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RESEARCH REPORT

Toxicological effects of caffeine on the antioxidant defense system and some biochemical responses in earthworm, *Allolobophora caliginosa*

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Abstract

Allolobophora caliginosa, an earthworm, was exposed to caffeine (CAF) *via* artificial soil to evaluate the effects on antioxidant enzymes in animals treated to 0, 10, 20, 40, and 80 mg CAF/kg soil after 7, 14, 28, and 56 d of exposure. There is evidence that antioxidant enzymes protect cells from free radical damage. A high CAF concentration generated changes in the activities of superoxide dismutase (SOD), catalase (CAT), and guaiacol peroxide (POD), but had slight effects on malondialdehyde (MDA) levels after 56 d of exposure. Earthworms′ MDA levels elevated somewhat after 7, 14, and 28 d. Earthworms treated with CAF were unable to induce the cytotoxic action over a very long period of time (56 d), as three enzymes [polyphenol oxidase (PPO), acetyl cholinesterase (AChE), and cellulose] were significantly inhibited. These data support the notion that oxidative stress plays a role in the response of earthworms to CAF poisoning.

Key Words: antioxidative enzymes; caffeine; growth rate; biochemical responses; earthworms

Introduction

Caffeine (CAF), a purine alkaloid (1, 3, 7-trimethyl xanthine), is found in leaves, seeds, and nuts of a number of plants. Also, it is a major component in tea, coffee, cocoa, chocolate, and energy drinks (Akomolafe *et al.,* 2017). This natural material has been authorized for use as a food additive. CAF is mostly utilised pharmacologically active medication in the world. It is one of the most widely the central nervous system (CNS), potential cognitive enhancement properties (Abrue *et al.,* 2011), antioxidant properties (Shi *et al.,* 1991; Noschang *et al.,* 2009), and has been employed as a cardiac and respiratory stimulant, as well as a diuretic (Akomolafe *et al.,* 2017). However, high dosages of CAF have been demonstrated to cause abnormalities in the animals (Christiani and Bent, 2001; O'Neil *et al.,* 2001).

Non-target animals suffer as a consequence. It, an environmental pollutant, has been identified in surface water near urban areas and is toxic to wildlife (Rodriguez del Rey *et al.,* 2012; Adamafio, 2013). A response to this concept, CAF was detected in different sites in the worldwide. For example, it was detected in streams and rivers within the San Diego region in the range 0.032-0.236 µg/L (Busse and Nagoda, 2015), in Terengganu river basins in Malaysia (384.0-426.0 ng/L) (Khalik *et al.,* 2020), and in wastewaters in Barbados (0.1-6.9 µg/L) (Edwards *et al.,* 2015). As consequences, after application, it lingers in the soil and has the potential to harm species other than the intended ones, such as earthworms (Hollingsworth *et al.,* 2003). It was earlier suggested for pest management (Nathanson, 1984; Hollingsworth *et al.,* 2002), but there are no updated investigations state this finding. On the other hand, other investigations evaluated the toxicity of CAF in marine organisms. It increased lipid peroxidation (LPO) in marine bivalves, *Mytilus californianus*, and *Ruditapes philippinarum* at concentrations: 0.5-8 µg/L (Li *et al.,* 2020). The same observation was conducted in an amphipod, *Ampelisca brevicornis* (Li *et al.,* 2020). Moreover, a decrease in the embryo-larval development was emerged in sea urchin, *Paracentrotus lividus* at CAF concentrations: 0.01-5.0 µg/L (Aguirre-Martinez *et al.,* 2015). Also, CAF was stated as neurotoxic agent in 6 species of marine invertebrates, including high inhibition of AChE (Baracchini *et al.,* 2024). Chronic exposure (28 d) of fish, *Danio rerio* to CAF (0.16-50.0 µg/L) induced alterations in antioxidant defence, and increased lipid peroxidation (LPO) levels (Diogo *et al.,* 2023).

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Table 1 Growth rate of *A. caliginosa* exposed to different concentrations of CAF after 0, 7, 14, 28, and 56 d

CAF (mg/kg soil)	Mean weight per earthworm (mg)					
	0 d	**7th d**	**14th d**	**28th d**	**56th d**	**Mean**
10	260.89^{k}±1.35	304.89^{b}±0.48	301.61^{c}±0.63	267.67^{j}±0.44	260.94^{k}±0.19	**279.20**
20	278.28^{h}±0.48	301.06^{c}±0.42	271.44^{i}±0.38	248.56^{n}±0.67	251.11^{m}±0.19	**270.09**
40	258.22^{l}±0.10	294.56^{e}±2.22	249.56^{m}±0.48	229.61^{o}±0.19	217.94^{q}±0.25	**249.98**
80	278.89^{gh}±0.10	258.67^{l}±2.25	231.72^{n}±0.54	223.00^{p}±0.58	200.06^{r}±0.54	**238.47**
Mean	**271.77**	**294.90**	**269.64**	**253.57**	**242.06**	**-**
Control	282.56^{g}±0.59	315.33^{a}±1.84	293.89^{e}±0.77	299.00^{d}±0.17	280.22^{g}±0.48	**294.20**

The same letters mean no significant difference at $P<0.05$
Each value indicates mean of 3 replicates±SE

Soil communities rely on earthworms, which are well-known for their involvement in the sustainability of soil life. They are used as a model organism for assessing environmental risks and soil toxicity (Miglani and Bisht, 2019*).* The earthworm, *Allolobophora caliginosa* (Savigng, 1826), is the prevalent local form in Egypt (EL-Duweini and Ghabbour, 1965). It lives in topsoil mineral strata in horizontal burrows and is categorized as an endogeic species. Burrowing endogeic earthworms consume vast quantities of dirt, which may boost soil particle contact with the earthworm's gut intestinal and lead to greater breakdown of contaminants. They could potentially improve the soil′s quality. After processing unclean dirt in their intestine, they add vermicast to the soil (Drake and Horn, 2007).

