



Biosynthesis of zinc oxide nanoparticles using *Mangifera indica* leaves and evaluation of their antioxidant and cytotoxic properties in lung cancer (A549) cells

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ABSTRACT

Green synthesis is an eco-friendly approach to nanoparticle production, which eliminates the use of toxic chemicals, high temperatures, and costly equipment needed for traditional physical and chemical synthesis methods. This eco-friendly approach was used in the present study to biosynthesize zinc oxide nanoparticles (ZnO NPs) from *Mangifera indica* (mango) leaves which were then evaluated for their antioxidant activity and cytotoxic effects on lung cancer A549 cells. Synthesized ZnO nanoparticles were characterized using UV–vis spectroscopy, XRD, SEM, and EDX analyses. The XRD and SEM analyses showed 45–60 nm as the size of synthesized nanoparticles, the pure crystal form of ZnO NPs, and the shape of the NPs as nearly spherical and hexagonal quartzite. The antioxidant potential of nanoparticles was estimated using a DPPH free radical scavenging assay. The percent of viable cells was inversely proportional to the concentration of ZnO nanoparticles at 25 µg/mL concentration. The MTT assay used for cytotoxicity evaluation depicted the significant cytotoxic effect of ZnO NPs against the A549 lung cancer cell line. The drop in the proportion of viable A549 cells after exposure to ZnO NPs was comparable to the effects of the standard drug used *i.e.* cyclophosphamide. Antioxidant activity of NPs was increased by increasing the concentration of NPs. The present biosynthesis approach is rapid, inexpensive and eco-friendly and it yielded highly stable ZnO NPs with significant antioxidant and anticancer potential. This is the first report of *M. indica* -mediated synthesis of ZnO NPs as antioxidant and anticancer agents for the treatment of lung cancer and subsequent therapeutic applications.

1. Introduction

Zinc oxide (ZnO) is an inorganic compound that is catalytic, semi-conducting, piezoelectric, optoelectronic and pyroelectric [1]. ZnO nanoparticles (ZnO NPs) exhibit unique properties such as better absorption of light, enhanced catalytic properties owing to their large surface area to volume ratio and a wide gap between their conduction and valence band [2]. Nanoparticles such as ZnO have been previously synthesized using various physical and chemical techniques including hydrothermal synthesis, vapor-liquid-solid (VLS), sol-gel process, chemical vapor deposition and microwave methods [3]. Comparatively little research has been conducted on the biosynthesis of nanoparticles,

which provides a more effective, rapid and eco-friendly approach to the synthesis of nanoparticles, reducing or completely eliminating the use of high temperatures, pressures, toxic chemicals, space, and capital required to set up equipment and heavy machinery necessary for the physical and chemical approaches to synthesis. Thus, green synthesis of nanoparticles has proven to be an alternative, cost-effective technique for the synthesis of NPs where plant phytochemicals act as both a reducing and capping agent [4]. Compounds present in the plant being utilized coat the nanoparticles during the synthesis process, which allows for varied biomedical applications depending on the plant compound [5].

The interactions of biological molecules with molecular oxygen

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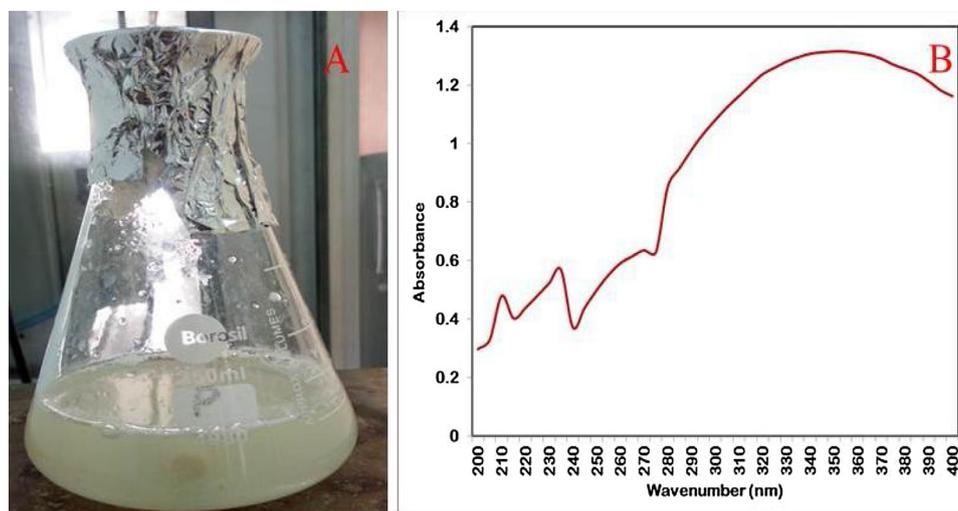


