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Assessment of the impact of green synthesized copper nanoparticles on freshwater snails (*Indoplanorbis exustus*) in comparison with field-control and lab-control snails

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Abstract

Background: Nanomaterials are used extensively because of their optical, electrical, catalytic, and mechanical properties. To understand the biological processes in an experimental organism, we aimed to highlight the comparative results obtained in the field-control, lab-control, and experimental freshwater snails (*Indoplanorbis exustus*). *Terminalia arjuna* bark extract was used for the synthesis of CuNPs and for characterization SEM, XRD, and FTIR techniques were used.

Results: The estimated LC₅₀ concentration for 96 h was to be 9.97 mg/L, thereafter animals were exposed further for 14 days to sub-lethal concentrations: 1/7th (1.42 mg/L), 1/5th (1.99 mg/L), 1/3rd (3.32 mg/L), and 1/2nd (4.98 mg/L). The data collected after conducting experiments on snails in the laboratory varied from the field-control snails. For comparison, the protein profile of field-control, lab-control, and experimental animals were displayed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then compared based on molecular masses. The bacterial isolates obtained were all belonging to streptococcus family. All isolates were Gram-positive and catalase-negative. A significant increase in bioaccumulation of CuNPs and dose-dependent necrosis was observed after 14 days.

Conclusions: The results obtained from the field control animals were different from those obtained in the lab control and experimental groups upon exposure to increasing concentration of CuNPs for 14 days.

Keywords: Copper nanoparticles, Gut microbiota, Streptococcus, Bioaccumulation, Field control, Protein profile

Background

Water pollution is one of the biggest threats to the entire world. The Major sources of water pollution include domestic sewage, discharge from industries, agriculture, electroplating mining, disposal of plastics, etc. Water purification is essential for the pharmaceutical and semiconductor industries because bacterial contamination may be responsible for system failure and can damage expensive materials. Nanomaterials are used extensively

because of their optical, electrical, catalytic, and mechanical properties. The physicochemical properties of copper nanoparticles (CuNPs) explain their use in various fields, including biological activities, antimicrobial, antifungal treatments in water bodies. Copper is an alternative to gold, platinum, and silver in microelectronic and thermally conductive materials. The green synthesis of nanoparticles includes harmless substances, such as plant extracts (Gondwal & Pant, 2018). The use of plant extracts for the synthesis of nanoparticles is emerging, and more attention is towards the development of engineered materials (Varshney et al., 2012). Green NPs, are used in drug supply, medicine, and cosmetic applications

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due to their antiviral and antibacterial properties (Corsi et al., 2018). Plant extract contains biomolecules that act as reducing agents (Gilavand et al., 2020). The inevitable use of nanoparticles ends up in an aquatic environment where they may represent a threat to organisms (Gomes et al., 2011; Chaturvedi & Dave, 2018). The toxicity of metals in aquatic bodies can change due to various factors (Rathore & Khangarot, 2003). Snails are the best model organisms used for toxicological studies (Ma et al., 2010, 2017; Xiang et al., 2021). Consumption of metals leads to various deformities such as a reduction in fertility rate, the delicate egg shell, etc. (Gall & Thompson, 2015). Pulmonate snails are sensitive to copper (Khangarot & Ray, 1988). Freshwater snail *Indoplanorbis exustus* is widely distributed all over Asia. The snail is hermaphrodite and feeds on the fresh and decaying aquatic plants, is found attached to floating vegetation in ponds, tanks, and stagnant pools of water. Copper nanoparticles are known for their inhibitory effect and ability to induce antiseptic reactions (Harris, 2003; Das & Khangarot, 2011; Monika et al., 2018). These properties have made CuNPs ideal for toxicity-related studies. Since the field animals are exposed to different environmental variables and variety of foods, we wanted to compare the stress of these variables on animals. Hence we have reared them in laboratory in controlled conditions, such as (pH (6.8 ± 6.9) temperature $(25 \text{ }^{\circ}\text{C} \pm 29 \text{ }^{\circ}\text{C})$, hardness $(50 \pm 60 \text{ ppm})$ and TOC $(25.76 \pm 0.29 \text{ ppm})$ and provided them only lettuce leaves as food and called them as lab control group, while animals collected from their natural habitat at the time of experimentation are named as field-control animals.

