International Journal of Research in Advent Technology, Special Issue, August 2018
E-ISSN: 2321-9637
International Conference on "Topical Transcends in Science, Technology and Management" (ICTTSTM-2018)
Available online at www.ijrat.org

Antibacterial and Cytotoxicity Studies Of Zno Nanoparticles Prepared By Bio-Fueled Solution Combustion Synthesis

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ABSTRACT

In this study we report the antibacterial and anticancer activity of ZnO nanoparticles prepared using aqueous fruit extract of *Ananus comosus* by solution combustion synthesis. The structure and morphology of the sample were determined by XRD, SEM and TEM. Antibacterial activity of ZnO nanoparticles was tested against *Clostridium perfringens* and *Salmonella enterica* by well diffusion method. The anticancer efficacy of ZnO nanoparticles was carried out on HeLa. The antibacterial results indicate that spherical ZnO nanoparticles constitute as an effective bactericidal agent and their plausible applications in antimicrobial products. Anticancer result indicates that ZnO nanoparticles exert dose dependent toxicity in HeLa cells.

Keywords— ZnO nanoparticels; *Clostridium perfringens*; *Salmonella enterica*; HeLa; MTT assay

INTRODUCTION

ZnO is a wide band gap semiconductor with Wurtzite structure. ZnO nanoparticles (NPs) have been extensively studied in the field of catalysis, paints, cosmetics, solar cells, gas sensors and food packaging materials [1-6]. It is due to their ease of preparation in different morphologies, low cost, UV shielding properties, large surface to volume ratio, chemically alterable physical properties. The effect of zinc oxide NPs on sex hormones and cholesterol in rat has also been reported [7]. Literature shows that ZnO NPs exhibit high toxicity against bacteria but minimum effect on human cells [8-9].

Clostridium perfringens is a ubiquitous pathogen that produces many toxins and hydrolytic enzymes. Because the toxin-encoding genes can be located on extra chromosomal elements or in variable regions of the chromosome, several pathovars have arisen, each of which is involved in a specific disease [10]. In addition to food poisoning of humans and apparently spontaneous cases of diarrhea of humans and other animals, the enterotoxin of *clostridium perfringens* is the cause of up to about 10 % antibiotic related diarrhea.

The Centers for Diseases Control and the Food and Drug Administration of the United States of America (USA) have estimated that over 30 million people each year get sick due to infections caused by consuming contaminated foods [11,12]. Earlier studies have shown that *Salmonella enterica* is one amongst the more prevalent bacterial pathogens that causes food borne infections [11]. According to official estimates the medical and productivity losses caused by these two enteropathogens in the USA were over the 3 billion dollars in 1995 [11].

Measurement of cell viability and proliferation forms the basis for numerous in vitro assays of a cell population's response to external factors. The MTT colorimetric assay determines the ability of viable cells to reduce the soluble, yellow tetrazolium salt [3-(4,5-dimehtylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) into an insoluble, purple formazan. This procedure is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells by the action of dehydrogenase enzymes to generate reducing equivalents such as nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH). The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometrically. The MTT assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. The MTT reagent yields low background absorbance values in the absence of cells. For each cell type the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation.

The interaction of NPs with microorganisms and bio molecules is an expanding area of research, which is still largely unexplored yet. Hence, the present study reports the synthesis of ZnO NPs by environmental friendly, simple and cost effective solution combustion synthesis (SCS) using aqueous fruit extract of *Ananus comosus* as bio fuel. The antibacterial efficacy of ZnO NPs has been studied on gram-positive *C. perfringens* and gram-negative *S. enterica.* Minimum inhibitory concentration (MIC) was determined by micro broth dilution technique. The cytotoxicity of ZnO NPs has been investigated on HeLa cell line by MTT assay.