Several bio-indicators of harmful compounds in earthworm ecotoxicity include detoxifying enzymes, antioxidant enzymes, acetyl cholinesterase (AChE), oxidative stress responses, and many others (Novais *et al.,* 2011). In the soil ecosystem, earthworms play an important role in nutrient mineralization, decompositions, and enhancing soil structure (Edwards and Bohlen, 1996; Bartlett *et al.*, 2010).

The antioxidant defence system and various metabolic responses in earthworms (*A. caliginosa*) under standard and stress conditions have never been explored for CAF′s toxicological effects. Generally, when exposed to environmental pollutants, living organisms can create reactive oxygen species (ROS) generation. Antioxidant enzymes protect the cells against ROS (Freng *et al.,* 2015). A variety of enzyme activities have been found as biomarkers of environmental pollution. Catalase (CAT), guaiacol peroxide (POD), and superoxide dismutase (SOD) are antioxidant enzymes, and this study investigates how CAF affects them. As indicators of pollutant oxidative stress, these enzymes have demonstrated to be useful in measuring the amplitude of reactions in diverse pollutants exposed to hazardous xenobiotics (Feret *et al.,* 2003). Glutathione-*S*-transferase (GST), an important detoxifying enzyme, and non-enzyme antioxidant, reduced glutathione (GSH), and malondialdehyde (MDA) levels were examined in the earthworm *A. caliginosa*. Some biochemical responses, such as AChE, polyphenol oxidase (PPO), and cellulase enzymes have been examined for CAF toxicity on worms. Antioxidant response study allows us to better understand the deleterious effects of CAF on earthworms in both normal and stressful settings.

Material and Methods

Chemical materials and soil amendment

CAF was the chemical product utilized in this study. It was purchased from Babatwadi on Kalbadevi Road in Mumbai′s Co. Building 30. All reagents were purchased from Sigma Chemical Co. (USA) and Beijing Chemical Co. (Beijing, China). Deionized water was used to spike the soil with CAF. A suitable solution was developed to obtain the following nominal soil concentrations. Limits: 10. 20, 40, and 80 mg CAF/kg dry weight. Three replicates were used for each one. After spiking, the soils were kept at 23±2 °C for 14 d before testing.

Earthworms and assay conditions

A. caliginosa is the species of earthworm used in this study. The worms were collected from dunghills in the Ismailia Governorate (Suez Canal region, East Delta Egypt), where they are common throughout Egypt. The individuals were acclimated for two weeks in a culture pot containing sterilized cow dung mixed with artificial soil at a temperature

of 23±2 °C. The soil contained 70% quartz sand, 20% Kaolin clay, 10% sphagnum peat, and (0.01% calcium carbonate to balance the pH between 6.0 and 5.0). Before adding distilled water, the dry components of the soil were carefully mixed to generate a moisture content of around 35 percent of the soil′s maximum water-holding capacity. In large plastic pots (38×60×10 cm) coated with soft cloth, they are raised in prepared soil to reduce evaporated water and block the worms from trying to flee and conserve moisture, even though characterization by Heimbach (1984). They are thus determined to keep in an incubator with controlled light-dark cycles (Preferably 16/8 h light: dark) during experiment′s periods. Every two d, 5 g of cow dung (supplied by Faculty of Veterinary, Suez Canal University, Egypt) is added to each container to keep the soil′s moisture level gravimetrically maintained. During 56-d time-points, meals were delivered once per week at no expense to the participants. A well-developed clitellum was discovered in the earthworms used in this experiment, which was performed on adults. Because they are hermaphrodites, no sexual distinctions were considered. After 24 h of taking healthy individuals from artificial dirt, and preserving them on moist filter paper in darkness at 23±2 ˚C, they were used for investigations. The organization for economic cooperation and development (OECD) requirements were followed in the production of mature earthworms (weighing roughly 200-350 mg) in artificial soils (OECD, 1984 and 2004).

Growth inhibition of earthworms

Prior to weighing, all of the earthworms were cleaned and dried with filter paper. They were then weighed using an electro-balance and released back into the ground. The weights of earthworms exposed for seven, fourteen, twenty-eight, and fifty-six d were tallied and compared to the controls. The body weight of earthworms in each sample was compared to that of the control. As demonstrated by Shi *et al.* (2007), the weight of earthworms for each dosage would have been utilized to determine the suppression of growth as given in equation.

$$GI_n\% = W_\circ - W_t/W^\circ \times 100 \qquad (1)$$

Where GI_n is the inhibition of growth for concentration n, W° is the weight on day 0 (control), and w_t is the weight after t d of exposure (treated sample).

Enzymatic assays

Approximately three g of worms (No., 10-12) were removed from the treatments, put in Petri plates on moist filter sheets, and incubated in the dark around 23±2 ˚C to prevent the worm′s gastrointestinal content. To reach a pH 7.0, a Teflon-coated pestle was used to ground the worm weight in a homogenizer in conjunction with a Teflon-coated mortar. For 20 min at 600 rpm, the homogenates were kept refrigerated with intermittent stirring and centrifuged at 4 ˚C. To perform the numerous enzymatic and non-enzymatic analyses, we divided the final supernatants into aliquots. To preserve the sample′s integrity, it was frozen at -80 ˚C until needed.

Acetyl cholinesterase (AChE) activity

The activity of enzyme was measured using the Ellman *et al.* (1961) approach with a spectrophotometer set at 25 ˚C and 412 nm. In each test tube, 2.5 ml of 0.1 M phosphate buffer, pH 8.0, 0.1 ml of ten times dilutes DTNB reagent solution [39.5 mg of 5, 5'- dithiobis (2-nitrobenzoic acid)] and 15 mg of sodium bicarbonate in 10 ml of 0.1M phosphate buffer pH 7.0, and 20 µl of the enzyme were mixed. An aliquot (0.02 ml) of the substrate, acetyl thiocholine iodide; (ASChI) (0.075 M) was added. The optical density of the developed yellow color was recorded after 10 min against the blank. The activity was measured in terms of µM acetylthiocholine hydrolyzed/mg protein per min.

