GREEN SYNTHESIS OF ZnO NANOPARTICLES AND ITS APPLICATION IN WOUND HEALING

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Abstract: Nanotechnology is a research hot spot in modern material science. Zinc oxide nanoparticles (ZnO NPs) are known to be one of the most multifunctional inorganic nanoparticles which have potential antimicrobial activity. Present study states a green approach for the synthesis of ZnO NPs employing aqueous extract of *Ocimum tenuiflorum* leaves. Leaves extract was used as the biological reducing agent for synthesizing ZnO NPs and were characterized by UV-Visible spectroscopy (UV-Vis), X-ray diffractometer (XRD) and Scanning Electron Microscopy (SEM). UV-Visible spectrum peak was obtained at 365nm clearly demonstrating the presence of ZnO NPs. XRD exhibited 2θ values corresponding to ZnO and particle size was estimated to be 100nm. ZnO NPs were tested for their antibacterial potential and found to be active against *Staphylococcus aureus* and *Escherichia coli*. Citric acid/ZnO NPs composite bandage was developed by impregnating it with the mixture of citric acid and ZnO NPs. The prepared composite bandage showed excellent antibacterial activity against *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae*. Hence an easy and effective green approach for synthesis of ZnO NPs with its biomedical capability is indicated in this study.

***Index Terms*: Zinc oxide nanoparticles, antibacterial effect, wound dressing.**

I. INTRODUCTION

Nanotechnology deals with the production and usage of material with nanoscale dimension. Nanoscale dimension provides nanoparticles with large surface area to volume ratio and thus very specific properties (Tabrez et al., 2016). Nanoparticles (NPs) are considered as nano antibiotics because of their antimicrobial activities (Sastry et al., 2003). Zinc oxide nanoparticles (ZnO NPs) is a bio-safe material that possesses large bandwidth and high excitation energy and has potential applications like antibacterial, antifungal, anti-diabetic, anti-inflammatory, wound healing, antioxidant and optic properties. Due to the large rate of toxic chemicals and extreme environment employed in the physical and chemical production of these NPs, green methods employing the use of plants, fungus, bacteria, and algae have been adopted (Agarwal et al., 2017). *Ocimum tenuiflorum* is an annual delicate herb cultivated extensively in tropical climate and also medicinally the leaves are used for various kinds of classical and home made preparations. There are reports on the use of leaves extract of *Ocimum tenuiflorum* for synthesis of metal oxide NPs (Raut et al., 2015). Hence in the current study it was chosen for green synthesis of ZnO NPs.

Wound healing is a complex physiological process involving tissue repair and regeneration. Nanoparticles are being studied for their possible use in wound healing (Mohandas et al., 2015). Wound healing products containing nano-silver primarily used for its antimicrobial properties are currently being utilized for wound management. However, other metal oxide nanoparticles, including zinc oxide (ZnO), have not been sufficiently tested for their ability to aid healing (Zhou et al., 2014).

Creating an acidic environment in a wound bed has an additional benefit that positively influences the wound healing process. Use of citric acid has been reported to help in wound healing by controlling wound infection and increasing antimicrobial activity and enhancing epithelialization and angiogenesis (Nagoba et al., 2015). Hence composite bandage was developed by impregnating it with the mixture of citric acid and ZnO NPs and its antibacterial activity was tested.

II. MATERIALS AND METHODS

Collection of leaves

Fresh leaves of *Ocimum tenuiflorum* (Holy Tulsi) were collected from the surrounding locality of Mithibai College, Vile Parle (W), Mumbai-400056, India. These were positively identified as *Ocimum tenuiflorum* by Botany Department of Mithibai College before it was used for preparation of ZnO NPs.

Preparation of Leaf Extract

Fresh leaves were washed several times and then sun dried. The leaves extract was prepared by placing 50g of washed dried fine cut leaves in 250 mL glass beaker along with 100 ml of sterile distilled water. The mixture was then boiled for 30 minutes until the color of the aqueous solution changes from watery to light yellow. The extract was cooled to room temperature and filtered using filter paper. The extract was stored in a refrigerator for synthesis of ZnO NPs.

