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Synthesis of zinc oxide nanoparticles, its characterization and anti-microbial activity assessment

Saarthak Khosla[1,2], Priyanka Singh[1], Mansi Malik[1], Saurabh Kumar Srivastava[3] & Anita Kamra Verma[1]*

[1]Nano-biotech Laboratory, Zoology Department, Kirori Mal College, University of Delhi, New Delhi-110 007, Delhi, India

[2]Vasant Valley School, Vasant Kunj, New Delhi-110 070, Delhi, India

[3]Department of Physics, IIT BHU Varanasi-221 005, Uttar Pradesh, India

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Zinc oxide nanoparticles (ZnO NPs) are one of the most abundant metal oxides nanoparticles. It provides excellent thermal, electrical and chemical stabilities with low biotoxicity; its photo-oxidising and photo-catalytic impact on biological and chemical species is of great importance, thereby making it a promising candidate to be used for *in vitro* and *in vivo* studies in biomedical field. Hereby, ZnO NPs were synthesized using precipitation method with zinc acetate and sodium hydroxide as starting materials. This study has characterized the synthesized ZnO NPs using different techniques such as UV-Visible spectroscopy indicating a peak at 365 nm wavelength, size of ZnO NPs was determined to be 286.7 nm by measuring hydrodynamic radii using Dynamic Light Scattering (DLS) phenomena. Further predominant charge existing at surface of the synthesised ZnO NPs was evaluated to be 31.6 mV. Anti-microbial activity of ZnO NPs was determined by Kirby-Bauer method for both Gram-positive and Gram-negative bacteria, *S. aureus* and *E. coli*, respectively. Anti-microbial activity was determined as Zone of Inhibition that measures both bactericidal and bacteriostatic activity of ZnO NPs and was found to be more potent for Gram-positive (*S. aureus*) bacteria and its activity increased with increasing concentration of nanoparticles. Growth kinetics was studied to determine percentage growth inhibition, for this optical density was recorded as a function of time in bacterial culture broth with and without treatment. Further DNA fragmentation assay was performed to determine genotoxicity caused by nanoparticles and its effect on genomic DNA of bacteria. Highlighting its potential role as a nano-carrier system for leading antibacterial drugs for enhanced effectiveness of the antibacterial therapies.

Keywords: Bacterial resistance, DNA fragmentation assay, Kirby-Bauer method, ZnO NPs

Nanoparticles (NPs) have nano-scale size in nanometer range (1-1000 nm) that generally confers large surface area is to volume ratio as compared to macro-sized particles[1]. Presence of notable percentage of atoms at the surface of nanomaterials offers them several unique applications in various fields like nanomedicines, biosensors, bio-nanotechnology, biological labelling, drug and gene delivery *etc*[2]. Nanoparticles exhibit size-related properties. Nanoparticles can be classified based on their morphology, size, physical and chemical properties. These are broadly categorized into organic and inorganic groups. Organic groups include polymeric nanoparticles, synthetic nanoparticles *etc*. and inorganic group includes carbon based nanoparticles, carbon nanotubes (CNTs), fullerenes, metallic/metallic oxide nanoparticles like gold (Au NPs), silver(Ag NPs), zinc oxide(ZnO NPs)[3-6]. Metallic nanoparticles are of extreme importance due to their diverse properties. ZnO NPs is one such metallic oxide nanoparticles that possess significant antibacterial activity. Synthesis method of ZnO nanoparticles is broadly divided in three categories as vapour state based methods, solution based method and biological methods. Solution based synthesis involves sonochemical method[7], co-precipitation method, solvothermal method[8-9], sol-gel method[10], micro-wave assisted method, microemulsion method. Vapor state synthesis involve laser ablation method, combustion based method *etc*. Biological approach for synthesis of ZnO NPs also include various bacterial/plant species which has significant role in reducing zinc precursor salts to zinc/zinc oxide nanoparticles, also stabilising the same. Surface morphology of ZnO NPs affects its bio-toxicity, where morphology itself is determined by the choice of the method adapted for synthesis of nanoparticles, different parameters such as precursor material type, solvents, physicochemical settings like temperature; pH *etc*. could be modulated for synthesizing desirable nanoparticles to reduce its toxicity levels[11]. Additionally, factors like

