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ORIGINAL RESEARCH

Green synthesized ZnO nanoparticles against bacterial and fungal pathogens

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Abstract Zinc oxide nanoparticles are known to be one of the multifunctional inorganic nanoparticles with effective antibacterial activity. This study aims to determine the antimicrobial efficacy of green and chemical synthesized ZnO nanoparticle against various bacterial and fungal pathogens. Various microbiological tests were performed using varying concentrations of green and chemical ZnO NPs with sizes 40 and 25 nm respectively. Results prove that green ZnO nanoparticles show more enhanced biocidal activity against various pathogens when compared to chemical ZnO nanoparticles. Also effectiveness of nanoparticles increases with increasing particle dose, treatment time and synthesis method. In addition, the current study has clearly demonstrated that the particle size variation and surface area to volume ratio of green ZnO nanoparticle are responsible for significant higher antimicrobial activity. From the results obtained it is suggested that green ZnO NPs could be used effectively in agricultural and food safety applications and also can address future medical concerns.

1. Introduction

Recent advances in the field of nanotechnology, particularly the ability to prepare highly ordered nanoparticulates of any size and shape, have led to the development of new biocidal agents. Nano-materials are called "a wonder of modern medicine". It is stated that antibiotics kill perhaps a half dozen different disease-causing organisms but nano-materials can kill some 650 cells [46]. Metal nanoparticles have various functions that are not observed in bulk phase ([43]; [45]) and have been studied extensively because of their exclusive catalytic, optical, electronic, magnetic and antimicrobial ([23,12]) wound healing and anti-inflammatory properties [48]. Over the past few decades, inorganic nanoparticles, whose structures exhibit significantly novel and improved physical, chemical, and biological properties and functionality due to their nano-scale size, have elicited much interest. Recent studies have shown that NP

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of some materials including metal oxides, can induce cell death in eukaryotic cells ([31,28,19,29]) and growth inhibition in prokaryotic cells due to cytotoxicity.

Among the metal oxide nanoparticles, zinc oxide is interesting because it has vast applications in various areas such as optical, piezoelectric, magnetic, and gas sensing. Besides these properties, ZnO nanostructure exhibits high catalytic efficiency, strong adsorption ability and are used more and more frequently in the manufacture of sunscreens [41], ceramics and rubber processing, wastewater treatment, and as a fungicide ([49,54]). In fact, nZnO usage may overtake nano-titanium dioxide ($nTiO_2$) in the near future as it can absorb both UV-A and UV-B radiation while $nTiO_2$ can only block UV-B, and thereby offering better protection and improved opaqueness [49]. Several physical and chemical procedures have been used for the synthesis of large quantities of metal nanoparticles in relatively short period of time. Chemical methods lead to the presence of some toxic chemicals adsorbed on the surface that may have adverse effects in medical application [24]. Currently, plant-mediated biological synthesis of nanoparticles is gaining importance due to its simplicity, eco-friendliness and extensive antimicrobial activity ([40,26]). Biosynthesis of zinc oxide nanoparticles by plants such as *Aloe vera* [36] and gold nanoparticles by alfalfa ([17,18]), *Cinnamomum camphora* [22], neem [42], *Emblica officianalis* [3], lemongrass [42] and tamarind [4] have been reported.

Antimicrobial activities of metal oxide (ZnO, MgO and CaO) powders against *Staphylococcus aureus*, *Escherichia coli*, or fungi were quantitatively evaluated in culture media ([38,39]). It is considered that the detected active oxygen species generated by these metal oxide particles could be the main mechanism of their antibacterial activity. On view to mycoses, NPs can be considered as potential antifungal agents [2]. However, only few studies have been performed to assess the effects of nanoparticles on fungal pathogens and against *S. aureus and E. coli* ([25,7]). As, nano-silver has been used for imparting antibacterial properties ([27,13]), nano-TiO_2 and the oxides of other nano-materials like CdO and ZnO have also been reported for antibacterial properties ([56,15,33,6,53,50,16]). This study, therefore, is aimed to evaluate the toxicity of biological and chemically synthesized ZnO nanoparticles along with bulk formulations against plant and human pathogens under laboratory conditions. Some of the species chosen have great ecological importance and the risk assessment associated with them should be highly relevant to practical applications. To the best of our knowledge, this is the first comparative toxicity evaluation study of biological and chemically synthesized ZnO nanoparticles against bacterial and fungal pathogens.