EXPERIMENTAL

Materials and methods:

Zinc nitrate hexahydrate [$Zn(NO_3)_2.6H_2O$), AR 99% SD Fine], Nutrient agar [Himedia], Dulbecco's Modified Eagle's medium [Gibco], Dimethyl sulfoxide [C_2H_6SO, AR 99% Merck], 2,2-diphenyl-1-picrylhydrazyl hydrate [$C_{18}H_{12}N_5O_6$, > 90% Merck], Mueller Hinton Agar [Sigma-aldrich] were used as such without further purification and pineapple fruit from the local market was purchased off the shelf.

Preparation of aqueous extracts of *Ananus comosus*:

30 g of the fresh fruits of *Ananus comosus* was subjected to soxhlet extraction for 72 h using 30 mL of double distilled. The aqueous solution obtained after extraction were filtered using Whatman No. 1 filter paper. Filtrate was collected, centrifuged to remove the solid particles if present any and stored for further use.

Synthesis of ZnO NPs:

Synthesis of ZnO NPs was carried out by SCS [13]. The procedure followed was as described in our previous studies [14-17]. 3.0 g of $Zn(NO_3)_2.6H_2O$ was dissolved in 10 mL of distilled water. Then 10 mL of the fruit extract was added to it. The reaction mixture was mixed well using with the help of a magnetic stirrer for 10 minutes and then crystallizing dish containing the reaction mixture was placed in a preheated muffle furnace at 375 ± 10 °C. Within a short while the solution boiled to form a gel followed by decomposition with the evolution of gases. The time span required for completion of the above reaction was about 5 minutes. White ZnO NPs thus prepared was ground in to a fine powder and characterized by various techniques.

Characterizations:

The crystal structure of ZnO was measured by X-ray diffraction using Panalytical X'pert diffractometer with Cu Kα radiation (λ=1.5418 Á) as the source. Surface morphology of the sample was studied by Scanning Electron Microscopy (SEM) performed on Hitachi S-3400-N. The particle shape was investigated by transmission electron microscopy (TEM) carried out on Philips CM200.

Evaluation of antibacterial efficacy of ZnO NPs by well diffusion method:

The antibacterial activity of ZnO NPs was carried out by well diffusion method in nutrient agar media as reported in the literature [14]. About 20 mL of sterilized and molten nutrient agar media was poured in to the sterilized petri dishes. The bacteria *C. perfringens* and *S. enterica* were cultured overnight at 37 °C in nutrient agar and adjusted to a final density of 10^7 CFU/mL by 0.5 McFarland standards. 100 µL of the pathogenic bacteria cultures were transferred onto plate and made culture lawn by using sterile L-rod spreader. Homogeneous dispersions of NPs with different concentrations ranging from 500 µg/mL to125 µg/mL were prepared by ultrasonication. Wells were cut and 50 µL of dispersions of ZnO NPs with different concentrations were loaded. The plates were then incubated at 37 °C for 24 h. The antibacterial activity was determined by measuring the diameter of the zone of inhibition formed around the wells.

Determination of MIC by micro broth dilution technique:

MIC determination was carried out as reported in our earlier studies [15]. Cell suspensions from bacterial cultures grown on trypticose soya broth (adjusted to 1-2 x 10^8 cells/mL) were used as inoculumns. Aqueous dispersions of ZnO NPs of 62.5-0.75 µg/mL (two fold dilutions) in MH broth were tested against the test cultures. RPMI media (MH broth) inoculated with culture and without ZnO NPs was used as the control. 75 µL of the dispersion of test compound of different concentrations was mixed with 10 µL inoculum in 96 well plates in triplicate. 90 µL of RPMI media (MH agar) without drug mixed with 10 µL inoculums was used as the control. Treated bacterial cultures were incubated at 35-37 °C. The test plates were observed after 24-48 h and optical density was measured at 600 nm in tecan plate reader.