The internal defense system of snails constitutes a soluble component, i.e. haemolymph consisting of hemocytes which work for the defense system against infectious agents (Van Der Knaap and Loker 1990). Circulating phagocytic cells are the principal cells for cellular defense. Snails have been important successful animals for over 600 years due to their ability to adapt to different environmental conditions because of some adaptive proteins that may help them survive in various stressful conditions (Ulagesan & Kim, 2018). Gut microbiota is an important part of all herbivores. The microbiota is responsible for secreting the enzymes needed for the digestion of plant parts to release energy. The GI (gastrointestinal) tract is rich in bacterial diversity, provides a micro-environment for bacteria to grow. Recent studies have shown a significant link between bacteria and the host during evolution. Any change in the microbiota can harm the organism from feeding and resulting in death (Nisbet, 1974). Gut microbiota from the pulmonate gastropod family has received little attention; therefore, we attempted to explore the difference between field control,

lab control and the potential of CuNPs by considering the following objectives. Examine the apoptosis, bioaccumulation, microbiota of gut, protein profiling and immune response.

Methods

Collection and rearing of snails

Indoplanorbis exustus were collected in sterile polypropylene bags from a pond ($18.5488^{\circ} \text{ N}$, $73.8244^{\circ} \text{ E}$) of Savitribai Phule Pune University's campus. After collection at around 11AM, the snails were washed, few snails were immediately dissected in a laminar airflow with sterile dissecting instruments for microbiota analysis (field control). The remaining snails were maintained in dechlorinated tap water in laboratory for acclimatization for 14 days and were dissected for microbiota and other parameters. Lettuce leaves served as food, support, and substratum to lay eggs. The pH (6.8 ± 6.9) and temperature ($25 \text{ }^{\circ}\text{C} \pm 29 \text{ }^{\circ}\text{C}$) were recorded as optimum.

Synthesis and characterization of CuNPs

Copper nanoparticles were, synthesized according to a method reported by (Yallapa et al., 2013) with minor modification using *Tarmanalia arjuna* plant bark extract in which 100 mL of 0.1 M Copper Sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, pH 7.) was added to the filtrate. After micro-waved at (2.0 GHz) for 4 min, 2 min interval time to remove phyto-constituents, the filtrate was taken in another flask and the formed CuNPs were monitored by UV–Vis spectra. The CuNPs were characterized by SEM (Scanning Electron Microscopy), FTIR (Fourier Transform Infrared Spectroscopy), and XRD (X-ray diffraction) microscopy.

Median lethal concentration (LC_{50}) of CuNPs on *Indoplanorbis exustus*

Small round tubs with 100 mL capacity, were used for acute toxicity tests. Snails were exposed to sublethal concentrations of CuNPs (1.4, 1.99, 3.32, 4.98 mg/L), represented as 1/7th, 1/5th, 1/3rd, and 1/2rd of the 96 h LC_{50} value. Parallel control group of snails in only water was maintained simultaneously. LC_{50} values and 95% confidence interval calculated by using the Finney table (1971).

Haemolymph collection and flow cytometry

The snails were cleaned with tissue paper and washed with 70% ethanol in the UV chamber. The blood was collected by pricking (needle) the foot. The snail retracts its body when pricked, and the red blood oozes out. The blood was collected by “Dispo Van Syringe” equal amount of anticoagulant was added to the sample. The anticoagulant containing (50 mM Tri-sodium citrate,

10 mM EDTA, 115 mM glucose, and 47 mM NaCl pH 7.2) to avoid agglutination (Chemin, 1970). For flow cytometry, a 100 µl haemolymph sample was diluted with 5 µl of freshly annexin V/PI (propidium iodide). The cell suspension was kept for incubation for 1 h at 40 °C. After incubation of the sample, flow-cytometry was performed to analyze the percentage of apoptosis due to induction of stress (Thermo fisher Attune NxT(USA)).

Protein profile

Sub-lethal concentrations of CuNPs were tested in three biological replicates. The snails ($n=4$) were cleaned, with 70% alcohol dried with absorbent tissue paper. Field controls were brought and dissected immediately. Four snails approximately, weighing (2.012 ± 2.990 g) were used for protein collection in a UV chamber. The snails were homogenized in homogenization buffer containing 20 mM Tris Hcl (pH-8.0), 100 mM NaCl, 20% glycerol, 0.5 mM EDTA, 0.5 mM PMSF (phenylmethylsulfonyl fluoride), and 0.5 mM DTT(Dithiothreitol). The homogenate was sonicated at 60% amplitude, for 30 s. (3 rounds on and off) and centrifuged at (1000 rpm) at 4 °C. The supernatant was collected for protein estimation by Bradford assay. The equal concentration (60 µg) of protein was resolved on 12% SDS-PAGE to check the protein profile (Biradar et al., 2013). Protein content was measured; according to Bradford (1976) taking, bovine serum albumin (BSA) as standard in the 96 well plates, multimode reader was used for absorbance.