Table 2 Acetylcholinesterase (AChE) activity (µM/mg protein/min) of *A. caliginosa* exposed to different concentrations of CAF during 7, 14, 28 and 56 d with well-matched control earthworms

CAF (mg/kg soil)	AChE activity (µM/mg protein/min)					
	7th d	14th d	28th d	% of control	56th d	% of control
10	49.83^{e}±0.26	55.08^{c}±0.11	44.93^{f}±0.29	87.80	30.89^{g}±0.08	56.54
20	30.88^{g}±0.24	27.93^{i}±0.15	27.27^{j}±0.06	50.43	20.78^{m}±0.21	38.04
40	28.68^{h}±0.27	23.57^{l}±0.17	20.29^{n}±0.26	42.50	18.18^{p}±0.18	33.28
80	25.80^{k}±0.08	20.99^{m}±0.15	18.58^{o}±0.05	38.30	15.91^{q}±0.21	29.12
Control	54.75^{cd}±0.36	56.95^{b}±0.10	58.97^{a}±0.17	100.0	54.63^{d}±0.38	100.0

% of control= treatment/control ×100

Table 3 Effects of CAF concentrations on CAT activity (U/mg protein) in earthworm, *A. caliginosa* after 7, 14, 28, and 56 d of exposure

CAF (mg/kg soil)	Activity (U/mg protein)					
	7th	14th	28th	% of control	56th	% of control
10	4.10^{jk}±0.01	4.74^{i}±0.06	5.08^{h}±0.15	118.37	6.83^{b}±0.07	164.98
20	4.19^{j}±0.01	5.20^{h}±0.07	5.85^{f}±0.03	129.59	7.62^{a}±0.01	184.06
40	5.89^{ef}±0.09	5.43^{g}±0.5	6.13^{d}±0.03	148.47	7.64^{a}±0.006	184.54
80	6.51^{c}±0.10	6.09^{de}±0.02	6.56^{c}±0.03	163.02	7.53^{a}±0.02	181.88
Control	3.74^{l}±0.08	3.90^{kl}±0.06	4.13^{jk}±0.002	100.00	4.14^{j}±0.01	100.00

The antioxidant enzymes activities

Superoxide dismutase (SOD) activity was evaluated based on the ability to suppress photo-chemical reduction of pyrogallol (Marklund and Marklund, 1974). The method builds up the use of 0.2 mM pyrogallol, and 50 mM tris-HCl containing 1 mM EDTA. The rates of pyrogallol reduction were recorded at 325 nm. The activity of the enzymes was quantified as U/mg protein. Peroxide (POD) activity was evaluated using Kochaba *et al.* (1977) methodology. When the supernatant has been introduced to the reaction mixture, the pH was adjusted to 6.0, the guaiacol concentration was 20 mM, and the H_2O_2 concentration was reduced from 0.2% (w/v). At 470 nm, a shift in absorbance was observed (Kochaba *et al.,* 1977). As shown by Claiborne (1985), the activity of CAT has been tested. The dismutation of H_2O_2 changed the absorbance at 240 nm. Following are the details of the assay: Acidic Tris buffer, pH=7.4, and H_2O_2 incubation with 100 µg of sample proteins in 3 ml of H_2O_2 was used to start the reaction, which was then incubated at 25 °C. Degradation of H_2O_2 was the basis for activity. It was expressed as U/mg protein.

Malondialdehyde (MDA) content

According to Ohkawa *et al.* (1979), MDA concentrations were tested using this technique. It was mixed with 1 ml of filtered water, 1.5 ml of 20% acetic acid, 1.5 ml of 1% thiobarbituric acid (TBA), and 0.02 ml of 8.1 percent sodium dodecyl-sulfate (SDS). The sample homogenate was added to the reaction mixture. The mixture was incubated for 1 h at 80-90 ˚C before centrifuged for 15 min at 3000 rpm. The MDA level was measured at 532 nm and expressed as nM MDA per mg protein.

Detoxication enzyme (Glutathione-S-transferase, GST)

Using the approach outlined by Habig *et al.* (1974), the activity of GST was determined. The conjugated complex of 1-Chloro-2, 4-dinitrobenzene (CDNB) and GSH was monitored at 340 nm during the test. 100 mM Tris-buffer, pH=7.0, 2 mM GSH, 1 mM CDNB, and 100 µg of sample proteins in 3 ml of incubation were used as the assay conditions. GSH was added to initiate the reaction, which was carried out at 25 °C. The enzyme activity was expressed as µM/mg protein/min.

Reduced Glutathione (GSH)

The individuals were weighed and then rinsed with an ice-cold NaCl (0.9 percent) solution. In 100 mM sodium phosphate buffer (pH 7.4), a 10% (w/v) homogenate was prepared. The homogenate was centrifuged for 30 min at 9000 rpm. The pellet was discarded, and the cell free supernatant was used to calculated GSH concentrations in the sample. The process contained three components: sodium phosphate buffer (100 mM, pH 7.4), DTNB (0.2 ml), and supernatant homogenate (1 ml, 10% by weight). It was centrifuged at 2500 rpm for 15 min, with 0.5 ml of supernatant recovered and 2.5 ml of DTNB added to the solution. The 412 nm reading was acquired after rapidly shaking the mixture. Unit, nM/mg protein was utilised to express the results of the experiment (Ellman and Fishes, 1959; Weckbeker and Groy, 1988).

Cellulase activity

Two earthworms from each concentration were taken at 7, 14, 28, and 56-d, placed on filter paper (moistened with deionized water) for 4 h to aid digestion of gut contents, and then homogenized with ice-cold deionized water. The homogenate was centrifuged for 10 min at 2500 rpm, followed by 5 min at 3000 rpm for the supernatant. The combined supernatants from the two runs were stored at -20 °C until the enzyme tested. Ghose (1987) stated that carboxymethyl cellulase (CMC) study was performed to assess the enzyme activity at 540 nm. The activity can be measured in mg glucose per mg protein per h.

