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Synthesis, characterization and evaluation of antimicrobial activity of zinc oxide nanoparticles

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Abstract

The study involved synthesis of Zinc Oxide nanoparticles using biological and chemical reducing agents. The aim was to compare the yield, nature and antimicrobial activity of nanoparticles synthesized by the two methods. In biological method, hot and cold Aloe vera leaf extract; and in chemical method, sodium hydroxide was used as reducing agents. Nanoparticles synthesized by the two methods were characterized by XRD and SEM. It was evident from SEM images that particles obtained by biological method were rod-shaped and those synthesized by chemical method were spherical. Antibacterial study was carried out on gram-positive and gram-negative bacterial strains by agar-well diffusion method and their MIC values were determined. ZnO-AC showed almost consistent activity on all the strains, whereas ZnO-AH and ZnO-C showed greater activity on some strains compared to others. ZnO-AH showed least overall activity on all strains as compared to ZnO- AC and ZnO-C.

Keywords: Zinc Oxide nanoparticles, biological method, XRD, SEM, antibacterial study, agar diffusion method..

Introduction.

Zinc oxide is an inorganic compound with the formula ZnO. It usually appears as a white powder, nearly insoluble in water. Zinc Oxide nanoparticles have been studied for their various applications in many fields including medicine. ZnO nanoparticles exhibits a high degree of cancer cell selectivity with the ability surpass the therapeutic indices of some commonly used chemotherapeutic agents (Hanley et al. 2008; Wang et al. 2009). It is used in paints, sunscreens, plastic and rubber manufacturing, electronics and pharmaceuticals products etc. It is also potentially used to treat leukemia and carcinoma cancer cell. It is also used as drug carrier.

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ZnO nanoparticles is also used in industrial sectors including environmental, synthetic textiles, food, packaging, medical care, healthcare, as well as construction and decoration.
It is known that zinc oxide exhibits antibacterial activity like many other metal oxide groups and like the others only few have been scaled down to the nano size and researched further, such as ZnO nanoparticles. The advantage of using inorganic oxides such as zinc oxide as antimicrobial agents is that they contain mineral elements essential to humans and exhibit strong activity even when administered in small amounts. ZnO nanoparticles exhibit strong antibacterial activities on a broad spectrum of bacteria (Sawai 2003; Atmaca et al. 1988; Jones et al. 2008). Synthesis of these particles has been carried out by three methods namely chemical, precipitation and physical methods.

In this study, we report the novel synthesis of ZnO nanoparticles by biological method using Zinc Nitrate and *Aloe vera* plant extracted solution. We have also synthesized ZnO by chemical method using sodium hydroxide as reducing agent for a comparative study of yield, nature of synthesized particles and antimicrobial activities of particles obtained by both methods. In the biological method for production of ZnO nanoparticles we have used hot and cold *Aloe vera* leaf extracts as reducing agent instead of chemical compounds. *Aloe vera* is a perennial succulent belonging to the Liliaceal family, and it is a cactus-like plant that grows in hot, dry climates. For many years, *Aloe vera* has been reported to possess immunomodulatory, anti-inflammatory, UV protective, antiprotozoal, and wound- and burn-healing promoting properties. The extract of *Aloe vera* plant has been successfully used to synthesize single crystalline triangular gold nanoparticle (~50-350 nm in size) and spherical silver nanoparticles (~15 nm in size) in high yield by the reaction of aqueous metal source ions (chloroaurate ions for Au and silver ions for Ag) with the extract of the *Aloe vera* plant. Spherical zinc oxide nanoparticles were successfully synthesized using *Aloe vera* extract and their optical properties was studied (Sangeethaa et al. 2011).

Materials and Methods

Synthesis Biological method

Aloe vera hot extract was prepared by boiling 35g of *Aloe vera* leaves in 100ml of distilled water. The resulting solution was filtered and the filtrate used as *Aloe* hot extract. *Aloe* cold extract was prepared by homogenizing 35g of *Aloe vera* leaves in 100ml

water and filtering it. 30ml of these extracts was used as reducing agents to synthesize nanoparticles. 3g of zinc nitrate was added to both extracts separately and kept at 60°C under vigorous stirring until dried. The resulting powder was ground and calcined at 570°C in muffle furnace (Maensiri S et al. 2008). Particles obtained using *Aloe* hot extract was labeled ZnO-AH and those obtained using *Aloe* cold extract was labeled ZnO-AC.

