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INVESTIGATIONS ON ANTICANCER AND ANTIBACTERIAL ACTIVITIES OF ZINC OXIDE NANOPARTICLES

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ABSTRACT

A facile and economical precipitation method was adopted to synthesise zinc oxide nanoparticles. The crystallography and optical properties of as synthesised nanoparticles were studied using X-ray diffraction, UV-Visible spectroscopy, Fourier transform infrared spectroscopy and the morphology of the nanoparticles were revealed via Scanning electron microscopy. The as synthesized nanoparticles were found to be remarkable in inhibiting both gram positive and gram negative pathogenic bacteria. The anticancer activity reveals the dose dependent influence of zinc oxide nanoparticles against human breast cancer cell line (MCF-7). The synthesized nanoparticles were proved to exhibit excellent cytotoxic effect on breast cancer cell line.

INTRODUCTION

Besides skin cancer, breast cancer is the most frequently diagnosed cancer among American women. According to a survey approximately under 30% of newly diagnosed cancers in women will be breast cancer in 2015 (www.breastcancer.org). The treatment of cancer involves diverse therapies which requires alklating agents, antimetabolites, biological agents, etc., that causes severe side effects due to complications in differentiating between cancerous and normal cells, producing systematic toxicity which is continued to be one of the daunting challenges worldwide, leading to the upsurge in the investigation of using nanoparticles aganist tumor formation, development and progression due to their intrinsic antitumor effect. Researchers are initiating periodically to unlock the enormous medical potential of nano-scale materials due to their stability, robustness and relatively low toxicity combined with effective antimicrobial properties. Concrete advances in developing promising therapeutic agent have been hard-won. In the context of combating cancerous cell and pathogenic bacteria noteworthy attention is directed towards metal oxide nanoparticles, which are proven to be effective for treating infectious diseases (Maria Pilar Vinardell *et al.,*2015).

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The pertinent metal oxide nanoparticles are predominantly interesting in that they can be synthesized with unusual morphologies and high surface areas that possess abundant reactive surface sites (Ravishankar Rai *et al.,* 2011, Peter K. Stoimenov *et al.,* 2002). The natural toxic properties of heavy metals determine the toxicity of metal oxide nanoparticles and the mechanism of toxicity depends on surface modification, intrinsic properties and composition (Mohammad J. Hajipour *et al.,* 2012, Agnieszka Kołodziejczak *et al.,*2014). Zinc oxide is a multifunctional, strategic, potential, bio-safe and versatile inorganic material with a broad spectrum of applications in sensors, energy harvesting and many electronic devices (Y. Abdi *et al.,* 2014, Nai-Feng Hsu *et al.,* 2013, Xiaoyong Lai *et al.,* 2012, Hsin-Ming Cheng *et al.,* 2008).

Several distinct applications are being explored in the biomedical and antibacterial areas due to their noxious nature to microorganisms and hold excellent biocompatibility to human cells (Paula Judith Perez Espitia *et al.,* 2012, Amna Sirelkhatim *et al.,* 2015, Guy Applerot *et al.,* 2009, Roberta Brayner *et al.,* 2010). Uptill now, a variety of synthetic strategies have been developed to synthesize ZnO nanoparticles differing in size, shape and crystalline structures via various techniques such as hydrothermal (Sonal Singhal *et al.,* 2012), precipitation (Shouli Bai *et al.,* 2011), sol-gel (Jayaseelana *et al.,* 2012), biosynthesis (Nasrin Talebian *et al.,* 2013), chemical vapour deposition (Protasova *et al.,* 2011),

solvothermal (Samanta Cimitan *et al.,* 2009) and polyol process (Buong Woei Chieng *et al.,* 2012). Among the aforementioned methods precipitation method is consider to be advantageous because of its simple cost competitive and environmentally benign nature. Keeping in view, the potential toxicity of ZnO nanoparticles, we report the anticancer and antibacterial efficacy of ZnO nanoparticles prepared via precipitation. We have investigated their strucutural and optical properties and their antibacterial activity against gram positive (*Staphylococcus aureus*, *Bacillus subtilis*) and gram negative (*Klebsiella pneumonia*, *Escherichia coli*) bacterial strains. The potential toxicity of ZnO NPs on human breast cancer cell line was evaluvated in this present work.

