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Biochemical studies in ZnO nanoparticle exposed *Eudrilus eugeniae*

Abbas M[1], Meeramaideen M[2], Mohamed Shamsudin[3]*
[1]Research Scholar, Department of Zoology, Jamal Mohamed College (Autonomous), Tiruchirappalli, Tamil Nadu, India
[2]Assistant Professor, Department of Zoology, Jamal Mohamed College (Autonomous), Tiruchirappalli, Tamil Nadu, India
[3]Head & Associate Professor, Department of Zoology, Jamal Mohamed College (Autonomous), Tiruchirappalli, Tamil Nadu, India

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***Corresponding author**
Mohamed Shamsudin

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Abstract: The adult earthworm *Eudrilus eugenia* are exposed to four sublethal concentrations such as 0.25gm/Kg, 0.5gm/Kg, 0.75gm/Kg and 1gm/Kg ZnO NPs. Biochemical and tissue damaging parameters are studied in Gut, Bodywall and Testis organs of both control and nanoparticles treated groups. The ANOVA results showed ZnO NPs treated groups results are significantly differed ($p<0.05$) than control group. Total Sugars, Total Cholesterol, Total Protein levels are significantly reduced whereas increased Urea, transaminase (GOT & GPT) and phosphatase (ALP & ACP) levels found in treated group organs. The results indicated that exposure of ZnO NPs through soil disturbed the basic biochemical compounds in tissues and their damages are indicated by increased enzyme levels.

Keywords: ZnO NPs, Earthworm, Biochemical, Enzyme studies.

INTRODUCTION

Nanoparticles are materials that have a typical size ranging from 1 to 100 nm. Nanotechnology has an increasing societal impact, with great expectations for the use of nanoparticles in a wide range of applications including food additives, medical devices and soil remediation [1-3] and applied almost all the latest inventions right from computers to drug delivery systems [4].

Increased use and improper disposal of products containing nanoparticles are inevitably resulting in dissemination of nano-waste into aquatic and terrestrial ecosystems [5].

During the last decade, the use and application of nanomaterials has shown an exponential growth, with nanoparticles increasingly being used in an increasing number of products [6]. Toxicity data and data quality gaps for nanoparticles are severely lags behind the development of nanotechnologies [7-8].

Zinc Oxide nanoparticles (ZnO NPs) are typical metal oxide nanoparticles and produced abundantly and applied in a range of products including sunscreens, cosmetics, paint, paper, plastics and building materials [9-10]. Various studies suggest that ZnO-NPs are bio-safe and biocompatible compound could be used in biomedical materials [11]. However, toxicological studies proved that ZnO NPs had deleterious effects on human health and environmental animal species [12-13]. Soils are the basis of terrestrial ecosystems and may serve as a sink for many pollutants [14], including nanoparticles, long-term exposure to nanoparticles is plausible which cause severe impacts on soil-living organisms and may also lead to a total extinction of organisms especially earthworms.

Earthworms are excellent subject for toxicological studies and provide an outline of the impacts caused by the produced nanoparticles [15]. Earthworms are physically aerators, crushers and mixers, chemically degraders and biologically stimulators in the decomposer system [16]. Earthworms gut is an effective tubular bioreactor, which accelerates various enzymes like acid and alkaline phosphatases, nitrate reductase, etc. Asparatate transaminase (AST) and Alanine transaminase (ALT) levels acts as markers of chloragogen cell activity and other biochemical parameters are used to study the nephridial activity in earthworms [17]. This study focuses on the biochemical changes in the ZnO nanoparticles treated *Eudrilus eugeniae.*

MATERIALS AND METHODS

Animal Preparation and Exposure

The earthworm *Eudrilus eugeniae* species are collected from Panikkam Patty and transported to the Environmental Research laboratory (Jamal Mohamed College, Tiruchirappalli). The worms are acclimatized for 30 days and separated into groups. Groups are maintained in plastic tubs (45x30x15cm) contains 8kg of soil. Experimental soil is prepared by mixing cow dung and degraded organic waste like dried leaves with 4.3% organic matter and pH 5. The soil is sieved through a 5mm sieve and transferred to plastic containers [18].