Chemical method

Zinc Oxide nanoparticles were synthesized by this method by adding 0.6g of zinc nitrate in 100ml of 0.1% starch solution and kept under constant stirring. To this solution, 60ml of 0.2M NaOH was added dropwise and the reaction was allowed to proceed for 2h after addition of NaOH. The solution was then allowed to settle overnight. Supernatant was discarded and the remaining solution was centrifuged and washed several times before drying at 80°C overnight (Behera J L 2009). These particles were labeled ZnO-C.

Characterization

ZnO-AH, ZnO-AC and ZnO-C were characterized by X-Ray Diffraction in order to identify the compound and determine its crystalline form. The particles were subject to Scanning Electron Microscopy to determine their size and morphology.

Evaluation of antimicrobial activity of Zinc Oxide nanoparticles Antibacterial study

Evaluation of antimicrobial activity of zinc oxide nanoparticles was carried out by agar-well diffusion method using Luria Bertani growth media (Sharath et al. 2008). To study the antibacterial activity, clinical isolates namely; *Bacillus subtilis Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi,* and *Staphylococcus aureus* were used. Luria-Bertani broth was prepared; the pH was adjusted to 7.2-7.5 and autoclaved. The prepared broth was poured into test tubes. Each strain was inoculated separately into culture broth. The culture test tubes were incubated overnight at 37°C in shaker-incubator.

10mg each of ZnO-AH and ZnO-AC from biological synthesis and 10mg of ZnO-C from chemical synthesis was dispersed separately in 1ml of 10% DMSO. Standard drug was prepared by dissolving 500mg of ciprofloxacin in 100ml sterile distilled water.

Luria-Bertani agar was prepared; the pH was adjusted to 7.2-7.5 and autoclaved. The agar was then poured into sterile petriplates and allowed to solidify. 50µl of the fresh bacterial culture was pipetted on the agar plate. Using a sterile cotton swab, the culture was swabbed on the agar. Using gel puncture, four wells were made on the agar plate. The labeled wells were loaded with samples of ZnO-AH, ZnO-C, control and standard drug, ciprofloxacin. This step was carried out for all the six bacterial strains for all 6 replicates. The plates were incubated for 16-18h in incubator at 37°C before the results were observed.

Diameter of zone of complete inhibition of the bacteria was measured around each well and readings were recorded in mm. The values were subject to analysis using ANOVA software to determine the mean value and standard error.

Minimum inhibitory concentration sample preparation

Different concentrations ranging from 10µg to 50µg of synthesized zinc oxide nanoparticles by biological (ZnO-AH and ZnO-AC) and chemical (ZnO-C) methods were dispersed in DMSO.

Plating

Luria-Bertani agar was prepared and about 25ml of agar was poured into each petriplate and allowed to solidify. 50µl of *Bacillus subtilis* cell suspension was pipetted onto agar plate. Using cotton swab, the culture was swabbed thoroughly to ensure uniform spreading of the culture. 5 wells were made on the agar using gel puncture ensuring sufficient space between the wells. The wells were labeled and samples of different concentrations were loaded in the wells respectively. The same procedure was carried out for the remaining five strains. The plates were incubated for 16-18h at 37°C in the incubator.

Diameter of zone of inhibition of the bacteria was measured around each well and readings were recorded in mm and the Minimum Inhibitory Concentration was noted. The values were subject to analysis using ANOVA software to determine the mean values and standard error.

Results and Discussion

Synthesis

ZnO-AH and ZnO-AC were both yellow in colour and crystalline in nature (Figures 1a, 1b). ZnO-C was white in colour and crystalline in nature (Figure 1c). Yield obtained from chemical method was three times more than biological method (Table 1).

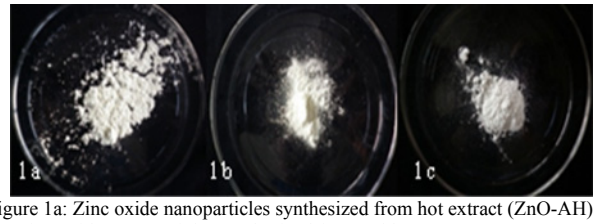


Figure 1a: Zinc oxide nanoparticles synthesized from hot extract (ZnO-AH)
1b: Zinc oxide nanoparticles synthesized from cold extract (ZnO-AC)
1c: Zinc oxide nanoparticles synthesized by chemical method (ZnO-C)