2. Materials and methods

2.1. Preparation of the materials and bacterial cultures

In biological method, *aloe* leaf broth extract were prepared with distilled water and made up to 250 ml. Zinc nitrate was then dissolved in the *aloe* extract solution under constant stirring using magnetic stirrer. After complete dissolution of the mixture, the solution was kept under vigorous stirring at 150°C for 5–6 h, allowed to cool at room temperature and the supernatant was discarded. The pale white solid product obtained was centrifuged twice at 4500 rpm for 15 min after thorough washing and dried at 80°C for 7–8 h.

In chemical method, Zinc nitrate was dissolved in distilled water under constant stirring with heating. While at room temperature, sodium hydroxide solution was added drop by drop. After completion of reaction, the solution was allowed to settle for overnight and the supernatant liquid was discarded. The white precipitate formed was washed thoroughly with double distilled water to remove all the ions and then centrifuged at 3000 rpm for 10 min. The obtained precipitate was dried in a hot air oven at 80 °C for 6 h. During drying, complete conversion of Zn $(OH)_2$ into ZnO takes place. The above resulting dried precursors was crushed into powder and stored in air tight container for further analysis [36]. The sizes of the prepared green and chemical ZnO nanoparticles were 40 and 25 nm. The bulk form of ZnO was purchased from Sigma–Aldrich, India. All metal oxide nanoparticles suspension (100 mM L^{-1}) were prepared analogously and resuspended in sterile distilled water and briefly sonicated for uniform dispersion and formed a colloidal suspension.

The following bacterial strains *S. aureus*, *Serratia marcescens*, *Proteus mirabilis*, *Citrobacter freundii*, and fungal strains *Aspergillus flavus*, *Aspergillus nidulans*, *Trichoderma harzianum*, and *Rhizopus stolonifer* were obtained from Department of Microbiology, Karpagam University, Coimbatore, Tamilnadu, India and used for antimicrobial assays. Bacterial and fungal strains were grown in the Luria–Bertani (LB) and Sabouraud dextrose (SDA) agar at 25 °C for 24 h and 72 h and maintained at 4 °C in a refrigerator. Throughout this study, the same nutrient media was used for all cultures.

2.2. Determination of antimicrobial activity of metal oxide nanoparticles

For antimicrobial assay, fresh microbial colonies were inoculated into 100 ml of nutrient broth medium. Growth was monitored at every 4 h intervals under a UV–Visible spectrophotometer (Shimadzu, UV-2550), till the optical density reached 0.8–0.1 at 595 nm. OD of 0.140, 0.182 corresponded to 1.95×10^8 cfu mL^{-1} and 7.3×10^7 cfu mL^{-1} for bacterial and fungal strains respectively.

In vitro antimicrobial activity of the synthesized green and chemical ZnO nanoparticles along with bulk formulations were determined using the agar well diffusion assay [32] and disc method. Approximately 20 ml of stertile molten and cooled media LB and SDA were poured in sterilized petri dishes. The plates were left overnight at room temperature to check for any contamination to appear. After inoculation and cultivation of different target bacteria on top of nutrient agar, discs and wells were placed in selected area on different plates. Each standard paper disk was impregnated with freshly prepared ZnO nanoparticles and agar wells of 5 mm diameter were prepared with a sterilized stainless steel cork borer and were properly labeled. About 0.05 and 0.1 ml of various concentrations (2, 4, 6 mM for bacteria and 4, 8, 12 mM for fungi) of two different ZnO nanoparticles and bulk ZnO were added in the discs and wells, respectively. The plates containing the microbes and ZnO nanoparticles were incubated at 37 °C and the antimicrobial activity was compared. The plates were examined for evidence of zones of inhibition, which appear as a clear area around the wells [8] and discs. The diameter of such zones was measured using a meter

ruler and the mean value for each organism was recorded and expressed in millimeter.

