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EFFECT OF RECKLESSLY DISPOSED SILVER NANOPARTICLES FROM LABORATORY ON *VIGNA RADIATA, BRASSICA JUNCEA,* RHIZOSPHERE FLORA *AND DROSOPHILA MELANOGASTER*

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ABSTRACT

Present work aims to examine the effect of chemically sythesized silver nanoparticles on *Vigna radiata*, *Brassica juncea*, their rhizobacteria and *Drosophila melanogaster*. Silver nanoparticles were synthesized by reducing silver nitrate with sodium borohydrate in the presence of sodium citrate. UV-Vis Spectroscopy, XRD and SEM confirmed the formation of spherical silver nanoparticles in the range of 16±4 nm. The germination potential of seeds was reduced to as low as 11.07% and 27.74% when *V. radiata* and *B. juncea* were treated nanoparticle solution. Length of roots, shoots and leaf number was reduced in treated plants. Confirming the antimicrobial activity of silver, significant reduction ($p<0.05$) was observed in rhizosphere bacteria. In culture independent Diphenylamine assay, treated soil samples reported only 3.84 µg as compared to 8.46 µg DNA of control. *Drosophila melanogaster* showed marked dose- and duration-dependent decline in treated adult flies, larva and pupa. Also, malondialdehyde, the end product of lipid peroxidation and catalase activity was higher in treated flies.

KEYWORDS: **Silver nanoparticles, toxicity, *Vigna*, *Brassica*, Rhizoflora, *Drosophila***

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INTRODUCTION

Interest on environmental impact of nanoparticles has rapidly increased over the past few years, because eventually these particles are released into the surroundings. Of late, implication of nanoparticles as carriers of insecticides/pesticides/manures and/or fertilizers has resulted in improved plant protection. However, on the flip side, we remain ignorant of the effects of nanoparticles that might be accumulating in our natural ecosystems like soil and water. Second mode of entry is the synthesis and reckless disposal of nanoparticles, in the laboratories world over. We don't have sufficient parameters and regulations to check what level is safe and what is unsafe, what is degrading and what is remaining and assuming what form and how is it affecting us, especially in developing countries like India. A critical evaluation of the fate of these particles is, therefore, very important because many particles like silver are toxic even in their bulk dimensions. Owing to the high surface to volume ratio these nanoparticles are highly reactive even if they are made from novel metal, hence nanoparticle present possible danger to the environment and its biota. Since they are small, these particles can enter cells passing the cell membrane and get deposited inside the cells. Their possible cytotoxicity is still in dark as their interaction with the biological system is relatively obscure. The nanoparticles localised in the soil or present in water may be taken up by plants and translocated. Hence plants can absorb nanoparticles and these particles may reach in the food chain and find their way to human and others animals. The bioaccumulation of these nanoparticles in the animals at the apex of the food chain will be significantly higher. Researches suggest that the size and the surface charge of the nanoparticles determine the uptake and translocation. Nanoparticles likely enter the environment through waste water, where they accumulate in bio-solids at waste water treatment plants. One of the ways in which the sludge is disposed off is through its land application, because it is valuable as a fertilizer[1]. Since the uptake of nanoparticle by food crops and their translocation, bioaccumulation and risk associated with it has not been greatly established, thorough research are needed to establish their biomagnification in the food chain. Quite a few reports have studied these aspects. Toxic effects were observed on root and shoot growth and seed germination. One study revealed that interaction of nanoparticles had various toxic effects on seed germination[2]. Root initiation, root growth and shoot growth mainly depends upon the shape, size and concentration of nanoparticles. Silver nanoparticles are shown to be effective against bacteria[3]. Silver nanoparticles exposure may cause toxic effects to skin, liver, lung, reproductive system and other parts of mammalian body including brain[4]. One study demonstrated apoptosis in *Drosophila* larvae and peroxide radicals produced by lipid peroxidation and catalase antioxidant enzyme activity were significantly higher in exposed *Drosophila melanogester*[5]. As such the aim of this research was to determine the effect of chemically synthesized silver nanoparticles on two important food crops: *Vigna radiata* and *Brassica juncea.* Additionally, we also examined how the rhizobacteria are affected. Finally, we directed our studies to *Drosophila melanogaster*. Its well documented genetics, fully studied developmental biology, less ethical objection, dimorphic sexuality, short generation time and closeness to human genome makes it a very good model for human systems[6].

