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In vivo toxicity of biogenic silver oxide- bismuth nanoparticles in the earthworm *Lumbricus Terrestris*: A naked eye approach

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Abstract
Earthworms are soil-based organisms of agricultural importance which have been referred by all as the "farmer's friend''. The exposure of soil to heavy metals are of serious concern. Chemically synthesized pesticides, heavy metals are of great concern to the environmentalists as well as the end users. Nanoparticles are materials which are one- billionth in size. Bimetallic nanoparticles are a combination of two different metallic core structures. Chemically synthesized nanoparticles have been reported to possess heavy toxicity to organisms including soil- based organisms. But, biogenic nanoparticles are the ones which are synthesized by environmentally friendly approaches using biologically derived products in agriculture. Hence, biogenic nanoparticles are being preferred over chemically synthesized nanoparticles. *Lumbricus terrestrius* is an earthworm which is commonly found in the Indian peninsula. The silver oxide- bismuth nanoparticles have been characterized by UV spectrometry. The natural soils were spiked with cow dung. The toxicity profiling was also carried out by naked eye analysis and movement analysis. The toxicity of nanoparticles biogenically synthesized were found to be less when compared to chemically synthesized ones. The anomalous weight gains and weight losses were seen in all concentrations selected in the earthworms *Lumbricus terrestrius*. Out of 22 test earthworms, 8 earthworms died whereas in the control, all three of the earthworms survived. This justifies the anomaly in the toxicity wherein random weight gains and weight losses and deaths were recorded even due to the supposedly biogenic nanoparticles.

Keywords: Earthworms, *Lumbricus terrestrius*, silver oxide, bismuth, nanoparticles, UV, anomaly, biogenic

Introduction
New materials are being developed in the realm of nanotechnology. Nanomaterials refer to materials which are one billionth in size. The nanomaterials have found exciting applications in batteries, automobiles, electronics and medical sectors but their impact on the living systems and the environment is a double- edged sword (Alves *et al* 2019) [1].

Earthworms are soil-based organisms which are referred to by mankind as the farmer's friend. Singh *et al* in the year 2022 [2] (Singh *et al* 2022) [2] have assessed the environmental impact of the silver nanoparticles on soil earthworms *Eudrilus euginae*. The silver nanoparticles were prepared by chemical method with the help of trisodium citrate. The morphology was determined with the help of scanning electron microscopy, transmission electron microscopy and the size of the nanoparticles was determined by X-Ray diffractometry (XRD). The FT- IR (Fourier transform- infra red) spectrometry was used to study the organic functional groups which encapsulated the nanoparticles [2].

Garcia- Velasco (Garcia- Velasco *et al* 2016) [3] *et al* have studied the uptake route of the silver nanoparticles in the earthworm *Eisenia fetida* through standard OECD tests. The study revealed higher the silver ion accumulation rate, greater the disruption of the tegument thereby leading to the mortality of the earthworms. The soils were prepared and spiked with manure and the nanoparticles thereby the uptake of the nanoparticles by earthworms was recorded.

Objective
From the above literature survey, it was observed that chemically synthesized nanoparticles were found to be toxic to the earthworms. Hence, a hypothesis was made to determine whether biogenically synthesized silver oxide- bismuth nanoparticles was found to be non- toxic or less toxic to the earthworm *Lumbricus terrestrius*.

Materials and Methods
Preparation of Bimetallic Silver Oxide- Bismuth Nanoparticles
Five grams of freshly processed *Curcuma longa* were taken and dissolved in 75 g of ethanol. The contents were filtered and the filtrate was concentrated *in vacuo*. 1 mL of the filtrate was then taken and dissolved in 99 mL of double-distilled water. To the resulting mixture, 0.1 M of silver nitrate and 0.1 M of Bismuth nitrate pentahydrate solutions were added and stirred continuously. The contents were heated at 42 °C at 35 minutes over a heating mantle and the liquid was decanted carefully. The resulting residue contained the nanoparticles.