Table 1: Yield of zinc oxide nanoparticles synthesized by biological and chemical methods

Method	Zinc nitrate (g)	Reducing agent	Qty. Reducing agent (ml)	Zinc oxide (g)	% Yield
Biological	3.0	Hot extract	30	0.7291	24.30
Biological	3.0	Cold extract	30	0.7400	24.66
Chemical	0.6	NaOH	60	0.4861	81.01

X-Ray Diffraction

The X-Ray diffraction pattern of ZnO nanoparticles prepared by biological and chemical method is shown in Figures 2(a), 2(b), 2(c) for ZnO-AH, ZnO-AC and ZnO-C respectively. It is evident from the graphs that, the 5 peaks observed in all the graphs are similar and their peak values coincide with that of reference values.

The 5 peaks noticed are in accordance with zincite phase of ZnO. No peaks due to impurity were observed, which suggest that high purity zinc oxide was obtained. The three strongest XRD peaks for ZnO were detected with Miller indices (100), (002) and (101) corresponding to bragg angles. All diffraction peaks can be readily indexed to wurtzite hexagonal ZnO structure with lattice constant a =3.2511(1)Å and c=5.2076(2)Å, consistent with the standard PDF database (JCPDS file no. 36-1451). The characteristic peaks are higher in intensity which indicates that the products are of good crystalline nature.

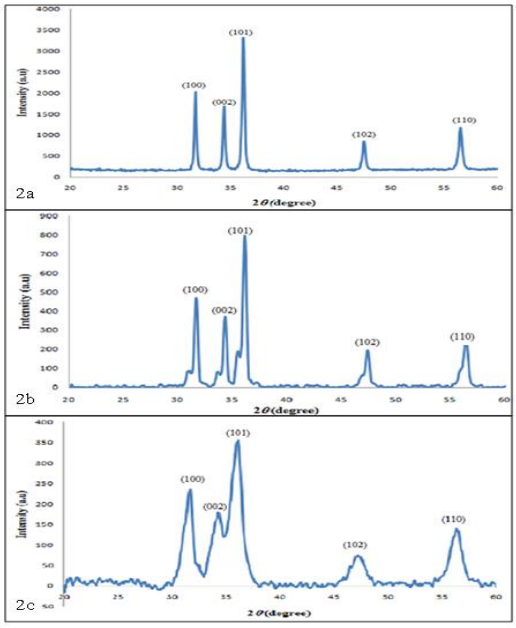
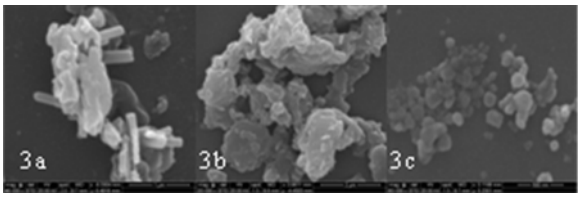


Figure 2a: XRD patter of ZnO-AH; 2b: XRD patter of ZnO-AC; 2c: XRD pattern of ZnO-C

Scanning Electron Microscopy

The SEM images of zinc oxide nanoparticles prepared by biological method (ZnO-AH and ZnO-AC) and chemical method (ZnO-C) are shown in the Figures 3(a), 3(b) and 3(c) respectively. From Figure 3(a) it can be inferred that the particles are rod-shaped with approximately 500nm length and width lesser than 100nm. Figure 3(b) indicates that the particles are clearly lesser than 100nm and are rod-shaped similar to ZnO-AH. From Figure 3(c) it is evident that ZnO-C particles are spherical lying within 100nm size range.



Evaluation of antimicrobial activity of Zinc Oxide nanoparticles
Antibacterial study

Antibacterial study was carried out on six clinically isolated strains namely, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Staphylococcus aureus*. Zinc oxide particles synthesized by chemical method and particles obtained using *Aloe vera* cold extract showed significant activity compared to that of particles synthesized from *Aloe vera* hot extract. The zone of inhibition readings for ZnO-AH, ZnO-AC and ZnO-C are indicated in Table 2.

ZnO-AC and ZnO-C have almost same amount of antibacterial activity, whereas, ZnO-AH showed lesser activity compared to the other two samples. It was observed that although ZnO-AH and ZnO-AC were both synthesized in a similar manner, there is significant difference in their antibacterial activities. This variation is because the size of ZnO-AH is much greater than that of ZnO-AC. The smaller the size of nanoparticles better is their activity (Yamamoto 2001a, Makhluf et al. 2005).