MATERIALS AND METHODS

Materials

Silver Nitrate ($AgNO_3$, 99.8%), Sodium Borohydride ($NaBH_4$, 99.8%) and Sodium citrate ($Na_3C_6H_5O_7.2H_2O$) were purchased from NICE chemicals, Chennai, India. The solutions were prepared in distilled water obtained from Easy pure system (PALL Bangalore India.). All chemicals were used without any further purification or treatment. Healthy seeds of *V. radiata* and *B. juncea* were selected and thoroughly washed with distilled water to remove adsorbed chemicals present on the outer coat of seeds.

Synthesis of silver nanoparticles

Synthesis of silver nanoparticles was carried out according to method described by Purcell (1998). Silver nitrate (0.01 M) was reduced with of sodium borohydride in the presence of sodium citrate as a stabilizing agent. The ratio of borohydride and silver ions was changed by volume of borohydride. Variation in the reductant concentration was done to achieve maximum possible yield of nanoparticles. Concentration of sodium citrate was 0.01M throughout the experiments. Silver nitrate and sodium citrate solutions were mixed and stirred for 60 sec. After heating the solution to boiling and vigorous stirring, freshly prepared 0.1M of sodium borohydride was added drop wise. The solution immediately turned yellow and was stirred for 60 sec and then stored.

Characterization methods

Characterization was done using UV-Vis spectroscopy, X-Ray diffraction and Scanning Electron Microscopy. Silver nanoparticles solutions were analyzed using a UV-Vis Spectrophotometer (Shimadzu Scientific Instruments, Japan). The absorbance was measured in the wavelength range 300-600 nm. Readings were taken immediately after the synthesis procedure and at regular intervals. X Ray Diffraction analysis (XRD) was done using. Powder form of the silver nanoparticles was prepared by centrifuging the particle solution. Pellet was collected and washed with thrice with triple distilled water and once with acetone to remove the impurities and then dried to get the powder form which was used for XRD analysis. Size of silver nanoparticles was determined by XRD using Debye-Scherrer formula. Scanning Electron Microscopy (SEM) analysis was done using Hitachi S-4500 SEM machine. Size and shape of silver nanoparticles were determined by SEM. A small drop of nanoparticle solution was coated on the copper grid in the form of thin layer and dried under mercury lamp for 5 min.

Treatment studies

Seed Germination

Three replicates of ten seeds each of *V. radiata and B. juncea* seeds were exposed to two different concentrations (OD= 0.5 and OD= 3.0) of silver nanoparticles. Seeds germinated in normal water without nanoparticles were kept as control. The presoaked seeds were raised in 60mm petriplates on Whatmann filter paper no. 1. The germination index was calculated using the formula given by Wang et al[7].

G.I. = (number of seeds germinated/ total no. of seeds) x 100

Where, G.I. is the germination index.

Plants

Healthy Seeds of *Vigna radiata* and *Brassica juncea* were selected. They were properly rinsed with tap water followed by distilled water. Soil was mixed with equal amount of fertilizer was taken from the VIT University Nursery. Disposable plastic glasses (height- 9 cm, diameter- 6cm) were taken for growth of plants. Seeds of *Vigna radiata* and *Brassica juncea* were soaked in water for 4h and transferred to pots containing 200g of soil. After the germination of seeds in pots twelve pots were kept as control, twelve pots were treated with low concentration (0.5 O.D at 420 nm) and twelve pots were treated with high concentration (3.0 O.D at 420 nm), of silver nanoparticle solution. The volume of nanoparticle solution disposed in each pot was 10 ml at three day interval. Plants were regularly watered. Initially, each pot was planted with two seeds, but after germination only one, which was physiologically healthier was used for the treatment studies. The morphological changes in the roots, stem and leaves of *V. radiata and B. juncea* caused by silver nanoparticles were documented upto thirty days. Readings were recorded for various parameters like shoot length, root length, number of roots and leaves, nature of root, shoot and leaves.