Bioaccumulation assay

Field-control, laboratory controls, and CuNPs treated experimental snails ($n=4$) were sacrificed by removing the shell and dried in an incubator at 60 °C. The sample was grounded in a fine powder. The digestion process performed by adding 60 mL of nitric acid (55%) and 10 mL of 70% Perchloric acid to the 100 mL flask to about 0.5 gm of tissue (dry mass). Digestion was done at 200–250 °C until the solution appeared clear. The solution was filtered, the final volume was made up to 100 mL by distilled water (Kotze et al., 2006). The concentration of the metal was determined using a nuclear absorption spectrophotometer (Spectra AA-220 Varian).

Isolation of gut microbiota from *Indoplanorbis exustus*

After exposure, the snails were wiped with alcohol in the UV chamber. To characterize the microbial populations in the snail's gastrointestinal tract, we sampled the bacterial contents within the gut and homogenized in a 9 mL sterile saline blank to get a gut content suspension. The gut suspension was diluted serially with sterile saline blanks after vortexing. The spread plate method was used; 0.1 ml of gut content suspension from each dilution

tube (i.e., 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4}) was spread on plates. Biochemical tests for characterization of bacteria were done by the method of (Kimberley & Elsa, 1994).

Statistical analysis

Differences among the control and experimental ones were determined by the ONE-way ANOVA, post-hoc by Tukey's test in SPSS and GraphPad Prism 6.

Results

Nowadays, there is a growing interest in extracting plant parts for the green synthesis of nanoparticles for various purposes. In our study, we have taken *T. arjuna* bark extract. As observed earlier by Das and Khangarot (2011) CuNPs were produced at pH 7, and copper hydroxide at pH 8–11. For the formation of CuNPs, the pH of our sample was 7, the color got changed from light yellow to dark brown, confirming a reduction in Cu^{2+} because the bark extract alone on irradiation could not have given such a color. SPR (Surface Plasmon Resonance) showed a peak of absorption above 530 nm (Fig. 1a). For characterization of shape, size and formation of nanoparticles SEM (Fig. 1b) XRD, and FTIR techniques were used (Fig. 2a, b).

FTIR analysis (Fourier transform infrared spectroscopy)

To investigate the formation of CuNPs and the functional group involved in the interaction between extract and copper sulfate pentahydrate, FTIR analysis ($400\text{--}4000\text{ cm}^{-1}$) was done by dissolving CuNPs in toluene. The width and peak formation is dependent upon particle size. When the size of NPs increases, the width of the peak decreases, and the intensity increases (Thomann et al., 2011). The peaks obtained in FTIR represent O–H stretching vibrations at 3349, C–H at 2987, C=C stretching at 1641, C=C aromatic ring stretching, at 1451, and C–OH stretching vibrations at 1383. These peaks confirmed the adsorption of phenolic compounds from the bark extract on the surface of nanoparticles produced by the interaction of π electrons (Nasrollahzadeh et al., 2014) and the reduction of copper ions to copper nanoparticles, was achieved under the effect of hydroxyl and carbonyl linkages in the constituents of the extract. Adsorbed Phenolic compounds can act as a capping agent, thus providing higher stability to nanoparticles (Elwy, 2020; Hassanien et al., 2018) (Fig. 2a).

XRD analysis (X-ray diffraction)

Phase identification and the structural properties of CuNPs identified using X-ray diffraction (XRD). The XRD pattern obtained by Bruker D8-advance X-ray diffractometer of synthetic CuNPs is shown in Fig. 2b.