The synthesized ZnO NPs method and their characterization are already studied and published previously [19]. Based on the previous work on acute toxicity of ZnO NPs to earthworm species *Eudrilus eugeniae*, four different concentrations (0.25gm/Kg, 0.5gm/Kg, 0.75gm/Kg and 1gm/Kg) which is below than LC50 values are fixed. By Dry mix method, the prepared soil is mixed with various concentrations of ZnO nanoparticles, and left undisturbed for 24h in room temperature. After 24h, mature earthworms (n=12, 50 days old) are introduced into the prepared tubs and observed. The control soil with worms is also maintained.

Biochemical studies

After experimental duration, the earthworms are collected and dissected. Gut, Bodywall and Testis from both control and exposed (0.25gm/Kg, 0.5gm/Kg, 0.75gm/Kg and 1gm/Kg) groups are removed and used for biochemical studies. Total sugar, Protein, Cholesterol and Urea levels are analyzed by standard spectrophotometer methods such as Anthrone method [20], Lowry *et al.*, [21] method, Zollner and Kirsch [22] method and Diacetyl Monoxime (DAM) method [23] respectively. For tissue damaging studies, tissue samples are homogenized in 0.1M cold phosphate buffer (pH 7.2) and then centrifuged at 10,000g for 20mins at 4°C. The clear supernatants are used for the determination of GOT, GPT, ALP and ACP activities [24]. The enzyme studies are done in the Diagnostic laboratory, SMS hospital, Tiruchirappalli.

Statistical analysis

The data are expressed as mean±SD. Differences between control and treatment groups are analyzed using one way ANOVA, with least significant differences. The ANOVA test carried out using SPSS (21 version) software.

RESULTS

Total Sugars and Cholesterol

Total Sugars and Cholesterol levels in ZnO NPs treated *Eudrilus eugeniae* organs showed significantly ($p<0.05$) reduced levels than control group (Table 1). In Gut organ, the total sugar level in control group is found as 4.35±0.10. ZnO NPs (0.25gm/Kg, 0.5gm/Kg, 0.75gm/Kg and 1gm/Kg) treated groups showed decreased sugar level (Table-1) as 3.95±0.10, 3.63±0.08, 3.15±0.10 and 2.70±0.08 respectively. Body wall is the major nanoparticle exposure route, so there is a significantly ($F=527.76$) decreased total sugar content (Figure 1) in the nanoparticles treated group than control. Reproductive organs like testis are easily gets disturbed by the nanoparticles by decreased total sugar content ($F=212.86$) in the ZnO NPs treated groups.

Table-1 showed decreased cholesterol levels in the treated groups due to the transamination reactions takes place in the earthworm organs. Among the three tested organs, body wall showed decreased cholesterol levels than other two organs. Body wall is the major nanoparticle exposure route, so there is a significantly ($F=827.53$) decreased cholesterol content (Figure-1) in the nanoparticles treated group than control.

Table-1: Total Sugars and Cholesterol levels (mean±SD) in various organs of Control and ZnO NPs treated *Eudrilus eugeniae*

Groups	Total Sugars (mg/dL)			Total Cholesterol (mg/dL)		
	Gut	Body wall	Testis	Gut	Body wall	Testis
Control	4.35±0.10	4.61±0.07	3.70±0.08	17.90±0.14	19.26±0.22	14.63±0.12
ZnO NPs 0.25gm/Kg	3.95±0.10	3.96±0.12	3.41±0.07	16.65±0.10	18.21±0.19	14.03±0.12
ZnO NPs 0.5gm/Kg	3.63±0.08	3.43±0.08	3.01±0.11	15.90±0.14	16.91±0.11	13.88±0.17
ZnO NPs 0.75gm/Kg	3.15±0.10	2.78±0.75	2.70±0.08	15.45±0.10	15.03±0.19	13.00±0.14
ZnO NPs 1gm/Kg	2.70±0.08	2.45±0.14	2.25±0.10	14.71±0.14	14.36±0.12	12.51±0.11