Rhizosphere bacteria/ microbes

To study the effect of silver nanoparticles on bacterial community, soil was collected from rhizosphere of nanoparticle treated plants, from 3 cm depth. Soil samples were serially diluted (10^{-2}, 10^{-4}, 10^{-6}) and inoculated on nutrient agar plate to count the colony forming units per milliliter (CFU/ml). Untreated soil was

used as standard. In another culture independent assay total DNA estimation of soil microbes was done by the standard Diphenyl amine method[8]). Calf thymus DNA was used as the standard. Equation generated from the calibration curve y= 0.0065x + 0.275, with R^2 value of 0.99 was used to calculate the amount of DNA in the test samples.

Drosophila melanogaster

The flies were exposed to different concentration of silver nanoparticles (0.0mg/ml, 0.01 mg/ml, 0.05mg/ml, 0.1mg/ml, 0.5mg/ml and 1 mg/ml), to study the effect on egg viability and reproduction capability. The flies were fed with standard cooked media that consisted of 500ml distilled water, 2.5gAgar, 17g maize powder, 15g sugar, 6g baker's yeast, 1ml propionic acid and1g antifungal agent (dissolved in 5ml of 90% ethanol). Flies were maintained in an air conditioned room (22±2ºC). Observations were made for 55 days at regular intervals. The flies were counted in their three developmental stages i.e. adult, larvae and pupa.

Cell destruction and catalase activity of Drosophila melanogaster

The larvae treated with silver nanoparticles were collected from the culture bottles and a larval extract was made for lipid peroxidation and catalase assay. The larval extract was used to determine the catalase activity by method of Sinha[9]. This method is based on reduction of dichromate/acetic acid reagent into chromic acetate in the presence of hydrogen peroxide (H_2O_2). The absorbance was taken at 570 nm using a spectrophotometer and amount of remaining H_2O_2 was measured using the standard equation y = 0.0409x– 0.0288.

Statistical analysis

The data were subjected to two-way analysis of variance (Anova) to determine the significance of different factors. p-values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Synthesis of silver nanoparticles

The reduction of silver nitrate by sodium borohydride in the presence of sodium citrate was observed to be a fast process. At boiling temperature, the colorless reaction mixture turned to yellow which was an indication of formation of silver nanoparticles. Figure 1 shows the image and UV-Vis spectra obtained with different concentration of borohydride solution. As expected, concentration of reduced silver increased with increase in reductant concentration and was found to be highest at 0.1M of $NaBH_4$. The maximum absorbance for silver nanoparticles was recorded in the range of 350-600 nm.

Characterization of silver nanoparticles

The absorption spectra of pale yellow colored silver colloids prepared by sodium borohydride reduction showed a absorption maxima at 410 ±10 nm indicating the presence of silver nanoparticles figure 1.

X-Ray Diffraction Analysis

Further confirmation for nanoparticle synthesis and structural properties was obtained by X-ray diffraction method. The XRD data of both experimental and standard sample indicates the formation of silver nano particles (Table 1). Considering the peak at 38.34 degrees, average particle size has been estimated by using Debye-Scherrer formula:

$$D = 0.9\lambda/W\cos\theta$$

Where, 'λ' is wave length of X rays (0.1541 nm), 'W' is FWHM (Full width of half maximum), θ-theta' is the diffraction angle and 'D' is particle diameter (size). The average particle size was calculated to be around 17 nm.

Scanning Electron Microscopy

SEM image recording from powder form of silver nanoparticles synthesized by chemical reduction method is represented in figure 1. The SEM image showed spherical or roughly spherical and relatively uniform shape of nanoparticles formation with diameter range of 16 – 20 nm.

Effect of silver nanoparticles on seed germination

The results of germination potential of *Vigna radiata* and *Brassica juncea* are represented in figure 2a. In low concentration OD (0.5), 62.07% and 55.54% seeds of *Vigna* and *Brassica* germinated whereas at higher OD (3.0) only 11.07% and 27.74% of seeds germinated for *Vigna* and *Brassica*, respectively. The results are in agreement of previous studies which reported a decline in germination of a variety of plant seeds and were also inhibitory to growth of roots, stems and leaves[10].

Morphological analysis of plants

Morphological examination was carried out using pot culture experiments of *Vigna radita* and *Brassica juncea* (Fig. 2b). Reduction in number of leaves, root and shoot length was observed in the silver nanoparticles treated plants (Fig. 2c, d and Fig. 3). Plants treated with higher concentration of particles were significantly affected as compared to untreated plants and plants treated with low concentration of nanoparticles. Particles also effected the formation of root hair. Additionally, stunted growth of plant parts and loss of root hairs were also observed. Adverse effects such as leaf curling, distortion, etiolation and yellowing were observed in the leaves of treated plants. Some studies suggest that silver nanoparticles may interact with proteins associated with photosystems, starch synthesizing machines and also affect carbohydrate translocation[11] , that may be the reason of such abnormalities.