The microbicidal activity of ZnO nanoparticles was checked by determining the MIC (minimum inhibitory concentration), MBC (minimum bactericidal concentration) and MFC (minimum fungicidal concentrations). To evaluate the MIC, an appropriate volume of pathogens in LB and SD broth was added to bulk and nanosized ZnO suspensions whose concentrations varied from 0.01 to 10 mM for bacteria and from 2 to 20 mM for fungi. The chosen nanoparticles were prepared with dimethyl sulphoxide (DMSO) and mixed with 450 µl/ml of broth and 50 µl of fresh microbial inoculum and the whole setup was allowed to grow overnight at 37 °C for 24 h and 72 h respectively. Negative and positive control tubes contained only inoculated broth and free ZnO solution. Compounds were tested three times and the results were averaged. The visual turbidity of the tubes was noted before and after incubation. The MIC was the lowest concentration of the nanoparticles that did not permit any visible growth of microbes during 24 h of incubation on the basis of turbidity [20]. To avoid the possibility of misinterpretations due to the turbidity of insoluble compounds if any, the MBC was determined by sub culturing the above (MIC) serial dilutions after 24 h in respective agar plates using 0.01 ml loop and incubated at 37 °C for 24 h and 48 h and the colonies were quantified. MBC was regarded as the lowest concentration that prevents the growth of bacterial colony on the solid media [20].

The protein leakage analysis was performed using the Bradford assay. The bacterial cells of *S. aureus, S. marcescens, P. mirabilis* and *C. freundii,* were treated with 2, 4, 6 and 8 mM bulk and nano ZnO solution for 6 and 12 h, while fungal cells of *A. flavus, A. nidulans, T. harzianum,* and *R. stolonifer* were treated with 4, 8, 12 and 16 mM of bulk and nano ZnO solution for 24 h and 48 h. After treatment, the tubes are centrifuged at 6000 rpm for 15 mins and the supernatant was collected. For each sample, 200 µl of the supernatant was mixed in 800 µl of Bradford reagent and kept for 10 mins incubation in the dark. The optical density of the sample was measured at 595 nm. Bovine serum albumin (BSA) was used as a standard protein.

To examine the microbial growth rate and behavior in the presence of the considered nanoparticles, various microorganisms were grown in liquid medium supplemented with varying concentrations (6 mM and 12 mM) of nanoparticle colloidal sus pensions. To avoid potential interference during optical