*Correspondence:
E-mail: akverma@kmc.du.ac.in

morphology, size, crystalline nature and composition *etc*. determines ZnO NPs intrinsic properties. ZnO NPs have large surface area due to small size; they possess a large percentage of atoms at their surface altogether enhancing reactivity of nanoparticles surface[12]. ZnO NPs are bio-safe material, also exhibits distinctive physico-chemical properties like photo-oxidising, photo-catalytic properties that impacts biological and chemical species, also exhibits optical properties like UV absorption, photoluminescence[13]. They provide excellent thermal, electrical and chemical stabilities. Thus through this study , the antibacterial activity of ZnO NPs have been evaluated in model test strains for gram negative and gram positive bacterial species, where antibacterial effect of ZnO NPs is an attribute to its nano size range. The drive for selecting these species is its wide ecological existence and requirement of moderate culture conditions, besides this there is another vital categories of drug-resistant microbial species such as MDSA, MDR *etc*., liable for causing numerous infectious illnesses in human species. This effort, comprehending the antibacterial activity of ZnO nanoparticles in dose dependent manner, its effect as % decrease in growth has also been assessed as function of time and toxicity mechanism was evaluated quantitatively using DPA assay and correlating it with nanoparticles properties that have been presented in terms of its interaction with bacterial surface, penetrate within bacterial membrane thus exhibiting distinct bactericidal or bacteriostatic mechanisms[14-15].

Materials and Methods

Materials required

Chemical Synthesis of ZnO nanoparticles, the salts of zinc acetate (ZnC_4H_6O4) (M.W 183.48), 2-methoxy ethanol($C_3H_8\ O_2$) (M.W 76.10), sodium nitrate ($NaNO_3.9H_2O$) (M.W 404), sodium hydroxide (NaOH) (M.W 40) from lobachem pvt. Ltd, Ethanol absolute from Changshu yangyuan chemical, China. For antibacterial assessment, bacterial model test strains of gram negative (*E. coli*) (MTCC-1677) and gram positive (*S. aureus*) (MTCC-3160) were used, procurement was from the Institute of Microbial Technology (IMTECH), Chandigarh, India. Chemical requirements such as Nutrient broth powder, nutrient agar powder, antibiotic (Gentamycin) were acquired from Hi-Media Laboratories Pvt. Ltd. Deionized water was used throughout the experiment. Instruments such as 2 MLH magnetic stirrer (220/230 V ac supply), UV visible spectrometer-Cary 60 (version 2.0), electronic balance of readability 0.001 g (model HK-220AB) laboratory heating plate employed in experiments and measurements.

Synthesis of ZnO nanoparticles

0.8 g of zinc acetate was added to 25 mL of distilled water(DW), dissolved using a magnetic stirrer, 200 mg of sodium hydroxide dissolved in 50 mL of DW and slowly added to the beaker with zinc acetate solution[16]. Solution was stirred at 700 rpm for another 2 h until turbidity appeared and aluminium foil was wrapped over it for reaction to complete. The dispersion was then removed from stirrer and kept overnight for sedimentation to occur. The clear liquid above the precipitate was removed using a pipette and remaining precipitate was centrifuged for 1 h at 900 rpm. The sample was then put in an oven for 4 h at 50°C. The obtained precipitate was then grounded with mortar and pestle and was stored at room temperature.

Characterization of the synthesised nanoparticles

Nanoparticles were characterized to determine its structural properties such as size, surface charge and UV visible spectra *etc*. Size, surface zeta potential was obtain using Zetasizer Nano ZS, Malvern Panalytical, this instrument works on the principle of dynamic light scattering (DLS). Further UV visible spectral peaks were determined using Spectrophotometer (Cary 60 Agilent Technology, U.S.A).

Antibacterial tests

Preparation of sterile broth: Nutrient broth powder was dissolved in deionised water as per manufacturer's instruction and sterilized with autoclaved machine at temperature of 120°C, 15 psi pressure for 20 min. Mother inoculum in ratios of 1:100 was inoculated in sterile broth which was kept at 37°C for 24 h in orbital shaker for turbidity to occur, this turbidity indicate culture growth, optical density of which was recorded at 600 nm by UV spectrophotometer (Cary 60, Agilent Technologies) and colony forming unit/mL represented as (CFU/mL) was calculated:

$$1OD = 0.8 \times 109\ CFU/mL.$$

Zone of inhibition (ZOI) measurement using Disc Diffusion Assay: Antibacterial susceptibility of nanoparticles was evaluated based on principle of Kirby Bauer method. Bacterial culture was initially mixed with semi solid soft agar and was spreaded uniformly on to the nutrient agar plate. Discs of diameter 5 mm was soaked with test samples *i.e*. different conc. of ZnO

NPs, gentamycin was used as positive control and Milli-Q water as negative control and was placed in a well defined section in the plate. The plates were then kept for incubation at 37°C and ZOI measurements were recorded next day *i.e.* post 24 h.