Selection of Earthworms
The earthworm selected was *Lumbricus terrestrius*.

Weight of the earthworms
Before the toxicity testing, five earthworms were randomly selected and were weighed prior to toxicity testing.

Preparation of concentrations of nanoparticles

1500 ppm, 750 ppm, 250 ppm, 125 ppm, 62.5 ppm concentrations of Biogenically synthesized Silver Oxide-Bismuth nanoparticles were prepared in dimethyl sulfoxide (DMSO) and they were dissolved each in 100 mL of double distilled water.

Preparation of soil samples and soil spiking

Natural soil samples were collected from Chennai City in 11 different plastic containers irrespective of dimensions. The soil samples were filled up to half of the containers' volume. Cow manure was collected from SVDD Temple Cowshed, Mylapore, Chennai, TN, India. 20 g of cow manure was inserted at the center of the plastic containers which were filled with the soil samples. The containers were segregated into two rows; each row containing 5 containers. The control had one container separately. The first container was filled with 1500 ppm nanoparticles, the second container with 750 ppm, the third container with 250 ppm, the fourth with 125 ppm and the fifth with 62.5 ppm. The procedure was repeated in the second row. The control did not contain nanoparticles, it contained 100 mL of water.

Insertion of earthworms

Three earthworms were inserted in the first container, the second container was inserted with three earthworms, the third container was inserted with three earthworms. The fourth container contained two earthworms and fifth container contained two earthworms. The procedure was repeated for the second row also. The control container was inserted with three earthworms. The containers were each sealed with Gaada cloth. The procedure was designed and carried out at CIMSER (Centre for Insect Molecular Science and Environmental Research) Unit, Department of Advanced Zoology and Biotechnology, Loyola College (Autonomous), Chennai under the supervision of Dr. M. Raja. Water i.e., tap water was sprinkled periodically. The containers were kept for 30 days undisturbed (Garcia-Velasco *et al* 2016) [3].

Characterization of Nanoparticles

The nanoparticles were characterized using UV at White Lab, Saveetha Dental College, SIMATS Deemed to be University, Chennai- 77.

Results

Table 1: Weight of the Earthworm before soil spiking

S.No	Weight (mg)
1	530
2	564
3	627
4	448
5	313

Table 2: Weight Gain/ Loss Toxicity test Table post spiking with cow dung and nanoparticles. Weights were recorded and the movements were seen

S. No	1500 ppm	750 ppm	250 ppm	125 ppm	62.5 ppm	Control (cow dung and water only)
1	360 mg	1000 mg	710 mg	1400 mg	1150 mg	750 mg
2	480 mg	960 mg	870 mg	1200 mg	680 mg	930 mg
3	70 mg	810 mg	600 mg	-	-	670 mg
1	Death	Death	Death	Death	690 mg	-
2	40 mg	Death	Death	790 mg	290 mg	-
3	360 mg	Death	Death	-	-	-

