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BIOSYNTHESIS, CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY ANALYSIS OF ZINC OXIDE NANOPARTICLES USING LEAF EXTRACTS OF *CLERODENDRUM INERME*

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ABSTRACT
Zinc oxide nanoparticles were synthesized using leaf extracts (aqueous, ethanolic and methanolic) of *Clerodendrum inerme*, a straggling shrub that grows both in the wild and as a garden hedge. Nanoparticles were characterized by FTIR, XRD, TEM and SEM. FTIR spectra of all the three samples exhibited vibrations in the region 400–600 cm^{-1}, attributed to the vibrations of Zn-O which confirms the formation of ZnO-NPs. XRD diffractogram confirmed the crystalline nature of ZnO-NPs. TEM and SEM images revealed the spherical and hexagonal shape of ZnO-NPs. The size of nanoparticles was measured between 15-100 nm from the TEM images. Antimicrobial activity of ZnO-NPs was investigated against *Klebsiella pneumoniae* (MTCC-3384), *Vibrio cholerae* (MTCC-3904) and *Staphylococcus aureus* (MTCC-84). Results revealed that the lowest concentration (10 mg/ml) was not effective against any tested pathogen. Minimum inhibitory concentration of AZnO-NPs was 25 mg/ml for each pathogen. In case of MZnO-NPs, Minimum inhibitory concentration was 25 mg/ml for *K. pneumoniae* and *S. aureus* whereas for *V. cholerae*, it was 50 mg/ml. Minimum inhibitory concentration of EZnO-NPs was 75 mg/ml for *S. aureus* and 25 mg/ml for *K. pneumoniae* and *V. cholerae*. Highest concentration (175 mg/ml) showed maximum zone of inhibition. It can be concluded that ZnO-NPs possess antimicrobial activity against infectious microorganisms.

KEYWORDS: *Clerodendrum inerme*, ZnO-NPs, FTIR, XRD, TEM, SEM, Minimum inhibitory concentration.

INTRODUCTION

Nanotechnology is an interdisciplinary science which deals with production, manipulation and use of materials ranging in nanometers (Kavitha *et al.*, 2013). It is an emerging field which can probably revolutionize pharmaceuticals and cosmetics industry. This technology is of great interest because several nanoparticles are being claimed to have good antibacterial properties as they are being used as an approach for killing or reducing the activity of numerous pathogenic microorganisms (Singh and Nanda, 2013). Nanoparticles have several applications in diverse fields of science and therefore this has become important topic of research including bio-medical, sensors, antimicrobials, catalysts, electronics, optical fibers, agricultural, bio-labeling and in other areas (Salam *et al.*, 2012). Nanobiotechnology is the integration between biotechnology and nanotechnology for developing ecofriendly technology for the synthesis of nanomaterials (Sobha *et al.*, 2010). It deals with the synthesis of nanostructures using living organisms (Kumar and Yadav, 2009). It has several useful applications in medical science. Implementation of nanotechnology in medicine and physiology means that mechanisms and devices are so technically designed that they can interact with sub-cellular as well as molecular levels of the cell with a high degree of specificity. Thus, therapeutic efficacy can be achieved to maximum with minimal side effects by means of the targeted cell or tissue-specific clinical intervention (Fakruddin *et al*., 2012). Nanoparticles can be synthesized using chemical methods but this conventional method of synthesizing nanoparticles were found to be more expensive and also involve the use of toxic and hazardous chemicals that can lead to a variety of biological risks. The use of toxic chemicals can be overcome by the use of biosynthetic methods which comprised of the use of microorganisms, plants and their components. The procedure of synthesizing nanoparticles using microorganisms were found to be more tedious and require more steps in maintaining cell culture, intracellular synthesis with more purification steps. Plant mediated biosynthesis shows better advancement over chemical and physical method as it is lesser toxic, economical and environmental friendly (Anand Raj and Jayalakshmy, 2015). Nanoparticles have emerged as novel antimicrobial agents owing to the high surface area to volume ratio, which is coming up as the current research interests due to the growing microbial resistances against metal ions, antibiotics and the development of resistant strains (Chan and HsunTsai, 2008). Zinc oxide nanoparticles have several properties with diverse applications which make them a subject of immense research. Zinc oxide nanostructures have applications in sensors, energy harvesting and many electronic devices. Zinc oxide nanoparticles are being currently explored in the biomedical & antiviral areas (Mishra *et al*., 2012). It was found that ZnO-NPs have found tremendous applications in the field of high sensitivity biomolecular detection, diagnostics, antimicrobials, catalysis and micro-electronics

(Sreenivasan *et al.*, 2012). Zinc oxide nanoparticles have received much attention among metal oxide nanoparticles due to their especial properties and wide applications (Rouhi *et al*., 2013). There are certain biochemicals found in plants and are regularly used as natural medicines to treat common bacterial infections because of their minimal side effects and cost effectiveness which provide scientific support to the therapeutic use of the plants in tribal medicine (Rajlakshmi *et al*., 2003).