Growth kinetic studies: Sterile broth was prepared and it was further inoculated with bacterial culture. Three different test samples were prepared by dissolving 1 mL of LB, 10 µL of bacteria cultures, 20 µL of different concentrations (2.5 mg, 10 mg, 15 mg) of nanoparticles, plain LB media with bacterial culture was used as control for the experiment. Absorbance values were measured at 600 nm from 0-5 h and data is represented as log CFU/mL as a function of time for both the treated, control groups to obtain the growth curves.

Quantitative measure of fragmented DNA using Diphenylamine (DPA) Assay: Fragmentation of bacterial genome was evaluated. Freshly prepared bacterial cultures were utilized in the assay and treatment was given at various concentration (2.5 mg/mL, 10 mg/mL and 15 mg/mL) for 24 h. Post treatment these test samples were centrifuged at 300 g for 10 min at 4°C, supernatant discarded, 800 µL of PBS buffer, 700 µL freshly prepared ice-cold lysis buffer added, further incubated on ice for another 15 min. Centrifugation was done at 13000 g at 4°C for 15 min, supernatant and pellet collected in different tubes, 1.5 mL TE buffer added to pellets, with further addition of trichloroacetic acid (TCA), centrifuge the same, separate the supernatant. Add DPA reagent to it and leave it for overnight incubation and record the OD values at 600 nm. The percentage DNA fragmentation was calculated by following formula:

% DNA Fragmentation= (OD of supernatant)/(OD of supernatant+OD of pellet)

Statistical analysis

The data is illustrated as mean values ± standard deviation. Comparison was done among groups and analysis was performed with one-way analysis of variance (ANOVA) under Tukey's test using Prism software (Prism software Inc. CA). Significance levels were accepted at $P \leq 0.05$ level.

Results

Nanoparticles size and surface zeta potential

The synthesised nanoparticles mean hydrodynamic diameter was obtained from DLS measurements, it shows nanoparticles size of 286.7 nm, polydispersity index (PDI) was 0.144, as shown in (Fig. 1A). The PDI value indicates homogenecity of the nanoparticles that are synthesised, apart from this it also indicate narrowness of distribution spectra for synthesised nanoparticles, thus it confirm the uniform size distribution that can easily be observed from further characterization using SEM technique, this can give the clear evaluation for surface morphology of the synthesised nanoparticles. Zeta potential was also determined as represented in (Fig. 1B) it was obtained to be 31.6 mV and signify its good stability in the medium[17]. Absorbance peak of ZnO nanoparticles was also determined to be at 365 nm wavelength in (Fig. 1C). This confirms the synthesis of nanoparticles.

Antibacterial Properties

Antibacterial activity determined as Zone of inhibition: ZnO nanoparticles antibacterial activity was determined against the model bacterial test strains *i.e. E. coli* and *S. aureus* using Kirby-Bauer method[18]. The results are summarized in graphical form (Graph 1), with the