Preparation of ZnO NPs

For the synthesis of ZnO NPs, 50 ml of *Ocimum tenuiflorum* leaves extract was taken and boiled to 60-80^{0}C using a stirrer heater. 5g of Zinc nitrate was added to the solution as the temperatures reached 70^{0}C. This mixture is then boiled until it reduced to a deep yellow colored paste. This paste was then collected in a ceramic crucible and heated in an air heated furnace at 400^{0}C for 2 hours. ZnO NPs were obtained in the form of pale yellow colored powder. This powder was mashed in a mortar & pestle to get finer nature for characterization purpose.

Characterization of biosynthesized ZnO NPs

UV-Visible Spectroscopy(UV-Vis): For UV-Vis spectroscopy, the light yellow nanopowder was re-suspended in equal amount of DMSO and spectrum scan was performed using Smart UV-Vis Double Beam Spectrophotometer 2203 from Systronics, in the wavelength range of 200-450nm.

X-ray diffraction (XRD): Washed and dried sample of ZnO NPs was used for XRD analysis to determine the structure of ZnO NPs. XRD analysis was carried out using X'pert PRO Analytical X-ray diffractometer (Philips) at Department of Chemistry, University of Mumbai, Kalina, Mumbai-400 098, India. XRD was performed in the 2θ range of 20-80 degree at 40 kV and 40 mA with a divergence slit of 10 mm in 2θ/θ continuous scanning mode.

Scanning Electron Microscopy (SEM): SEM analysis of ZnO NPs was performed using JEOL JSM-7600F Field Emission Gun-SEM at Sophisticated Analytical Instrument Facility, Indian Institute of Technology Bombay, Powai, Mumbai-400 076, India.

Antibacterial activity of synthesized ZnO NPs

Antibacterial activity of the synthesized ZnO NPs was performed against both Gram positive (*S.aureus*) and Gram negative (*E. coli*) bacteria. The antibacterial activity was done by agar cup method. 0.2 ml of 18 hour old actively growing culture of test organism was added in sterile molten Mueller-Hinton agar (MHA) (Hi-Media, Mumbai, India) butts and poured in sterile petri dishes. After the plates were solidified, 4 cups were made/ plate with the help of a sterile borer (8mm). 70% Ethanol, saline, dilute HCl and Dimethylsulfoxide (DMSO) were used as solvent to prepare suspension of ZnO NPs. Strength of NPs used was 20 mg/ml i.e. 2% solution of NPs was used for *in-vitro* antibacterial assessment. 50 µl of nanoparticle solution was loaded into each well. After addition of nanoparticles, plates were incubated at 4^{0}C for 30 min to allow effective diffusion of ZnO NPs and control. Later, they were incubated at 37±1^{0}C for 24 hours. After overnight incubation, the zone of inhibition was measured. Solvent blank was maintained as negative control.

Ditch plate method

Ditch (6 cm X 1 cm) was made using a sterile scalpel in the sterile MHA plate. 50 mg of ZnO NPs was added in 5 ml sterile MHA butt and poured in the ditch. A plate containing bulk ZnO particle was maintained as negative control. After solidifying, 18 hour old actively growing culture of test organism (*S. aureus* and *E. coli*) was streaked across the ditch and incubated at 37±1^{0}C for 24 hours.

Preparation of composite bandage impregnated with the mixture of citric acid and ZnO NPs

The bandage was prepared by 'Pad-drying method'. To prepare bandage cotton gauge strip of 4.5 cm X 1.5 cm dimension was taken and sterilized by autoclaving. 2% ZnO solution was prepared using 1% citric acid as solvent. Citric acid acts as a binding agent & aids in the entrapment of ZnO NPs in the prepared cotton gauge. These gauges are immersed in nanoparticle solution and are dried in air without washing. After drying, these strips are sterilized by UV rays in Laminar Air Flow for 10-15 min.

In-vitro antibacterial activity evaluation

Antibacterial activity was evaluated against Gram positive (*S. aureus*) and Gram negative (*E. coli* and *K. pneumoniae*) organism. Bandages impregnated with 1% citric acid was used as a control. Saline suspension of 18 hr old actively growing test culture was swabbed on a sterile MHA plate followed by that UV sterilized composite bandage was placed on plate and incubated at 37±1^{0}C for 24 hours. Bandages impregnated with 1% citric acid was used as a control. The zone of inhibition was noted to determine the antibacterial effect of the composite bandage.