Genus *Clerodendrum* is widely distributed in tropical and subtropical regions of the world and is comprised of small trees, shrubs and herbs. There are several species of the genus which have been documented in traditional systems of medicine (Moldenke, 1985). *Clerodendrum* is a large and diverse genus which confined to more than five hundred species including small trees, shrubs and herbs. Ethno-medical importance of various species of *Clerodendrum* genus has been reported in various indigenous systems of medicines and as folk medicines (Shrivastava and Patel, 2007). The genus is being used as medicines specifically in Indian, Chinese, Thai, Korean, Japanese systems of medicine for the treatment of various life threatening diseases such as syphilis, typhoid, cancer, jaundice and hypertension. Few species (*Clerodendrum inerme*, *C. thomosonae*, *C. indicum* and *C. speciosum*) of the genus are ornamental and being cultivated for aesthetic purposes. The powder/paste form and the various extracts of root, stem and leaves are reported to be used as medicine for the treatment of asthma, pyreticosis, cataract, malaria, diseases of blood, skin and lung (Shrivastava and Patel, 2007). *Clerodendrum inerme* is a common shrub that grows in India, both in the wild and as a garden hedge (Thirumal *et al*., 2013). The green synthesis of nanoparticle is of great interest due to some specific advantages over chemical synthesis that includes eco-friendliness, economic prospects and feasibility. The synthesis of nanoparticles depends on the plant extracts as they may act both as reducing agents and stabilizing agents. The characteristics of the nanoparticles are influenced by the source of plant extract (Kumar and Yadav, 2009). In plant extract-mediated bioreduction, mixing of the aqueous extract with an aqueous solution of the relevant metal salt usually takes place at room temperature and is generally complete within a few minutes (Mittal *et al.*, 2013). The present study was completed with the biosynthesis of zinc oxide nanoparticles (ZnO-NPs) using leaf extracts of *Clerodendrum inerme* and their characterization. Evaluation of antimicrobial potential of ZnO-NPs was done against infectious microorganisms.

MATERIALS & METHODS

For synthesis of ZnO-NPs, zinc sulphate heptahydrate ($ZnSO_4.7H_2O$) (RM 695-500G), sodium hydroxide (NaOH) (MB095-500G), nutrient agar media (M001-500G), nutrient broth media (M002-500G) and Muller Hinton's agar media (M173-500G) of Hi-Media laboratories Pvt. Ltd. Mumbai, India were used in this study and all these chemicals were of analytical grade. The pathogenic strains *Klebsiella pneumonia* MTCC-3384, *Vibrio cholera* MTCC-3904 and *Staphylococcus aureus* MTCC-84 were procured from Microbial Type Culture Collection (MTCC) IMTECH, Chandigarh, India.

Extraction of powdered material and Preparation of leaf extracts

The leaves of *C. inerme* were rinsed twice with tap water followed by distilled water to remove the dust and other contaminants. Then the leaves were shade dried and powdered using electric grinder.10 g of the leaf powder was added into three Erlenmeyer flasks having 100 ml of solvents *viz.* distilled water, ethanol and methanol for making aqueous, ethanolic and methanolic extracts, respectively and kept in shaker for 48 h at 190 rpm. After 48 h, flasks were removed from the shaker and solutions were filtered using Whatman no. 1 to get the extract.

Biosynthesis of zinc oxide nanoparticles

ZnO-NPs were synthesized using the leaf extracts (aqueous, ethanolic and methanolic extracts) of *C. inerme*. Three hundred ml of 4mM aqueous zinc sulfate heptahydrate solution was prepared and 5 ml of the each extract was added to the separate zinc sulphate solution. The solution agitated using magnetic stirring for 5 min followed by drop by drop addition of 1M NaOH solution resulted change in the colour of solution. Synthesized ZnO-NPs in solvents (methanol, ethanol and water) were separated by centrifugation at 10,000 rpm for 15 min at 5°C. Supernatant was discarded and pellet was dispersed in distilled water thrice in order to remove extra debris.

Characterization of zinc oxide nanoparticles

ZnO-NPs were characterized using Fourier transform infra-red (FTIR) spectroscopy, X-ray diffraction (XRD), Transmission electron microscopy (TEM) and Scanning electron microscopy (SEM). The FTIR spectrum was recorded using a FTIR (Perkin Elmer - Spectrum RX-I FTIR) spectrophotometer within range of 4000 cm^{-1} to 250 cm^{-1}. X-Ray diffraction of ZnO-NPs was done using Panalytical's X'Pert Pro**.** TEM reveals particle size and morphology of ZnO-NPs and were characterized using the instrument Jeol/JEM 2100. SEM was used to deduce the particle size and morphology of the synthesized ZnO-NPs using JEOL Model JSM – 6390LV.

Antimicrobial assay

The antimicrobial activity of ZnO-NPs was tested against pathogenic bacteria (*Staphylococcus aureus, Klebsiella pneumoniae* and *Vibrio cholerae*) using agar well diffusion method (Senthilkumar and Sivakumar, 2014). Wells were made on the solidified media plates seeded with test organisms using sterile cork borer (8 mm diameter). A loop full of bacteria from the bacterial strains were transferred to 100 ml sterilized nutrient broth and incubated in incubator shaker for 18 h at 37±1°C. Muller Hinton's agar media plates were prepared by pouring about 25 ml of sterile Muller Hinton's agar media in the Petri-plates and were left as such for 15 min for solidification of agar. The test bacteria were spread on media and the plates were kept undisturbed for 15 min. Two Muller Hinton agar's media plates of each micro-organism were prepared. Four wells were made on the solidified media plates seeded with test organisms using sterile cork borer (8 mm diameter). The concentration of samples used in wells was kept 10 mg/ml, 25 mg/ml, 50

mg/ml, 75 mg/ml, 100 mg/ml, 125 mg/ml, 150 mg/ml and 175 mg/ml. Equal volume of samples were transferred into the respective wells with the help of micropipette. The plates were allowed to remain undisturbed for 1 h to ensure even diffusion of samples into agar. The plates were incubated at 37±1°C for 18-24 h. At the end of incubation, zone of inhibition formed around the wells was measured with the help of antibiotic zonescale (Hi-Media laboratories Pvt. Ltd. Mumbai, PW096-3NO) expressed in mm. All experiments were performed in triplicates. Mean and standard deviation were calculated using Microsoft Excel 2007.

