

Biosynthesis and safety evaluation of ZnO nanoparticles

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Abstract The secrets gleaned from nature have led to the development of biomimetic approaches for the growth of advanced nanomaterials. Biological methods for nanoparticle synthesis using microorganisms, enzymes, and plants or plant extracts have been suggested as possible eco-friendly alternatives to chemical and physical methods. Here, we report extracellular mycosynthesis of ZnO-NPs by *Alternaria alternata* (Fr.) Keissl (1912). On treating zinc sulfate solution with fungal culture filtrate, rapid reduction of ZnSO₄ was observed leading to the formation of highly stable ZnO-NPs in the solution and up-to-date literature survey showed this was the first report of biosynthesis of ZnO-NPs using this fungus. The particles thereby obtained were characterized by different analytical techniques. EDX-spectrum revealed the presence of zinc and oxygen in the nanoparticles. FTIR spectroscopy confirmed the presence of a protein shell outside the nanoparticles which in turn also support their stabilization. DLS and TEM analysis of the ZnO-NPs indicated that they ranged in size from 45 to 150 nm with average size of 75 ± 5 nm. But potential

negative impacts of nanomaterials are sometimes overlooked during the discovery phase of research. Therefore, in the present study, bio-safety of mycosynthesized ZnO-NPs were evaluated by using cytotoxicity and genotoxicity assays in human lymphocyte cells, in vitro. Cytotoxicity studied as function of membrane integrity and mitochondrial dehydrogenase activity revealed significant ($P < 0.05$) toxicity at treatment concentration of 500 µg/ml and above. Additionally, DNA damaging potential was also studied using comet assay. The results revealed significant genotoxicity at the highest concentration (1,000 µg/ml).

Keywords *Alternaria alternata* · Biomaterials · ZnO nanoparticles · Spectroscopy · Comet assay

Abbreviations

FCF	Fungal culture filtrate
DLS	Dynamic light scattering
XRD	X-ray diffraction
EDX	Energy dispersive X-ray
FTIR	Fourier transform infrared
TEM	Transmission electron microscopy
ZnO	Zinc oxide
ZnO-NPs	Zinc oxide nanoparticles

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Introduction

Nanotechnology is the production and use of materials at the smallest possible scale. Nanotechnology has considerably improved and revolutionized several technology and industrial sectors including medicine, food safety, and many others. The use of man-made nanoparticles ranges from the use of carbon black and fumed silica in plastic

fillers, car tires to microgram quantities of fluorescent quantum dots used as markers in biological imaging [1]. At nanoscale, the property of materials differs substantially from that of the respective bulk forms. As per the March 2011 market survey [2], about 1,317 engineered nanoparticle based products or product lines are in use. Zinc oxide nanoparticles with 31 products are only next to silver, titanium dioxide and carbon nanoparticles.

While conventional synthesis methods of ZNO-NPs have involved a number of chemical methods [3] the procedures generate a large amount of hazardous by-products. Thus, there is a need for ‘green chemistry’ that includes a clean, nontoxic and environment-friendly method of nanoparticle synthesis. Among different bio-organisms, fungi in general possess some distinctive advantage over others, because of their high metal tolerance, easy to scale-up, low cost downstream processing, handling of biomass and economic viability. Furthermore, fungi are extremely efficient secretors of extracellular enzymes and possible to easy large-scale production. The cell free culture filtrates of different fungi were used for biosynthesis of different nanoparticles like silver [4–10].

While interaction of nanomaterials with cells and its macromolecular components is critical in many applications such as imaging and drug/gene delivery, the same features are a concern with respect to their safety. The potential of nanoparticles to interact with biological systems has been acknowledged in recent years by the scientific community. There has been increasing scientific evidences that physical and chemical properties that impart nanoparticles their characteristic properties, also lead to an increase of bioavailability and toxicity [11]. Nanoparticles are capable of crossing most biological barriers including blood–brain barrier [12]. Until recently nanosized materials were treated as variations of the technical material or existing formulation and thus not requiring a separate registration [13]. Barnard [14] summarized the problem of potential hazard of nanoparticles: “nanohazards are different because nanomaterials do not behave in a predictable way. They are the Jekyll and Hyde of materials science, giving us unique chemical, electrical, optical and physical properties; as well as a new range of possible carcinogens, poisons and allergens...”. Evaluation of toxicological effects of nanoparticle exposure is hence crucial prior to industrial/biomedical application. Therefore, in the present study, apart from biosynthesis of the nanoparticle special attention was given to the safety aspect.