III. RESULTS AND DISCUSSION

Preliminary observation

ZnO NPs have attracted great attention because of their superior optical properties. Visual colour change is the preliminary test for NP synthesis (Santoshkumar et al., 2017). Figure 1 represents the synthesis of ZnO NPs synthesized using freshly prepared *Ocimum tenuiflorum* fresh leaf extract. Color change from white to pale yellow represents the synthesis of ZnO NPs.

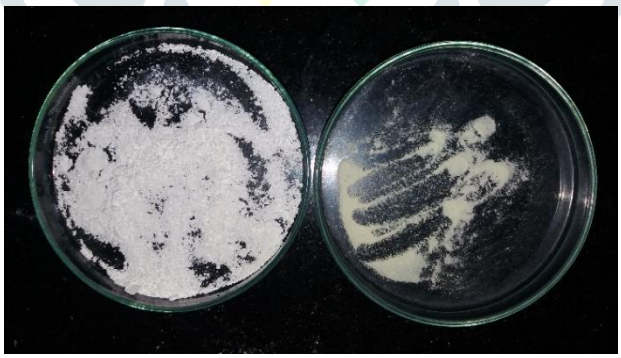

Figure 1 Bulk ZnO (Left) and synthesized ZnO nanoparticles (Right)

UV-Visible Spectroscopy

UV-Vis spectroscopyis usually conducted to confirm the synthesis of ZnO NPs. Figure 2 represents the UV-Vis spectra of freshly prepared ZnO NPs. Peak obtained at 365nm clearly demonstrates the presence of ZnO NPs in the reaction mixture.

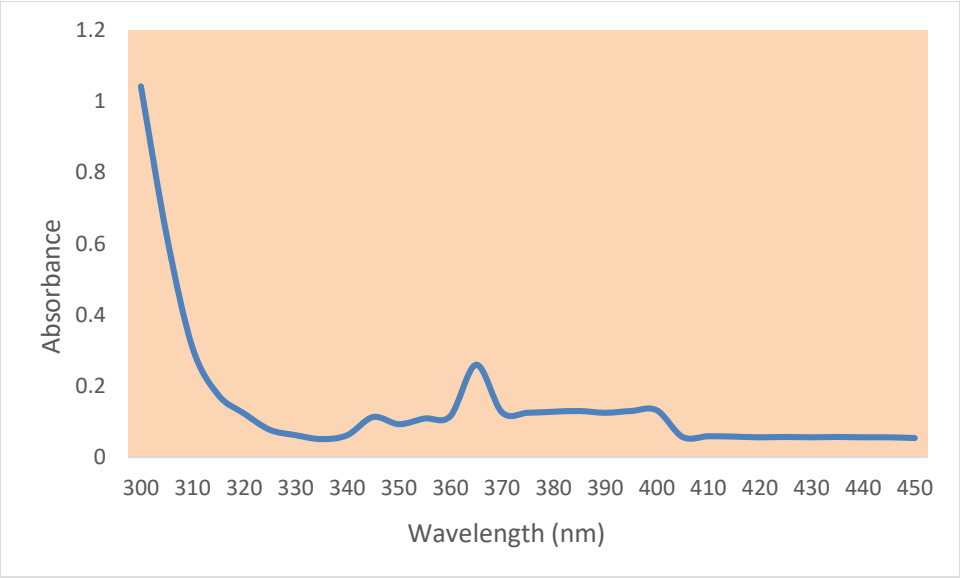

Figure 2 UV-Vis spectrum of ZnO NPs

X-ray diffraction

XRD Spectra provides an insight about the crystallinity of NPs. Figure 3 represents XRD spectra of ZnO NPs synthesized using *Ocimum tenuiflorum* leaf extract. XRD peaks obtained at 2θ value ranging from 31.73°, 34.38°, 36.22°, 47.50°, 56.56°, 62.81°, 66.34°, 67.91°, 69.03°, 72.6° and 76.90° values corresponding to crystal structure of ZnO NPs. The locations of peaks were compared to literature values and the presence of ZnO NPs was confirmed (Bagheri et al., 2013).