RESULTS & DISCUSSION

Biosynthesis of ZnO nanoparticles

The plant mediated synthesis of nanoparticles is more advantageous than the other biological processes due to its easy and one step synthesis (Bansal *et al*., 2014). Plant-mediated biological synthesis of nanoparticles is gaining importance due to its simplicity, eco-friendliness and extensive pharmaceutical effects (Khandelwal *et al*., 2010). Singh *et al.* (2011) used sap from *Calotropis procera* and zinc acetate to produce nearly spherical ZnO-NPs with average size between 5 and 40 nm. Vidya *et al.* (2013) described that heating aqueous solution of zinc nitrate aqueous solution and leaves extract of *Calotropis gigantea* produced ZnO nanocrystallites. Awwad *et al.* (2014) reported an eco-friendly and simple method for the synthesis of zinc oxide nanosheets using *Olea europea* leaf extract. Zinc oxide nanoparticles AZnO-NPs, MZnO-NPs and EZnO-NPs were synthesized using the aqueous leaf extract (Figure 1 A), methanolic extract (Figure 1 B) and ethanolic extract (Figure 1 C) respectively. The synthesis of zinc oxide nanoparticles resulted in the colour change of solution.

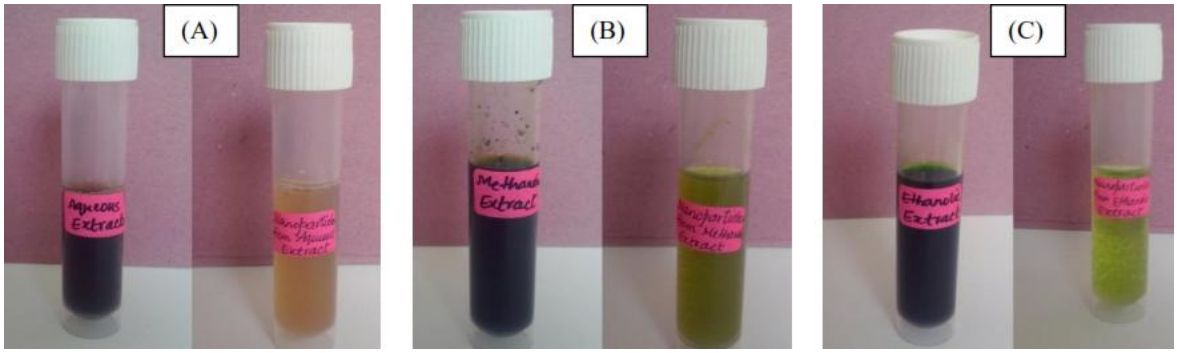

FIGURE 1: A- AZnO-NPs, B- MZnO-NPs, C- EZnO-NPs

Characterization of ZnO nanoparticles

Fourier transform infra-red (FTIR) spectroscopy

The biosynthesized ZnO-NPs were subjected to FTIR analysis to detect the various characteristic functional group associated with the synthesized nanoparticles. FTIR spectra of all the three samples were studied. FTIR spectra of AZnO-NPs (Figure 2), MZnO-NPs (Figure 3) and EZnO-NPs (Figure 4) showed band at 3392 cm^{-1}, 3391 cm^{-1} and 3398.0 cm^{-1} which is due to stretching vibrations of O–H groups in water, alcohol and phenols and N–H stretching in amines. The band at 2136.1 cm^{-1} is due C=C=O Stretching. The strong bands at 1645.2 cm^{-1}, 1632.16 cm^{-1} 1640.3 cm^{-1} are assigned to the stretching vibration of (NH)C=O. In the FTIR spectrum of AZnO-NPs, Small peaks are recorded on 1102.17 cm^{-1} and 1020.18 cm^{-1} which might be due to C-H group. A peak appeared at 466.17 cm^{-1} is the characteristic peak of ZnO molecules. In the FTIR spectrum of MZnO-NPs Peak at 2126.14 cm^{-1} and 1632.16 $cm^{-}1$ are attributed to C=C=O stretching and due to Zn-O stretching, respectively. Peak at the 477.23 cm^{-1} is attributed to ZnO molecules. FTIR spectra of all three nanoparticle samples exhibited vibrations in the region 400–600 cm^{-1}, which can be attributed to the vibrations of ZnO which confirms the formation of ZnO-NPs (Azam *et al*., 2012).