MATERIALS AND METHODS

The starting materials used in this work were analytical grade Zinc nitrate (hexahydrate) (Zn $(NO_3)_2$ $6H_2O$), sodium hydroxide (NaOH). Soluble starch was obtained from merck. Double distilled water was used for the preparation of all aqueous solutions.

Preparation of ZnO nanoparticles

The nanoparticles of zinc oxide was synthesized by the facile precipitation method using 0.1M of Zinc nitrate, 0.3 g of soluble starch as nontoxic capping agent and 0.2M of NaOH as base catalyst. Typically, the starch aqueous solution was stirred in a magnetic stirrer for 20 mins followed by the addition of zinc nitrate solution. The mixture was constantly stirred for 2 h and the prepared NaOH solution was injected dropwise resulting in the formation of white precipitate. After 24 h the setteled white precipitate was washed three times in double distilled water and two times in ethanol to remove the by products. The obtained sample were dried in an oven at 100 º C for 2 h and subsequently the product was crushed into fine powder.

Analysis of ZnO nanoparticles

The optical absorption spectra of ZnO nanoparticles were obtained using Perkin Elmer Lambda 25 UV-Vis spectrometer from 200 to 800 nm. The FTIR spectrum of as synthesized NPs converted into pellet form were recorded between 4000 to 400 cm^{-1}.The X-ray diffraction pattern were recorded on Rich Seifert diffractometer using monochromatic CuK_α (λ=1.5406 Å) radiation in the 2θ range from 10-80º. The morphology of the ZnO nanoparticles were visualized using a field emission scanning electron microscope without sputter coating because the NPs were self conducting.

Antibacterial assay

The antibacterial activity of the synthesized ZnO nanoparticles were evaluvated using the quantitative well diffusion assay. All glassware, media and reagents used were sterilized in an autoclave at 121ºC for 15 min. Nutrient agar was used as a test medium for antibacterial susceptibility testing. *Staphylococcus aureus* (Gram positive), *Bacillus subtilis* (Gram positive), *Klebsiella pneumonia* (Gram negative), *Escherichia coli* (Gram negative) were used as model test strains for both gram classes of bacteria. Five wells of 8 mm diameter were bored aseptically by using a sterile cork borer seeded with test organisms in different concentrations (250, 500, 750, 1000 µg) of ZnO nanoparticles. The plates were incubated at 37 ºC for 24 h and they were examined for the effectiveness of ZnO nanoparticles by measuring the zone of inhibition, which appear as a clear area around the wells. The diameter of the clearing zones was measured in mm using the ruler scale.

Cell culture

MCF-7 human breast cancer cell lines were obtained from the National Centre for cell science, Pune, India. The cells were maintained in Minimal Essential Medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO_2 at 37 °C (Hi Media laboratories, Mumbai, India). All of other chemicals and reagents were obtained from Sigma Aldrich.

MTT assay

To evaluate the cytotoxicity of ZnO nanoparticles dimethyl thiazolyltetrazolium bromide (MTT) assay was performed. Cells (1×10^5/well) were plated in 96-well plates and incubated for 24 h at 37ºC in a humidified atmosphere of 95% air and 5% CO_2. The human breast cancer cells were treated with a series of 10-200 µg/ml concentration of zinc oxide nanoparticles after they reach the confluence. 100 µl/well MTT was added to the cultures and incubated for 4 h and then 1 ml of DMSO was added in all the wells. The absorbance at 570-620 nm was measured using UV-Spectrophotometer with DMSO as blank. The viability was calculated using the following formula and the concentration required for a 50% inhibition (IC_{50}) has been determined graphically.