Effect of silver nanoparticles on rhizosphere soil microbes

In the present study significant reduction in number of colony forming units was observed ($p<0.05$), in the soil treated with silver nanoparticles solution (Fig. 2e). The effect was also observed on the total DNA content of microbes present in soil. In diphenyamine assay, 3.84 µg/ml of DNA was present in treated soil as compared to 8.46µg/ml of DNA in untreated soil. Toxicity of silver nanoparticles has been reported in heterotrophic and chemolithotrophic soil forming bacteria[12] . Reports suggest bactericidal effect of silver nanoparticles is due to the destruction of enzymes that transport cell nutrients and weakening of cell membrane leading to increased cell permeability and cell death[12] .

Effect of silver nanoparticles on egg viability and reproduction capability of Drosophila melanogaster

Result clearly revealed significant reduction in reproductive capability of flies on prolonged exposure to nanoparticles. The effect was conspicuous from the 20th day of treatment, before this there was little or no effect in all treatment groups. Also, higher concentration of 1.0 mg/ml was most toxic as compared to the lower concentrations. The toxic effects were more visible from 0.05 mg/ml. This was evident from relative decreased number of flies, affected transition ability between their developmental stages and the egg viability. The graphs in figure 4 represent number of survivors at an interval of ten days; more detailed, day-by-day analysis of observation is listed in table 2. Earlier studies also reported toxic effects of silver nanoparticles on reproduction[13] whereas the other demonstrated decrease in egg laying capacity of treated fruit flies[14].

Effects of silver nanoparticles on cell destruction via lipid peroxidation

Lipid peroxidation was found to be significantly higher in silver nanoparticles treated flies as compared to untreated group (Table 3). Similar results were obtained in a study in which malondialdehyde, the end product of lipid peroxidation was found significantly higher in treated flies as compared to the control[5] .

Effects of silver nanoparticles on catalase activity of Drosophila melanogaster

Catalase activity was also significantly higher in silver nanoparticles treated flies (Table 3). The specific activity of catalase is calculated and represented in figure 5. The results are in agreement of previous study in which activity of antioxidant enzyme catalase was significantly higher in treated flies as compared to the control[5].

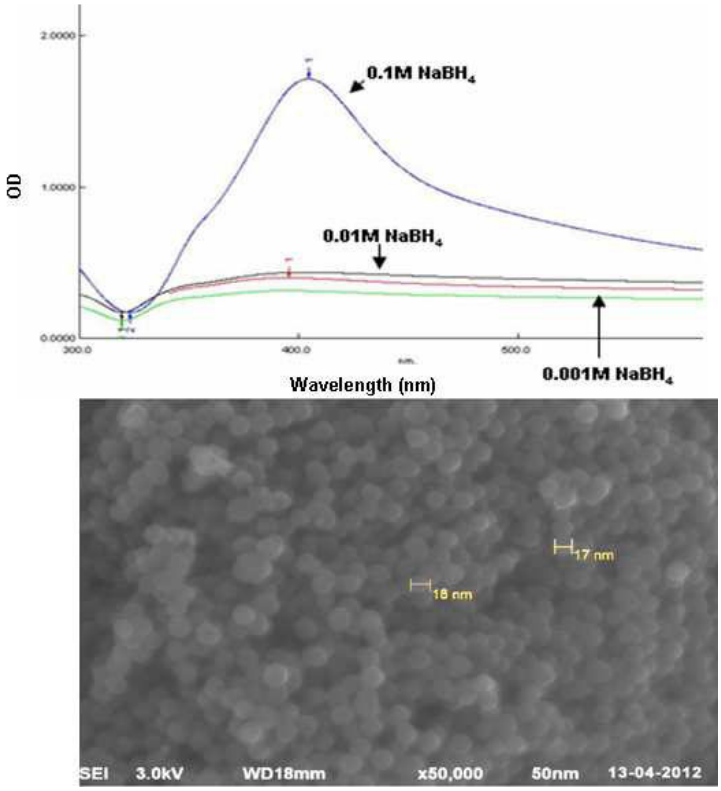

Figure 1

Characterization of silver nanoparticles a) UV-Vis absorption spectra measured with different amounts of borohydride solution b) Scanning electron micrograph of silver nanoparticles