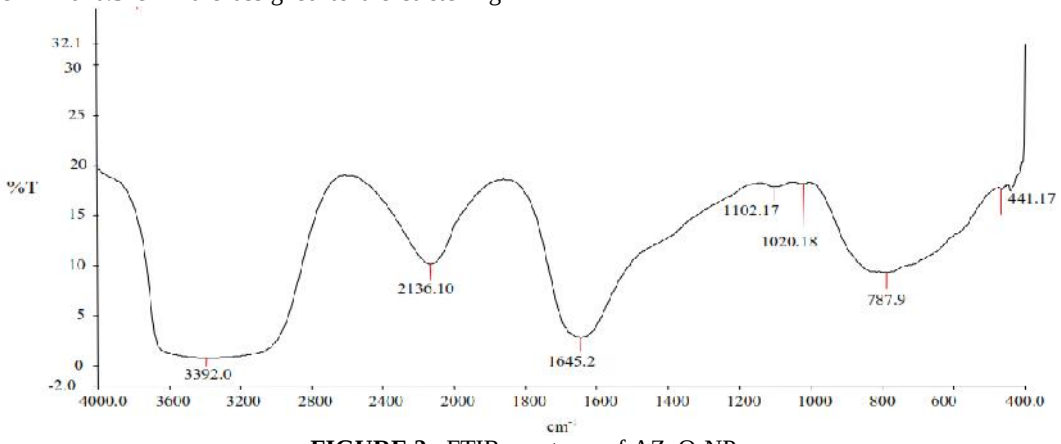

FIGURE 2: FTIR spectrum of AZnO-NPs

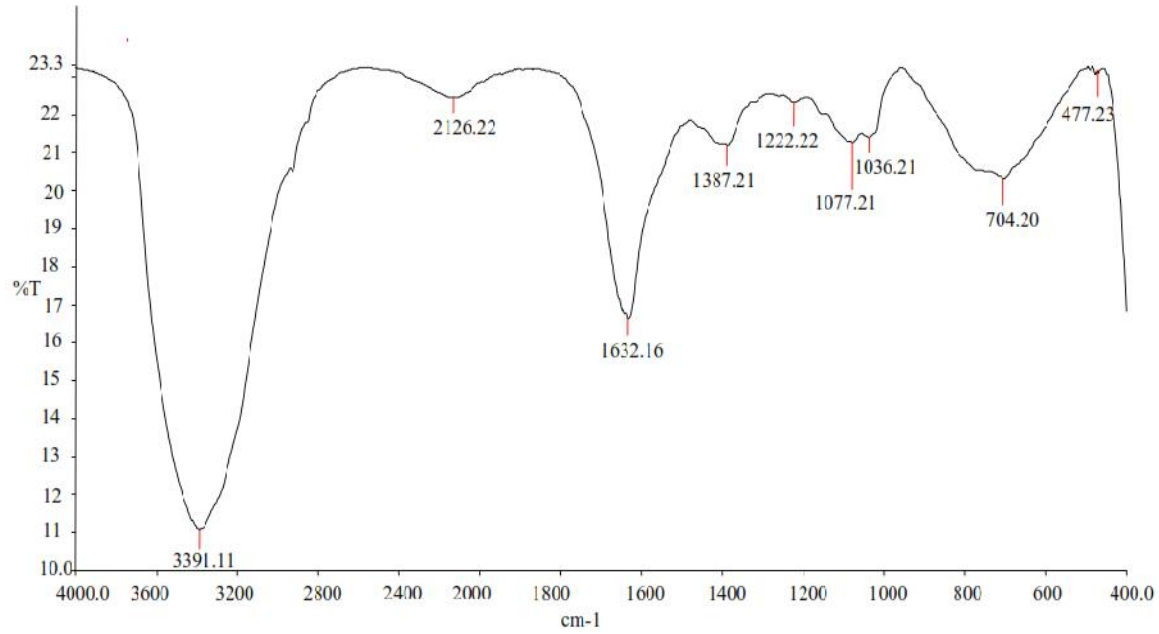

FIGURE 3: FTIR spectrum of MZnO-NPs

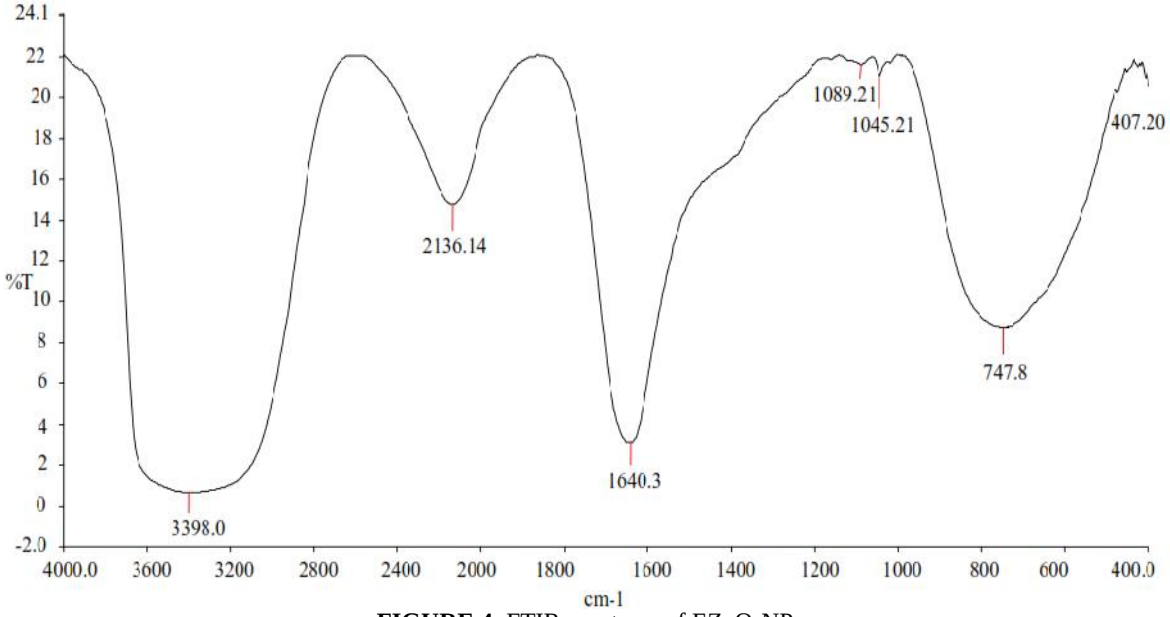

FIGURE 4: FTIR spectrum of EZnO-NPs

X-ray diffraction (XRD) measurement

The phase identification and composition of the products obtained by the biosynthesis using leaf extracts of *Clerodendrum inerme* was examined by XRD. XRD pattern of AZnO-NPs (Figure 5) shows several peaks at different intervals. Peaks are recorded at the position 2θ values of 31.7332°, 34.3959°, 36.2295°, 47.5351°, 56.5112°, 62.8409° and 69.0194°. MZnO-NPs have shown peaks recorded on the positions with 2θ values of 31.7662°, 34.4555°, 36.3326°, 47.5428°, 56.5328°, 62.8588° and 69.1263° (Figure 6). EZnO-NPs have shown peaks on the positions with 2θ values of 31.6912°, 34.3705°, 36.1934°, 47.547°, 56.5087°, 62.7624°, 67.9126° and 69.0285° (Figure 7). Peaks observed at 2θ values for all three samples (AZnO-NPs, MZnO-NPs and EZnO-NPs) were found to be same with slight variations. Miller indices values for all the three XRD patterns correspond to (100), (200), (101), (102), (110), (103), (200) and (112) planes which confirmed face-centered cubic structure and crystalline nature of ZnO-NPs. Mainly broad peaks at about 31° and 36° are indicative of nano-crystalline nature of the ZnO phase (Gnanasangeetha and Sarala, 2014).

