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Metallic nanoparticles as alternative antimicrobials

Abstract

Nanoparticles (NPs) of ZnO TiO_2 & ZVI were synthesized by sol–gel method and precipitation route. XRD analysis showed that all synthesized nanoparticles were 25–55 nms in size. TEM analysis provided shape of synthesized nano ZnO, TiO_2, and ZVI. They were evaluated for their ability to minimize or deteriorate microbial load. For assessment of antimicrobial effect of synthesized nanoparticles, agar well diffusion method was used. Inhibition zone was measured and further minimum inhibitory concentration (MIC) of nanoparticle was evaluated. The obtained results showed that all of the three synthesized nanoparticles possess antimicrobial activity. The experimental results also provided information about changes of microbe growth curve upon the treatment with nanodose.

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Introduction

The infections caused by numerous microbes present a number of uncommon challenges. Microbial infections are still a major cause of morbidity and mortality.[1,2] The growing concern regarding multidrug–resistant bacterial strains and biofilm–associated infections calls for the development of additional bactericidal agents.[3] Conversely, many methods for treating these and other bacterial infections currently exist; there is still an essential need for new and improved approaches for bacterial destruction. Nanomedicine is one approach to overcome challenges of conventional drug delivery systems based on the development and fabrication of nanostructures. Various types of nanoparticulate systems have been tried as potential drug delivery systems, containing metal nanoparticles, biodegradable polymeric nanoparticles, nanogels, solid lipid nanoparticles (SLN), nanoliposomes.[4] Out of these all, inorganic metallic nanoparticles have received increasing attention due to their high stability.[5] Nanoparticles (NPs) are typically no greater than 100 nm in size and their biocidal effectiveness is recommended to be due to a combined effect of their smaller size and high surface–to–volume ratio, which facilitate intimate interactions on microbial membranes.[6] Most used metal oxide nanoparticle include silver (Ag), iron oxide (Fe_3O_4), titanium oxide (TiO_2), copper oxide (CuO), and zinc oxide (ZnO).[7] Anti–microbial properties of Metal oxide nanoparticles exhibit through reactive oxygen species (ROS) generation. The deterioration of Gram–negative bacteria (*E. coli*) and Gram–positive bacteria (*S. aureus*) with TiO_2 and Ni doped TiO_2 nanoparticle was investigated in the absence and presence of irradiation by.[8] Antibacterial activities of iron oxide nanoparticles were reported against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Serratia marcescens* by.[9] The results suggested by.[10] that the use of ZnO NPs as an efficient fungicide in agricultural and food safety applications. They investigated activity of zinc oxide nanoparticles against two pathogenic fungi (*Botrytis cinerea* and *Penicillium expansum*). In this research, TiO_2, ZnO and ZVI nanoparticles were prepared and their potentiality to inhibit growth of various microbes was assessed. The synthesized nanoparticles were characterized by X–ray diffraction (XRD), transmission electron microscopy (TEM). The antimicrobial effect of three nanoparticles against *Bacillus subtilis*, *Staphylococuus aureus* & fungi *Aspergillus niger* was evaluated.

Materials and methods

Synthesis of nanoparticles (NPs)

For nanoparticles synthesis, all reagents were purchased from Sigma–aldrich and used as received without further purification. All solutions used in the experiments were prepared using double distilled water. For synthesis of ZnO NPs, the precipitation process of.[11] was used. The solution NaOH (1M) was first heated to 50 °C and then $ZnCl_2$ was added drop by drop under high magnetic stirring. After addition of $ZnCl_2$, it was allowed to stir for further 10 min till white precipitates formed. The ZnO nanoparticles containing pellet was washed three times with distilled water followed by washing with ethanol twice. Finally, ZnO nanoparticles were collected after drying the pellet at 50 °C. For synthesis of TiO_2 NPs, a sol– gel method based on.[12] was followed. 12 mL titanium isopropoxide was added to 23 mL acetic acid with continuous stirring. Hydrolysis of titanium tetraisopropoxide solution was carried out by adding distilled water (72 ml) slowly at the rate of 0.5 ml/min with continuous stirring. The solution was kept stirring for 6 h until achieving a clear transparent sol. Sol was dried at 100 °C, followed by calcination at 600 °C for 2 h. For synthesis of ZVI NPs, the precipitation process of.[13] was used. The Sodium borohydride 0.16 M ($NaBH_4$, 0.6053 g) was dissolved in 100 mL of 0.1 M NaOH solution.0.1 M $FeCl_3.6H_2O$ was prepared into 100–mL pure water. Addition of the $NaBH_4$ to the $FeCl_3$ solution in the presence of vigorous magnetic stirring resulted in the rapid formation of fine black precipitates. The particles were washed 3 to 4 times with Distilled water and then collected after drying.