Body tissue homogenate protein concentration has been evaluated according to Lowry *et al.* (1951) with bovine serum albumin (BSA) as a reference standard.

Table 4 SOD activity (U/mg protein) of *A. caliginosa* exposed to different concentrations of CAF during 7, 14, 28 and 56 d with well-matched control earthworms

CAF (mg/kg soil)	Activity (U/mg protein)					
	7th d	14th d	28th d	% of control	56th d	% of control
10	2.22^{h}±0.01	2.09^{i}±0.01	2.34^{g}±0.01	124.72	3.01^{c}±0.001	149.75
20	2.44^{f}±0.03	2.32^{g}±0.01	3.03^{c}±0.02	146.07	3.47^{b}±0.01	172.64
40	2.63^{e}±0.03	2.60^{e}±0.001	3.53^{b}±0.01	164.05	3.52^{b}±0.10	175.12
80	2.60^{e}±0.01	2.84^{d}±0.04	3.70^{a}±0.01	171.35	3.68^{a}±0.05	183.08
Control	1.65^{m}±0.10	1.77^{l}±0.04	1.91^{k}±0.04	100.00	2.01^{J}±0.03	100.00

Polyphenol oxidase (PPO) activity

Polyphenol oxidase (PPO) activity has been evaluated by monitoring the oxidation of the pyrocatechol substrate at 420 nm for 5 min at 0.1 M phosphate buffer (pH 6.8) and 30 °C (Fottouch *et al.,* 2010). PPO's specific activity was reported as nM/mg protein/min.

Statistical analysis

The results are shown as mean ± standard deviation (SD). All statistical observations were based on the results of three replicates. The statistical analysis of variance (two-way ANOVA) was performed using the costate software (Version 6.311). Steel *et al.* (1997) used LSD<0.05 to compare treatment and control groups for mean differences.

Results and Discussion

Acute toxicity

After 56-d of treatment with 75, 150, 300, 600, and 1200 mg/kg of artificial soil, there was no death. After these time-points, the LC_{50} levels exceeded 1200 mg/kg soil. Earthworm mortality could be a reliable indicator of environmental contamination. Kokta (1992) demonstrated that pesticides with LC_{50s} greater than 1000 mg/kg were safe for earthworms in the field. CAF appeared to have no effect on earthworm mortality, according to this study. According to the literature, similar results have been observed in *Aporrectodea caliginosa*, an earthworm with an LC_{50} of more than 1200 mg/kg soil for the herbicide, iosproturon (Mosleh, 2008).

Survival and growth rates of earthworm A. caliginosa

In contrast to the control, *A. caliginosa* exposed to varied concentrations of CAF across varying exposure durations reached complete maturity in the current investigation. Earthworms thrived in filthy soil gratitude to their ability to detoxify it. The average weight of individual earthworms in the control group increased significantly after exposure to 0, 10, 20, 40, and 80 mg/kg at varied exposure intervals (0, 7, 14, 28, and 56 d) as shown in Table 1. After 28 and 56 d of treatment with increasing concentrations of CAF, *A. caliginosa* demonstrated a significant decrease (P<0.05) in worm body weight, with values of 217 and 200.06 mg for dosages of 40 and 80 mg/kg, respectively. Table 1 displays the weights of earthworms. For 14 d, the weight of the worms in the same 10 mg/kg treatment had increased relative to their starting weight. After 28 and 56 d of treatment, high doses (40 and 80 mg/kg soil) reduced development by 20.04 and 28.27 percent, respectively. According to the study's findings, earthworms grew faster at low CAF concentrations than at higher concentrations. A positive growth inhibition rate % means that the worms gained weight in the control. Over a seven-d period, it was demonstrated that even the lowest dose of 10 mg/kg had a deleterious effect on growth. Artificial soil containing 20 and 40 mg CAF/kg reduced growth negatively over seven days, although positive growth inhibition was seen after 14 and 56-d of exposure. After 7, 14, 28, and 56-d of exposure, the highest dose (80 mg/kg) had a positive effect on growth rate. Mosleh *et al.* (2003) found that the insecticides, aldicarb, cypermethrin, profenofos, and chlorfluazuron as well as metalaryl and endosulfan showed *A. caliginosa* growth. According to Zhou *et al.* (2007), chlorpyrifos had a negative impact on earthworm development following eight weeks of exposure to 5 kg/ha. Also, Booth and O'Halloran (2001) discovered that exposure to diazinon and chlorpyrifos at 60 and 28 kg/ha greatly inhibited the development of *A. caliginosa*. Inhibition of development can be a valuable indicator of chemical stress, a chemical action that can be linked to dynamic energy and ultimately restrict the growth of tested earthworms. Because earthworm control had gained a little weight, it was apparent that the soil nutrients were adequate for further growth. Lindane and deltamethrin hinder growth in ways that are typical

Table 5 POD activity (nM/mg protein/min) in earthworm, *A. calignosa* exposed to concentrations of CAF for different time intervals