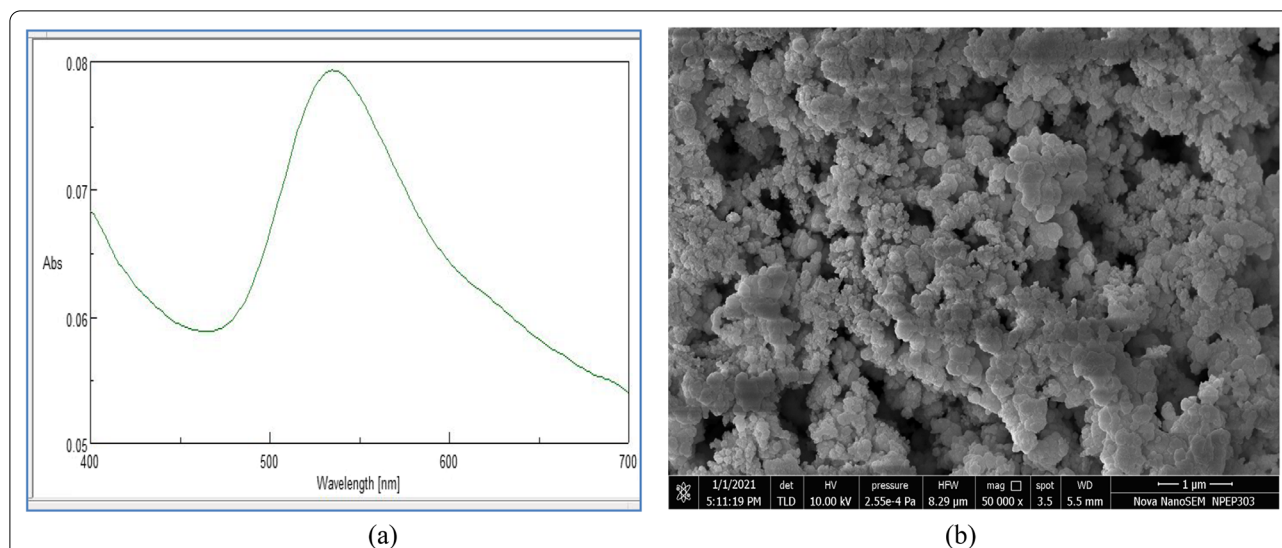


Fig. 1 **a** Absorption peak of CuNPs, showed peak at 530 nm, **b** scanning electron microscopy images of CuNPs showed the topography of nanoparticles particles

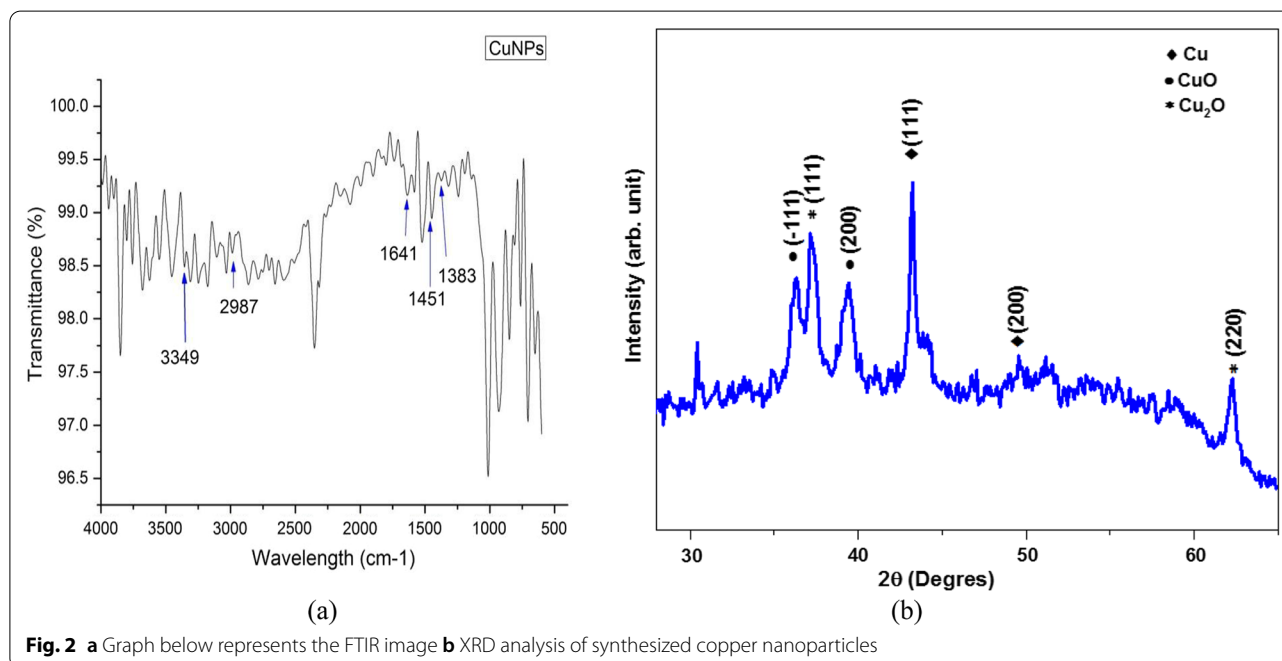


Fig. 2 **a** Graph below represents the FTIR image **b** XRD analysis of synthesized copper nanoparticles