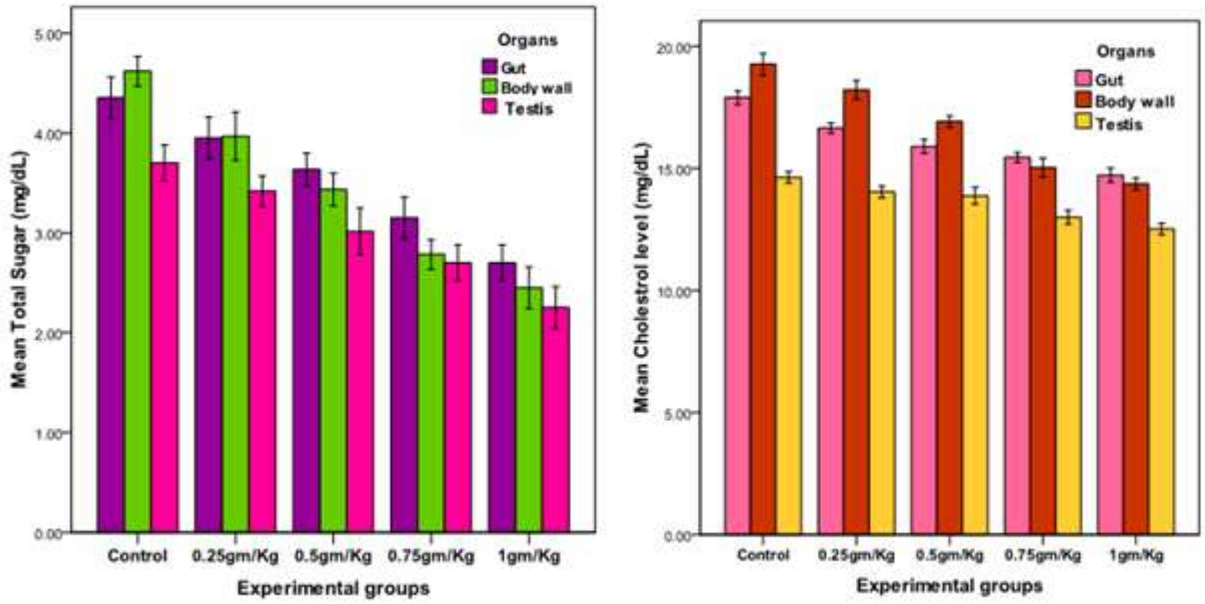

Fig-1: Total Sugars (mg/dL) and Cholesterol (mg/dL) levels (mean±SD) in control and ZnO nanoparticles treated *Eudrilus eugeniae* earthworm

Total Urea and Protein

Figure-2 showed increased urea levels in the ZnO NPs treated group organs due to the increased transamination reactions takes place in the earthworms than control group organs. The comparison between the mean values (Table-2) showed that the absorption and accumulation in the earthworm tissues are enhanced when there is an increased NPs concentration resulted in increased utilization of biomolecular contents which resulted in increased urea production. ZnO NPs (0.25gm/Kg, 0.5gm/Kg, 0.75gm/Kg and 1gm/Kg) treated groups showed increased urea levels and there F values are 183.35, 663.91 and 23.93 for Gut, Bodywall and testis organs respectively.

Increased urea level indicated the increased utilization of protein from the cells, it leads to decreased protein levels in ZnO NPs treated group organs and there F values are 166.57, 113.02 and 9167.1 for Gut, Bodywall and testis organs respectively.

Table-2: Total Urea and Protein levels (mean±SD) in various organs of Control and ZnO NPs treated *Eudrilus eugeniae*

Groups	Total Urea (mg/dL)			Total Protein (gm/dL)		
	Gut	**Body wall**	**Testis**	**Gut**	**Body wall**	**Testis**
Control	5.98±0.11	7.25±0.10	7.21±0.11	0.08±0.007	0.05±0.005	0.040±0.001
ZnO NPs 0.25gm/Kg	6.21±0.07	7.66±0.08	7.48±0.07	0.05±0.005	0.03±0.004	0.030±0.001
ZnO NPs 0.5gm/Kg	6.50±0.06	8.55±0.10	7.50±0.17	0.03±0.005	0.03±0.005	0.020±0.001
ZnO NPs 0.75gm/Kg	6.81±0.07	9.33±0.20	7.73±0.13	0.01±0.005	0.01±0.005	0.010±0.001
ZnO NPs 1gm/Kg	7.28±0.11	11.01±0.17	7.83±0.05	0.01±0.004	0.007±0.001	0.008±0.001