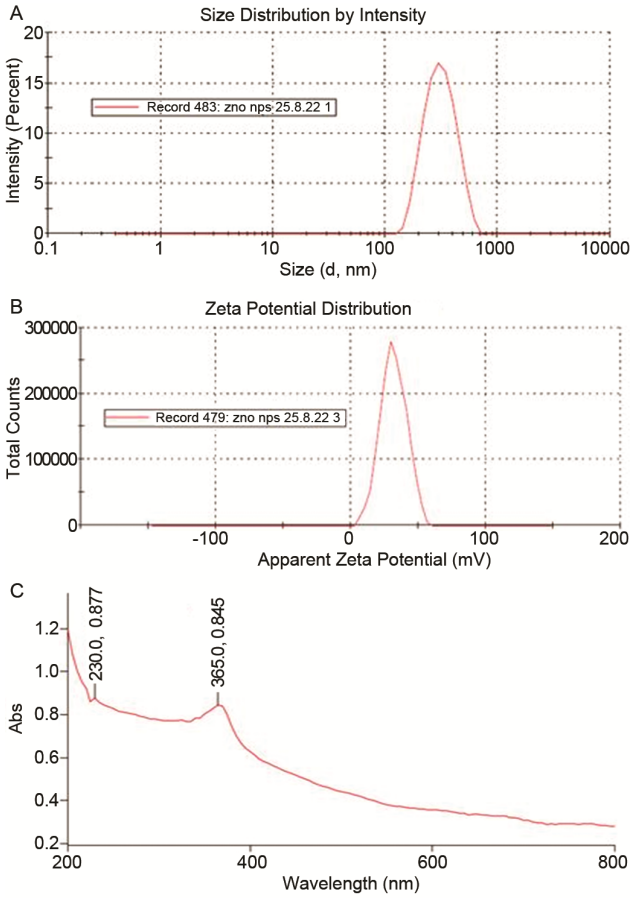
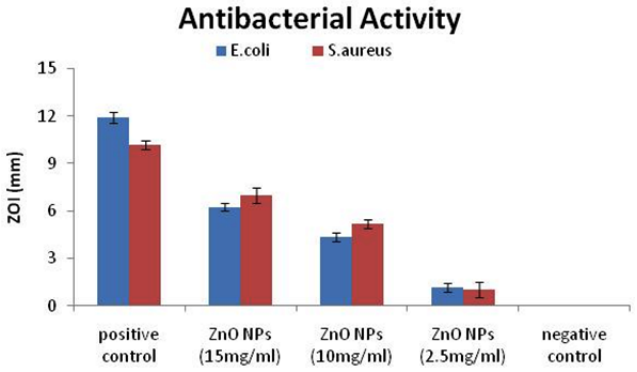


Fig. 1 — (A) Size distribution obtained from DLS; (B) Zeta potential of the ZnO nanoparticles; and (C) absorbance peak of ZnO nanoparticles is at 365 nm wavelength

subsequent images of plate represented in (Fig. 2). At different nanoparticles concentration such as 2.5 mg/mL the ZOI values are 1.16±0.28 mm and 1.00±0.50 mm, for 10 mg/mL it is 4.33±0.28 mm and 5.16+0.28 mm, 15 mg/mL- 6.23±0.25 mm and 7.00±0.50 mm, positive control 11.9±0.36 mm and 10.16+0.28 mm, negative control there was no ZOI value observed in *E. coli* and *S. aureus* bacterial species, respectively, suggesting nearly higher susceptibility of gram positive bacteria than gram negative bacteria for the synthesized nanoparticles[19].



Graph 1 — Representation of data as ZOI values depicting antibacterial activity of the synthesised ZnO NPs

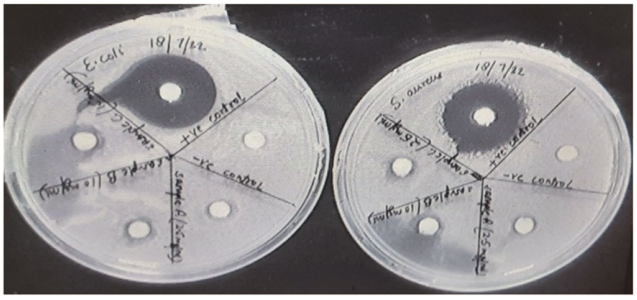


Fig. 2 — Comparative analysis of antibacterial activity of ZnO NPs against Gram positive (*S. aureus*) and Gram negative (*E. coli*) bacteria with gentamycin (5 mg/mL) as a positive control and sterilized discs as negative control

Growth kinetics studies: This was done to investigate the inhibitory outcome of ZnO nanoparticles on bacterial growth. Figure 3 noticeably depict variation in growth of treated group on comparison to the growth of untreated culture. Treatment with ZnO nanoparticles at different concentration (2.5 mg/mL, 10 mg/mL, 15 mg/mL) depicted inhibition of 20%, 26%, 35% in the growth of *S. aureus.* But for *E. coli* these values are 16%, 24%, 33% after 24 h of incubation. This was enhanced by successive application of increased concentration of nanoparticles. This can be attributed to effect introduced by nanoparticles causing bacterial damage[20].