Multiple peaks observed indicate the polycrystalline nature of CuNPs with secondary phases as CuO (cupric oxide) and Cu₂O (cuprous oxide). Major diffraction peaks of CuNPs at 43.24 and 49.55 corresponds to the planes (111) and (200), respectively, having FCC (face-centered cubic) structure of pure Cu (JCPDS (Joint Committee on Powder Diffraction Standards) No. 71-4610). Crystallite size for CuNPs found to be 10.68 nm, evaluated applying the Scherrer equation as followed

$$D_{111} = \frac{K\lambda}{\beta \cos \theta_B}$$

where D = Crystallite size, $K=0.9$ (Scherrer constant), $\lambda=0.15408$ nm (Wavelength of the x-ray source), β = Full Width Half Maxima (FWHM) (radians), θ_B = Bragg angle.

Flow cytometry analysis of haemolymph

Flow cytometry of cells is a rapid and valuable technique to detect live, necrotic, and apoptotic cells in a single cell population. In necrosis, the cell viability is lost and evidenced by the breakdown of the plasma membrane. Annexin VI binds phosphatidyl-serine group while propidium iodide dye detects necrosis because it binds DNA (Bortner & Cidlowski, 2001). The haemolymph of field, lab controls, and exposed snails were examined by annexin V-FITC (Fluorescein isothiocyanate) and PI. Flow cytometry analysis revealed that Copper NPs induced cell death in experimental groups. The quadrant graph below indicates, the lower left quadrant live cells, the upper left quadrant revealed necrotic cells, the lower right quadrant represented early apoptotic, the upper right quadrant showed late apoptotic cells. The percentage of live cells was high in the field control and low in the treated groups. The average percentage viability values of cells observed were as 77.33%; 76.76%; 76.96%; 68.25%, 56.27%, 58.09% and the concentration dependent apoptosis percentages were 16.6%, 18.3%, 10.4%, 26%, 32.5% and 32.5%, respectively (Fig. 3).

Bio-accumulation

After 14 days of exposure, the whole body 3 g (gram) was dried and approximately, 0.5 g of dried tissue was taken for bioaccumulation assay. A significant ($p < 0.05$) increase in the bioaccumulation potential of CuNPs was observed in experimental animals compared to both controls. The concentration of copper in pond water was below detection level (Fig. 4).

Morphological identification of gut microbiota

Six different isolates of microbiota were observed, showing variations in color and colony formation. All isolates had circular colony shape, convex colony elevation, entire colony edge, and different colored colonies. These isolates were subjected to colony characterization on NA plates at 37 °C and grown for 24–48 h. The colonies formed by each species of bacteria were different from other bacterial distribution of streptococcus obtained by 16S RNA sequencing.

Characterization of bacteria

Biochemical tests:

Gram staining

Gram-positive bacteria stain violet due to the presence of a thick layer of peptidoglycan in their cell walls. However, gram-negative bacteria stain red due to the thinner peptidoglycan wall. All isolated obtained were gram-positive.

Endospore test

After 24–48 h of incubation, sample disturbance was observed in inoculated tubes which were placed at 80 °C for 10 min. in water bath, however no turbidity was observed in control tubes that were inoculated but not received heat treatment. So, this confirmed isolate was of spore-forming type.

Catalase test

Not a single isolate showed catalase activity, thus all isolates were catalase-negative.

Oxidase test

After rubbing isolates with the wet filter paper with tetramethyl p-phenylenediamine deep purple color was observed. This evidenced that all isolates were oxidase-positive.

Oxidation fermentation test (Hugh and Leifson test)

All isolates (except control) changed color from green to yellow indicated that the isolates were HL positive.

Sugar (glucose) fermentation test

After incubation at 37 °C for 18–24 h tubes were observed for color change from red to yellow and gas production in inverted Durham tube which evidenced positive reaction.