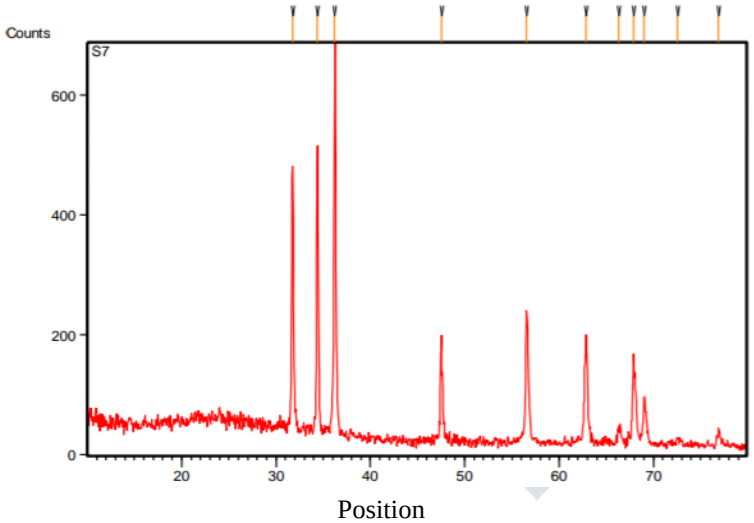

Figure3 XRD Spectrum of ZnO NPs

Scanning Electron Microscopy

SEM studies is done to visualize the size and shape of ZnO NPs. Figure 4 shows the typical bright –field SEM micrograph of the synthesized ZnO NPs. SEM images were seen in different magnification range from 1µm-100nm which clearly demonstrated the presence of spherical shaped NPs with mean average diameter of 100nm (Santoshkumar et al., 2017).

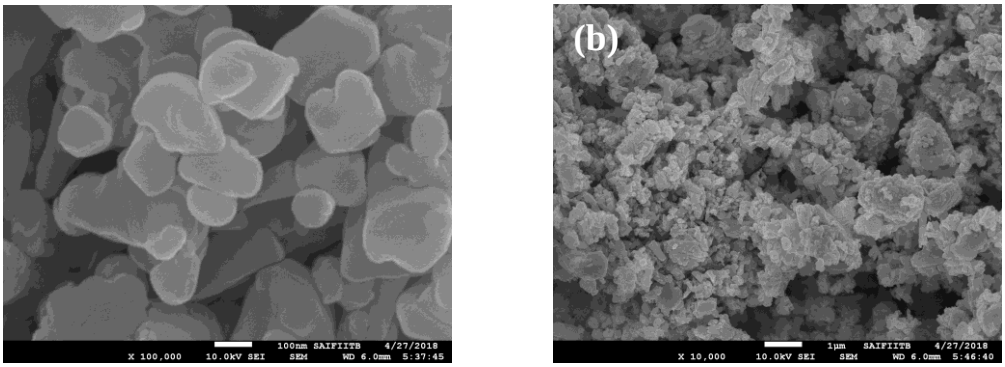

Figure 4 SEM Images of ZnO NPs in different magnification ranges (a) 100nm (b) 1µm.

Antibacterial activity of synthesized ZnO NPs

Antibacterial effect of ZnO NPs was visualized against both Gram positive (*S. aureus*) and Gram negative (*E. coli*) bacteria.Various solvents were used for the dispersion of the synthesized nanoparticles like dilute HCl, 70% ethanol, DMSO and saline as well. Only solvents were maintained as control. Along with nanoparticles, bulk ZnO dispersed in the same solvent was also maintained for checking its activity as control. Nanoparticles showed best antibacterial activity with dilute HCl when used as a solvent for dispersion of the nanoparticles. The following table 1 depicts the zone of inhibition (mm) by ZnO NPs in various solvents used for both *S. aureus* and *E. coli.* No antibacterial effect was observed on using ethanol and saline as a solvent to prepare suspension of ZnO NPs.

S. aureus showed greater susceptibility towards the synthesized NPs compared to *E. coli.* There have been reports of increased sensitivity of *S. aureus* to ZnO NPs (Santoshkumar et al., 2017 and Premanathan et al., 2011), which may be attributed to, the affinity of ZnO for the membrane of *S. aureus* and this microorganism's sensitivity to stress caused by H_2O_2. Previous work has indicated that ZnO NPs generate H_2O_2 (Premanathan et al., 2011).