CAF (mg/kg soil)	Activity (nM/mg protein/min)					
	7th	14th	28th	% of control	56th	% of control
10	0.49^{g}±0.01	0.52^{f}±0.01	0.57^{e}±0.02	112.77	0.58^{e}±0.03	123.40
20	0.53^{f}±0.01	0.58^{e}±0.01	0.61^{d}±0.02	121.28	0.63^{d}±0.01	134.04
40	0.57^{e}±0.01	0.62^{d}±0.03	0.72^{c}±0.02	136.17	0.73^{bc} ±0.01	155.32
80	0.63^{d}±0.01	0.70^{c}±0.01	0.75^{b}±0.02	147.52	0.80^{a}±0.01	170.21
Control	0.45^{h}±0.001	0.49^{g}±0.01	0.47^{g}±0.01	100.00	0.47^{g} ±0.004	100.00

with many other organic contaminants (Shi *et al.,* 2007). Reinecke and Venter (1985) demonstrated that the development of *Eisenia fetida* treated with dieldrin at various sublethal dosages was dose-dependent. Pyrene, a non-carcinogenic polycyclic aromatic hydrocarbon (PAH), inhibited the growth of the earthworm, *Lumbricus rubellus* (Burrow and Edwards, 2002). Earthworms typically use this approach to avert poisoning from organic chemicals and heavy metals. These poisons are typically transported across the body through the skin, where they enter the coelomic fluid. Earthworms′ epidermis has previously been proven to be a significant conduit for the absorption of toxicants from polluted soils (Jager *et al.,* 2003; Vijver *et al.,* 2003). Jeyanthi *et al.* (2016) found that low concentrations of carbaryl and lead accelerated earthworm development, whereas increasing amounts slowed growth. Methyl parathion was found to be the most toxic to endogeic (*Metaphire posthuma*), with mortality ranging from 36.0 to 57.1 percent and weight loss of 1.2-11.0 percent at different test doses (Suthar, 2014). There is a lack concern effect of CAF on earthworm growth, but some previous investigations focused on its effect on mammals. For example, Emmanuel *et al.* (2017) discovered that rats administered varying dosages of CAF for 28 d showed a significant decrease in body weight and percentage weight rise compared to control rats. Also, CAF ingestion reduced mice body weight (Choi *et al.,* 2002; Muroyama *et al.,* 2003; Zheng *et al.,* 2004).

Acetyl cholinesterase (AChE) activity

There was a significant reduction in earthowrm′s AChE activity when CAF was administered the dosages of 10, 20, 40, and 80 mg/kg soil compared to the control (0 mg/kg soil). Over 56 d, a dosage of 10 mg/kg decreased AChE activity by 56.54% of control (activity; 30.89±0.08 µM/mg protein/min) compared to the control value (54.63±0.38 µM/mg protein/min). At 20 mg/kg, AChE activity dropped by 38.04%, whereas high dosages at 40 and 80 mg/kg lowered activity to 33.28 and 29.12%, respectively, during the entire 56-d reached period compared to control. We noticed that all of the results were significantly different from the relevant controls at P=0.05. Many enzymes′ activity has been utilised as a measure of environmental pollution. As documented, AChE catalyses the hydrolysis of ACh, which leads to the termination of nerve impulses in the animals ending to death at acute doses (Pretto *et al.,* 2011). In the present work, all individuals were treated to CAF dosages ranging from 10, 20, 40, and 80 mg/kg for 56-d indicated that AChE activity decreased with increasing concentration. After 28 and 56 d, AChE activity was low, although not as low as after 7 d of exposure. During the first seven days of exposure, a large amount of ACh substrate accumulated, which could explain these findings. With a significant amount of ACh, CAF′s inhibitory effects can be mitigated by increasing the exposure period. According to Rao and Kavitha (2004), after 7 d of exposure, the percent inhibition of AChE increased rapidly, and after 14 d the maximum dose of 250 mg/kg of azodrin had 90 percent inhibition. It was proposed that the earthworm *E. fetida* is poisoned by azodrin accumulation and AChE inhibition. Similar results were found on the earthworm *E. fetida* following short-term exposures of 7, 14, 21, and 28 d, where the concentration was in dependent reduction of AChE activity (Sameena, 2017). The current study′s findings are consistent with those of Laura *et al.* (2007), who discovered that pesticides reduce AChE, another marker associated with behavioral disturbance.

The earthworm′s muscle wall contains AChE, an enzyme that modulates synaptic transmission, as well as cholinergic neuromuscular junctions similar to those seen in vertebrates. Anti-AChE compounds, such as insecticides reduce muscle AChE activity, however the link between this and changes in movement activity is thin. The few studies that exist do not discuss earthworms. AChE inhibition activated nicotinic and muscarinic AChE receptors, leading to cholinergic hyperactivity (Abou-Donia, 2003). The AChE is an important enzyme in the neurological system because it plays a role in nerve signal transmission. One of the finest

Table 6 MDA level (nM/mg protein) in *A. calignosa* exposed to different concentrations of CAF for different times intervals

CAF (mg/kg soil)	MDA (nM/mg protein)					
	7th	14th	28th	% of control	56th	% of control
10	17.03^{j}±0.01	17.96^{h}±0.01	18.67^{e}±0.08	120.57	14.92^{kl}±0.02	100.07
20	18.10^{g} ±0.09	17.29^{i}±0.07	19.70^{c}±0.03	125.22	14.95^{k}±0.01	100.27
40	18.35^{f}±0.03	18.90^{d}±0.02	20.63^{b}±0.04	130.07	14.89^{kn}±0.03	99.86
80	14.85^{ln}±0.05	19.73^{c}±0.06	21.42^{a}±0.13	125.89	14.82^{n}±0.01	99.39
Control	14.84^{mn}±0.12	14.94^{k}±0.04	14.72^{o}±0.05	100.00	14.91^{km}±0.02	100.00

and quickest ways to evaluate when chemicals are dispersed in the routine functioning of the body is by examining toxicant repercussions on the CNS. In this regard, specialists believe that AChE activity is a valid biomarker of pesticide toxicity (Devi and Fingerman, 1995; Labrot *et al.,* 1996). Earthworm *E. fetida* exposed to chlorpyrifos at the LC_{50} concentration shown a decrease in AChE activity, which Rao *et al.* (2003) discovered to be more severe with extended exposure times. In another finding, the *Caenorhabditis elegans* AChE assay indicated that monocrotophos inhibited AChE activity even at dosages (Joshi *et al.,* 2018). According the present findings, AChE activity was dramatically reduced after 7 and 14 d of exposure, but also significantly reduced after 28 and 56 d of exposure to 80 mg/kg soil. This can be explained that, there are a variety of ROS including hydroxyl radicals (OH^-), O_2^-, and H_2O_2, generated by mitochondria (Essick and Sam, 2010). Under normal conditions, an organism's ROS concentration is maintained in equilibrium. This balance is disrupted when an organism comes into touch with pollutant residues or contamination (Fazio *et al.,* 2014). The antioxidative enzymes system, which includes CAT, SOD, and POD will significantly reduce excess ROS. Hydrogen peroxide (H_2O_2) is largely scavenged by CAT, an enzyme that creates H_2O_2 and O_2^- (Zhang *et al.,* 2015). Such radicals are produced when SOD decomposes the superoxide radical (Mittler, 2002), and variability in its activity are used to determine the cell's oxidation-reduction balance. POD may prevent ROS production and H_2O_2 breakdown (Wen *et al.,* 2011).