$$\%\ of\ viability = \frac{OD\ value\ of\ experimental\ sample\ (ZnONPs\ treated)}{OD\ value\ of\ experimental\ control\ (untreated)} \times 100$$

RESULTS AND DISCUSSION

The powder XRD analysis was carried out to analyse the structure and crystalline nature of the as synthesised ZnO NPs. The XRD spectrum shown in Fig.1 reveals the single phase formation of ZnO with three prominent peaks at 31.9°, 34.5° and 36.4° corresponding to (100), (002) and (101) lattice planes. The obtained results are in good agreement with the hexagonal wurtzite structure of ZnO reported in JCPDS Powder Diffraction File no. 01-075-0576.No characteristic peak associated with the impurities can be observed in the XRD spectrum of the obtained sample, indicating high purity of ZnO products and also, the sharp and intense peaks in the XRD patterns of the product indicate good crystallinity. The average crystallite size was estimated from the peak width at half maximum using Scherrer formula and the calculated crystallites size is 32.7 nm. UV–Vis absorption spectrum, has been studied to reveal the energy structures and optical properties of ZnO NPs. As can be seen, Fig.3 shows the diffuse reflectance spectrum of the sample revealing a sharp increase at 377 nm. The band gap energy was determined using Kubelka-munk function.

$$K=(1-R)^2/2R$$

Where (R) is the absolute value of reflectance and F (R) is equivalent to the absorption coefficient. The direct band gap of ZnO was estimated by plotting (F (R). hυ) ^{2} vs hυ (EV) as

shown in Fig.3 (inset). The obtained bandgap energy (Eg) value was 3.32 eV which is comparatively lower to the reported values of the bandgap energy of bulk ZnO (Eg=3. 37 eV) indicating that the optical absorption edge slightly red shifted which may be attributed to the increase in grain size.