Figure 2

a) Effect of silver nanoparticles on seed germination

b) Pot culture experiment showing all the plants of Vigna radiata and Brassica juncea before treating them with a silver nanoparticle solution.

c) Plants of Vigna radiata and d) Brassica juncea uprooted after treatment with silver nanoparticles

e) Effect of silver nanoparticles on soil microbes

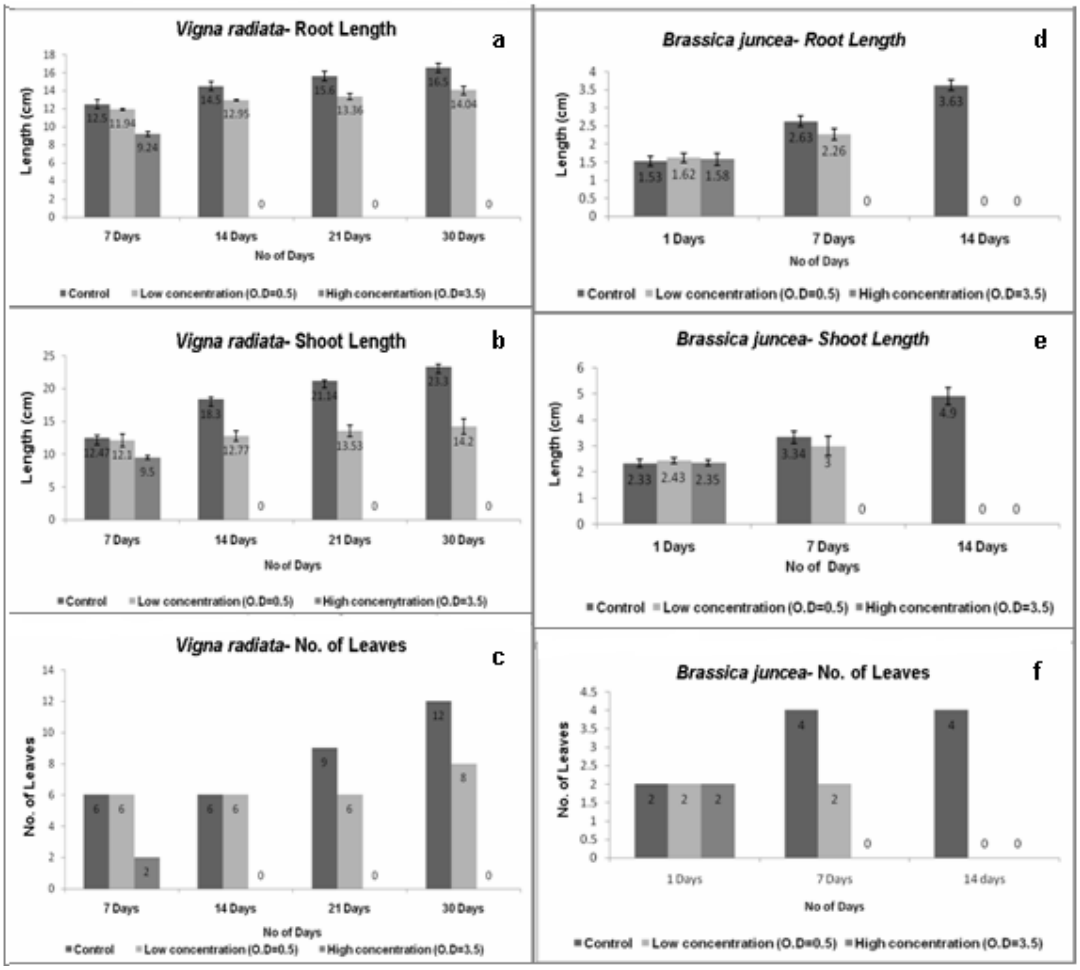

Figure 3
Effect of silver nanoparticles on number of shoot, root and leaves of a, b, c Vigna radiata and d, e, f Brassica juncea