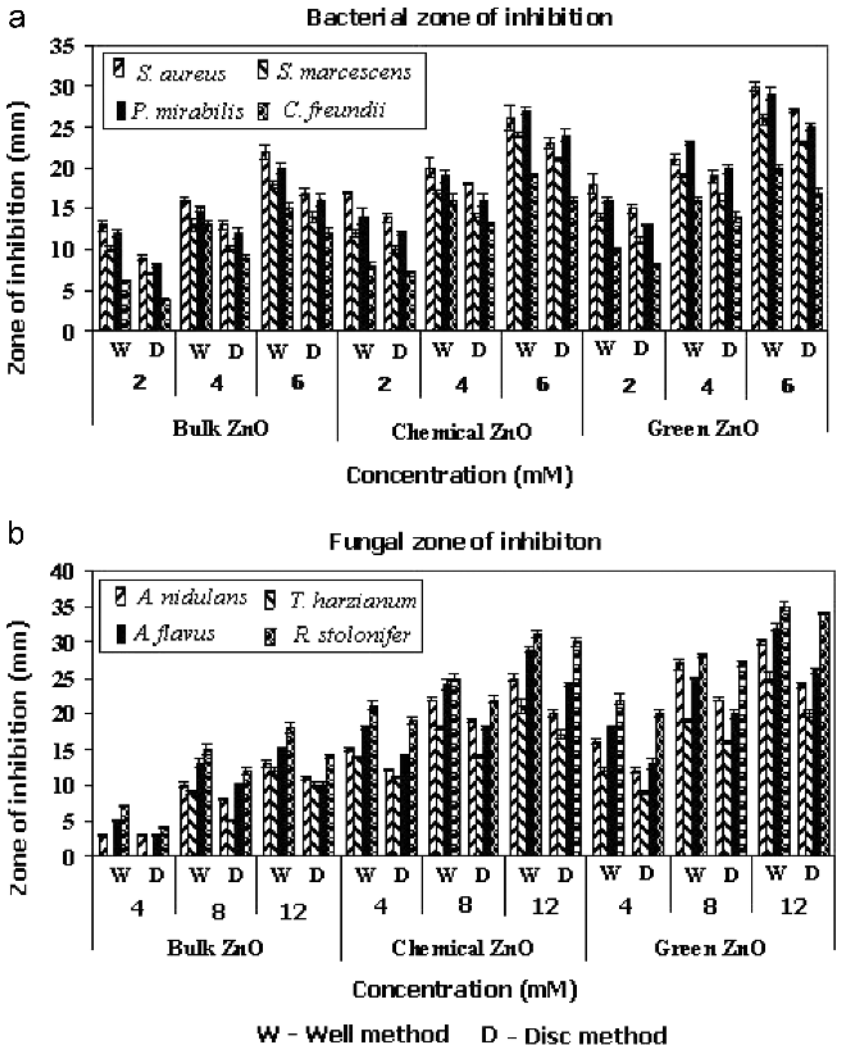

Fig. 1 Graphical representation of well and disc method of inhibition of bulk and nano ZnO nanoparticles against various microbes.

Table 1 Interaction effect of bacterial and fungal pathogens treated with different concentrations of green and chemical ZnO nanoparticle along with bulk formulations by well and disc diffusion agar methods.

SOURCE	BACTERIA				FUNGI			
	DISC		WELL		DISC		WELL	
	SED	CD (0.05)	SED	CD (0.05)	SED	CD (0.05)	SED	CD (0.05)
Z	0.061	0.122	0.099	0.197	0.063	0.127	0.067	0.134
C	0.061	0.122	0.099	0.197	0.063	0.127	0.067	0.134
O	0.071	0.141	0.114	0.228	0.073	0.146	0.077	0.154
ZC	0.106	0.212	0.171	0.341	0.110	0.219	0.116	0.232
CO	0.123	0.245	0.198	0.394	0.127	0.253	0.134	0.267
ZO	0.123	0.245	0.198	0.394	0.127	0.253	0.134	0.267
ZCO	0.212	0.424	0.342	0.683	0.220	0.438	0.232	0.463

Z - Treatment (Chemical, green ZnO nanoparticle and bulk formulations).
C - Concentration O – Various Organisms.

Table 2 Determination of MIC and MBC for bacterial and fungal pathogens treated with green and chemical ZnO nanoparticle.

Name of bacteria	Chemical ZnO		Green ZnO	
	Microbicidal concentration (mM)			
	MIC	MBC	MIC	MBC
S. aureus	0.80	8.00	0.40	7.40
S. marcescens	1.60	9.60	1.40	8.40
P. mirabilis	1.20	8.60	1.0	7.80
C. freundii	2.00	10.80	1.80	10.20
Name of fungi	**MIC**	**MFC**	**MIC**	**MFC**
A. nidulans	3.20	18.80	2.80	18.00
T. harzianum	3.80	20.40	3.40	19.20
A. flavus	2.80	17.50	2.20	17.00
R. stolonifer	2.40	16.80	2.0	16.00

measurements of the growing cultures caused by the light-scattering properties of the nanoparticles, the same liquid medium without microorganisms, but containing the same concentration of nanoparticles cultured under the same conditions was used as blank controls. All the fresh cultures were inoculated into respective growth medium and then the flasks were put on rotatory shaker (180 rpm) at 37 °C. Following innoculum, the OD of the cultures was serially monitored at every 3 h interval up to 18–24 h for bacteria and for fungi assessment was carried out every 7 h up to 49–72 h by using a UV–visible spectrophotometer [34] at 595 nm.