Characterization of NPs

All three NPs were characterized using X–Ray Diffraction (XRD) & Transmission Electron Microscopy (TEM) techniques. The shape and size of the particles were obtained through TEM acquired with a Philips Tecnai 20 Electron Microscope operating at an accelerating voltage of 100 kV. Samples for TEM were prepared by drop casting on carbon coated copper TEM grids and left solvent evaporate at room temperature. Afterwards, more than 500 particles from different images were computer–analyzed and measured for size distribution analysis. The crystallinity was acquired by Powder XRD (Phillips X'pert MPD system, Holland) using Cu–Ka radiation (λ = 1.5405A°)

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in a 2y range of 5–601 at a scan speed of 0.11 s^{-1}, maintaining applied voltage at 40 kV and current at 40 mA. About 0.5 g of the dried particles was deposited as a randomly oriented powder onto a Plexiglas sample container, and the XRD patterns were recorded between 20° and 80° angles. XRD patterns were compared with the standard anatase diffractograms.[14]

Evaluation of antimicrobial activity of synthesized nanoparticles

Evaluation of nanoparticles associated antimicrobial activity was carried out by agar–well diffusion method.[15]

Organism preparation for antimicrobial study: To study the antimicrobial activity, clinical isolates namely; *Bacillus subtilis*, *Staphylococuus aureus* & fungi *Aspergillus niger* were used as these are opportunistic pathogens. Nutrient broth was prepared in conical flask for bacteria and PDA was prepared for fungus and sterilized. Above mentioned isolated strain of species was inoculated in media. These bacterial cultures inoculated in nutrient broth were placed on rotary shaker for overnight at 100 rpm at 37 °C in shaker–incubator. The grown cells were then re–suspended in nutrient medium and optical density (OD) was adjusted to 0.1, corresponding to 10^8 CFU/ml at 600 nm.

Preparation of nanoparticles for antimicrobial activity: Nanoparticles were autoclaved in distilled water to make stock solution of 1000 ppm. Solution was sonicated for 20 minutes. Stock solution was dispersed in 1ml of 10% Dimethyl Sulfoxide (DMSO) with different concentration to make 50, 100, 150, 200 ppm nanodose. They were inoculated in plates separately.

Inoculation of test plate: Nutrient agar (2%) was prepared and autoclaved. The agar suspension was poured into plate and allowed to solidify. A 100 µL sample of bacterial suspension cultured in broth medium with a concentration of 10^8 CFU/mL of bacteria was placed on a nutrient agar plate. The inoculum was spreaded evenly over the entire surface of the plate by spread plate technique. With the help of gel puncture, wells were prepared in agar plate. Plates were allowed to dry before applying nanoparticle dose.

Nanoparticles with different doses were placed in the wells of agar. The inoculated plates were incubated at 37 °C for 24hours. DMSO as a control was placed in the well to ensure its non–antimicrobial property. Finally, after incubation the antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms. After which, the zone of inhibition was recorded. Based on the inhibition zone after incubation, the results were interpreted either as positive or negative. Further minimum inhibitory concentration (MIC) was evaluated.

Results and discussion

Synthesis and characterization of nanoparticles

The structure identity and purity of the prepared nanoparticles were verified by XRD & TEM. The crystalline sizes of the synthesized NPs were determined by Debye Scherer's formula according to the equation and summarized in Table 1:

$$(d=k\lambda\ /\beta\ cos\theta)$$

Where λ is the wavelength of incident X–ray; β is the Full Width Half Maximum (FWHM) of diffracted peak and θ is diffracted angle. XRD spectra of synthesized particles are shown in Figure 1. XRD peaks of the nanocrystallite match well with standard ZnO, TiO_2, ZVI and no other crystalline phases were detected.