The present study has two fold objectives: firstly the biological synthesis and characterization of ZNO-NPs by using the culture filtrate of a phytopathogenic fungus *Alternaria alternata* (Fr.) Keissl (1912) (strain number: MAMP/C/51) and secondly, to find the genotoxic response of ZnO-NPs to human lymphocyte cells in vitro using

cytotoxicity and genotoxicity testing endpoints over a wide dose range (0–1,000 µg/ml).

Materials and methods

Fungal culture

The pathogen, *A. alternata* (strain number: MAMP/C/51) was isolated previously by Maiti et al. [15]. The strain of the fungus was maintained by sub-culturing in PDA medium [potato extract (40 %), glucose (2 %) and agar (2 %)] for further use.

Preparation of fungal culture filtrate

The fungal strain was grown in PDB medium which was composed of potato extract (40 %), glucose (2 %) at pH 7.4 and kept for 15 days at 37 °C temperature. After the incubation, the media was filtered with Whatman filter paper no. 1. Hundred ml of that FCF was taken in a sterilized Erlenmeyer flask and used to synthesize ZnO-NPs.

Synthesis of ZnO-NPs

In the present study, we used the FCF as reducing agent to reduce zinc sulfate (ZnSO_4). Hundred ml of FCF was taken in each different Erlenmeyer flask and mixed with zinc sulfate solution (Sigma, St. Louis, MO, USA) solution (1 mM final concentration). The FCF containing flask was agitated for 24 h at room temperature. Simultaneously, only the culture filtrate of *A. alternata* and only zinc sulfate solution were maintained under same conditions. The reaction mixture was routinely monitored by visual color change.

The ZnO-NPs were separated out by centrifugation at 12,000g for 10 min, and the settled nanoparticles were washed with deionized water (three times). The purified ZnO-NPs were resuspended in deionized water and ultrasonicated by Piezo-u-sonic ultrasonic cleaner (Pus-60w). Synthesis of ZnO-NPs was repeated for three times ($n = 3$) and subsequently utilized for characterization of the particles.

Size measurement by DLS experiment

Particle size was measured by laser diffractometry using a nano size particle analyzer (Zen 1,600 Malvern USA) in the range between 0.6 and 6.0 µm.

Energy-dispersive X-ray (EDX) analysis

Vacuum-dried samples were used for the EDX analysis which was carried out by the Hitachi S 3400N instrument

(Japan) and employed to know the elemental compositions of the particles. Samples were filtered and dried before measurements. The results presented in this study were reproducible with the accuracy of $\pm 5\%$ error.

FTIR spectroscopic analysis

FTIR spectroscopic measurements were carried out to identify the capping agents responsible for the stability of the biogenic nanoparticle solution. Bio-reduced ZnO-NPs solution was centrifuged at 12,000g for 10 min and resulting suspension was redispersed in deionized water. The purified pellet was dried and mixed with potassium bromide (KBr) at a ratio of 1:100 which was further analyzed by Shimadzu 8400S fourier transform infrared spectrophotometer in the diffuse reflectance mode at a resolution of 4 cm^{-1} in KBr pellets. The scanning data were obtained from the average of 50 scans in the range between 4,000 and 400 cm^{-1} .

TEM observation of ZnO-NPs

TEM samples of the ZnO-NPs synthesized by the biological reduction were prepared by placing a drop over carbon coated copper grids and allowing the water to evaporate. TEM measurements were performed on a Tecnai G² spirit Biotwin (FP 5018/40) TEM instrument operated at an accelerating voltage at 80 kV.

Isolation of mononuclear cells from blood and treatment

Lymphocyte cells were isolated from fresh blood according to the method of Boyum [16], using Histopaque. The cells were washed with PBS and resuspended in RPMI-1640 media at a concentration of 10^6 cells/ml for further use.

Human lymphocytes were incubated for 3 h at 37 °C in RPMI-1640 media with different concentrations of ZnO-NPs (0, 125, 250, 500 and 1,000 $\mu\text{g/ml}$). For cytotoxicity assays, cells with treatment concentrations of ZnO-NPs were seeded onto 96-well culture plates at 1×10^5 cells per well, incubated at 37 °C for 3 h. Following treatment, the lymphocytes were processed for cytotoxicity and DNA damage by the alkaline comet assay.