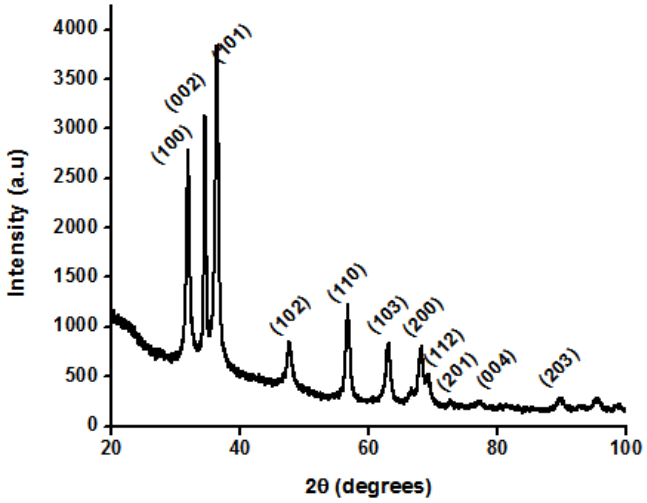

Fig. 1. XRD Spectrum of ZnO nanoparticles

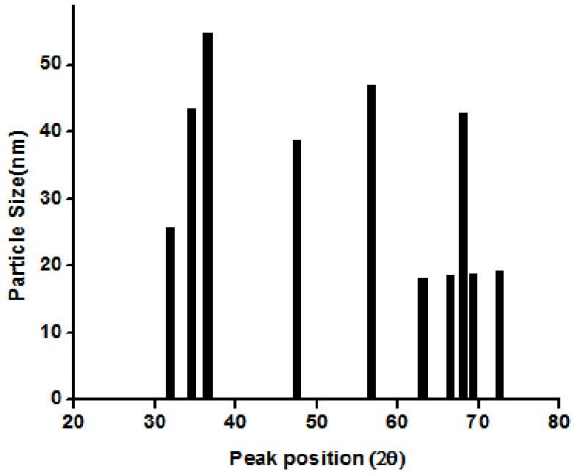

Fig. 2. Peak position Vs particle size plot of ZnO nanoparticles

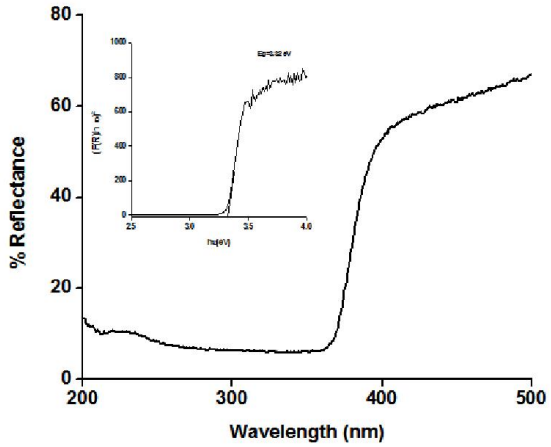

Fig. 3. Diffuse reflectance spectrum of ZnO nanoparticles: Inset: Band gap energy plot

To further support the formation mechanism of ZnO NPs FT-IR studies were performed and the results were shown in Fig 4. The fundamental mode of vibration at 3404.13 cm^{-1} corresponds to the O-H stretching vibration due to the water molecule present in the sample. The peaks at 2928.23 cm^{-1} and 1007.06 cm^{-1} are due to the presence of C-O and C-H stretching vibrations indicating the saccharide strucuture of starch. The peak at 759.92 cm^{-1} is attributed to the C-O bond stretching.