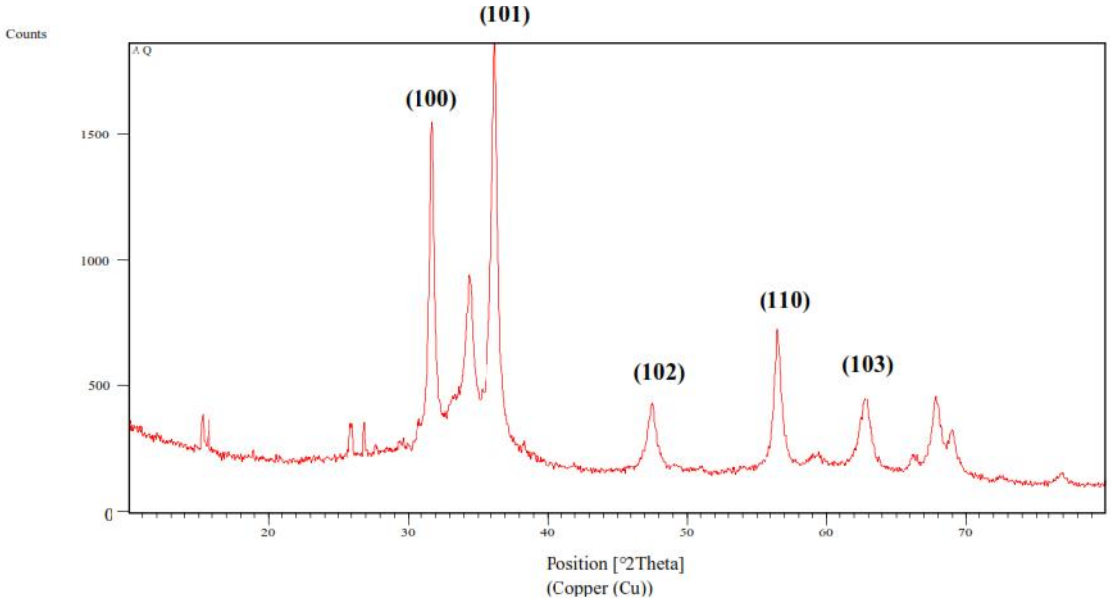

FIGURE 5: X-Ray Diffractogram of AZnO-NPs

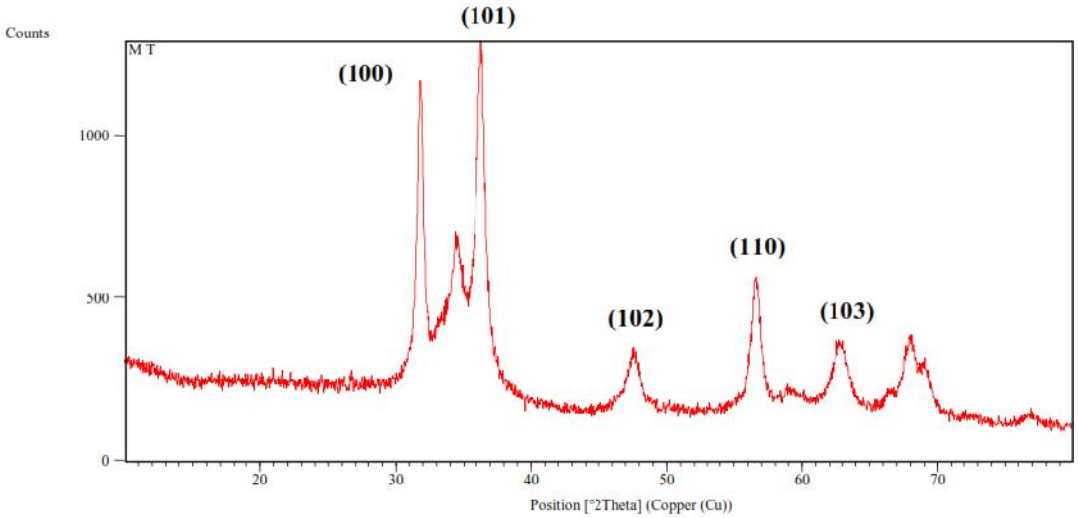

FIGURE 6: X-Ray Diffractogram of MZnO-NPs

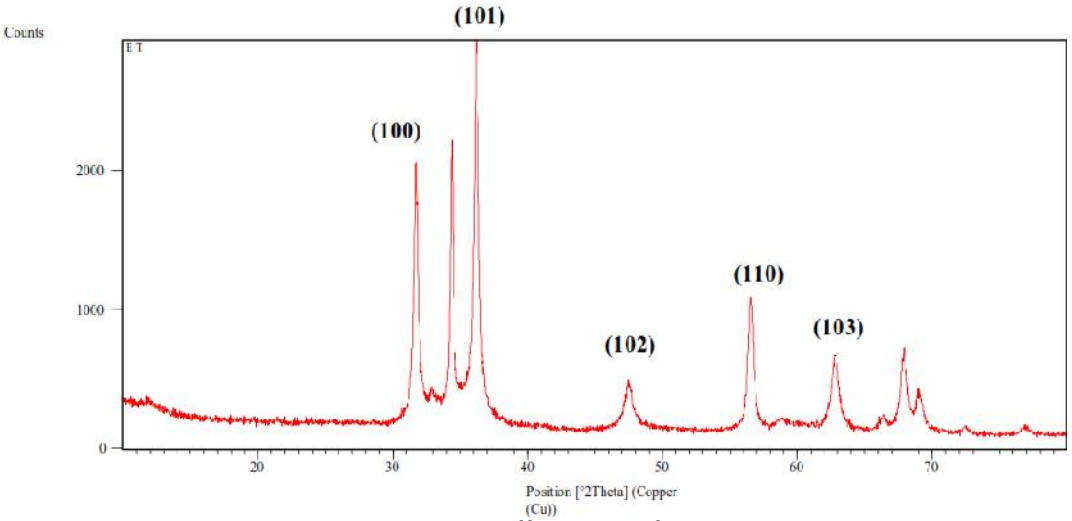

FIGURE 7: X-Ray Diffractogram of EZnO-NPs

Transmission electron microscopy (TEM)

Transmission electron microscopy is a useful technique to study particle shape and size of the nanoparticles. TEM images were recorded for measurement of different sizes of AZnO-NPs, MZnO-NPs and EZnO-NPs. TEM images revealed the spherical shape of AZnO-NPs and EZnO-NPs and hexagonal shape of MZnO-NPs. The size of AZnO-NPs was 50 -100 nm, MZnO-NPs was 25 nm and EZnO-NPs was 15 nm. Difference in the size of nanoparticles might be due to different extracts used in the synthesis.

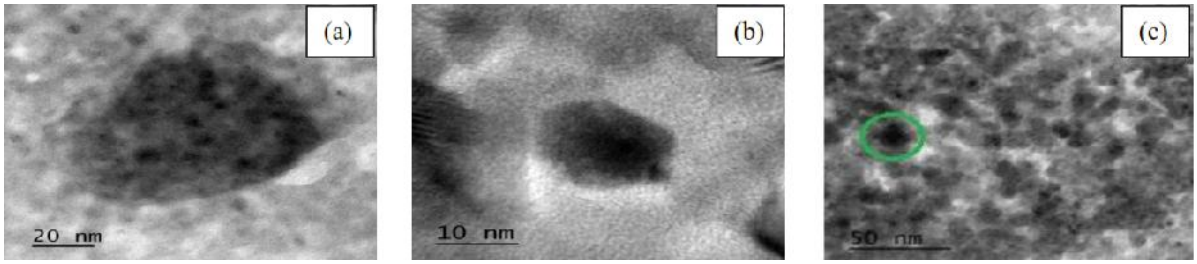

FIGURE 8: Transmission electron micrograph of AZnO-NPs (a), MZnO-NPs (b) and EZnONPs (c)

Scanning electron microscopy (SEM)

