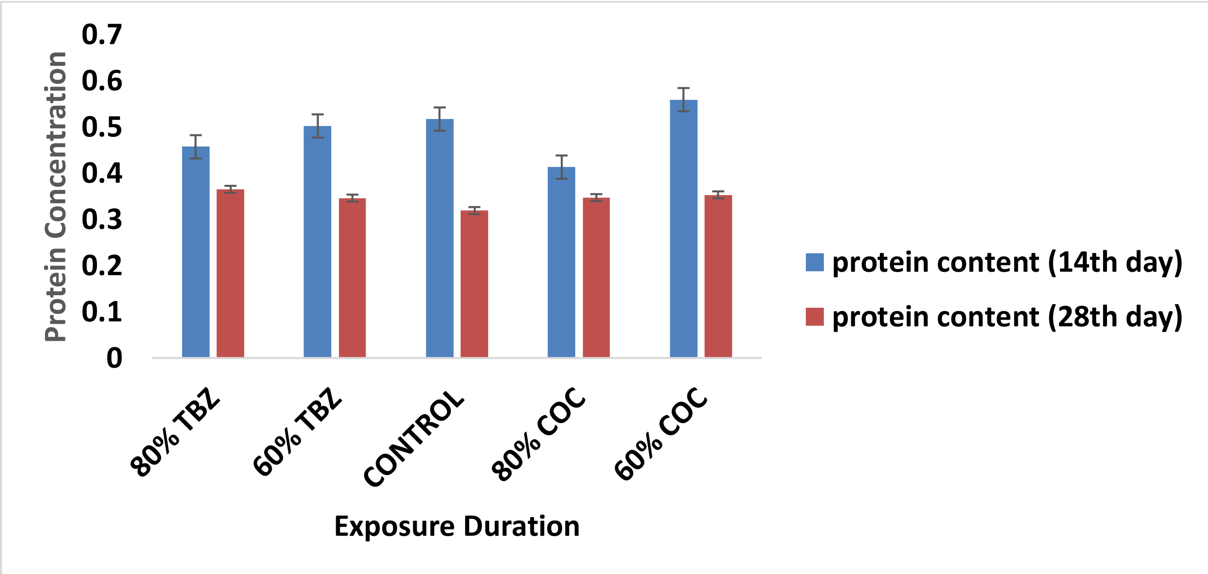

Figure 8: Graphical presentation of protein content in control and fungicides treated groups. Significant difference was observed between the control and treated groups p << 0.05

DISCUSSION

In our investigation, coelomic fluid concentrate from *Eisenia fetida* shows a striking measure of phenolic compound. Methanol as the solvent is the best used phenolic derivative master. Phenolic molecules are exceptionally soluble in methanol. Without any disturbance in the earthworm's defense system, it could balance produced reactive oxidative stress and the prodefense mechanism when an organism's body is exposed to various chemicals, reactive oxygen species (R.O.S) form in the body. When the levels of R.O.S exceed the average level, the body is said to be in a state of oxidative stress. The enhanced level of R.O.S may cause severe damage to cells like oxidation of proteins, inhibition of enzymes, disintegrations of the body wall, etc. Hence, we can say the defense mechanism can neutralize stress. To withstand the increased ROS, the worms are provided with a capable defense mechanism consisting of SOD, CAT, POD, and many more enzymes to counteract the free radicles. These enzymes area biomarker for indicating the toxicity induced using pesticides even at the sublethal doses. Scavenging free radicles by these enzymes ultimately protect the organism. Our study focused on the protein content of earthworms and the defense mechanism against free radicles. Various antioxidant enzymes like superoxide dismutase remove these R.O.S. Under oxidative stress caused by chlorpyrifos increase in value determines that the body is trying to remove these free radicals. But with further and increasing pesticide concentration, the deals start decreasing as the body cannot fight more. These R.O.S irreversibly damage proteins, and these damaged proteins activate signaling pathways and try to adapt to elevated R.O.S. But cannot do these the same for a prolonged period as R.O.S are highly harmful and results in cell death. The increasing concentrations indicate that the body tries to cope with the stressed condition, but this ability decreases.