Table 1 Crystallite size of prepared nanocatalyst.

Nanoparticles	Crystallite Sizes (NMS)
ZnO	26
TiO_2	24
ZVI	55

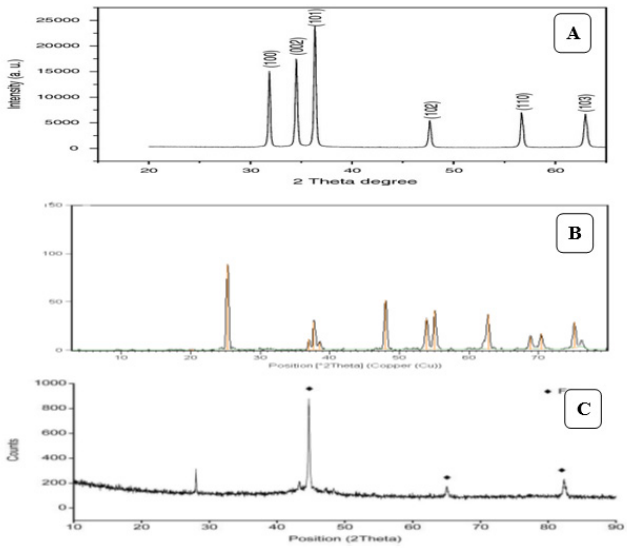

Figure 1 XRD spectra of (A) ZnO; (B) TiO_2; (C) ZVI nanoparticles

TEM pictures of the sample were taken to resolve the morphology and size of the nanoparticles. The TEM micrograph of the ZnO nanoparticles showed hexagonal structure while TiO_2 nanoparticles showed the uniformity of particles size ranging from 20 –30 nm with cuboidal shape of particles (Figure 2) whereas ZVI nanoparticles exhibited spherical structure of catalysts (Figure 2). It is further evident that all the crystals were completely separated from each other. The crystallites had sets of clearly resolved lattice fringes giving evidence that the nano material was highly crystalline. Similar phenomenon was observed by other researchers.[13,16] TEM results showed good agreement with the particle sizes of XRD results.

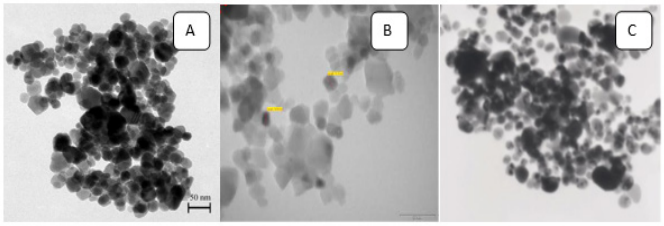

Figure 2 TEM images of synthesized (A) ZnO; (B)TiO_2;(C) ZVI nanoparticles.

Determination of antimicrobial activity of synthesized nanoparticles

Stock cultures of microorganisms (*Bacillus subtilis, Staphylococuus aureus and* fungi *Aspergillus niger*) were incubated on the nutrient agar or PDA plates, the colonies of the two replicates were counted, and then colony of particular organism was transferred to autoclaved nutrient broth and allowed to incubate for 16–18 hours. The O.D. of inoculated culture was tested at 600 nms to be 0.1. If O.D. was higher than 0.1 then broth was diluted with appropriate amount of sterile media and O.D. was adjusted to 0.1 in the order to attain microbial load 10^8 cfu/ml. On the other hand, if O.D. was less than 0.1, media was allowed to incubate for more time period to get optimum microbial load.

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Antimicrobial activity of nanoparticles: Synthesized three nanoparticles exhibited antibacterial activities against above selected microbes upon agar diffusion assay. The preliminary antimicrobial activity study as diameter of inhibition zones (in millimetres) around each well loaded with NPs is represented in Figure 3. It is evident from the zone of inhibition that TiO_2 nanoparticles possess potent bactericidal activity as compared to other two nanoparticles. TiO_2 NPs were able to inhibit microbial growth by producing a maximum zone of inhibition of 9 mm against *S. aureus* and a minimum zone of inhibition of 7 mm against *A. niger* at 250 ppm.