The scanning electron microscopic (SEM) images confirm the size and shape of the synthesized ZnO nanoparticle (Anand Raj and Jayalakshmy, 2015). SEM images revealed the spherical shape of AZnO-NPs and the size ranged from 161.25-228.04 nm. MZnO-NPs showed small amount of agglomeration. The length of EZnO-NPs ranged from 379.47-496.79 nm with the width below 250 nm. EZnO-NPs were present in hexagonal shape.

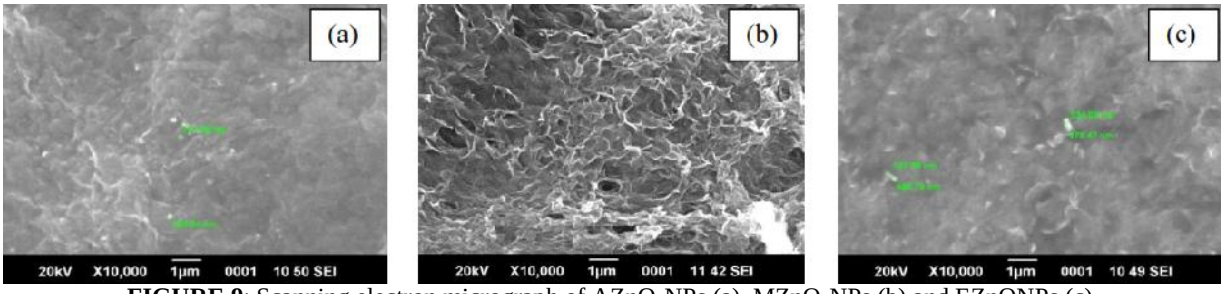

FIGURE 9: Scanning electron micrograph of AZnO-NPs (a), MZnO-NPs (b) and EZnONPs (c)

Antimicrobial activity

There are several studies which have demonstrated that specially formulated metal oxide nanoparticles have good antimicrobial activity. These compounds have strong inhibitory antimicrobial activities against fungi, virus, and bacteria since ancient times (Saha *et al.*, 2011).

TABLE 1: Zone of inhibition against different concentrations of ZnO-NPs (mm)