DNA Fragmentation assay: Further the diphenylamine (DPA) reaction was performed. It is a kind of quantitative estimation that takes advantage of the bonds between purines and deoxyribose, which are very labile. Once these bonds are broken, inorganic phosphates are liberated from the DNA and provide the substrate w-hydroxylevulinylaldehyde which under acidic condition reacts with DPA & forms blue-coloured complex, absorbance recorded at 600 nm. The estimated amount of fragmented DNA is slightly higher in gram positive bacteria as compared to gram negative bacteria on increasing the concentration of nanoparticles[21]. Figure 4, represents the percentage of fragmented DNAs in 2.5 mg/mL treated group is 44% ± 0.97%, 43%± 0.11%, in 10 mg/mL treated groups the values are 57% ± 0.29%, 58% ± 0.38%, in *E. coli* and *S. aureus* but for 15 mg/mL nanoparticles treatment the fragmented DNAs % is 61% ± 0.47%, 64% ± 0.94% in *E. coli* and *S. aureus*, respectively. It

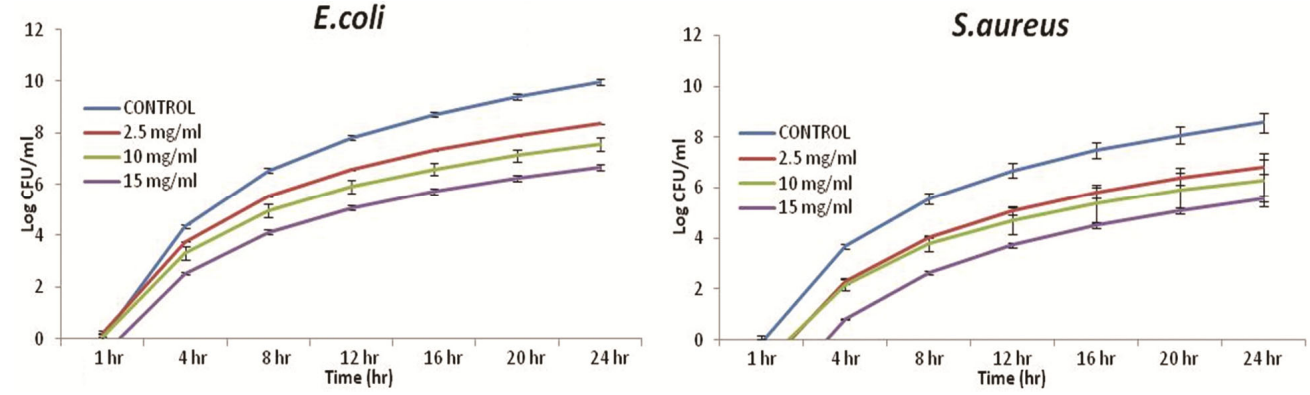


Fig. 3 — Growth kinetics curve (A) Gram negative bacteria *E. coli*; and (B) Gram positive bacteria *S. aureus*, when treated with ZnO NPs at three different concentrations 2.5 mg/mL, 10 mg/mL, 15 mg/mL. Log CFUs plotted as a function of time data represented as mean ± S.D