All experiments were performed in triplicate and the averages were obtained. Three factor analysis of variance (ANOVA) was performed to the entire bacteriological test to assess the efficacy of ZnO preparations against various bacterial and fungal pathogens. The statistical significance between values was accepted at CD(0.05) and the values are drawn as mean $\pm$CI with 95% confidence intervals.

3. Results and discussion

3.1. Microbicidal activity of ZnO nanoparticle

The antimicrobial activity of bulk, green and chemical synthesized ZnO suspensions of different concentrations (2, 4, 6 mM and 4, 8, 12 mM) towards various bacterial and fungal pathogens were tested by the well and disc diffusion agar methods and are represented in the Fig. 1. The presence of inhibition zone clearly indicates that the mechanism of the biocidal action of ZnO nanoparticles which involves disruption of the membrane with high rate of generation of surface oxygen species and finally lead to the death of pathogens. Interestingly, the size of the inhibition zone was different according to the type of pathogens, synthesis method and the concentrations of ZnO nanoparticles. As it was shown in the study of [35], it has been found in this study that by increasing the concentration of ZnO nanoparticles in wells and discs, the growth inhibition has also been increased consistently because of proper diffusion of nanoparticles in the agar medium.

Both nano and bulk ZnO nanoparticles showed antimicrobial activity against selected pathogens but maximum activity (26/23 mm) was observed in *S. aureus* followed by *P. mirabilis* (27/24 mm), *S. marcescens* (24/21 mm) and *C. freundii* (19/16 mm) (Fig. 1a). Among fungal pathogens maximum activity was noticed for *R. stolonifer* > *A. flavus* > *A. nidulans* > *T. harzianum* (Fig. 1b). [10], have described that the release of Zn^{2+} ions is responsible for the antibacterial activity. In our study, green ZnO nanoparticles showed a greater significant zone inhibition when compared to chemical ZnO nanoparticles. However, low enhancement of the antimicrobial activity was recorded in the cases of bulk ZnO at lower concentration (2 and 4 mM) but medium inhibition was noticed at higher concentrations (Fig. 1a). All the treatments (Z) namely bulk, green and nano ZnO particles showed significant difference on different organisms (O) at critical difference (CD 0.05) with varying concentration (C). While considering the methods (well and disc), the pathogens were more sensitive to well method when compared to disc method of zone inhibition. The interaction effect (ZCO) for bacteria is found to be 0.424/0.683 CD(0.05) for disc and well method of inhibition whereas for fungi CD(0.05) was found to be 0.438/0.463 for disc and well method Table 1).

3.2. Determination of minimum inhibitory and microbicidal concentration

The relative antibacterial activity of ZnO suspensions towards various pathogens was studied qualitatively by disk diffusion and quantitatively in terms of MIC, MBC and MFC. Bacterial and fungal growth was studied by visually inspecting the broth for turbidity. A standard testing protocol was employed that is applicable to inorganic metal oxides and composite materials such as AgBr-polymer and Ag–SiO_2 ([37,51]). The microbicidal efficacies of bulk, green and chemical synthesized ZnO nanoparticle suspensions are shown in Table 2 and the lowest concentration range being 0.5–4 mM and 1–8 mM and the highest concentration range being 4–11 mM and 8–21 mM for bacterial and fungal species respectively. To establish whether the suspensions were microbistatic or microbicidal, 150 µL aliquots were taken from the incubated broth, each containing ZnO and