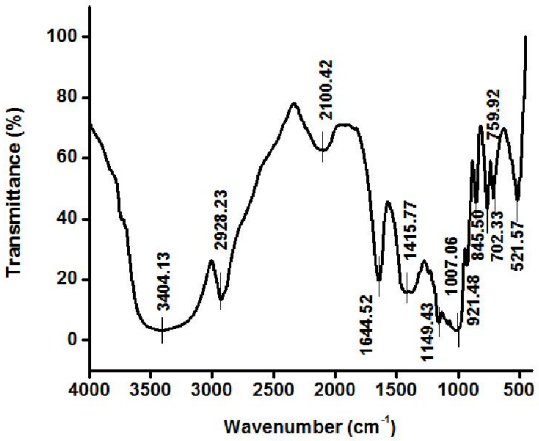

Fig.4. FT-IR spectrum of ZnO nanoparticles

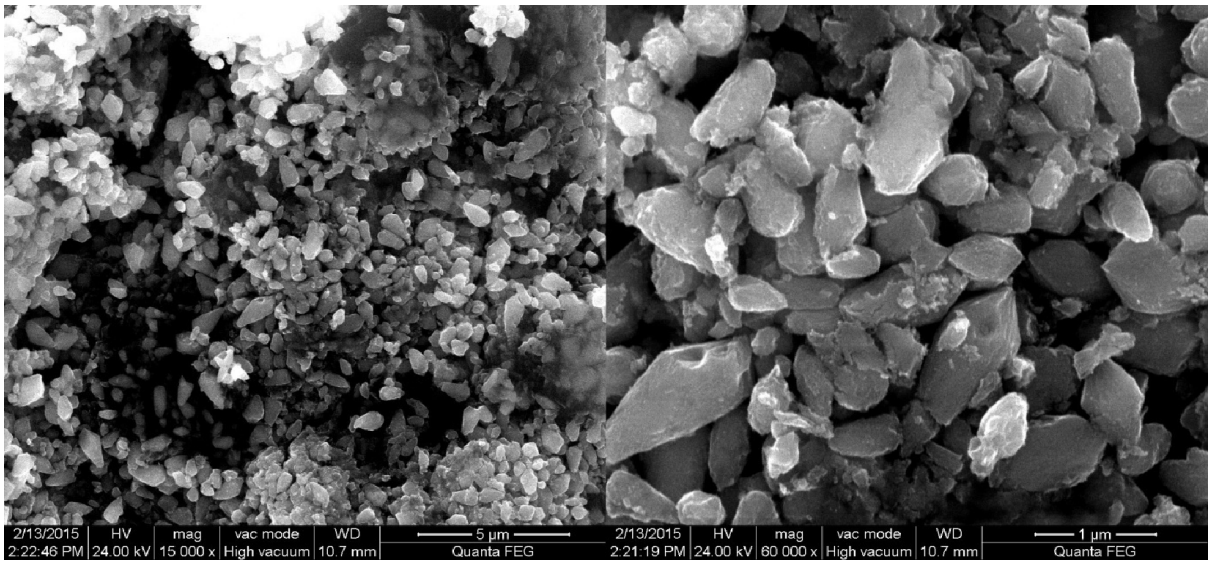

Fig. 5. FESEM images of ZnO nanoparticles

The band at 1415.77 cm^{-1} and 1644.52 cm^{-1} corresponds to C=O asymmetric C=O symmetric stretching vibration. The prominent peaks at 702.33 cm^{-1} and 521.57 cm^{-1} indicates the stretching vibrations of ZnO nanoparticle. The FESEM analysis is employed to identify the morphology of the synthesised ZnO NPs. The micrograph of ZnO nanoparticles shown in Fig.5 reveals the particles having rice like morphology with the formation of network of aggregated particles which can be interpreted as the characterisitic of the particle synthesised in water medium.

Evaluation of antibacterial properties

In the current study, the relative antibacterial activity of ZnO nanoparticles towards gram positive (*Staphylococcus aureus*, *Bacillus subtilis*) and gram negative (*Klebsiella pneumonia*, *Escherichia coli*) bacterial strains were studied using well diffusion assay method. Four different concentration of ZnO suspensions were tested and the results are given in table 1. A higher inhibition zone of 19 mm was observed in the gram positive *Bacillus subtilis* compared to other gram-negative strains employed in this antibacterial susceptibility assay. For all bacterial strains, the highest activity was observed for 1000 µg/ml. Gram positive (*Staphylococcus aureus, Bacillus subtili)* bacterial strain depicted highest sensitivity to ZnO nanoparticles compared to gram negative strains. Based on these results, it can be concluded that the synthesized ZnO nanoparticles had significant antibacterial action on both the gram classes of bacteria. Clear Zone of inhibition indicates the boicidal action of ZnO nanoparticles which involves distrupting the bacterial membrane and formation of surface oxygen species from ZnO leading to bacterial cell death (Nagarajan Padmavathy *et al.,* 2008). Fig.6 shows the antibacterial activities of ZnO nanoparticles in pathogenic bacterial strains.