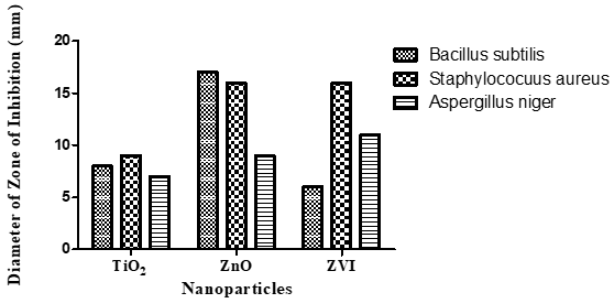

Figure 3 Disc diffusion of synthesized nanoparticles against microbes.

The inhibition zone diameter of ZnO nanoparticles comes out to be almost equal in *B. Subtilis* (16mm) *and S. Aureus* (17mm). Therefore, the ZnO nanoparticles worked equally well against both these bacteria. Antifungal activity of ZnO was observed in the range of 9 mm. ZnO NPs showed consistent inhibition on all the strains showing significant antimicrobial activity against a broad–spectrum of microbes. Antimicrobial study results revealed ZVI as potential antifungal agent among other two particles. Figure 3 shows the inhibition zone produced by ZVI against *A. niger* (11 mm). Diffusion of bacteria in presence of ZVI was restricted in the form of zone at 6 & 16 mm against *B. subtilis & S. aureus*. ZVI showed to be potent antimicrobial against *S.aureus.*

Minimum inhibitory concentration (MIC): After confirming the antimicrobial activity of nanoparticles, further it was needed to identify, the lowest concentration of nanoparticles that produce complete inhibition of microbial growth. Concentrations of nanoparticles from 50–250 ppm were taken into account and were loaded on microbe seeded agar plate to find out its Minimum Inhibitory Concentration (MIC). The susceptibility of microbes against synthesized nanoparticles is recorded in Table 2.

Table 2 MIC of nanoparticles against microbes.

Microbe	Nanoparticles	MIC
	TiO_2	150ppm
Bacillus subtilis	ZnO	50ppm
	ZVI	100ppm
	TiO_2	200ppm
Staphylococcus aureus	ZnO	100ppm
	ZVI	100ppm
	TiO_2	200ppm
Aspergillus niger	ZnO	100ppm
	ZVI	100ppm

It is evident from our study that the MIC values of TiO_2 nanoparticles against all three taken microbes are much higher than the other two nanoparticles. 150ppm for *B. subtilis* and200ppm TiO_2 nanoparticle against *S. aureus* and *A. niger* showed complete inhibition of growth. It was observed that ZnO nanodoses have noticeable variation in activity on all the three strains and found that *B. subtilis* growth was inhibited at lower concentration as 50ppm. Whereas, 100 ppm concentration of ZnO retarded the growth of *S. aureus* and *A. Niger.* While, ZVI was able to inhibit the growth of all strain at 100 ppm.

Growth curve study of three microbes in the presence and absence of TiO_2, ZnO & ZVI nanoparticles was studied. Three varying concentration of nanoparticles were taken into account. MIC concentration based growth curve was also studied. Experimental result provided the effect of sub–inhibitory and inhibitory concentration of synthesized nanoparticles against microbes till stationary phase. In control of *B. subtilis* cell lag phase ended within 2 hour after which log phase started which ended within 8 hours (Figure 4). However, when cells were treated with TiO_2, ZnO & ZVI nanoparticles, there occurred delay in the lag phase by 4 hours. At MIC concentration lag phase ends up to 7–8 hours. MIC value showed minimum growth that proves its inhibitory action.

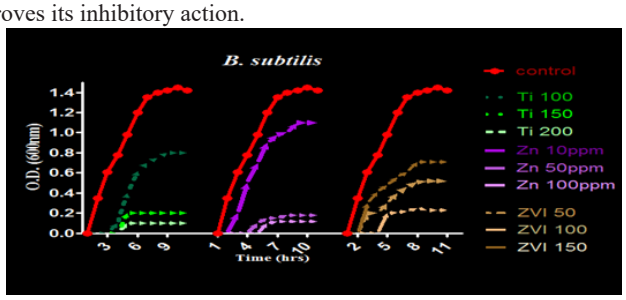

Figure 4 Growth curve of *B. subtilis* against nanoparticles.