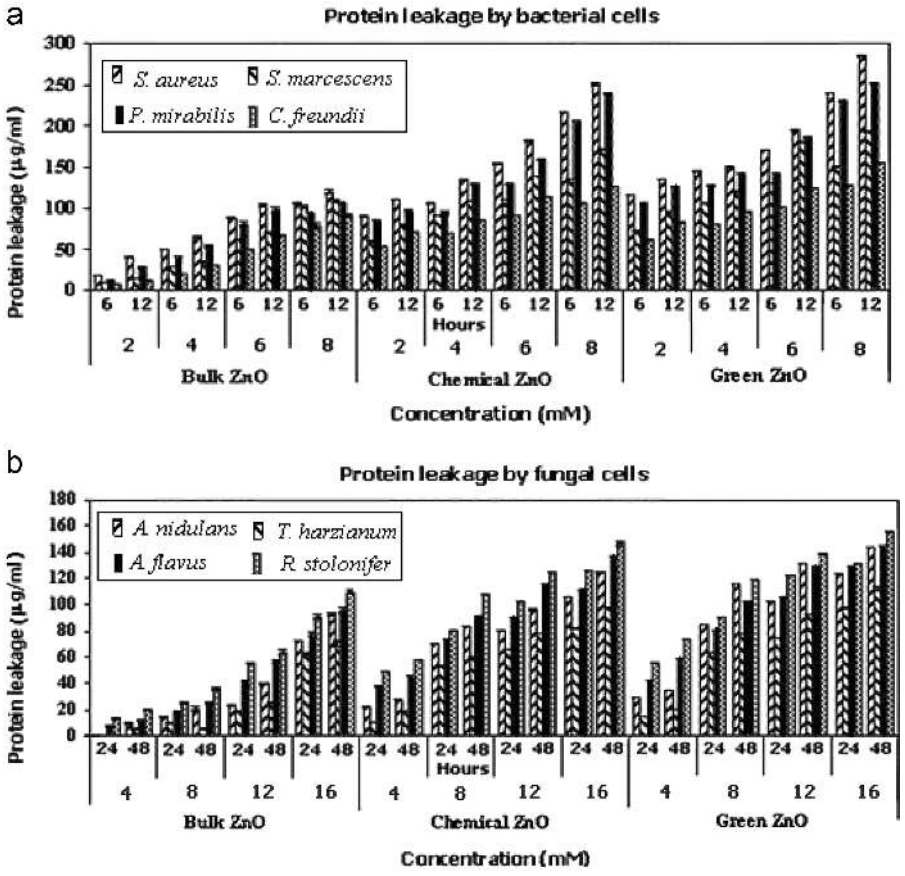

Fig. 2 Bradford assay for protein leakage analysis on treatment of pathogen with ZnO nanoparticles.

Table 3 Interaction effect of bacterial and fungal pathogens treated with different concentrations of green and chemical ZnO nanoparticle along with bulk formulations by protein leakage analysis.

SOURCE	BACTERIA				FUNGI			
	6		12		24		48	
	SED	CD (0.05)	SED	CD (0.05)	SED	CD (0.05)	SED	CD (0.05)
Z	0.128	0.255	0.137	0.271	0.098	0.195	0.128	0.255
C	0.148	0.294	0.158	0.313	0.113	0.225	0.148	0.294
O	0.148	0.294	0.158	0.313	0.113	0.225	0.148	0.294
ZC	0.257	0.509	0.273	0.542	0.196	0.389	0.256	0.509
CO	0.296	0.588	0.315	0.626	0.226	0.449	0.296	0.588
ZO	0.257	0.509	0.273	0.542	0.196	0.389	0.256	0.509
ZCO	0.513	1.019	0.546	1.084	0.392	0.778	0.513	1.018

Z - Treatment (Chemical, green ZnO nanoparticle and bulk formulations).
C - Concentration O – Various Organisms.

pathogens and were plated on nutrient and SDA agar plates and incubated for 24 h and 72 h respectively. From the results summarized in the Table 2, it is clear that ZnO suspension with concentration in the range of 4–10 mM and 8–20 mM effectively inhibits bacterial and fungal growth. No significant antibacterial and antifungal activity was observed at concentrations less than 0.5 mM for all the ZnO samples. This may be due to the possible presence of fewer Zn^{2+} ions, which might act as nutrient [38]. However, the microbicidal efficacy at 1 mM is higher for green ZnO suspension than for the chemical ZnO suspensions.