Lipase and xylanase

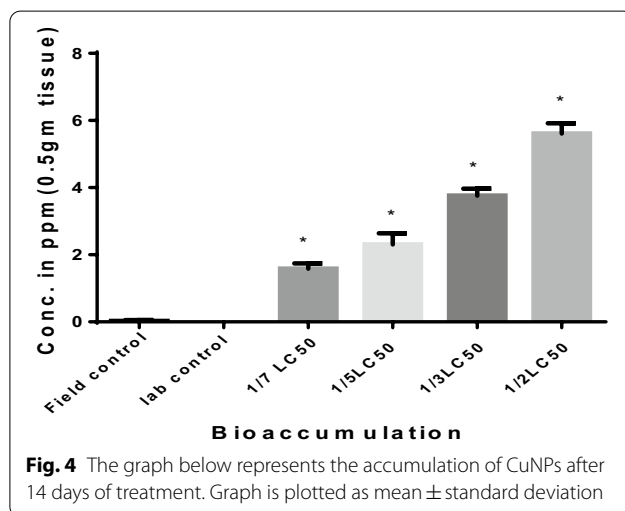
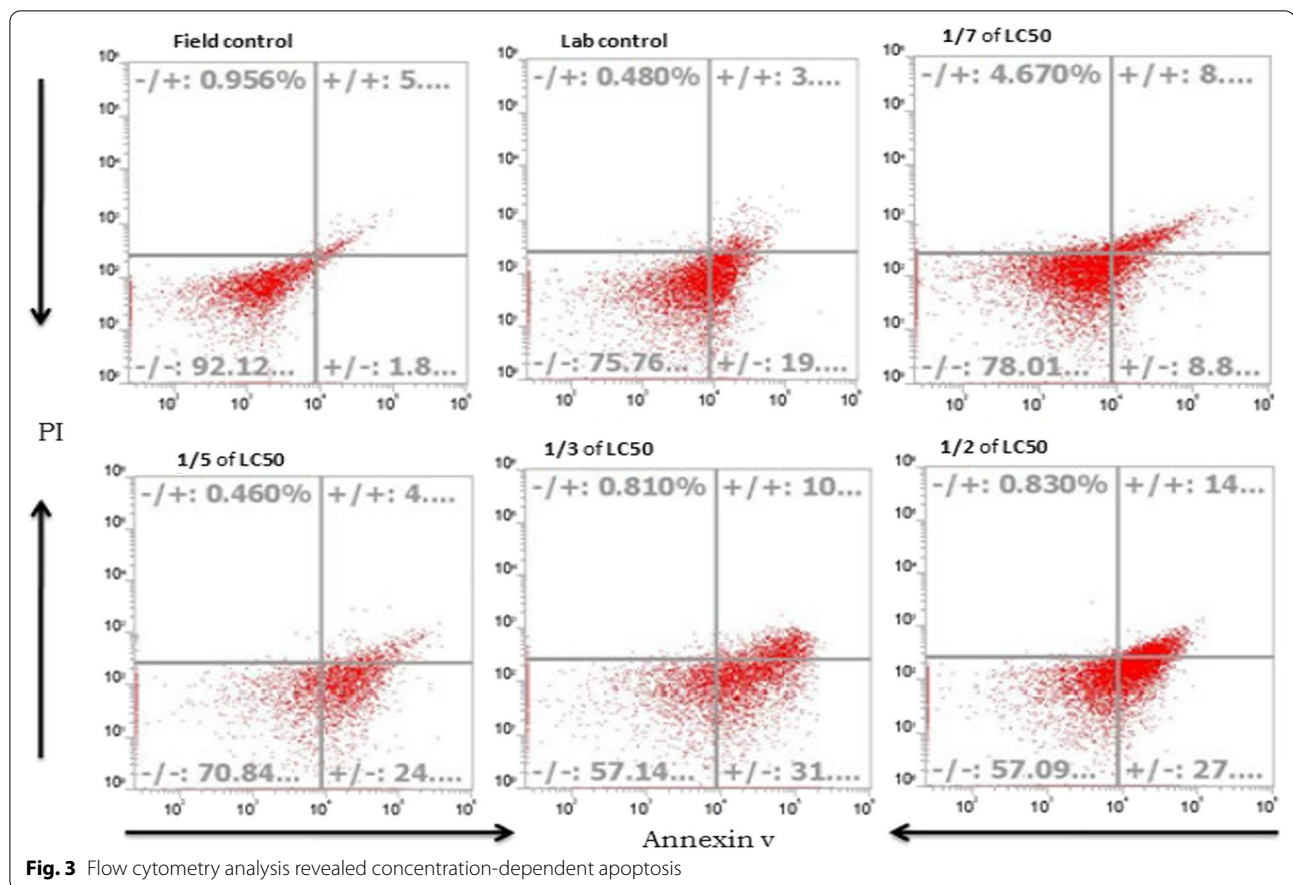
Not a single isolate showed its activity on lipase and xylanase medium. No zone of hydrolysis around the bacterial colony indicated the absence of lipase and xylanase enzyme production.

Protein profile

Whole-body protein was extracted and estimated using the Bradford method, and the protein was displayed using SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis). The red arrows marked protein bands in the lab control (2nd lane) are different as compared to field control (1st lane). Whereas the proteins marked by blue arrow are differential within different treatment groups representing that these proteins either are not expressed in all treatment groups or if expressed not in the same amount in any of the treated groups as compared to lab control group (Fig. 5).