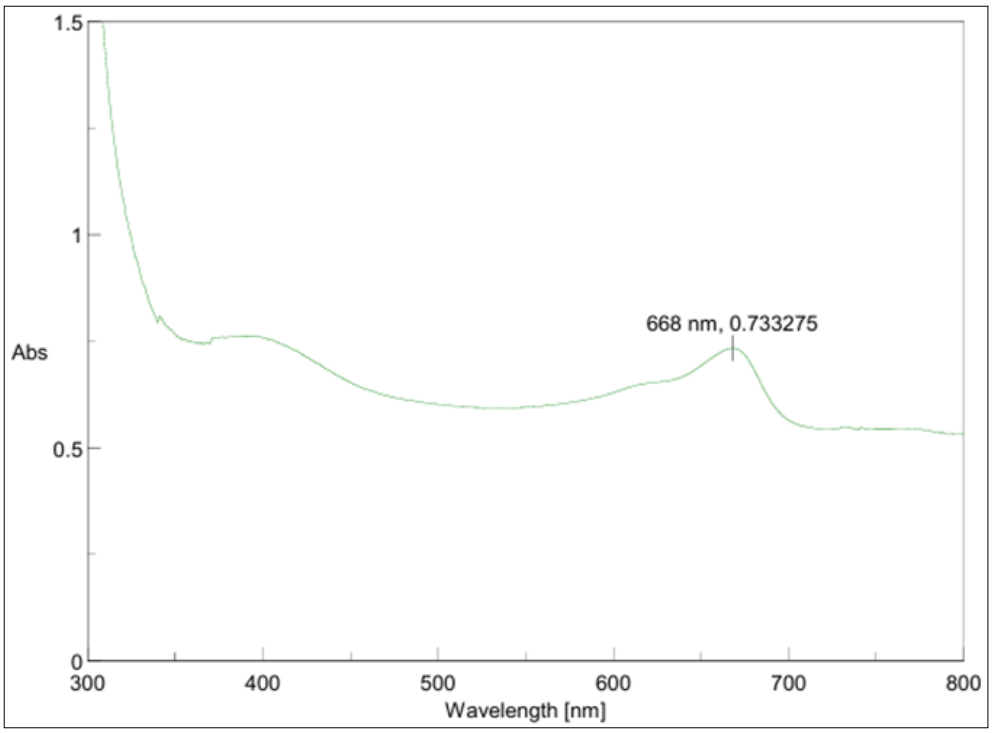

Fig 1: UV- Vis of the Biogenic Ag_2O- Bi nanoparticles. Bathochromic shift could be observed because the bimetallic NPs were formed at 668 nm.

Fig 2: Image of the earthworm *L. terrestrius*. (Source: CABI Digital Library)

Discussion

From the above results, it is seen that an anomalous weight gain and weight loss in the earthworms could be observed. From the table, 8 deaths overall were observed. Non-Uniform weight loss/weight gain was observed from the table and 62.5 ppm was the threshold concentration at which all the earthworms survived. This is comparable with the study done by Topuz and van Gestel in the year 2017 (Topuz and Van Gestel 2017) [4]. Except that the authors have carried out the study on silver nanoparticles in *E. crypticus* which is another species of earthworm. The present study involves a combination of silver oxide and bismuth and we have not carried out the bioaccumulation studies as there have been only 8 deaths out of 22 earthworms tested. From the UV Vis spectrometry, it can be inferred that the silver nanoparticles were formed at 668 nm indicating a bathochromic shift. Moreover, this study involved the use of natural soil and biogenic nanoparticles. From the table (1) above it can be inferred that there are contrasting values indicating weight losses and weight gains when compared to the table (2) and the control. The contrasting weight losses and weight gains at 1500 ppm, 750 ppm, 250 pm, 125 ppm and 62.5 ppm indicate the effect of the bimetallic nanoparticles. At 62.5 ppm all the earthworms survived. Whereas the earthworms died at 1500 ppm, 750 ppm, 250 ppm and 125 ppm.

Conclusion

From the above study, we infer that the potential use of the nanoparticles in the agrarian sector has implications on soil organisms. Hence, the toxicity study on the earthworms was carried out. With this study, it is concluded that the nanoparticles were found to be less toxic. No deaths were found to be at 62.5 ppm concentration. Further, this study involved a one pot solvo thermal biogenic synthesis and it did not involve the use of toxic chemicals as reducing agents for the preparation of the nanoparticles. Future and potential application of the nanoparticles might be conducted in the preparation of the antifeedant agents as against insect pests.

Ethics Statement

This study did not involve the use of animals as it involved the use of earthworms only.

Conflict of Interest

The authors do not have any conflict of interest.

Authors Contribution

Adithya CG Performed the assays. Dr. M.F. Valan and Dr. M. Raja designed the work and supervised the study.

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