Earthworms have also been a very effective drug for various diseases in different countries. Effects of multiple pesticides (including fungicides, insecticides, and herbicides) have been intensively studied over the last decade due to their negative impact on soil macro and micro-organisms. Earthworms are affected by pesticides at all organization levels. Therefore, it's not possible to review all the published literature. So, an attempt is made to review some appreciable studies.

The conclusion drawn from the enantioselective accumulation experiment was that S-(-)-tebuconazole, less toxic to targeted fungi, was more easily accumulated in *E. fetida* (Cui et al., 2018).Individual & consolidated impacts of TEB on *E. fetida* showed on day 14, TEB exhibited a very high level of toxicity to *Eisenia* as per OECD. When observed on day 14 with a lethal concentration (LC_{50}) of 287 mg kg^{-1}. Administration of earthworm paste into albino rats (having inflammation) was found to decrease inflammation, decrease GST, SOD, CAT, and LPO, and regularize the values of red blood cells, white blood cells, differential degrees of neutrophils, lymphocytes, corrosive & basic phosphatase, electrolytes (Balamurugan et al., 2007). A conclusive remark from this study is that the polyphenolic content is responsible for earthworms' antioxidant and anti-inflammatory properties.

Earthworms paste possesses many curative properties like antioxidant, antiphlogistic, antineoplastic, and antibacterial activities (Aldarraji et al., 2013). Antioxidant activity was directly associated with phenolic compounds. Earthworm paste (EP) of *Eudrillus Eugenia* provides more phenolic compounds than *Lumbricusrubellus*. Antioxidant (DPPH) assay demonstrated that *Eudrillus eugenia* showed more significant inhibition than that of *Lumbricusrubellus* in methanol. In contrast, in ethanol, the inhibition activity of *Eudrillus eugenia* was not so much different from that of *Lumbricusrubellus* extract. This study concluded that earthworm paste possesses a remarkable quantity of phenolic compounds that have significant antioxidants—impacts on antioxidative enzymes and DNA damage in *E. fetida* induced by herbicide atrazine (Song et al., 2009). The outcomes demonstrated that the CAT movement was animated at 2.5 mg/kg treatment aside from on the 14thday, and quelled at 5, 10 mg/kg atrazine barring 5 mg/kg on the 28th day and 10 mg/kg on the 21st day; the overall SOD activity was restricted, whereas all atrazine concentrations stimulated the POD activities in 28 days.

Once the no-effect concentration of copper oxychloride was exceeded, a drastic inhibition in the growth of *Aporrectodea caliginosa* was reported using a toxicokinetic model (Bart et al., 2020).

Copper oxychloride showed influenced characteristic solubilization of copper from fungicide due to accumulation of copper in agricultural fields due to anthropogenic activity (Pose et al., 2009). Soil pH, acidity, and ion exchange capacity define the amount of dissolved Cu. *E. fetida* exposed to sulfamethazine (SMZ) either alone or in combination with copper presented that they cause protein oxidation, elevated synthesis of ubiquitin, heat shock protein, and aburst of ROS in worms' body (Rong et al., 2020).

CONCLUSION

The current investigation shows that the coelomic fluid concentrate of *Eiseniafetida* contains anoticeable amount of phenolic content. This phenolic content can be used as a natural antioxidant that can treat various disorders like neurological disorders such as Parkinson's disease and other inflammatory or ischemic conditions. Also, the fungicides have a noticeable effect on the total phenolic content of earthworms. Only the certified fungicide concentration must be used until an appropriate alternative is discovered. Further steps are required for the proper delivery of fungicide so that less or no residue of the same remains in the soil so that it doesn't harm non targeted soil fauna. Some biodegradable substitute for these harmful agrochemicals needs to be found. Different variables influence the science of earthworms. These diverse factors were physical, chemical, and natural soil property, type of plant litter, animal excrement, and microorganisms and nematodes. Earthworms can also store and concentrate many inorganic and biological toxins.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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