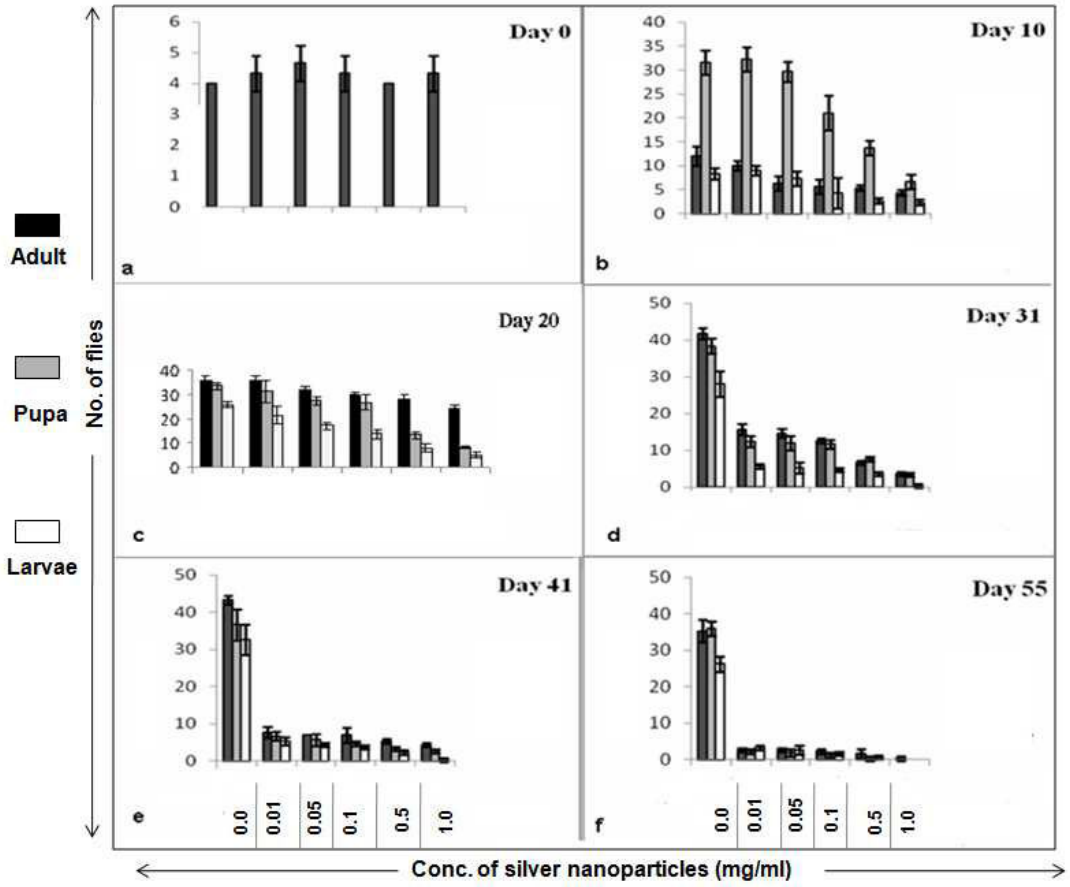

Figure 4
Graphs showing decreased number of flies with respect to increased number of days of treatment a) day 0 b) day 10 c) day 20 d) day 31 e) day 41 f) day 55

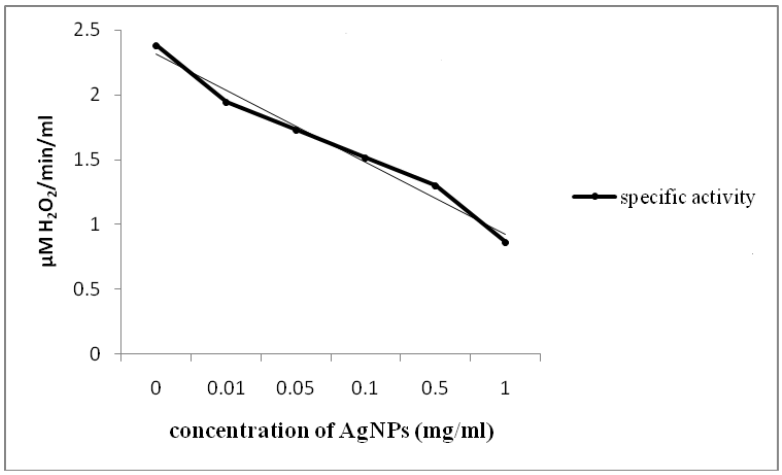

Figure 5
Specific activity of Catalase

Table 1
X Ray diffraction data of test and standard silver nanoparticles

Experimental diffraction angle (2θ degrees)	Standard diffraction angle (2θ degrees)
38.34	38.17
44.51	44.31
64.76	64.50
77.67	77.05