Inhibitory activity of nanoparticles against microorganisms		Concentrations of ZnO-NPs (mg/ml)							
		10	25	50	75	100	125	150	175
AZnO-NPs	*K. pneumoniae*	NZ	16.67 ±1.15	17.67 ±0.58	18.33 ±1.53	20.00 ±1.73	21.00 ±1.73	21.67 ±1.53	22.67 ±2.3
	V. cholerae	NZ	13.67 ±1.53	14.33 ±0.58	17.00 ±1.73	17.67 ±0.58	19.67 ±0.58	21.33 ±1.15	23.00 ±1.73
	S. aureus	NZ	14.33 ±0.58	15.67 ±1.15	16.67 ±0.58	17.33 ±0.58	18.33 ±1.15	19.67 ±0.58	21.67 ±0.58
MZnO-NPs	*K. pneumoniae*	NZ	15.33 ±0.58	15.67 ±1.53	17.33 ±2.08	18.33 ±0.58	19.67 ±1.15	20.33 ±0.58	21.33 ±1.53
	V. cholerae	NZ	NZ	13.67 ±1.53	14.67 ±1.15	15.33 ±0.58	16.33 ±1.15	18.33 ±2.08	18.67 ±0.58
	S. aureus	NZ	15.33 ±1.15	15.67 ±0.58	16.33 ±1.15	17.33 ±0.58	18.67 ±1.15	19.67 ±1.15	20.67 ±1.53
EZnO-NPs	*K. pneumoniae*	NZ	14.67 ±1.15	15.67 ±1.53	16.33 ±1.15	17.00 ±1.73	18.33 ±1.15	19.33 ±0.58	20.33 ±1.15
	V. cholerae	NZ	13.67 ±0.58	14.00 ±1.73	15.33 ±0.58	15.67 ±1.53	17.67 ±0.58	18.33 ±1.15	19.67 ±0.58
	S. aureus	NZ	NZ	NZ	14.67 ±1.15	15.33 ±0.58	15.67 ±1.53	17.33 ±0.58	18.33 ±1.53

*Mean ±SD, NZ- No zone of inhibition

Evaluation of antimicrobial activity of zinc oxide nanoparticles was carried out by agar-well diffusion against *B. subtilis*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. typhi* and *S. aureus*. 10 mg each of ZnO-AH and ZnO-AC (from biological synthesis using hot and cold *Aloe vera* extracts, respectively) and 10 mg 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. The preliminary antibacterial activity study results indicated that ZnO-AH showed maximum activity against *K. pneumoniae* (8.33±1.87) while ZnO-AC and ZnO-C showed maximum activity against *P. aeruginosa* (ZnO-AC 16.00±0.26 and ZnO-C 19.00±0.68) compared to other five strains. ZnO-AH exhibited least activity against *B. subtilis* and *S. aureus* (Lakshmi *et al.*, 2012). The antibacterial activity of ZnO-NPs was performed with varying concentrations (0.5 mg/ml, 1 mg/ml, 1.5 mg/ml, 2 mg/ml, 2.5 mg/ml, 3 mg/ml and 4 mg/ml) against gram negative (*E. coli*, *P. aeruginosa*, *K. pneumoniae*) and gram positive (*S. aureus*, *B. subtilus*) bacteria. The antibacterial activity yielded a higher zone of inhibition at 2mg/ml (Raj and Jayalakshmy, 2015). In the present study, the antimicrobial activity of ZnO-NPs was investigated against pathogenic bacteria *Klebsiella pneumoniae*, *Vibrio cholerae* and *Staphylococcus aureus*. Antimicrobial activity was determined by agar well diffusion method. Different concentrations (1 mg/ml, 25 mg/ml, 50 mg/ml, 75 mg/ml, 100 mg/ml, 125 mg/ml, 150 mg/ml and 175 mg/ml) of ZnO-NPs was introduced into wells and allowed to diffuse at room temperature. The bacterial plates were incubated at 37°C for 24 h. After the incubation period, the clear zone of growth inhibition diameter was recorded in mm.