The preliminary antibacterial activity study results indicate that ZnO-AH shows maximum activity against gram-negative, *K. pneumoniae* (8.33±1.87) while ZnO-AC and ZnO-C showed maximum activity against gram-negative, *P. aeruginosa* (ZnO-AC 16.00±0.26; ZnO-C 19.00±0.68) compared to other five strains. ZnO-AH exhibited least activity against gram-positive strains, *B. subtilis and S. aureus*. ZnO-AC showed consistent inhibition on all the strains showing no significant variation amongst gram-positive and gram-negative bacteria. It was observed that ZnO-C has noticeable variation in activity on all the strains, but similar to ZnO-AH, these nanoparticles have least effect on gram-positive strains, *B. subtilis and S. aureus*. It can hence be concluded that zinc oxide nanoparticles irrespective of synthesis method has antibacterial activity against a broad-spectrum of bacteria.

Although the mechanism of action of zinc oxide particles on bacteria is still under investigation, it has been suggested that the electrostatic interactions between the bacteria surface and nanoparticles may be responsible for the inhibitory effect. The presence of ZnO nanoparticles leads to damages to the membrane wall of the bacteria. Also, when particles are in the form of large agglomerates, they are less likely to penetrate through the cell wall and damage the bacteria from the interior (Yamamoto et al. 2000).

Table 2: Zone of inhibition of ZnO-AH, ZnO-AC and ZnO-C

Bacterial strains	Zone of Inhibition (in mm)			
	ZnO-AH	ZnO-AC	ZnO-C	Standard drug (Ciprofloxacin)
Bacillus subtilis	5.83± 0.95	15.17± 0.40	12.17± 0.91	40.33±0.33
Escherichia coli	7.00± 0.37	15.17± 0.40	14.17± 0.54	34.33±0.21
Klebsiella pneumoniae	**8.33± 1.87**	15.17± 0.97	16.17± 0.57	38.67±0.21
Pseudomonas aeruginosa	7.67± 0.21	**16.00± 0.26**	**19.00± 0.68**	41.83±0.17
Salmonella typhi	7.33± 0.33	15.17± 0.40	15.83± 0.17	40.17±0.17
Staphylococcus aureus	4.50± 0.34	15.17± 0.40	11.17± 0.40	39.33±0.21

The value of each constituents consisted of ±S.D. of 6 replicates, $p < 0.01$

Minimum Inhibitory Concentration

The MIC of ZnO-AH, ZnO-AC and ZnO-C for all six strains are as shown in Table 3. It is evident from our study that that the MIC values of particles synthesized by biological and chemical methods for E.coli, K.pneumoniae and S.aureus are much higher than nanoparticles obtained from non- hydrolytic solution method (Wahab et al. 2010). Studies have revealed that size and shape of nanoparticles depend upon the method of synthesis used (Phuriwat et al. 2011). The smaller the size of nanoparticles better is their activity (Yamamoto 2001a, Makhluf et al. 2005).

Our study indicated MIC values in the rage 3-5mg/ml for all three nanoparticle samples. What is evident from this study is that nanoparticles synthesized by biological method are as efficient as that synthesized by chemical method making the former competent enough to replace the latter. Green synthesized zinc oxide nanoparticles could be a potential antibacterial agent to treat diseases caused by bacteria. Green synthesis offers a number of advantages over other methods. The method is eco-friendly and provides bio-compatibility in pharmaceutical, biomedical and cosmetic applications as they do not use toxic chemicals for the synthesis protocol. Chemical synthesis generally leads to formation

of by-products that get adsorbed onto the surface of nanoparticles that prove to be toxic in medical applications. Green synthesis has also proved to be cost-effective.

Table 3: MIC of ZnO-AH, ZnO-AC and ZnO-C for all six bacterial strains

Bacterial strain	MIC (mg/ ml)		
	ZnO-AH	ZnO-AC	ZnO-C
Bacillus subtilis	3.00	4.00	5.00
Escherichia coli	3.00	4.00	3.00
Klebsiella pneumoniae	4.00	4.00	3.00
Pseudomonas aeruginosa	5.00	3.00	3.00
Salmonella typhi	5.00	5.00	5.00
Staphylococcus aureus	4.00	3.00	3.00

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