CAT activity

Table 3 shows that CAF had a substantial effect on CAT activity in high-concentration CAF treatment as compared to the control group. After 7 d of exposure in *A. caliginosa*, there was no significant difference in CAT activity ($P>0.05$) between the control and different treatments. However, at 80 mg/kg soil CAT activity was significantly induced ($P<0.05$) and increased at a rate of up to 163.02 percent compared to the control after 28 d. After 14 d of exposure, *A. caliginosa*'s reactions to various CAF concentrations were relatively similar. Activity of CAT significantly increased at dosages ranging from 10 to 80 mg/kg of artificial soil. Even after 56 d, the CAF dosage that began to effectively boost CAT activity was significantly higher than in 7 and 14 d of exposure. After 56 d, dosage 80 mg/kg resulted in 181.88 percent compared to the control.

SOD activity

Earthworms exposed to CAF dose of 40 mg/kg soil showed a significant increase in SOD activity after 28 d, which represented positive effect with 146.07% of control. However, the highest dosage of 80 mg/kg soil resulted in positive effect with increase representing 171.35%. The SOD activities of earthworms in the control and CAF treatments did not differ substantially after seven d (Table 4). After 56-d, CAF induced positive effects with %of control: 149.75, 172.64, 175.12, and 183.08% for dosages: 10, 20, 40, and 80 mg/kg soil, respectively. In actuality, SOD is an antioxidant enzyme that scavenges and eliminates free radicals in the organisms and protects them from damage caused by superoxide anion free radicals (Liu *et al.,* 2019). The CAF inhibits hydroxyl and alkyl radicals. Therefore, SOD and CAF were combined to determine the antioxidation impact; nonetheless, the data revealed the oxidative stress role of CAF in earthworm subjected to the studied quantities.

POD activity

According to the data in Table 5, large dosage of 80 mg/kg positively increased POD activity. It exhibited the greatest activities: 0.75 and 0.80 nM/mg/min after 28 and 56 d representing % of control (170.21 and 147.52%), compared to control (0.47 nM/mg protein/min). Dosage of 40 mg/kg induced activity (0.73 nM/mg protein/min) after 56 d (155.32%), followed by (0.72 nM/mg/min) after 28 d. The least activity was noticed for dosage 10 mg/kg after all time-points, which did not exceed 0.58 nM/mg protein/min (123.40%). Oxidative damage

Table 7 Effects of CAF concentrations on Glutathione-*S*-transferase (GST) activity (µM/mg protein/min) in *A. caliginosa* for different time's intervals

CAF (mg/kg soil)	Activity (µM/mg protein/min)					
	7th	14th	28th	% of control	56th	% of control
10	122.64^{e}±0.02	115.57^{j}±0.01	115.49^{k}±0.04	105.35	130.99^{l}±0.007	116.31
20	123.14^{d}±0.02	118.62^{i}±0.01	119.46^{h}±0.03	107.59	149.83±0.01	133.04
40	129.58^{c}±0.02	120.58^{g}±0.02	120.56^{g}±0.05	110.42	165.05±0.01	146.55
80	133.62^{b}±0.01	138.47^{a}±0.01	155.89^{n}±0.002	127.48	180.59±0.007	160.35
control	121.60^{f}±0.05	112.58^{m} ±0.03	101.56^{n}±0.03	100.00	112.62^{lm}±0.04	100.00

was reduced by activating the enzymes: CAT, SOD, and POD which were discovered early in the experiment. Oxidative damage was prevented by activating the enzymes CAT, SOD, and POD, which were observed in the early phases of the experiment. Phase I radicals are scavenged by the CAT-SOD antioxidant system, which protects cells from oxygen damage (Yang *et al.,* 2012). Enhanced ROS removal was facilitated by an increase in SOD activity. Antioxidant defenses may have grown because of increased oxygen free radical generation (Torres *et al.,* 2002) in order to counteract the increased oxidative stress and prevent cell damage. An increase in SOD activity may result in the buildup of H_2O_2, which would augment CAT activities to promote the removal of H_2O_2, allowing earthworms to adjust to environmental changes and maintain ROS balance, according to Lee and Lee (2000). Prompting the elimination of H_2O_2 is also facilitated. Accumulating evidence suggests that CAF is hazardous to *A. caliginosa* earthworms. To test this, researchers used the antioxidant enzymes CAT, SOD, and POD, which convert superoxide anion (O_2^-) and H_2O_2 into water, respectively, in the current study. Caffeine (CAF) may activate the body's natural antioxidant defense system, resulting in an increase in SOD activity. This rise suggests that the detoxication process against pro-oxidation forces was driven by this enzyme (Elia *et al.,* 2007).