Fig. 1. (A) Synthesis of zinc oxide nanoparticle from *M. indica* leaves (B) UV-vis spectroscopic analysis of zinc oxide nanoparticles synthesized using *M. indica* leaves extract.

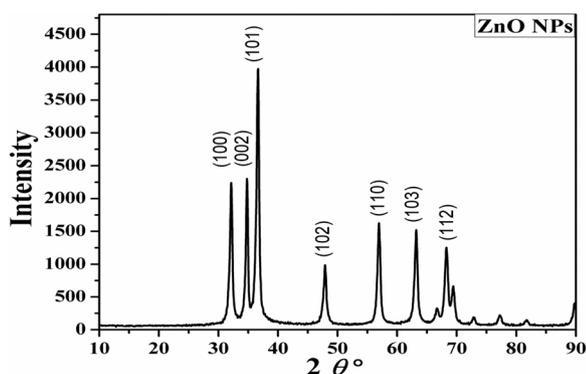


Fig. 2. XRD Analysis of zinc oxide nanoparticles synthesized using *M. indica* leaf extract.

leads to the formation of free radicals. These free radicals denature proteins by either direct fragmentation or by providing them with denatured substrates that later activate their intracellular lysis pathway [6]. The 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) assay is the most extensively used assay for determining the antioxidant potential of a substance. DPPH absorbs at a wavelength of 515 nm in its radical form but when it interacts with an antioxidant, absorption decreases and this decrease is measured in the assay [7]. Cytotoxicity testing is of the utmost importance when screening a compound of pharmacological applications. Hemolytic properties of nanoparticles were studied as hemolysis could lead to jaundice, anemia and lethal pathological conditions in extreme cases [8]. Previous studies have shown the antioxidant activity of ZnO NPs synthesized using neem leaf (*Asadirachta indica*) extract [9], green tea leaf (*Camellia sinensis*) extract [10], dog rose fruit (*Rosa canina*) extract [11], and red clover flower (*Trifolium pretense*) extract [12]. In contrast, no cytotoxicity effect was seen in human breast cancer MCF-7 and colon cancer cell HT-29 cell lines observed for ZnO NPs that were synthesized using Chinese goldthread (*Coptidis chinensis*) rhizomes [13], and palmyra palm fruit (*Borassus flabellifer*) extract, respectively [14]. Also *Rhodococcus pyridinivorans* NT2 extracellularly synthesized NPs showed no effect on HT-29 colon carcinoma cell lines [15].

Mangifera indica (mango) belongs to the family Anacardiaceae and is native to South Asia. Pattanayak and Nayak (2013) reported the biosynthesis of iron oxide nanoparticles using *M. indica* leaves. They extracted the compound mangiferin from leaves of the plant in high quantity. Mangiferin has been shown to possess antioxidative,

antibacterial, antifungal, antiviral, antiinflammatory, anticancer and antiallergic pharmacological activities [16]. However, the efficiency of *M. indica* leaves in ZnO NPs synthesis and their pharmacological properties have yet to be studied. Thus, this investigation aimed to synthesize ZnO NPs using *M. indica* leaves extract and to characterize NPs using various techniques. The DPPH assay was used to assess the free radical scavenging activity of synthesized nanoparticle. Cytotoxic effect of ZnO NPs was determined on lung cancer A549 cell lines.

2. Materials and methods

2.1. Plant extract preparation

Fresh *M. indica* leaves were collected from Vellore district, Tamil Nadu, India. Leaves were washed with distilled water and 5 g of chopped leaves were added to 100 mL double distilled water. The mixture was boiled in a water bath at 80 °C for 30 min. After cooling, the solution was filtered using Whatman filter paper and used as an extract for the synthesis of ZnO NPs.