Effect on membrane integrity (trypan blue dye exclusion test)

The trypan blue dye exclusion test was performed according to the method of Tennant [17]. Failure to exclude trypan blue reflected a loss of plasma membrane integrity associated with necrosis [18]. The cut-off point suggested by Henderson et al. [19] was 70 %.

Effect on mitochondrial dehydrogenase activity (MTT assay)

The MTT assay was colorimetric technique that allowed the quantitative determination of cell viability. The assay was based on the capability of viable cells to transform the MTT into formazan dyes. Following incubation, cells were treated with 0.5 mg/ml solution of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT; 100 $\mu\text{l/well}$) at 37 °C for 3 h. The number of viable cells was determined by uptake of MTT. Optical density was read on iMarkTM microplate absorbance reader (BIO-RAD, USA) at 570 nm, with 630 nm as a reference wavelength. All experiments were performed at least in triplicates. In both the assays cell viability was expressed as percentage in relation to controls and data presented as mean \pm SD.

Effect on metabolic activity of lymphocyte (resazurin assay)

The Resazurin system measured the metabolic activity of living cells [20]. Resazurin was reduced to resorufin (highly fluorescent) in the medium by cell activity and a direct correlation exists between the reduction of resazurin in the growth medium and the metabolic activity of living cells. Cells after incubation were washed twice in PBS. Resazurin assay using resazurin based in vitro toxicology assay kit (TOX-8) was carried out according to the supplier's instructions.

Single cell gel electrophoresis (comet assay)

Human lymphocytes incubated with ZnO-NPs (0, 125, 250, 500 and 1,000 $\mu\text{g/ml}$) for 3 h at 37 °C were processed for DNA damage following the method of Singh et al. [21] with modifications [22, 23]. Slides were prepared in triplicates per concentration. Slides were immersed in cold lysis solution at pH 10. The lysis solution consisted of 2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Trizma base, 1 % Triton X-100, 10 % DMSO and kept at 4 °C for 60 min. After lysis the DNA was allowed to unwind in the electrophoresis buffer (300 mM NaOH: 1 mM Na₂EDTA at pH 13.5) for 20 min. This was followed by electrophoresis conducted at a constant voltage of 25 V and 300 mA at 4 °C. Slides were neutralized in 0.4 M Tris (pH 7.5) for 5 min and finally rinsed in water. Each experiment was repeated thrice.

The slides were stained with EtBr (20 $\mu\text{g/ml}$) and rinsed in water to wash off excess stain. Slides were scored using image analysis system (Kinetic imaging; Andor Technology, Nottingham, UK) attached to a fluorescence microscope (Leica, Wetzlar, Germany) equipped with appropriate filters (N2.1). The microscope was connected

to a computer through a charge-coupled device camera to transport images to software (Komet 5.5) for analysis. The final magnification was 100X. Among the comet parameters we reported the % of DNA in the tail [tail DNA (%)]. This would give us a clear indication of the extent of DNA damage induced by the test chemical. Images of 150 (50 × 3) cells per concentration were analyzed for human lymphocytes. The median values of each concentration with respect to the comet parameter were calculated.

Statistical analysis

For comet assay and other cytotoxicity tests one way analysis of variance (ANOVA) was performed. For statistical tests Sigma Stats.3 software (Systat Software Inc., Chicago, Illinois, USA) was used. The level of significance was established at $P \leq 0.05$.

Results and discussion

Production and characterization of ZnO-NPs

The culture filtrate mediated synthesis of ZnO-NPs was validated by visually monitoring three flasks containing only the culture filtrate of *A. alternata*, reaction mixture of the culture filtrate with zinc sulfate and only zinc sulfate solution. Only the reaction mixture displayed a time dependent color change, where as the fungal culture filtrate and the zinc sulfate solution were observed to retain their original color (data not shown). At the beginning, the reaction mixture was light yellow and the light yellow color changed to yellowish green color gradually at 24 h and then the color did not change with increasing incubation time. This finding was similar to the result of Sangeetha et al. [24]. The appearance of the yellowish green color indicated the occurrence of the reaction and the formation of ZnO-NPs.

Particle size measurement

Particle size was determined by dynamic light scattering measurement. Laser diffraction revealed that particle size obtained in the range of 45–150 nm (Fig. 1).