Effects of CAF on lipid peroxidation

The MDA is a good biomarker in determining the level of cellular oxidative damage (Ma *et al.,* 2017). The MDA content gradually increased for all dosages until 28 d of exposure. The greatest level (21.42 nM/mg protein) was noticed after 28 d for dosage 80 mg/kg, followed by 40 mg/kg (20.63 nM/mg protein), and 20 mg/kg (19.70 nM/mg protein), respect to the control (14.72 nM/mg protein). The mean values of MDA level after 28th d represented 120.57, 125.22, 130.07, and 125.89% for dosages: 10, 20, 40, and 80 mg/kg, respectively. while MDA levels in the treatment groups declined after 56 d, where no significant difference between values compared with control one (Table 6). The peroxidation of lipids can be determined using MDA (Zheng *et al.,* 2016). MDA, an intermediate molecule that is quickly eliminated, is a result of the breakdown of unsaturated fatty acid peroxides in cellular membranes (Martin-Diaz *et al.,* 2009). The use of MDA as a biomarker for the impact of pollutants on earthworms has proved successful in the past (Lin *et al.,* 2010; Liu *et al.,* 2011). According to the findings of this investigation, earthworms exposed to all recorded CAF dosages maintained MDA levels at the control level after 56 d of exposure. It accumulates in earthworm tissues due to an oversupply of peroxide radicals, which are scavenged by activated antioxidant enzymes, possibly relieving oxidative stress (Schmit *et al.,* 2007). It is possible that the inability of the antioxidant defence system under CAF stress, which also results in excessive ROS production, and eventually LPO in membranes, is the cause of the MDA accumulation in the exposed earthworms 56 d after exposure began to accumulate in a concentration -dependent manner.

GST enzyme and GSH content

Table 7 illustrates how CAF influences GST activity and in *A. caliginosa* earthworms after 7, 14, 28, and 56 d. The GST activity of *A. caliginosa* was compared with that of the control group. After 7 and 14 d of treatment at a maximal dosage of 80 mg/kg, GST activity was 133.62±0.01 and 138.47±0.01 µM/mg protein/min, compared to the control value of 121.60±0.05 and 112.58±0.03 µM/mg protein/min. After 28 d of treatment to 10, 20, and 40 mg/kg, there was gradually increase in GST activity ranged from 115.49±0.04 to 155.89±0.002 µM/mg protein/min compared to control which did not exceed 101.56±0.03 µM/mg protein/min. It increased by 160.35 percent compared to the control with a very high CAF treatment (80 mg/kg soil). The biotransformation enzyme, GST converts dangerous compounds (xenobiotics) into benign conjugates with an electrophilic substrate GSH (Bernard *et al.,* 2015). Toxic effects of pollutants can be better protected by increased GST activity, which

Table 8 Effects of CAF concentrations on glutathione (GSH) content (nM/mg protein) in *A. caliginosa* after 7, 14, 28 and 56 d of exposure

CAF (mg/kg soil)	GSH (nM/mg protein)					
	7th	14th	28th	% of control	56th	% of control
10	7.51^{d}±0.01	7.39^{e} ±0.01	7.28^{f} ±0.01	90.65	7.08^{h}±0.02	84.49
20	7.25^{g}±0.004	7.04^{i} ±0.01	6.53^{k}±0.01	83.21	6.23^{m}±0.02	74.34
40	6.77^{j}±0.01	6.54^{k}±0.01	6.45^{l}±0.001	79.02	6.00^{n}±0.02	71.60
80	6.02^{n}±0.01	5.91^{o}±0.01	3.64^{p}±0.001	62.23	3.33^{q}±0.02	39.38
Control	8.30^{c}±0.01	8.34^{b}±0.01	8.37^{a}±0.02	100.00	8.38^{a}±0.01	100.00

may serve as a bio signal for the contamination (Oruc *et al.,* 2004). The current study found that CAF significantly increased GST activity in earthworms. Toxic CAF in *A. caliginosa* earthworms is transformed into a non-toxic compound by the enzyme, which may explain an increase in GST activity.

Reduced glutathione (GSH) is an electrophilic molecule that helps the GST enzyme converts CAF into a non-toxic product. The biggest reduction occurred at a GSH concentration of 80 mg/kg (Table 8). The negative effects of CAF were significantly induced by dosage 80 mg/kg after 28 d (3.64±0.001 nM/mg protein), and 56 d (3.33±0.02 nM/mg protein), with respect of controls: 8.37±0.02 and 8.38±0.01 nM/mg protein. Such effects of CAF (% of control) represented the values: 90.65, 83.21, 79.02, 62.23%, and 84.49, 74.34, 71.60, 39.38% after 28 and 56 d for dosages: 10, 20, 40, and 80 mg/kg, respectively. In addition, our findings are consistent with those of other investigations (Tiwari *et al.,* 2016; Vandavas *et al.,* 2016; Marcano *et al.,* 2017). As previously documented, CAF induced an increase in GST activity, which may be due to the activation of the natural antioxidant defence system by these particles; however, the detoxication process against the pro-oxidation forces was mediated by this enzyme (Elia *et al.,* 2007). Canesi *et al.* (1999) demonstrated that copper treatment increased GST activity, which reflected greater utilisation of GSH conjugation in lipid hydroperoxides and carbonyl compound metabolism following metal peroxidation of cellular membranes. Similarly, the current findings are consistent with those obtained by Radwan *et al.* (2010) and Abdel-Halim *et al.* (2013), who found that GST activity increased in snails: *Theba pisana* and *Helix aspersa* subjected to heavy metal contamination in two Egyptian urban areas.