2.2. Biosynthesis of zinc oxide nanoparticle

80 mL of 0.1 M zinc nitrate was added to 20 mL of plant extract and the solution was then stirred constantly for 6 h by a magnetic stir bar. After stirring at room temperature, the solution was left to settle for 2 h. The solution was then centrifuged at 10,000 rpm for 5 min. The resulting pellet was washed twice with double distilled water then dried in a hot air oven at 80 °C. The pellet was then calcified in a muffle furnace at 450 °C. The powdered ZnO NPs produced were used for characterization and application purposes.

2.3. Characterization techniques

The synthesis of nanoparticles was confirmed through UV-vis spectrophotometric analysis. X-ray diffraction (XRD) analysis was conducted at an operating voltage of 40 kV and current of 30 mA to determine the crystalline nature and purity of the nanoparticles. XRD analysis was also be used to determine the size of the nanoparticles using the Debye-Scherrer equation. Scanning electron microscopy (SEM) operating at a voltage of 20 kV was used to determine the surface morphology and size of the nanoparticles. The nanoparticles were then coated on a copper grid for Energy Dispersive X-ray (EDX) analysis.

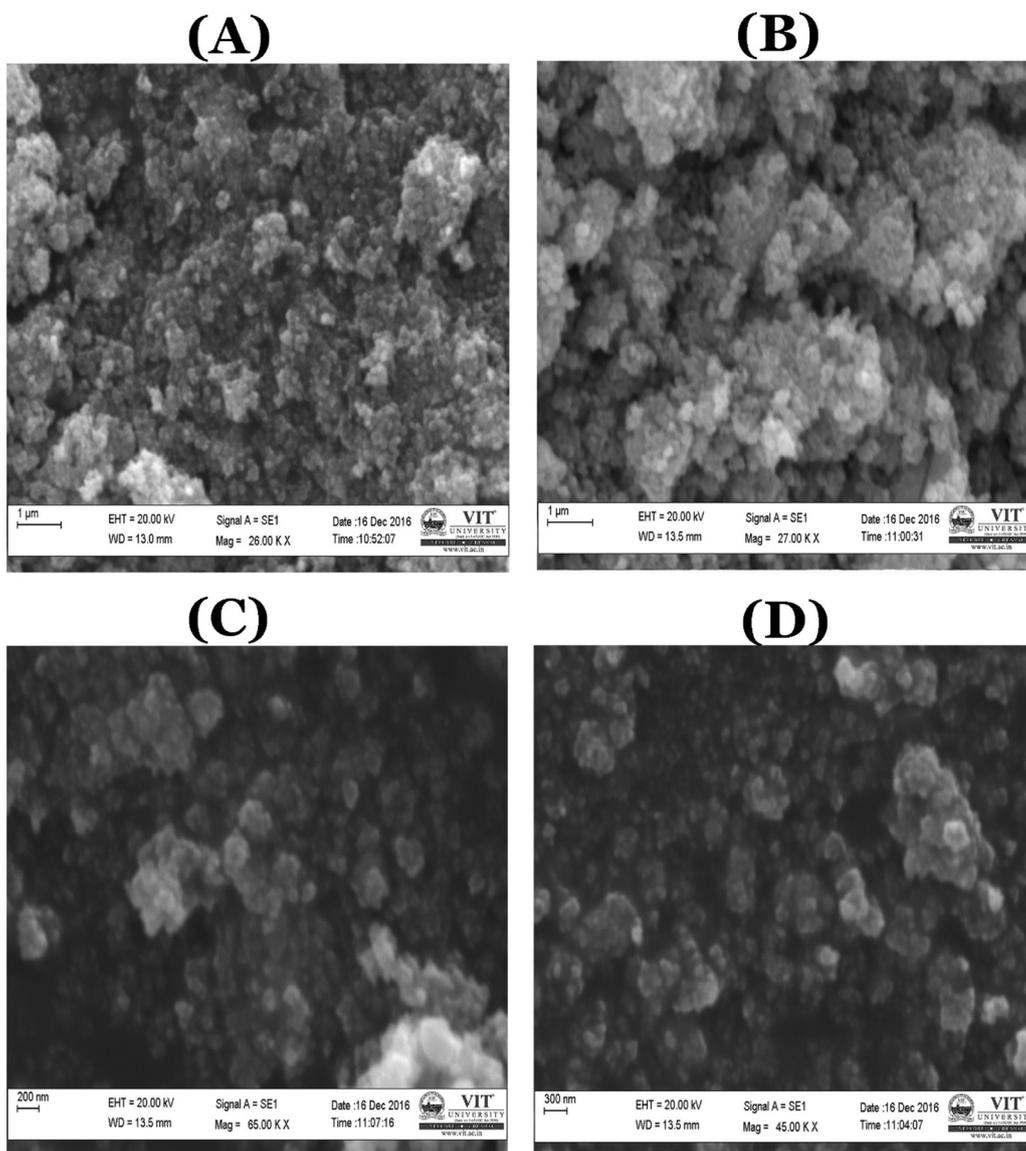


Fig. 3. SEM analysis of synthesized zinc oxide nanoparticles in different magnification ranges (A) 26 KX (B) 27 KX (C) 65 KX (D) 45 KX.

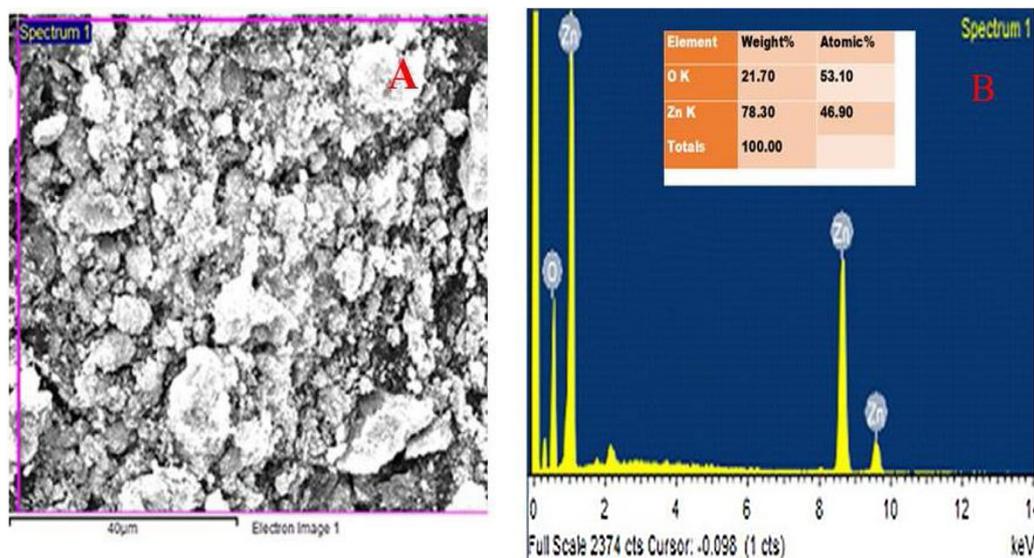


Fig. 4. (A) SEM image of scanned area (B) EDX analysis of zinc oxide nanoparticles using *M. indicia* leaves extract.

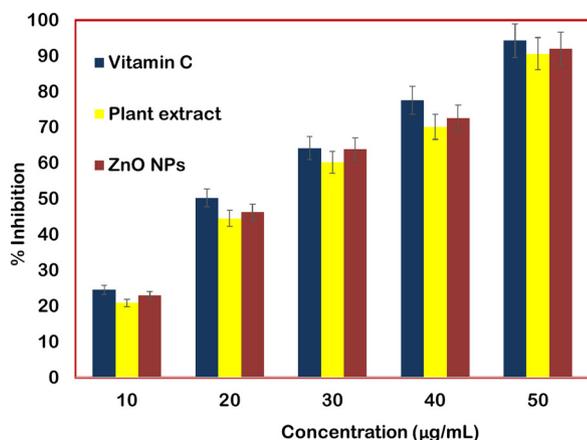


Fig. 5. The antioxidant activity of zinc oxide nanoparticles, mango plant extract, and vitamin C.