EDX observation of ZnO-NPs

Figure 2 showed the EDX spectrum recorded in the spot-profile mode from one of the densely populated ZnO-NPs area. Strong signals of zinc and oxygen in the examined field were observed. The sharp optical absorption peak in the range of 1–2 keV signified the presence of zinc and 0–1 keV signified the presence of oxygen in the nanoparticles.

The signals from C, P, S, Cl and K atoms were also recorded. The C, P, S, Cl and K signals were likely due to X-ray emission from carbohydrates/proteins/enzymes present in the cell wall of the fungal mycelium.

FTIR analysis of ZnO-NPs

FTIR absorption spectra of biosynthesized vacuum-dried ZnO-NPs were shown in the Fig. 3. The spectra showed the presence of bonds due to O–H stretching (around $\sim 3,430 \text{ cm}^{-1}$) and aldehydic C–H stretching (around $\sim 2,920 \text{ cm}^{-1}$) [25]. These peaks indicated the presence of proteins and other organic residues, which might have produced extracellularly by *A. alternata*. FTIR spectrum of ZnO-NPs showed absorption band at $1,627 \text{ cm}^{-1}$, corresponding to the amide I of polypeptides [25]. The FTIR

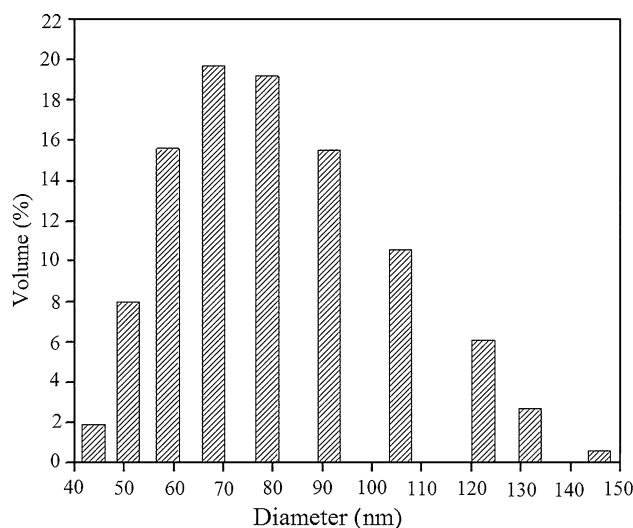


Fig. 1 Bar diagram of particle size distribution as obtained from dynamic light scattering of the ZnO-NPs produced by *Alternaria alternata*

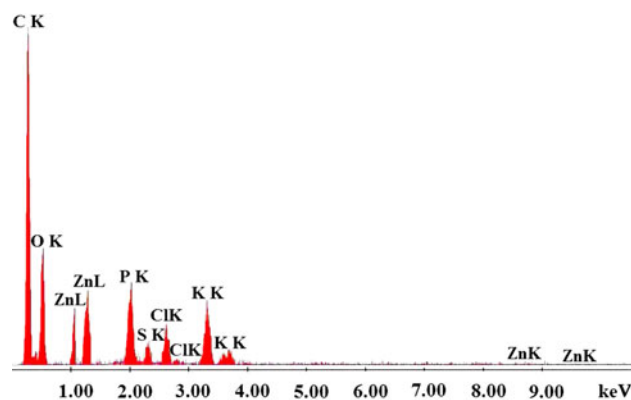


Fig. 2 EDX spectrum recorded showing sharp peaks in between 1–2 keV and 0–1 keV confirming the presence of zinc and oxygen respectively

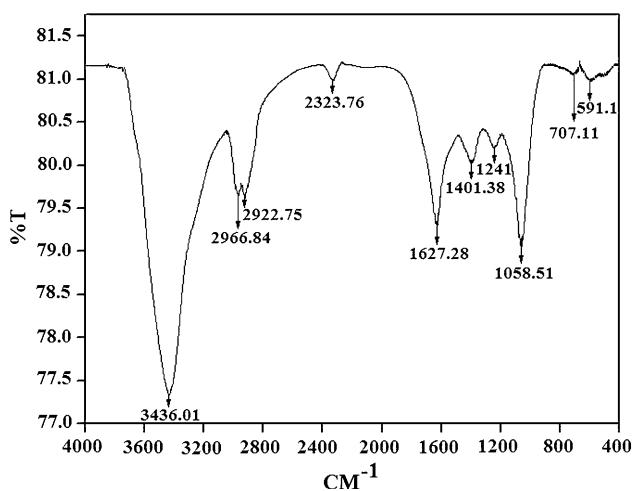


Fig. 3 FTIR absorption spectra of ZnO-NPs showing different peak values to identify the different bonds which were responsible for the stability of the biogenic nanoparticle solution