Table 2
Effect of different doses and duration of silver nanoparticles on Drosophila melanogaster

Days	Number of adults					
	0.0	0.01	0.05	0.1	0.5	1.0
0	4.0±0.0	4.3±0.5	4.6±0.5	4.3±0.5	4.0±0.0	4.3±0.5
3	4.0±0.0	4.3±0.5	5.0±0.0	4.3±0.5	4.0±0.0	4.3±0.5
6	4.0±0.0	4.3±0.5	5.0±0.0	4.3±0.5	4.0±0.0	4.3±0.5
10	12.0±2.0	10.0±1.0	6.3±1.5	5.6±1.5	5.3±0.0	4.3±0.0
12	29.0±1.0	30.6±1.1	27.6±1.5	25.6±3.2	17.6±1.5	14.3±0.5
14	32.0±1.0	32.3±2.5	28.0±1.0	27.6±1.5	24.3±0.5	17.6±1.5
17	33.6±1.5	34.6±1.5	30.0±1.0	28.3±0.5	26.0±1.0	19.6±1.5
20	36.3±1.5	36.0±2.0	32.0±1.0	30.0±1.0	28.3±1.5	24.0±2.0
24	39.3±0.5	32.3±3.2	27.6±1.5	26.3±1.5	26.3±1.1	19.6±2.0
27	42.0±1.0	20.3±0.5	16.3±1.5	12.6±2.0	7.3±1.1	3.6±0.5
31	41.6±1.5	15.6±1.5	14.6±1.1	12.6±0.5	6.6±0.5	3.6±0.5
34	42.0±1.0	12.6±2.0	9.3±0.5	8.6±1.1	6.6±0.5	3.6±0.5
38	42.6±1.5	8.6±0.5	8.3±0.5	7.3±0.5	5.3±1.1	3.3±0.5
41	43.3±1.1	7.6±1.5	7.0±0.0	7.0±2.0	5.3±0.5	4.3±0.5
45	43.3±1.1	7.0±1.0	6.6±0.5	6.0±1.0	5.3±0.5	4.3±0.5
48	43.3±1.1	4.3±0.5	4.0±1.0	3.3±0.5	2.6±0.5	2.3±0.5
52	35.3±4.0	2.6±0.5	2.3±0.5	2.3±0.5	1.6±1.1	0.3±0.5
55	35.3±3.0	2.6±0.5	2.3±0.5	2.3±0.5	1.6±1.1	0.3±0.5

Number of larvae					
0.0	0.01	0.05	0.1	0.5	1.0
0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
5.3±0.5	4.6±0.5	4.3±1.1	3.3±0.5	3.0±1.0	2.3±0.5
15.0±1.0	15.3±1.5	11.6±1.5	9.3±0.5	6.6±0.5	4.0±1.0
8.3±1.1	9.0±1.0	7.3±1.5	4.3±3.2	2.6±0.5	2.3±0.5
14.3±2.0	12.3±1.5	7.3±1.5	5.6±2.5	2.6±0.5	2.3±0.5
16.3±1.5	15.3±2.3	8.6±0.5	6.3±1.5	3.3±0.5	2.6±0.5
21.6±1.5	18.3±2.0	13.3±0.5	8.6±0.5	6.3±1.5	4.0±1.0
26.0±1.0	21.6±3.5	17.3±1.5	14.0±2.0	8.0±1.7	5.0±1.0
26.3±0.5	21.6±3.5	17.3±1.5	14.0±2.0	8.0±1.7	5.0±1.0
30.0±6.9	7.3±2.5	9.6±2.0	7.6±0.5	5.3±1.5	0.6±0.5
28.0±3.4	5.6±0.5	5.3±1.5	4.6±0.5	3.6±0.5	0.3±0.5
28.6±3.0	5.3±1.1	4.3±0.5	3.6±0.5	2.3±0.5	0.3±0.5
29.3±3.5	5.3±1.1	4.3±0.5	3.6±0.5	2.3±0.5	0.3±0.5
32.6±4.0	5.3±1.1	4.3±0.5	3.6±0.5	2.3±0.5	0.3±0.5
29.6±4.7	5.3±1.1	4.3±0.5	3.6±0.5	2.3±0.5	0.3±0.5
29.6±4.7	4.6±1.1	3.6±0.5	3.3±1.1	2.3±0.5	0.3±0.5
26.3±2.0	3.3±0.5	2.6±1.1	1.6±0.5	0.6±0.5	0.3±0.5
26.3±2.0	3.3±0.5	2.6±1.1	1.6±0.5	0.6±0.5	0.0±0.0