From the results of MIC, we confirm that the green ZnO with smaller particle size showed enhanced activity due to the large surface area to volume ratio and surface reactivity when compared to the ZnO nanoparticle prepared by chemical method, whereas ZnO suspensions with lower concentration range (0.5–4 mM) seems to exhibit less antimicrobial activity. We also noticed in all the cases, ZnO suspension prepared from green synthesis method is more effective than the suspension with other preparations. This can be explained on the basis of the oxygen species released on the surface of ZnO, which cause fatal damage to microorganisms [44]. They react with hydrogen ions to produce molecules of H_2O_2. The generated H_2O_2 can penetrate the cell membrane and kill the bacteria [14]. The generation of H_2O_2 depends strongly on the surface area of ZnO, which results in more oxygen species on the surface and the higher antibacterial activity of the smaller nanoparticles [57]. The results of this study may be applicable to medical devices that are coated with nanoparticles against microbes.

3.3. Protein leakage analysis and microbial growth rate determination

Several factors related to the antimicrobial activity of metal oxides have been investigated such as size, the mixture concentration, pH, exposure time and the surface properties

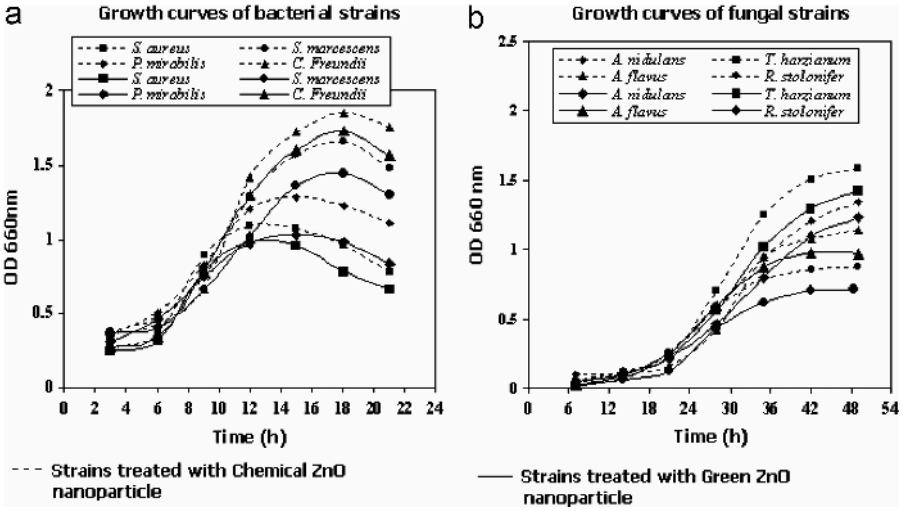

Fig. 3 Growth curves of various bacterial and pathogens strains treated with ZnO nanoparticles.

of the powder, the active oxygen generation and the particles of metal oxides ([30,59]). The Fig. 2 show the amount of protein released in the suspension by the treated cells which was estimated by the Bradford assay. The amount of protein released from the cells increased along with increasing concentration and contact period of ZnO nanoparticles. However, the particle size plays a vital role in the antibacterial activity. The cellular membranes in the bacterial cells contain pores in nanometer range. The possible mechanism is the nanoparticles which have a size less than that of pore size in the bacteria have a unique property of crossing the cell membrane without any hindrance [47]. Moreover, the antibacterial activity of the metal oxide nanoparticles mostly appeared on the surface bind with the thiol (-SH) groups of protein present in the cell wall. This interaction decreases the cell permeability which leads to cell lyses [58].