peak at around $1,240\text{--}1,260\text{ cm}^{-1}$ present in both the cases signified amide III band of the random coil of protein [26]. Peak at $1,401.38\text{ cm}^{-1}$ may be assigned to the symmetric stretching of the carboxyl side groups in the amino acid residues of the protein molecules [27]. Band at around $1,052\text{ cm}^{-1}$ indicated C–O stretching [28]. The bands visible in between $500\text{ and }749\text{ cm}^{-1}$ signified the presence of R–CH group [29]. Thus, it could be concluded that the ZnO-NPs are stabilized by surface bound protein molecules that also prevent aggregation [30]. In addition to that microscopic fungi can generate different extracellular nanoparticles by a process involving the enzyme NADH-reductase [9].

TEM image of ZnO-NPs

The morphology and size of the synthesized nanoparticles were determined by the transmission electron microscopic images. TEM image shown in the Fig. 4 recorded different sizes of ZnO-NPs which arose from the bio-reduction of chloroauric acid by FCF at room temperature ($37\text{ }^{\circ}\text{C}$) for 24 h. These observations revealed that spherical, triangular as well as hexagonal structures of the ZnO-NPs were formed in the reaction solution. The diameters of these ZnO-NPs were measured and the size was in the range of $45\text{--}150\text{ nm}$. The average diameter of these ZnO-NPs was of $75 \pm 5\text{ nm}$.

Bio-safety evaluation of ZnO-NPs

With the increase in use of ZnO-NPs and incorporation into various consumer products, safety evaluation of the nanoparticle is essential. Over the last decade, researches had reported ZnO-NPs to be toxic to many species, including

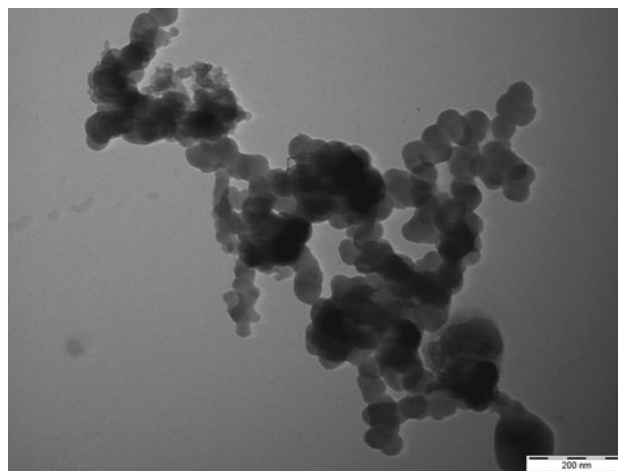


Fig. 4 Transmission electron micrograph of ZnO-NPs after bioreduction of zinc sulfate solution (accelerating voltage 80 kV)

microbes, algae, plants and animal cells. Moreover environmental levels of ZnO-NPs were expected to increase continually and are predicted to be in the range of $76\text{--}760\text{ }\mu\text{g/l}$ in water and $3.1\text{--}31\text{ mg/kg}$ in soil, depending on market penetration [31]. Hence with an ever increasing concentration of ZnO-NPs in the environment, a wide range of concentrations ($0, 125, 250, 500$ and $1,000\text{ }\mu\text{g/ml}$) were selected for the present study to assess safety of the nanoparticles synthesized.

Cytotoxic effect of ZnO-NPs on human lymphocyte cells

In the present study cytotoxicity was evaluated simultaneously by multiple assay endpoints that detected cell viability as a marker of cell membrane integrity, mitochondrial dehydrogenase activity and metabolic activity. Trypan blue dye exclusion method was used to study the effect of ZnO-NPs on membrane integrity of human lymphocytes. Statistically significant ($P < 0.05$) change was observed in the treated sets ($500\text{ }\mu\text{g/ml}$ and above) as compared to control (Fig. 5). The MTT assay result demonstrated a concentration dependent decrease in mitochondrial activity, significant at concentrations of $500\text{ }\mu\text{g/ml}$ and above (Fig. 5). Decrease in mitochondrial dehydrogenase activity could be attributed to decrease in the reduction of tetrazolium salts to formazan dyes [32]. Resazurin assay however did not reveal any significant alteration in metabolic activity as compared to control (data not shown). Previous cytotoxicity study on BEAS-2B and RAW264 cells have shown induction of the intracellular Ca^{2+} flux, lowering of the mitochondrial membrane potential, and loss of membrane integrity after exposure to 20 nm ZnO-NPs [33]. Studies of ZnO-NPs ($32\text{--}95\text{ nm}$, with a TiO_2 shell) on A549 lung cells have revealed similar