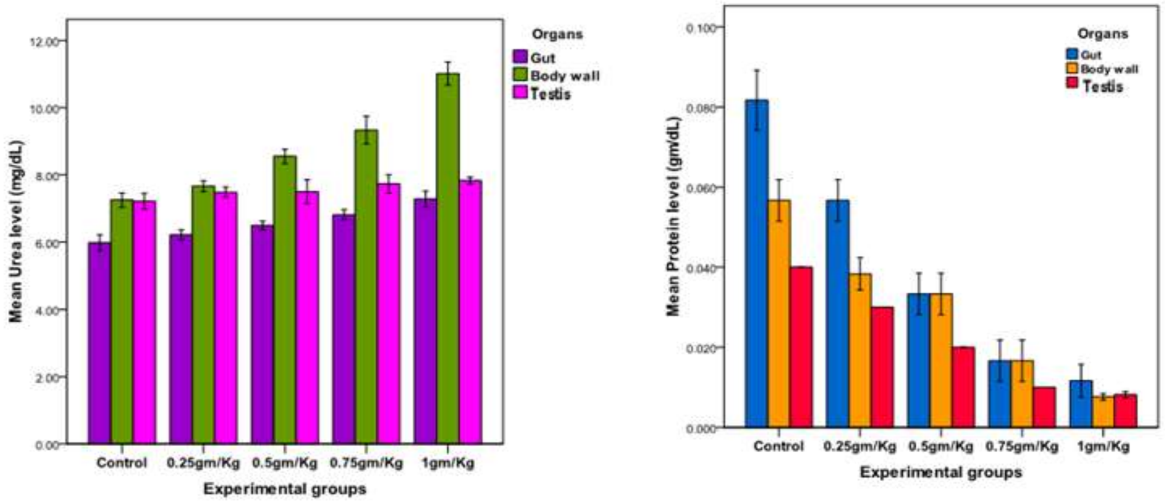

Fig-2: Total Urea (mg/dL) and Protein (gm/dL) levels (mean±SD) in control and ZnO nanoparticles treated *Eudrilus eugeniae* earthworm

Transaminase enzymes

Glutamate oxaloacetate transaminase (GOT) and Glutamate pyruvate transaminase (GPT) enzymes are produced during transamination reactions in protein and carbohydrate assimilation process. ZnO NPs treated earthworm showed increased transaminase enzyme production than control group. Treated earthworms body wall produced significantly ($p<0.05$) high GOT (Table-3) and GPT (Figure-3) enzymes production than other two organs.

Table-3: Transminases (GOT and GPT) levels (mean±SD) in various organs of Control and ZnO NPs treated *Eudrilus eugeniae*

Groups	GOT (IU/L)			GPT (IU/L)		
	Gut	Body wall	Testis	Gut	Body wall	Testis
Control	97.83±1.16	90.00±1.41	85.67±1.21	246.00±1.09	266.17±2.92	244.83±1.16
ZnO NPs 0.25gm/Kg	104.50±3.27	114.83±1.72	97.67±1.50	252.50±5.68	273.17±4.57	247.83±1.16
ZnO NPs 0.5gm/Kg	114.33±1.50	138.17±1.16	106.17±1.72	266.17±2.13	296.00±1.41	256.50±1.64
ZnO NPs 0.75gm/Kg	116.83±1.83	147.67±1.36	121.67±1.63	285.00±2.28	315.33±2.25	261.17±3.65
ZnO NPs 1gm/Kg	121.00±1.67	172.50±1.87	129.67±1.03	293.50±1.76	329.50±1.87	275.50±1.04