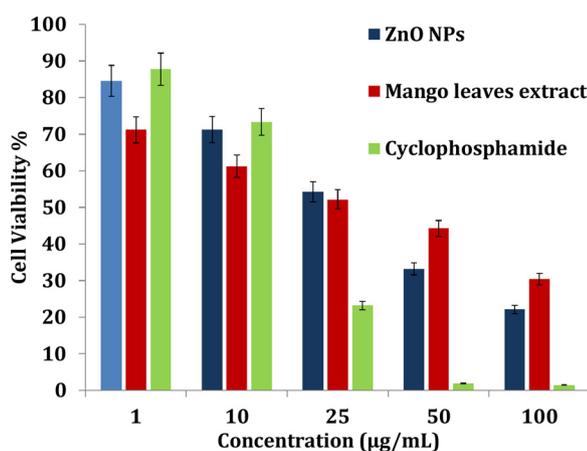


Fig. 6. Anticancer activity of zinc oxide nanoparticles and mango *M. indica* leaves extract.

2.4. Measurement of antioxidant activity

The antioxidant potential of synthesized ZnO NPs was estimated as described [17]. The experiment was carried out using DPPH activity estimation. The deep violet color of DPPH turns yellow in the presence of an antioxidant compound. When DPPH is mixed with a hydrogen donor substance, free radicals are reduced and a color change occurs. The different volume of plant extract was added to 1 mL of 0.1 mM DPPH solution in methanol. The solution mixture was incubated for 30 min at room temperature in the dark. The absorbance was measured at 517 nm after the incubation period to estimate the reduction in DPPH free radical number. Methanol solution mixed with DPPH was used as a control, vitamin C was used as the standard and methanol plus plant extract solution was used as a blank. All the experiments were performed in triplicate. Origin pro 8.5 software was used for statistical analysis. DPPH free radical scavenging activity was calculated by the following formula;

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of Sample}}{\text{Absorbance of control}} * 100$$

2.5. Anticancer activity of zinc oxide nanoparticle against lung cancer cell lines

The cytotoxicity of ZnO NPs was tested against lung cancer A549 cell lines. Cell lines were procured from the National Centre for Cell Science, Pune, India. The cell lines were maintained at 5% CO₂ in a CO₂

incubator at 37 °C. Cell lines were transferred to 96 well plates at a concentration of 1 × 10³ cells per well and incubated for 24 h. Cells were later washed with 100 µL of serum-free medium and were starved for one hour in a CO₂ incubator at 37 °C. Cells were then treated with different concentrations of ZnO NPs (1–100 µg / mL) and incubated for 24 more hours in a CO₂ incubator. The 96 well plates of cells were wrapped in aluminum foil to avoid light exposure. After the incubation period was over, MTT reagent (0.05 mg / mL) was added to each well and incubated for 4 h in a CO₂ incubator at 37 °C. After the incubation, MTT reagent was discarded and cell lines were washed with 200 µL of phosphate buffer saline (PBS). 100 µL of DMSO was used to dissolve the crystals. The absorbance value was recorded at 570 nm. The absorbance value was plotted against cell density concentration. The experiment was performed in triplicate.

3. Results and discussion

3.1. Visual identification and UV–vis spectrophotometry

The white precipitate formed after the addition of plant extract to zinc nitrate hexahydrate was the preliminary indicator of successful ZnO NP synthesis (Fig. 1A). Previous studies report that the size and shape of the nanoparticles could also be predicted through UV–vis spectra [18]. UV–vis spectrophotometric spectra obtained after 6 h of incubation depicted an absorption peak at 355 nm demonstrating the presence of ZnO NPs (Fig. 1B). Similar results were obtained for *Plectranthus amboinicus* leaf extract mediated synthesis of ZnO NPs [19].

3.2. XRD analysis

XRD analysis was conducted to determine the crystalline nature of the nanoparticle. XRD peaks obtained at 2θ values 31.86°, 34.72°, 36.57°, 47.66°, 56.89°, 61.74°, 68.69° corresponded to lattice plane (100), (002), (101), (102), (110), (103), (112) according to JCPDS card (NO 36-1451) and depicted the hexagonal wurtzite crystal structure of the nanoparticles (Fig. 2). Lattice planes (100), (002) and (101) indicate the presence of a pure form of nanoparticles. Nanoparticle size was calculated using Debye-Scherrer equation

$$D = \frac{K\lambda}{\beta \cos\theta}$$

Where, D = average particle size (nm), K = Shape factor, λ = X-ray wavelength (1.5406 Å), β = full width at half maximum (FWHM).