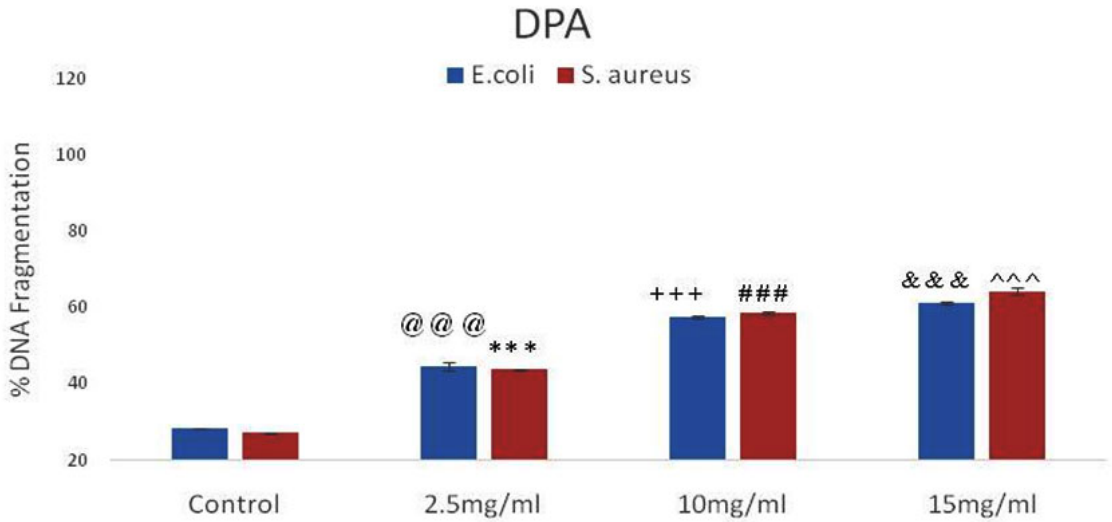


Fig. 4 — Percentage DNA fragmentation and its comparison for different conc. of ZnO nanoparticles prepared against negative control. Data were expressed in mean ± Standard deviation (n=2) @ denote significant difference between control *vs* 2.5 mg/mL ZnO NPs treated group in *E. coli* * denote significant difference between control *vs* 2.5 mg/mL ZnO NPs treated group in *S. aureus* + denote significant difference between control *vs* 10 mg/mL ZnO NPs treated group in *E. coli* # denote significant difference between control *vs* 10 mg/mL ZnO NPs treated group in *S. aureus* & denote significant difference between control *vs* 15 mg/mL ZnO NPs treated group in *E. coli.* ^ denote significant difference between control *vs* 15 mg/mL ZnO NPs treated group in *S. aureus* **P* <0.05; ***P* <0.01; ****P* <0.001

can thus be inferred that modification in the synthesized nanoparticles substantially improves the antibacterial potential hence it can potentiate its utilization as an antibacterial formulation against bacterial growth.

Percentage of DNA fragmentation of both *S. aureus* and *E. coli* bacterial genome represented in (Fig. 4.) for different concentrations of ZnO NPs against negative control. Literature review suggests that ZnO NPs induce their antimicrobial properties through different mechanisms. ZnO NPs are capable of inducing morphological changes in bacteria as these particles are nano-sized, these are capable of passing through peptidoglycan layer of bacteria making them susceptible to damage, and they can also directly contact cell walls of bacteria thereby damaging bacterial cell wall integrity[22-24]. Bacterial cell wall carries negative charge and ZnO NPs carries positive charge thus creating electrostatic force of attraction between nanoparticles and bacterial surface so damaging cell membrane, disturbing bacterial cell integrity and induces morphological changes in bacteria. Upon cell wall disorganisation, nanoparticles get internalized in bacterial cells and causing destruction of cellular components like lipids, DNA, proteins *etc*. ZnO NPs gets dissolved in medium, released Zn^{+2} ions inhibit through multiple mechanisms of bacterial cells death pathways such as through active transport, amino acid metabolism, enzymatic system disruption[24]. Dissolution of ZnO NPs in Zn^{+2} ions is size dependent and is effected by two parameters like physico-chemical properties of particles like porosity, concentration, particle size and morphology of NPs along with chemistry of media like pH, UV illumination, time exposure[26]. Metal ion uptake into bacterial cells also leads to depletion of ATP production; also leads dissipation of proton motive force due to membrane disruption. Production of Reactive Oxygen Species (ROS) is also one of the major causes of nano-toxicity[27-28]. Photocatalytic generation of ROS is major contributor of antibacterial activities of various metal oxides. Several literatures suggests that ZnO NPs leads to formation of ROS like superoxide anions, hydrogen peroxide, hydroxide ions *etc*. in both UV and dark conditions that leads to oxidative stress in various bacterial species[29].

Conclusion

The synthesis of ZnO NPs using precipitation method is the most cost effective, bio safe, nontoxic method with good yield of nanoparticles. The literature review related to ZnO NPs and its prominent role as an antimicrobial agent can be best

utilized for nanocarrier systems. In both Gram positive and Gram-negative bacterial strains, different concentrations of ZnO NPs are reported to have antibacterial activities with enhanced effect on Gram negative bacterial strains. Whereas the growth kinetics data was found to be more prominent for *S. aureus* as compared to *E. coli.* The DPA results were also reported for both bacterial strains. Thus it can be said that it effectiveness can be enhanced against bacterial infection that can lead to an advent on loading with potential antimicrobial drug. Future direction may focus on mechanism of action responsible for antimicrobial activity of ZnO nanoparticles.

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Conflict of interest

All authors declare no conflict of interest.

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