Discussion

Water bodies from all over the world have suffered irreparable damage from anthropogenic and industrial activities. Various research studies are in progress to



come near efficient processes to minimize the contamination of aquatic bodies. Different factors such as property dosage, duration of exposure, pH, and temperature may affect the ecosystem (Ezemonye et al., 2008). Most of the experiments are performed in a lab environment

to make it easier to interpret the data and obtain clear information to investigate the real causes behind various phenomena. The high level of copper is common place in agriculture and industries. The synthesis of CuNPs was confirmed after incubation when the color of the sample changed from pale yellow to dark brown, absorption spectra were observed, at 520 nm, absorption bands for copper nanoparticles earlier reported to be in the range of 500–600 nm (Sathyavathi et al., 2010). CuNPs are proven to be phototoxic in plants; a high concentration of Cu is responsible for creating reactive oxygen species by activating molecular oxygen (Contreras et al., 2009). In plants, Cu reduces the photosynthesis rate and leads to oxidative stress (Han et al., 2008). The apoptosis percentage of control was lower than the experimental groups therefore, the cells beyond repair got expelled out through apoptosis. The protein bands based on the molecular weight on the SDS page confirmed more proteins expressed in lab control and experimental than the field controls, which confirmed the lab and the experimental groups showed different results from that of the field control group. The proteins with high molecular weight more than ~63 kDa were not observed in field

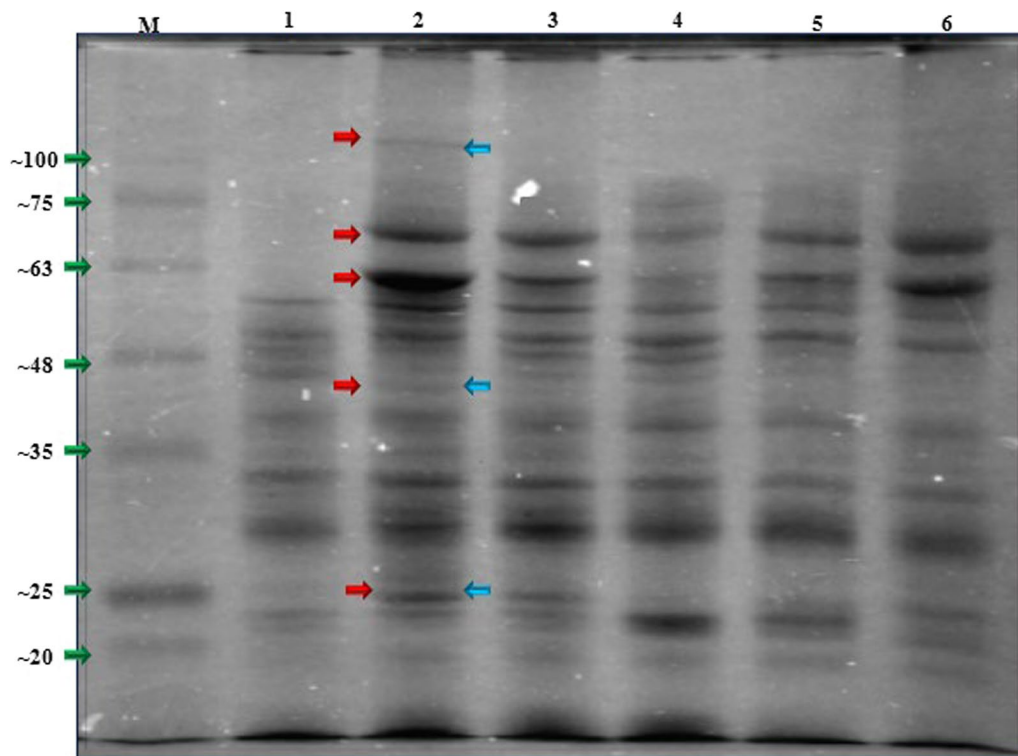


Fig. 5 The protein profile of snail under different concentration of CuNP: The 60 µg proteins were resolved on 12% SDS-PAGE analysis of protein extracts of *Indoplanorbis exustus*. Lane M = protein ladder (11–245 kDa (kilodalton)), Lane 1 = Field control; Lane 2 = Lab control; Lane 3 = 1/7th of LC_{50} ; Lane 4 = 1/5th of LC_{50} ; Lane 5 = 1/3rd of LC_{50} ; Lane 6 = 1/2th of LC_{50}