The damage to the cell membrane directly leads to the leakage of minerals, proteins and genetic materials, causing cell death. From the Fig. 2 it is observed that the leakage of protein from the bacterial pathogens was higher than the fungal pathogens. These results indicate that most of the nanoparticle treated cells were ghost cells from which intracellular material was released into the cell suspension. This stress in the cell wall produces more lactate dehydrogenase enzymes and leads to damage the cell membrane and the severity depends upon the exposure time [55]. The study clearly represents that the antibacterial effect of green synthesized nanoparticle was severe to *S. aureus* and *P. mirabilis* than *S. marcescens* and *C. freundii* (Fig. 2a), whereas among fungal pathogens *R. stolonifer* and *A. flavus* (Fig. 2b), showed more obvious protein leakage when compared to other species. The intrinsic toxic properties of metal oxide NPs, as well as the types of microbial cells are associated with the species sensitiviy of metal oxide NPs. Statistical comparison between different treatments and concentrations showed significant differences at CD (0.05) among varying pathogens (Table 3). The protein leakage for bacteria with varying treatment concentrations showed significant CD (0.05) values of 1.019/1.084 for 6/12 h in case of bacteria whereas fungi showed 0.778/1.018 for 24/48 h respectively. We also noticed the existence of quantitative relationships among the bulk and nano metal oxide nanoparticles for different organisms.

3.4. *Effect of ZnO nanoparticles on microbial growth*

Fig. 3 shows the effect of green and chemical synthesized ZnO nanoparticles on the growth of bacterial and fungal pathogens where, time dependent changes in microbial growth were monitored by measuring OD at 595 nm. The antimicrobial activity is probably derived, through the electrostatic attraction between negatively charged cell membrane of microorganism and positively charged nanoparticles ([21,9,11]), interaction of metal ions including zinc with microbes [5] and orientation of ZnO [52]. Both green and chemical ZnO treatments exhibited significant inhibitory effect on the growth of pathogens during 24 and 72 h of incubation. The optical density of the medium was investigated as the number of microbes after contact with the nanoparticles. The growth inhibition of the pathogens by both treatments recorded as a function of time, suggested significant differences in antibacterial and antifungal activity of the nanoparticles (Fig. 3a,b). To make the figure clear, growth inhibitions of the tested pathogens are not shown for all the used ZnO concentrations. Only representative concentrations (6 mM and 12 mM) leading to the continuous inhibition of growth of the tested organisms during 24 and 52 h cultivation have been used.

As seen from the growth inhibition rates in Fig. 3, green ZnO has a stronger inhibitory effect than chemically synthesized NPs. The measurement of OD was carried out at 595 nm to avoid strong absorption due to the ZnO nanoparticle in the region 380–450 nm and from bacterial cellular components such as nucleic acids (A260), proteins (A280) and molecules present in the medium. The OD at 595 nm is due to the scattering of light by the bacterial and fungal cells. It is a function of bacterial cell density and thus correlates with the growth of the colonies. It is clear that ZnO nanoparticle at a concentration 8 mM and 16 mM inhibited growth of bacterial and fungal pathogens, whereas the effect was much less at lower concentration. Increasing concentration of ZnO nanoparticle decreases the growth of microbes, and the concentration at which growth stopped altogether was higher in green ZnO than chemically synthesized nanoparticle. According to [1], ZnO nanoparticles inhibited growth of gram-positive by 90% but gram-negative were much more resistant.

4. Conclusion

Overall, the nano-materials, based on the metal oxide ions, exhibit broad-spectrum biocidal activity towards different bacteria, fungi, and viruses and have a distinct advantage over conventional chemical antimicrobial agents. In this study, we have demonstrated the enhanced bioactivity of green synthesized ZnO nanoparticles by studying the antimicrobial activity of suspensions with various other formulations using a standard microbial method. The growth inhibition was solely higher in biologically synthesized ZnO than chemical ZnO nanoparticle and other common antimicrobials. The enhanced bioactivity of smaller particles is attributed to the higher surface area to volume ratio. Based on the herein proved antibacterial and antifungal activity, it can be concluded that the ZnO nanoparticles constitute an effective antimicrobial agent against pathogenic microorganisms.

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