The effects of CAF on cellulase enzyme activity in worms

Table 9 reveals that cellulase activity reduced considerably in all CAF concentrations after 7, 14, 28, and 56 d of exposure. The greatest decline (5.61 mg glucose/mg protein/h) (9.04%) was noticed for dosage 80 mg/kg after 56 d, followed by 40 mg/kg (9.71 mg/mg protein/h) (15.65%), compared to control (62.03 mg/mg protein/h). End to 28 d of exposure, dosages of CAF induced the declines with mean values: 62.00, 36.41, 20.88, and 20.24% for 10, 20, 40, and 80 mg/kg. Earthworms, the primary soil invertebrates, could serve as a biomarker of pollution if their cellulase activity is diminished (Patnaik and Dash, 1993). The researchers discovered that sublethal doses of malathion inhibited cellulase activity in three different species of earthworm. Shi *et al.* (2007) found that acute exposure to deltamethrin reduced cellulase activity, indicating that it has a negative impact on earthworm metabolic metabolism. Hydrolytic fermentation inside these organisms can also hydrolyze a material. This allowed the *E. fetida* enzyme to recover from cellulase activity reduction for 4 h. Furthermore, cellulase activity was shown to be considerably decreased in comparison to the control. Some herbicides, such as acetochlor and fomesafen have been found to decrease soil earthworm cellulase activity in a way comparable to our own (Xiao *et al.,* 2006; Zhang *et al.,* 2015). CAF appears to be less dangerous to earthworms once a certain length of time has passed. Long-term exposure does not have any detrimental effects. In this regard, CAF might be called an environmentally favorable substance. Because of this, it can be called an environmentally safe substance. Conclusion: Cellulase may be utilised to identify chemical pollution in agricultural areas as a biomarker. Toxic compounds (xenobiotics) are converted into non-toxic conjugates with an electrophillic substrate by GST, a phase II biotransformation enzyme (Bernard *et al.,* 2015). The toxic effects of pollutants can be better shielded by higher GST activity, which may serve as signal for pollution (Oruc *et al.,* 2004). The current study found that CAF significantly increased GST in earthworms. Toxic CAF in *A. caliginosa* earthworm is transformed into a non-toxic compound by the GST enzyme, which may explain an increase in its activity. Reduced glutathione (GSH) is an electrophilic molecule that helps the GST

Table 9 Effects of CAF concentrations on cellulase enzyme activity (mg glucose/mg protein/h) in earthworm, *A. caliginosa* after 7, 14, 28 and 56 d of exposure

CAF (mg/kg soil)	Activity (mg glucose/mg protein/h)					
	7th	14th	28th	% of control	56th	% of control
10	41.01^{c}±0.01	39.27^{d}±0.15	34.62^{e}±0.26	62.00	29.43^{g}±0.04	47.44
20	30.63^{f}±0.21	17.38^{j}±0.15	19.46^{h}±0.04	36.41	14.53^{k}±0.12	23.42
40	19.07^{i}±0.17	14.66^{k}±0.13	12.90^{l}±0.05	20.88	9.71^{m}±0.10	15.65
80	14.61^{k}±0.09	13.06^{l}±0.12	9.83^{m}±0.07	20.24	5.61^{n}±0.47	9.04
Control	60.93^{b}±0.12	62.17^{a}±0.14	62.22^{a}±0.05	100.00	62.03^{a}±0.05	100.00

converts CAF into a non-toxic product. The present finding stated significant decline in GSH was induced by CAF in high dosage. Such finding is consistent with those of other investigators (Tiwari *et al.,* 2016; Vandavas *et al.,* 2016; Marcano *et al.,* 2017), where they stated the same pattern.

Polyphenol oxidase (PPO) enzyme

Earthworms were treated to a range of CAF doses for 56 d to examine the effects on PPO activity (Table 10). The enzyme activity in all treatment groups was significantly different from the control group (P<0.05). PPO enzyme activity was decreased to 2.18 and 1.16 nM/mg protein/min, respectively, in CAF-treated groups after 28 and 56 d of exposure to 80 mg/kg, compared to their controls: 5.10 and 5.16 nM/mg protein/min. Generally, PPO activity declined gradually independent on dosage and time-point exposure. The mean values of activity represented % of control as follows: 75.89, 66.14, 55.58, 52.99%, and 46.71, 35.08, 29.46, 22.48% after 28 and 56 d for dosages: 10, 20, 40, and 80 mg/kg, respectively. PPO has the critical role to catalyze the oxidation of *O*-diphenols to produce *O*-quinones, which have the main role in producing black, brown, or red color (polyphenols) in tissues of plants and animals (Eichen *et al.,* 1999). Several earthworm species, notably *A. caliginosa*, have been shown to have PPO activity inhibited (Wang *et al.,* 2012; Badawy *et al.,* 2013; Suthar, 2014).

Conclusion

Until now, no original research has been done on the effects of CAF on the *A. caliginosa* earthworm at sublethal dosages. Multiple criteria were selected to assess the toxic effect on *A. caliginosa* of CAF in the fake soil (soil polluted with CAF). It has a modest mortality on *A. caliginosa* earthworms, according to the findings of many studies. AChE, cellulase, and PPO activity were all inhibited by CAF, as was GST activity at 56 d after exposure. This indicates that phase II detoxication mechanisms are still active, and that CAF induces oxidative stress, which leads to the induction of antioxidant enzymes like CAT, SOD, and POD as well as LPO, which raises MDA levels. Because they were sensitive, these biochemical indices might serve as useful biomarkers for CAF non-lethal effects. When enzyme activity is altered it may have an impact on other physiological processes such as growth inhibition. Lastly, the cellulase enzyme may be employed as a biomarker to identify contamination in an agro-ecosystem. Because of this, the effects of CAF on these earthworm disturbances and the biomarker acquired should be researched in order to give some environmental significance.

Table 10 Polyphenol oxidase (PPO) activity (nM/mg protein/min) in *A. caliginosa* after exposure to different concentration s of CAF for 7, 14, 28 and 56 d

CAF (mg/kg soil)	Activity (nM/mg protein/min)					
	7th	14th	28th	% of control	56th	% of control
10	4.25^{e} ±0.03	4.00^{f} ±0.01	3.18^{h} ±0.01	75.89	2.41^{k} ±0.01	46.71
20	3.98^{f} ±0.03	3.32^{g} ±0.01	2.65^{ij} ±0.01	66.14	1.81^{n} ±0.01	35.08
40	3.35^{g} ±0.02	2.70^{i} ±0.03	2.32^{l} ±0.01	55.58	1.52^{o} ±0.01	29.46
80	3.16^{h} ±0.03	2.63^{j} ±0.03	2.18^{m} ±0.01	52.99	1.16^{p} ±0.01	22.48
Control	4.96^{d} ±0.12	5.01^{c} ±0.01	5.10^{b} ±0.03	100.00	5.16^{a} ±0.01	100.00

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