control group as compared to that of lab control and experimental groups this may be due to the disturbance in homeostasis of an organism. After 14 days of exposure, the higher bio-accumulation potential was observed at 1/2th concentration of CuNP, which indicated that snails are the best indicators for determining bioaccumulation potential of CuNPs, as earlier reported by Dummee et al. (2012) in golden snails. The weight of snails was noted every day from Day 1 to Day 14. No significant change was observed in weight after 14 days (Additional file 1: Fig. S1). At the highest concentration of CuNPs, the snails secreted mucus that might serve as a protective device in stressful conditions (Additional file 1: Fig. S2). After 96 h of treatment in hard water, no mortality was reported, however, 100% mortality was observed in the soft water (Ebrahimpour et al., 2010). Concentration-dependent reduction in the percentage of live cells was observed, cells stained blue with trypan blue were considered as dead and cells without stain were considered as alive (Additional file 1: Fig. S3).

GI microbiota includes members, of all three main life domains (Archaea, Bacteria, and Eukaryotes), with bacteria being the most abundant and metabolically active

group. The composition of the microbial community is host-specific, evolving throughout an individual's life.

The most common types of bacteria isolated were those belonging to the genus *Streptococcus*. *Streptococcus mitis*, *S. pneumoniae*, *S. oralis*, *S. anginosus*, *S. sanguinis* and *S. Psedopneumoniae*; The colony morphology showed variation in shape, size, elevation, color, and biochemical tests. The dominant bacteria present in the digestive tract of garden snail (*Helix aspersa*) revealed enterobacteria (Lesel et al., 1990) and enterococci species (Charrier et al., 1998). Phylum proteobacteria dominated the esophagus, crop, intestine, and rectum in Gaint African snail (Kiran et al., 2012). The gut microflora of *Geukensia demissa* (ribbed mussel) shows presence of *Mycoplasma spp.*, *Spiroplasma spp.*, *Entoplasma somnilux*, and *Pseudomonas sp.* and the Eastern oyster *Crassostrea virginica*, showed presence of *Wolbachia sp.*, *Ralstonia spp.*, *Haemophilus sp.*, and *Escherichia coli* (Loggans and Wang 2007). Recent research has revealed that the communities of microorganisms living within the guts of animals can largely affect the physiology, development and fitness of their animal hosts (Mcfall-Ngai et al., 2013).

Conclusions

In conclusion, our data demonstrated that the environment in which the organism thrives has a substantial influence on the extent to which toxins affect the organisms by various means. Furthermore, the techniques like SEM, XRD and FTIR for the characterization of nanoparticles were reinstated, during the experiments. Concentration dependent stress of CuNP was observed in snails with respect to bioaccumulation and cell death. Flow cytometry results confirmed concentration-dependent apoptosis in experimental animals. There was a distinct difference between the protein expressions observed in the field control and experimental groups with respect to lab-control group. No significant difference was observed in microbiota among three groups.

Abbreviations

BSA: Bovine serum albumen; CuNP: Copper nanoparticles; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$: Copper(II) sulfate pentahydrate; CuO: Cupric oxide; Cu_2O : Cuprous oxide; DTT: Dithiothreitol; EDTA: Ethylene diamine tetra-acetic acid; FC: Field control; FTIR: Fourier-transform infrared spectroscopy; FITC: Fluorescein isothiocyanate; FWHM: Full width at half maximum; GI: Gastrointestinal tract; g: Gram; JCPDS: Joint Committee on Powder Diffraction Standards; kDa: Kilodalton; LC50: Median lethal concentration; LC: Lab control; ppm: Parts per million; PI: Propidium iodide; S.1.2.3: Supplementary figures; SDS-PAGE: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SEM: Scanning electron microscopy; SPR: Surface plasmon resonance; XRD: X-ray diffraction.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s41936-022-00276-9>.

Additional file 1. S.1 Weight of snails in gms (13 snails in groups), no significant difference was observed. **S.2** Higher concentration of copper nanoparticles had a negative effect on the snails as they secrete more mucus. The arrows represent the mucous secretion. **S.3** The haemocytes showed a decline in viability after trypan blue staining.

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Authors' contributions

SVP conceived and planned the project. AM carried out the experiment, wrote the manuscript, under the supervision of SVP. Both authors contributed in analysis and finalization. Both authors read and approved the final manuscript.

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Availability of data and materials

All data analysed during this study are included within the paper and in supplementary file.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

We have the consent for this publication.

Competing interests

Both authors declare that they have no competing interests.

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