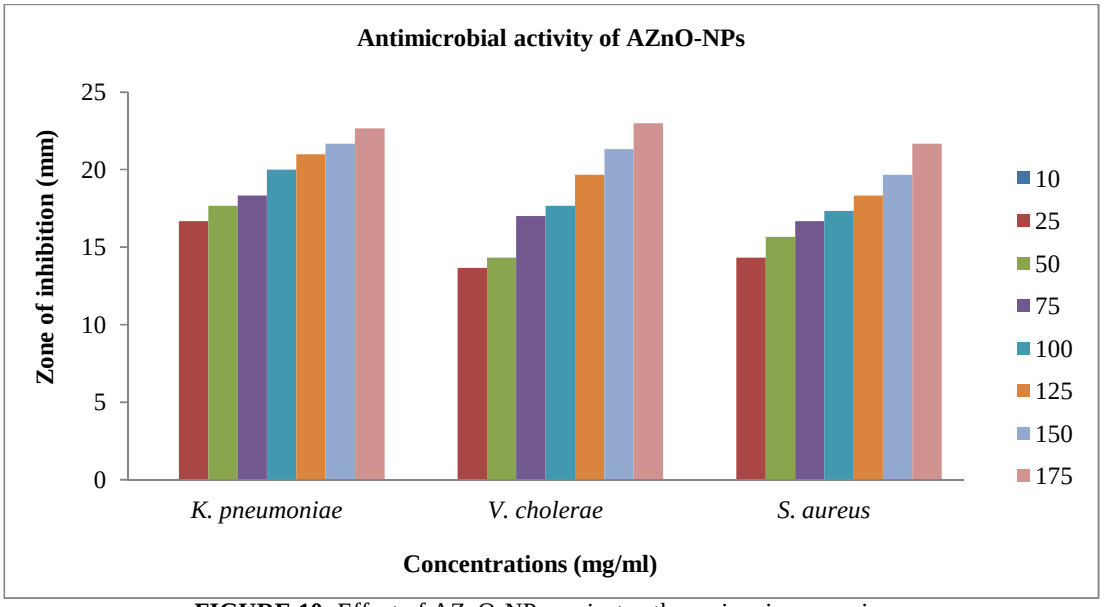

FIGURE 10: Effect of AZnO-NPs against pathogenic microorganisms

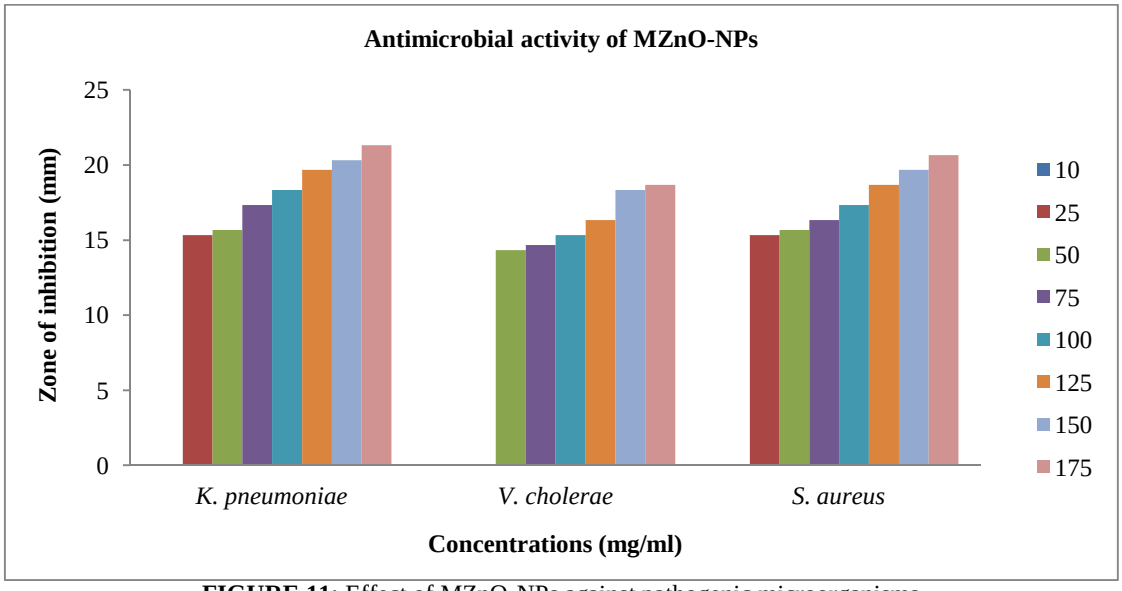

FIGURE 11: Effect of MZnO-NPs against pathogenic microorganisms

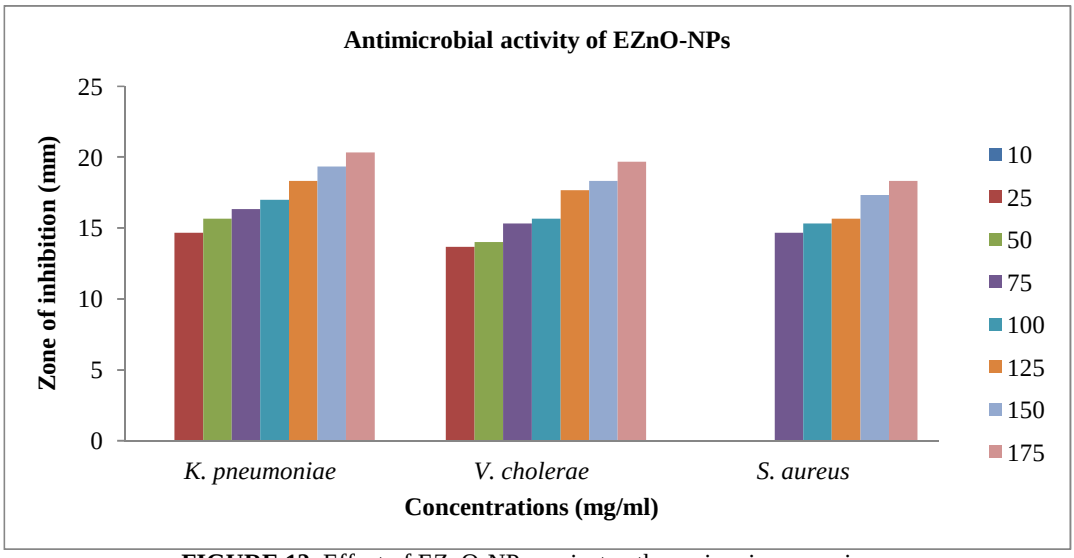

FIGURE 12: Effect of EZnO-NPs against pathogenic microorganisms

CONCLUSION

Nanotechnology is of great interest because several nanoparticles are being claimed to have good antibacterial properties (Singh and Nanda, 2013). Zinc oxide nanoparticles have gained very much attention due to their unique properties and extensive applications. Several methods have been described for the synthesis of zinc oxide nanoparticles but biosynthesis of nanoparticle is found very interesting due to some specific advantages over chemical synthesis that includes eco-friendliness, economic prospects and feasibility. Synthesis of zinc oxide nanoparticles using the leaf extracts (aqueous, ethanolic and methanolic extracts) of *Clerodendrum inerme*, was confirmed by colour change of the extracts and characterized by various techniques. FTIR spectra of all the three samples were studied which showed stretching vibrations of O–H groups in water, alcohol and phenols and N–H stretching in amines, C=C=O stretching, stretching vibration of (NH)C=O. FTIR spectra of all three nanoparticles samples exhibited vibrations in the region 400–600 cm−1, which can be attributed to the vibrations of Zn-O which confirms the formation of ZnO-NPs. XRD diffractogram confirmed the crystalline nature of ZnO-NPs. Miller indices values for all the three XRD patterns confirmed face-centered cubic structure. TEM and SEM images revealed the spherical shape and hexagonal shape of ZnO-NPs with variations in size. Antimicrobial activity of ZnO-NPs was investigated against pathogenic bacteria *Klebsiella pneumonia* (MTCC-3384), *Vibrio cholerae* (MTCC-3904) and *Staphylococcus aureus* (MTCC-84). It can be concluded from the results that ZnO-NPs possess antimicrobial potential against infectious microorganisms.

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