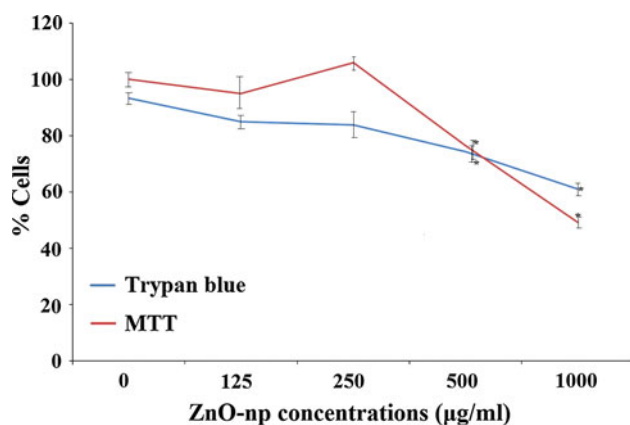


Fig. 5 Trypan blue and MTT cell viability assay; cytotoxicity induced by ZnO-NPs in human lymphocyte cells; error bars indicate standard deviation. * $P < 0.05$

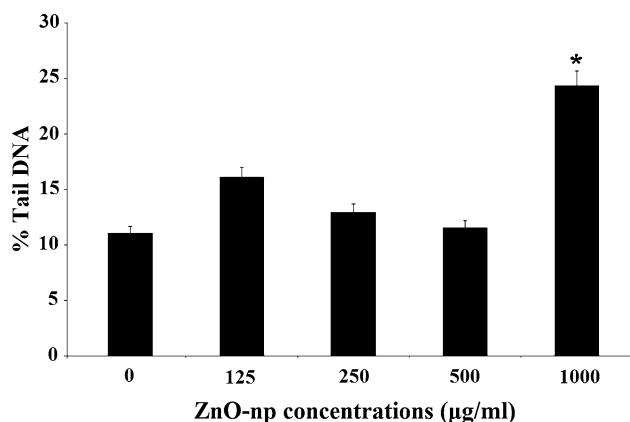


Fig. 6 Comet data (% tail DNA) of ZnO-NPs induced genotoxicity in human lymphocyte cells; error bars indicate standard deviation. * $P < 0.05$

results of Hsiao and Huang [34] and Vandebriel and De Jong [35].

DNA damage induced by ZnO-NPs in human lymphocyte cells

A significant ($P < 0.05$) increase in DNA fragmentation was induced by ZnO-NPs in human lymphocytes at treatment concentration 1,000 µg/ml (Fig. 6). Though an increase in comet parameter was observed for the other ZnO-NPs concentrations the change was not significant compared to control. Genotoxic potential of ZnO-NPs (<20 nm) and their ability to perturb the mitochondrial membrane potential, possibly through oxidative stress, in human peripheral blood mononuclear cells have been reported by Javed et al. [36]. Several other studies had reported cellular uptake and genotoxicity of ZnO-NPs had also been reported in vitro and in vivo [37–39]. From the

results thus obtained the toxicity of the ZnO-NPs were observed only at a concentration of 1,000 µg/ml, which is much higher than the predicted concentration. The ZnO-NPs thus synthesized can be considered safe up to concentrations of 500 µg/ml.

Conclusion

A simple, rapid biological procedure was developed to synthesize ZnO-NPs directed by particle size using the culture filtrate of *A. alternata*. The ZnO-NPs showed distinct polydispersity and the particle size ranged from 45–150 nm with an average size of 75 ± 5 nm. The advantage of biosynthesis of nanoparticles using this protocol over other methods currently in use was that the nanoparticles were quite stable in solution. FTIR characterization confirmed the presence of protein matrix as a stabilizing agent. Here, we also demonstrated that biosynthesized ZnO-NPs could induce cytotoxic response and interacted with DNA at concentrations of 500 µg/ml and above. And hence monitoring toxicity of biosynthesized nanoparticles should be taken into account for safety assessment. Based on the results on genotoxicity study, caution should be exercised on the application of ZnO-NPs on human health.

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