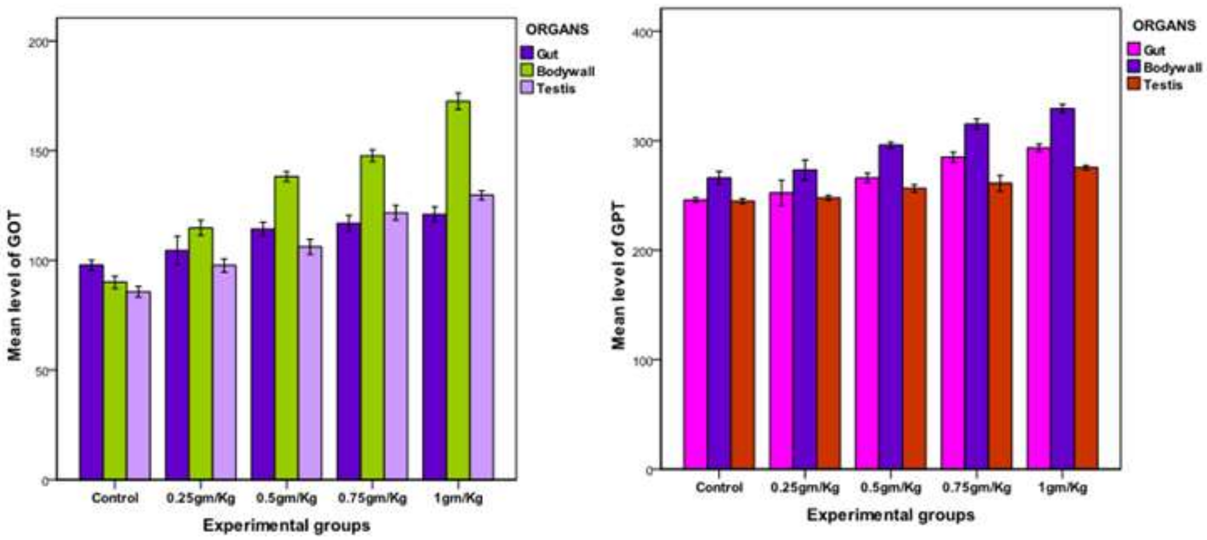

Fig-3: Transaminases levels (mean±SD) in control and ZnO nanoparticles treated *Eudrilus eugeniae* earthworm

Phosphatase enzymes

Increased ALP levels in ZnO NPs treated earthworm organs indicates increased glycogenesis or glycogenolysis to meet the energy demand. Earthworm's body wall showed significantly high ALP levels when compared to other two organs due to the frequent exposure to various compounds in the soil. Similarly, the ZnO NPs treated earthworms body wall tissues produces high amount of ALP enzymes (Table-4) especially when the concentration goes up the enzyme levels also increases.

Invading nanoparticles develops severe cellular damage which is evidenced by increased ACP levels in ZnO NPs treated earthworm tissues (Figure-4). A significantly increased ACP levels found in the treated groups, increased nanoparticle exposure increasing the lysosomal activity by producing more amount of ACP enzymes in higher concentration exposed earthworm tissues.

Table-4: Phosphatases (ALP and ACP) levels (mean±SD) in various organs of Control and ZnO NPs treated *Eudrilus eugeniae*

Groups	ALP (IU/L)			ACP (IU/L)		
	Gut	Body wall	Testis	Gut	Body wall	Testis
Control	291.67±4.84	382.83±12.28	165.33±2.80	315.83±4.53	453.83±1.72	115.00±2.61
ZnO NPs 0.25gm/Kg	346.83±4.79	466.33±11.57	196.50±1.22	493.67±2.42	489.50±5.46	191.17±5.03
ZnO NPs 0.5gm/Kg	381.17±4.16	498.33±9.77	246.67±1.21	556.00±2.00	538.67±4.80	257.83±1.94
ZnO NPs 0.75gm/Kg	474.50±2.25	591.33±7.76	292.00±5.40	614.33±2.94	582.33±6.59	293.67±4.92
ZnO NPs 1gm/Kg	551.67±3.83	686.50±3.88	347.17±3.48	705.67±2.06	614.83±3.97	333.00±2.60