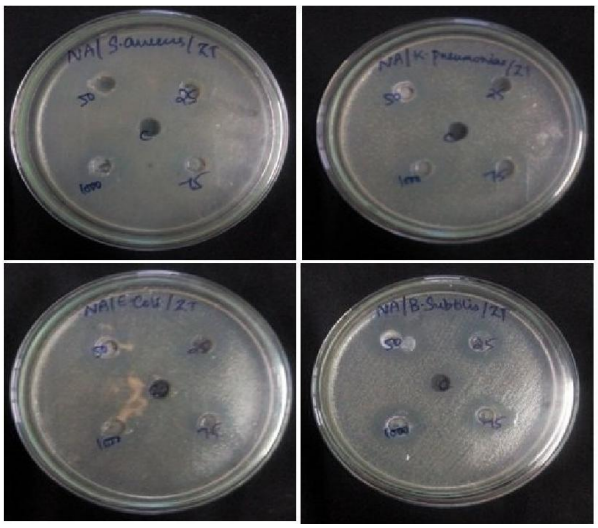

Fig. 6. Antibacterial activity of ZnO nanoparticles

Cytotoxic activities of synthesized ZnO NPs against MCF-7 cells

The as synthesized ZnO NPs have been tested for their potent cytotoxic activity against MCF-7 (breast cancer cells) using MTT assay. Fig.7 shows the cell viability evaluated after 24 h exposure to ZnO NPs of various concentrations ranging from (10 µg/ml- 200 µg/ml). ZnO NPs induced cytotoxicity on MCF-7 cell lines was found to be increasing with an increase in concentration of ZnO NPs. The 50% viability happens at the concentration of 40 µg/ml which is the half maximal Inhibitory Concentration (IC_{50}). The metabolic activity of MCF-7 cells presented a dose-dependent characteristic, which decreased with the decreasing of the dose of ZnO NPs incubated with the MCF-7cells.

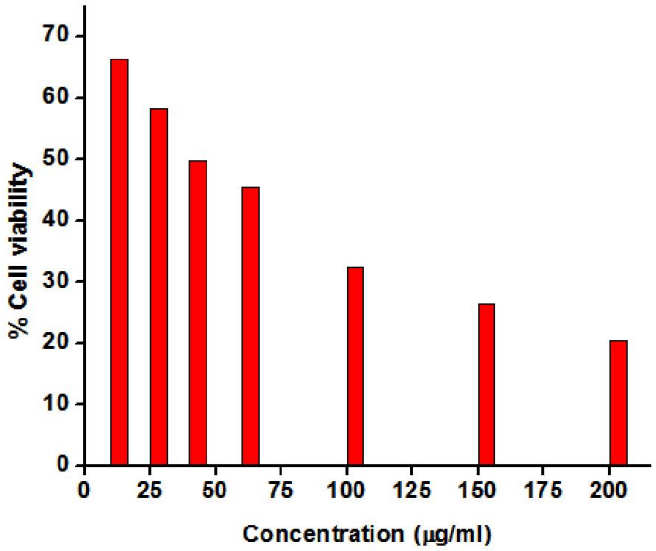

Fig.7 Percentage of viability on MCF-7 cell line

The alterations in the morphology of the cells treated with ZnO NPs were observed, however no such effects were observed in untreated cells. As the concentration of ZnO NPs increases majority of the cells appeared to be shrunken due to mitochondrial dysfunction leading to cell death. Fig. 8

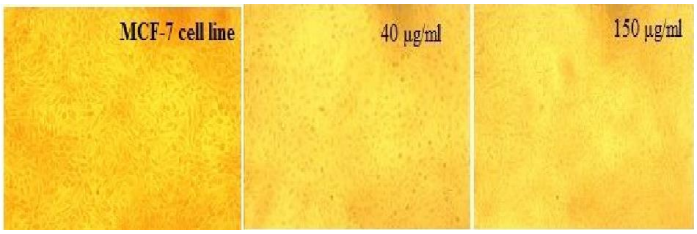

Fig. 8. Toxic effect of synthesized ZnO NPs on MCF-7 cell line

Table 1. Zone of inhibition of synthesized ZnO NPs against various pathogenic bacteria (NZ- No Zone)

S.No	Concentration (µg/ml)	Zone of inhibition (mm)			
		Staphylococcus aureus	*Bacillus subtilis*	*Klebsiella pneumoniae*	*Escherichia coli*
1	Standard	14	13	14	14
2	250	11	16	NZ	NZ
3	500	13	17	NZ	NZ
4	750	15	18	11	11
5	1000	18	19	12	12

Conclusion

The present study revealed the strucutural, optical and morphological properties of synthesised ZnO nanoparticles. The XRD results indicated that the synthesised ZnO NPs have hexagonal wurtzite structure with the crystallite size of 32.7 nm. The diffuse reflectance spectrum showed a sharp increase at 377 nm with the band gap energy value of 3.32 eV. The formation mechanism of ZnO NPs were confirmed by FT-IR spectrum. The as synthesised ZnO NPs could be used as a potential antibacterial agent because of its effective toxicity to pathogenic bacterial strains. ZnO NPs might be a promising alternative agent for cancer therapy aganist human breast cancer cell line.

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