Days	Number of pupa					
	0.0	0.01	0.05	0.1	0.5	1.0
0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
3	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
6	12.0±2.0	13.6±1.5	9.3±0.5	7.3±0.5	4.3±1.1	2.3±0.5
10	31.6±2.5	32.3±2.5	29.6±2.0	21.0±3.6	13.6±1.5	6.6±1.5
12	25.0±1.0	26.0±1.7	25.6±1.5	22.6±6.4	21.3±1.5	13.3±1.5
14	30.3±1.5	33.0±1.0	26.0±1.0	25.3±4.1	10.0±2.0	7.6±0.5
17	33.6±1.5	31.3±4.7	27.6±1.5	26.6±3.0	13.3±1.5	8.6±0.5
20	33.6±1.5	31.3±4.7	27.6±1.5	26.6±3.0	13.3±1.5	8.6±0.5
24	36.3±1.5	31.3±4.7	27.6±1.5	26.6±3.0	13.3±1.5	8.6±0.5
27	38.0±2.0	19.6±1.5	18.6±1.5	15.0±2.6	6.6±0.5	3.3±0.5
31	38.3±2.0	12.3±1.5	12.0±2.0	11.6±1.1	7.6±0.5	3.3±0.5
34	39.3±1.1	9.3±0.5	8.3±1.5	6.3±0.5	5.3±1.5	3.3±0.5
38	39.6±1.5	8.6±0.5	6.3±0.5	5.6±1.1	4.3±1.5	2.6±0.5
41	36.6±4.1	6.6±1.1	5.6±1.5	4.6±0.5	3.3±0.5	2.6±0.5
45	43.6±1.5	6.6±1.1	5.6±1.5	4.6±0.5	3.3±0.5	2.6±0.5
48	37.6±1.1	4.0±0.0	3.3±0.5	3.3±0.5	2.3±0.5	1.6±0.5
52	35.3±3.0	2.3±0.5	2.0±1.0	1.3±0.5	0.3±0.5	0.0±0.0
55	36.0±2.0	2.3±0.5	2.0±1.0	1.3±0.5	0.3±0.5	0.0±0.0

Table 3
Effects of silver nanoparticles on cell destruction

(a) Cell destruction via lipid peroxidation	AgNPs (mg/ml)	O.D.	MDA concentration (nM)	(b) Catalase activity	O.D.	H_2O_2 concentration (µM)
	0.0	0.000	1.638		0.425	11.095
	0.01	0.267	2.322		0.322	8.577
	0.05	0.346	2.525		0.303	8.112
	0.1	0.676	3.370		0.293	7.867
	0.5	1.394	5.210		0.233	6.400
	1.0	2.183	7.232		0.202	5.643

CONCLUSION

The present study was undertaken to observe what happens when we thoughtlessly dispose off nanoparticles. As mentioned in the introductory section, this usually happens when these particles are used as carriers or simply when workers synthesize and throw the particle solution after use, in labs. Unaware of the fate of these particles, which may be accumulating in our natural ecosystems like soil and water bodies, finally affecting the organisms in adverse way, we just tried to replicate the condition in our model organisms. The results of the study demonstrated that interaction of nanoparticles exerted stress condition on the growth and metabolism of *Vigna radiata and Brasssica juncea.* These nanoparticles have also shown significant toxic effect on seed germination and rhizosphere microbes. Exposure of the *Drosophila melanogaster* to silver nanoparticles demonstrated the toxic effects on all the developmental stages (adult, larvae and pupa). Chronic exposure may lead to cell destruction and irreversible damage to reproductive capabilities of fruit fly. The study, although preliminary, is a sufficiently good indicator of why stringent regulations are mandatory in handling and employing these particles for practical purposes.

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