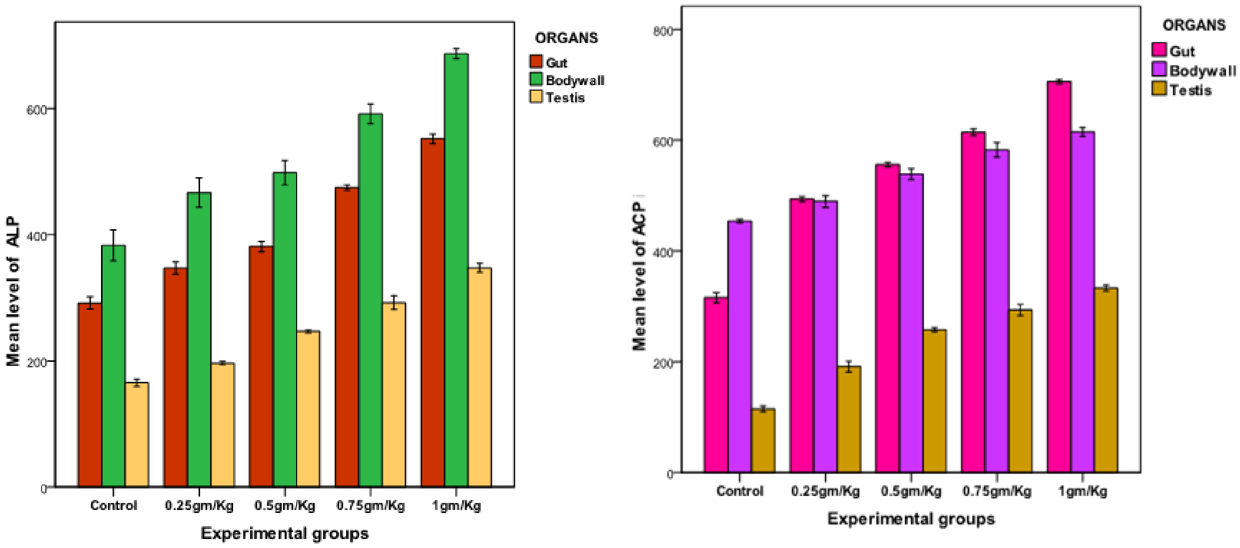

Fig-4: Phosphatases levels (mean±SD) in control and ZnO nanoparticles treated *Eudrilus eugeniae* earthworm

DISCUSSION

The living organisms exposed to environmental pollution can produce Reactive oxygen species (ROS) and the accumulation leads disturbed the cellular components such as proteins, nucleic acids, and lipids [25-27] and its metabolism. When metal oxide nanoparticles are directly taken up by organisms, they may cause cellular-level damage by disrupting biological processes [28].

During stress, organisms need more energy to detoxifying, biotransforming and to excrete the toxicants with the view of minimizing the toxic effects. This is achieved by the use of carbohydrate, the principal and immediate energy source [29]. The depletion of protein fraction in organisms may be due to the degradation of carbohydrate [30].

The circulatory level of glucose (a readymade source of energy) and protein was found significantly high than tissues in exposed groups due to the assimilation of stored contents from the tissues into the circulation to meet the energy demand in exposed organisms. When the growth and reproductive activities of the worms are gradually reduced, the protein content is also reduced to intermediate level [31].

Urea is the end product of the protein metabolism. It is synthesized in the liver from the ammonia produced by the catabolism of amino acids. Increased urea level indicates renal diseases, shock, and congestive heart failure in the treated organism. Decreased protein content in the ZnO NPs treated earthworm is due to the increased protein assimilation resulted in increased urea production in the earthworm. Various research indicates that GOT and GPT can be used as biomarkers of cellular damage in blood plasma, protein degradation and organ damage [32].

Disturbed enzyme levels in ZnO NPs treated earthworms are due to the altered physiological condition resulted from decreased biochemical composition in the organs. Hu *et al.,* [33] studied ZnO NPs effect on *Eisenia fetida* and showed increased accumulation when the NPs concentration goes up which supports this study results. Similar to our results, increased transaminase levels, increased acid and alkaline phosphatase enzymes with respect to decreased protein content in different pollutant exposed earthworms are reported by Ahmed *et al,*. [34], Habiba and Ismail [35].

GOT and GPT are two key function enzymes known for their role in the utilization of proteins and carbohydrates. Transamination reactions are prominent under stress conditions [36] and these enzymes are released during cellular damage or lysis and hence used to measure the stress intensity developed by the exposed toxicant to the organisms [37].

Prabha *et al.,* [16] reported higher activity of various enzymes including acid phosphatase in the gut of *E. eugeniae* and *E. fetida*. Due to pollutant exposure, there is a high energy demand, so the levels of glucose found increased. Also the parameters of protein metabolism (like urea, ammonia) are found to be significantly high. Theories have been proposed to explain the toxicity of NPs in organisms, that includes: generation of reactive oxygen species (ROS) which can disrupt cell structures, interference with normal metabolism [38, 39]; binding with macromolecules making them dysfunctional [40]; and functioning as a source for soluble metal ions, enhancing their bioavailability [41].

CONCLUSION

Gut, Bodywall and Testis are selected due to their nanoparticle exposure from surrounding soil which is bioagumented. Exposure of four different concentrations of ZnO NPs (0.25gm/Kg, 0.5gm/Kg, 0.75gm/Kg and 1gm/Kg) decreases the biochemical constituents of the tested earthworm organs due to balance the energy demand developed by the nanoparticle toxic stress. It is further evidenced by increased transaminase and phosphatase enzymes due to tissue damages in the ZnO NPs organs than control. The severities of the effects are increased while there is an increased NPs concentration.

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