Table 1 Antibacterial activity of synthesized ZnO NPs against pathogenic bacteria

Sample Name	**Zone of inhibition (mm)**	
	S. aureus	***E. coli***
A. ZnO NPs NPs + HCl NPs + DMSO	 23±0.88 18±0.58	 21±0.88 16±1.0
B. Bulk ZnO ZnO + HCl ZnO + DMSO	 13±0.58 15±0.88	 13±0.58 08±0.58
C. Control plate (solvent) HCl DMSO	 08±0.88 08±0.58	 08±0.88 08±0.58

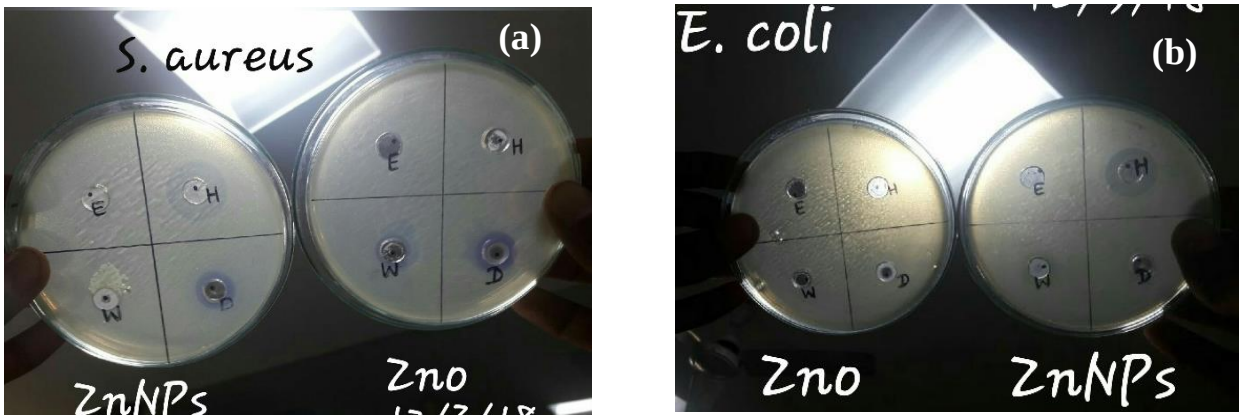

Figure 5 Antibacterial activities of ZnO NPs and bulk ZnO against (a) *S. aureus*and (b) *E. coli*

Ditch plate method

The results of the ditch plate method (fig. 6) showed that the test organisms *S. aureus* (Gram positive) and *E. coli* (Gram negative) grew over the MHA which consisted of bulk ZnO and did not grow over the MHA which consisted of ZnO nanoparticles in it. This clearly indicates the inhibitory effect of nanosized ZnO on the survival of the test isolate.

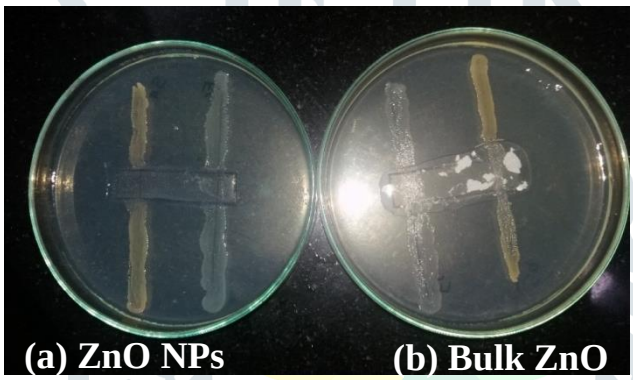

Figure 6 Antibacterial activities of (a) ZnO NPs and (b) bulk ZnO using ditch plate method

***In-vitro* antibacterial activity evaluation of the composite bandage**

The antibacterial activity of the prepared composite bandages was evaluated against Gram positive (*S. aureus*) and Gram negative (*E. coli* and *K. pneumoniae*) organisms. Table 2 shows the zone of inhibition (mm) by composite bandage and control bandage (impregnated with 1% citric acid).