The average crystal size of the nanoparticle was found to be 47.70 nm using the above-mentioned equation. Similar results were obtained by Das et al. (2013) [20].

3.3. SEM analysis

SEM analysis was typically conducted to mark the surface morphology. Fig. 3 consists of 4 SEM image of ZnO NPs at different magnification ranges. Nearly spherical and hexagonal shaped nanoparticles are clearly visible in the picture. The average size estimated by SEM analysis was 60 nm. These results were consistent with the previously reported study of coconut water-mediated synthesis of ZnO NPs where they found the average size range of 20–80 nm [21].

3.4. EDX analysis

EDX analysis was conducted to determine the elemental composition of the nanoparticles. Fig. 4 includes the EDX image of synthesized nanoparticles and the atomic weight percentage of the nanoparticles. Distinct peaks obtained for zinc and oxygen atoms represent the formation of ZnO NPs. No additional peaks were found, demonstrating the purity of the ZnO nanoparticles. XRD analysis results reveal the pure form of synthesized nanoparticles, which was later confirmed by EDX

analysis results. Similar results were obtained by seaweed mediated synthesis of ZnO NPs, where they found a pure form of nanoparticle clearly depicted through EDX imaging [22].

3.5. Antioxidant activity analysis

The role of antioxidants is to scavenge free radicals. The Fig. 5 graph depicts antioxidant activity of ZnO NPs, mango plant extract and Vitamin C in different concentrations in triplicate. These results demonstrate that the radical scavenging activity of plant extract was increased when it was used to synthesize ZnO NPs. Antioxidant activity of ZnO NPs was almost comparable to standard Vitamin C and if further functionalized or engineered, the activity could increase. Antioxidant activity of ZnO NPs synthesized from *Cassia fistula* plant extract has been previously reported [23].

3.6. Cytotoxicity analysis of zinc oxide nanoparticles on lung cancer cell lines

Reduction of 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) reagent to its insoluble formazan crystal form by metabolically active cells was assayed. Mitochondrial lactate dehydrogenase aided in the reduction process. Dimethyl sulfoxide (DMSO) is a solubilizing buffer added to dissolve MTT formazan crystal. Formazan crystal turns a purple color when dissolved in an appropriate solvent. The intensity of the color is recorded spectrophotometrically and is proportional to a number of viable cells.

The *in vitro* cytotoxic effects of green synthesized ZnO NPs at concentrations ranging from 1 to 100 µg/mL was assessed using the MTT assay (Fig. 6). Cyclophosphamide was used as a standard. DMSO was used as a control for the activity and no effect was seen. The anticancer activity of ZnO NPs increased with the increasing concentration of NPs and is comparable to the cytotoxic effects of cyclophosphamide in low doses. The concentration of the administered nanoparticle plays a crucial role in the anti-cancerous property. The nanoparticle may penetrate the cell membrane through ion channels of cell membranes and interact with nitrogen bases of DNA and intracellular proteins. Similar results were reported for biosynthesized silver nanoparticles on lung cancer cell lines [24].

4. Conclusion

A very simple, one-step procedure for the synthesis of ZnO NPs was developed where *M. indica* leaves extract acted as both the reducing and stabilizing agent. Nanoparticles were characterized using XRD, which estimated the average size of the nanoparticle to be 47.7 nm. Nearly spherical and hexagonal quartzite shaped nanoparticles were visualized through SEM analysis. SEM analysis estimated the average size of nanoparticles to be 60 nm. SEM and XRD results were consistent with each other. XRD and EDX analysis confirmed the synthesis of pure zinc oxide nanocrystals. The antioxidant activity of ZnO NPs was found to be comparable to standard vitamin C as estimated through DPPH activity. The antioxidant activity of nanoparticles was increased by increasing the concentration of nanoparticles. *In vitro* cytotoxicity studies carried out using the MTT assay depicted dose-dependent activity

fluctuations in the cytotoxic activity of ZnO NPs. This was the first time *M. indica* leaves extract was synthesized and ZnO NPs were stabilized for use as an antioxidant agent. This would be a cost-effective, simple and environment-friendly approach to ZnO NP production, which could expand its use into various pharmaceutical industries. Our study requires *in vivo* experiments to better understand ZnO NPs toxicity and future biomedical applications.

Conflict of interest

The authors declare that they have no conflict of interest.

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