Similar result was observed with treatment of synthesized nanoparticles against *S. aureus* (Figure 5). In control lag phase started at 5 hrs and ended within 10 hours. Whereas, it was delayed by 10 hours upon treatment with nanoparticle. MIC value showed reduced growth when compared with control. Growth curve by *A. niger* also gave delayed lag phase on treatment (Figure 6). Growth of fungi was highly inhibited by three nanoparticles and reduced to 72%, 75% and 87% by TiO_2, ZnO & ZVI, correspondingly. The delay in lag phase upon exposure to different concentration of nanoparticles, resulted in prominent growth reduction of microbe.

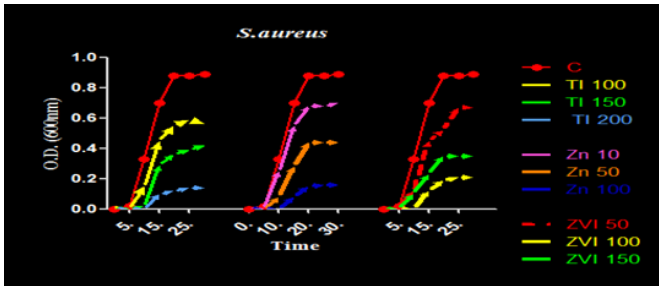

Figure 5 Growth curve of *S. aureus* against nanoparticles.

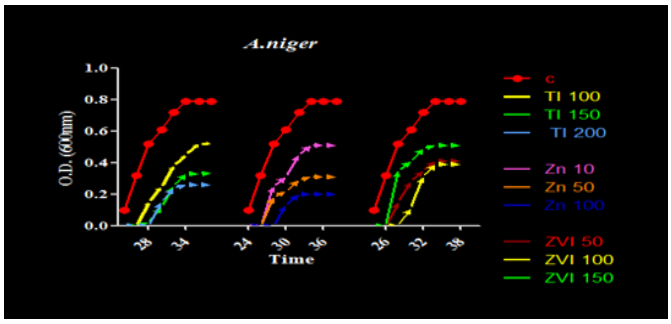

Figure 6 Growth curve of *A. niger* against nanoparticles.

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Rai & Bai.[17] studied metal oxide nanoparticles as promising antimicrobial agents by virtue of their physico–chemical properties. Nataraj et al.[18] experimented the effect of Titanium Oxide nanoparticles on *Staphylococcus* and found descent antibacterial effect. In the present study same results of antibacterial effect was achieved. Study of Roy et al.[19] suggested that TiO_2 NPs exhibited no antibacterial activity, but it was able to reduce microbe growth upon recombination with antibiotics. Our results were collaborative with the findings of Azam et al.[20] who documented ZnO nanoparticles as more significant than iron nanoparticles. Report on ZVI based inactivation of microbes was presented by Lee et al.[21] and suggested inactivation might occur as a result of the penetration of the small particles into cell membrane. In the present experiment, for potent anti– bacterial activity, the nanoparticles concentration was considered as a major factor. A similar behavior of concentration–dependence was observed by Yamamoto.[22] Kim.[23] observed that smaller the size of nanoparticles better is their antimicrobial activity. Adams & Lyon et al.[24] suggested the mechanism of the inhibition effect of cation metals upon investigation in numerous bacteria. They further suggested that binding of metal ions to different sites in the respiratory chain inhibits NADH oxidase activity and also proposed that the antimicrobial effect of metal oxides such as ZnO, TiO_2 and SiO_2, which are photocatalysts, and responsible to produce reactive oxygen species (ROS) in the presence of light, which leads to disruption of the cell membrane and leakage of cellular material.

Conclusion

The ZnO, TiO_2 & ZVI nanoparticles with size around 25–55 nm were successfully synthesized in the laboratory using precipitation & sol–gel method with hexagonal, cuboidal & spherical shape, correspondingly. Our study indicated MIC values in the rage of 100–200ppm for all three synthesized nanoparticle samples. The synthesized nanoparticles are efficient antimicrobials making them competent enough to replace the existing antimicrobials. Zinc oxide nanoparticles are found to be intensely effective among others as a potential antimicrobial agent.

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Conflicts of interest

None.

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