Results were in accordance with the antibacterial activity shown by ZnO NPs, where higher sensitivity was demonstrated by Gram positive organism compared to Gram negative organism. However, much larger zone of inhibition was observed for composite bandage compared to ZnO NPs in isolation against the pathogenic organisms. This may be attributed to the literature indicating higher antibacterial activity at acidic pH (Saliani et al., 2015). One of the reasons for NPs toxicity is the release of toxic ions from ZnO NPs under aqueous conditions. As a result acidic pH increases the dissolution of ZnO NPs, which leads to higher toxic properties (Zhao et al., 2013). The results clearly demonstrate (fig. 7) the potential of these synthesized ZnO NPs against the test organisms.

Table 2 Antibacterial activity of composite and control bandage against pathogenic bacteria

Test organism	**Zone of inhibition (mm)**	
	Composite Bandage (ZnO NPs + 1% Citric acid)	**Control Bandage (1% Citric acid)**
S. aureus	40	17

E. coli	25	15
Klebsiellapneumoniae	21	15

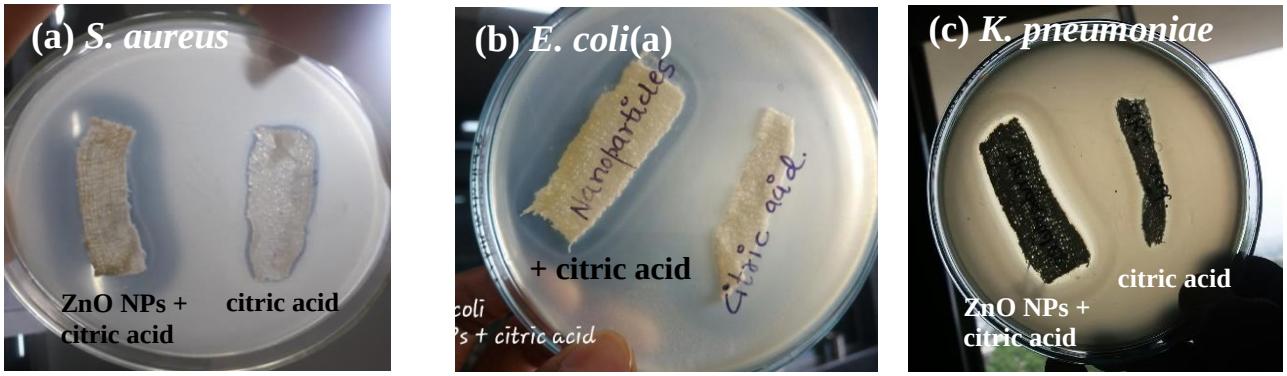

Figure 7 Antibacterial activity of composite bandage (ZnO NPs + citric acid) and control bandage (citric acid) against (a) *S. aureus* (b) *E. coli* (c) *K. pneumoniae*

IV. CONCLUSION

ZnO NPs were synthesized by the green method using *Ocimum tenuiflorum* leaf extract i.e. the eco-friendly method which is preferred over the conventional chemical & physical methods. After the synthesis of ZnO NPs, these nanosized materials were characterized by UV-Vis spectrophotometry, XRD and SEM that confirmed nanocrystallinity of ZnO NPs and an average size of 100nm. By agar cup method and ditch plate method the inhibitory activity of ZnO NPs was observed against both Gram positive (*S. aureus*) and Gram negative (*E. coli*) organism. *S. aureus* was found to be more susceptible compared to *E. coli*. One of the few reasons for this may be due the difference in their cell membranes.

Composite bandage prepared using mixture of ZnO NPs and citric acid showed good *in-vitro* inhibitory activity against *S. aureus* (Gram positive), *E. coli* and *K. pneumonia* (Gram negative) indicating its broad spectrum of activity. Interestingly, ZnO-NPs are reported by several studies as non-toxic to human cells, this aspect encourages their usage as antibacterial agents that hold good biocompatibility to human cells (Mohandas et al., 2015). Thus, the eco-friendly approach of synthesizing ZnO NPs and using it for composite bandage preparation can be a novel procedure leading to increased contact of NPs with skin, which might help in better absorption of ZnO NPs and enhance healing of deep wounds.

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