Percent inhibition was calculated as follows:

$$\frac{\text{[absorbance of control (untreated) - absorbance (treated)]}}{\text{absorbance (control)}}$$

MIC was determined as minimum concentration of ZnO NPs giving a minimum of 50 % inhibition of OD as compared with the control.

Evaluation of anticancer activity on HeLa by MTT assay:

Anticancer activity of ZnO NPs was carried out by MTT assay as reported in the literature [15]. In brief, Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 11% fetal bovine serum at 37 °C with 5% CO_2. Cells were plated in 96-well plates (10^5 cells/well) containing 100 µL of medium. ZnO NPs with different concentrations were dispersed in After 24 h, ZnO NPs with different concentrations dispersed in dimethyl sulfoxide (DMSO) were added to each well and incubated for 24 h. Similar amount of DMSO was added to control also. Growth of the cells was quantified by the ability of living cells to reduce the yellow dye MTT to a blue formazan product. At the end of 24 h of incubation the medium in each well was replaced by fresh medium (100 µL) containing 0.5 mg/mL of MTT. Four hours later, the formazan product of MTT reduction was dissolved in DMSO and absorbance was measured using a microplate reader. Effect of ZnO NPs was quantified as the percentage of control absorbance of reduced dye at 570 nm.

RESULTS AND DISCUSSION

Crystal structure:

The PXRD pattern of ZnO NPs is presented in Fig.1. The result was examined with Crystallographica Search-Match (CSM). PXRD of the sample showed the crystalline nature of the sample having hexagonal structure with the standard Joint Committee on Powder Diffraction Standards (JCPDS) No. [36-1451] corresponding to zincite pattern and can be indexed as hexagonal wurtzite type of ZnO. The crystallite size was calculated using Debye Scherrer equation, $D = k \lambda / \beta \cos \theta$, where D is the crystallite size, k is the Scherrer constant (0.9), λ is the X -ray wavelength, θ is the Bragg angle and β is the corrected half-peak width of the sample. The average crystallite size of ZnO NPs was found to be ~28 nm.

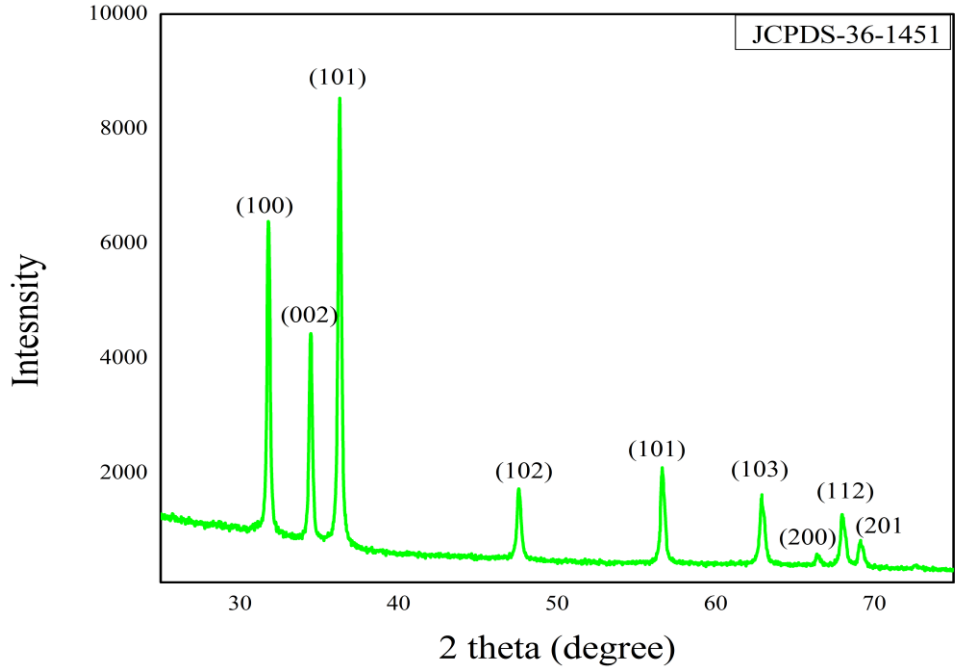

Fig. 1 PXRD pattern of ZnO NPs

Morphology:

The FE-SEM micrograph of ZnO NPs is shown in Fig. 2. The SEM micrograph of ZnO NPs shows that the morphology of particles is spherical. Micrograph reveals that besides the spherical crystals the powders also contain voids and pores, the reason for which can be traced to the large amounts of hot gases that escape out of the reaction mixture during combustion. The TEM study was carried out to understand the crystalline characteristics of the NPs. The particle size of powders can be determined from the TEM picture. The TEM method is better than X-ray line broadening in that it is direct and less likely to be affected by experimental errors and/or other properties of the particles such as internal strain or distribution in the size of the lattice parameter [18]. The HRTEM image of ZnO NPs is shown in Fig. 3. The mean particle size by histogram was found to be 30 nm. Various SAED patterns were obtained from the ZnO NPs. These patterns and the tilting angles matched well with the hexagonal ZnO crystal as shown in Fig. 3 (b-c).

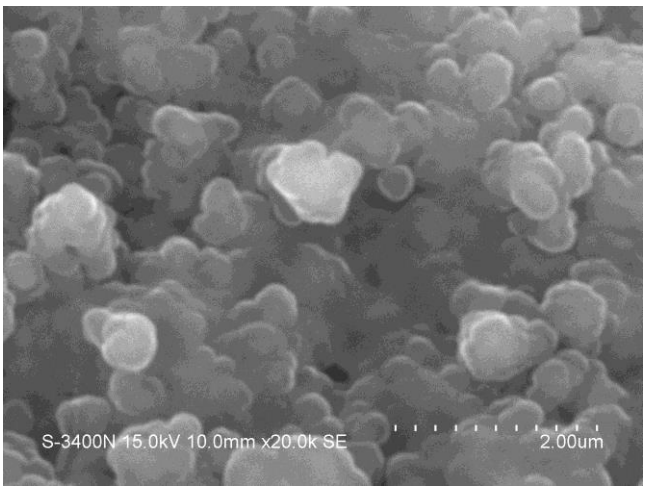

Fig. 2 SEM image of ZnO NPs

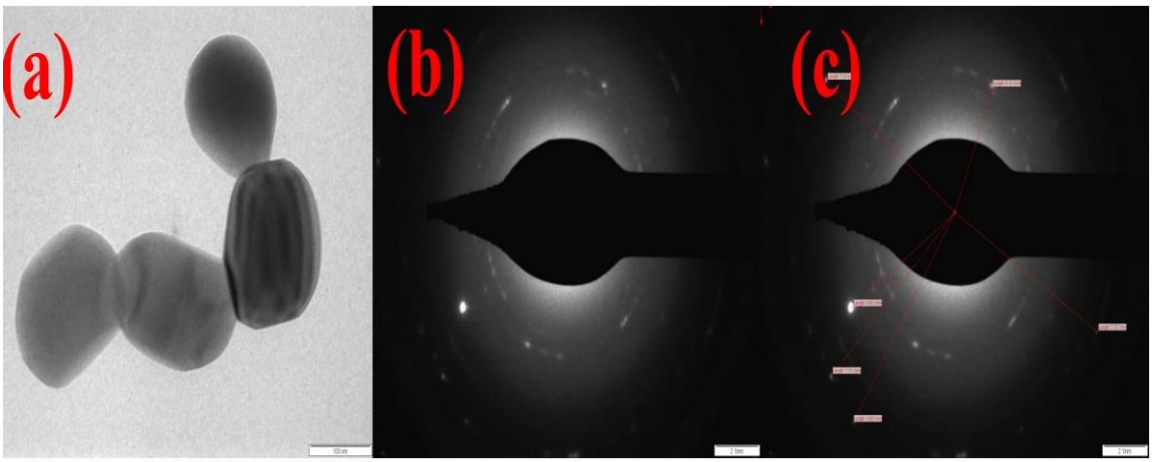

Fig. 3 (a) TEM image (b-C0, SAED patterns of ZnO NPs

Antibacterial activity:

The antibacterial results on ZnO NPs against *C. perfringens* and *S. enterica* are presented in Table 1 and the zone of inhibitions is shown in Fig. 4. These results indicate that the zone of inhibition is maximum for ZnO powder at a concentration of 500 µg/mL against *C. perfringens* and *S. enterica*. The results show that ZnO induced higher toxicity in gram-positive *C. perfringens* than the gram-negative *S. enterica*. The detailed mechanism of the bioactivity of ZnO is still under discussion. The main two probable mechanisms involved in the interaction between NPs and bacteria suggested by several investigations are (a) the production of increased levels of ROS, mostly hydroxyl radicals and singlet oxygen [19,20-23] and (b) Zinc toxicity of cell membrane by adhesion of ZnO particles which inhibits the bacterial growth [24,9].

As can be summarized from the antibacterial results, ZnO NPs exhibited higher zone of inhibitions for Gram-positive bacterial strain than the Gram-negative bacterial strain. This result might be indicative of higher Gram-negative strain tolerance against ZnO NPs over Gram-positive bacterial strains. Our these results are in agreement with the literature which reports that the ZnO NPs effect is more pronounced against Gram-positive bacterial strains than Gram-negative bacterial strains [25,26]. Results of MIC studies of ZnO NPs against *C. perfringens* and *S. enterica* are presented in Table 2. According to the MIC values *S. enterica* was more resistant to ZnO NPs than *C. perfringens.*

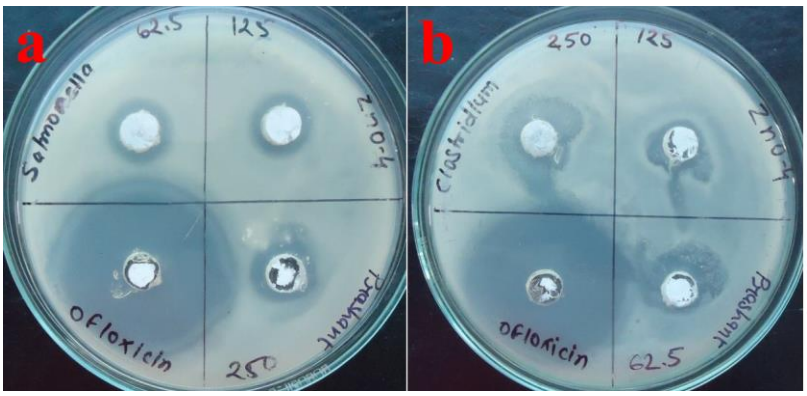

Fig. 4 (a-b) Zone of inhibition produced by ZnO NPs against *S. enterica* and *C. perfrinens* respectively

Anticancer activity:

Cytotoxic effect of ZnO NPs on HeLa cells is shown in Fig. 5. The determination of IC_{50} value of ZnO NPs HeLa using Graph Pad Prism 5 is shown in Fig. 6. The IC_{50} value for ZnO NPs on HeLa after 24 h was found to be 132.9 µg/mL. These antiproliferative studies clearly demonstrate that treatments with ZnO NPs sensitize cancer cells. A dose dependent decrease was observed in the cell viability. The results indicate that ZnO NPs at their highest concentration of 320 µg/mL tested in the present studies indicated around 21 % cell viability. Literature shows that ZnO NPs induce cytotoxicity in a cell specific and proliferation dependent manner by rapidly dividing cancer cells being the most susceptible and quiescent cells being the least sensitive [25,27]. However, the anticancer activity of ZnO NPs, in particular the mechanism of apoptosis in cancer cells due to ZnO NPs is still not clear.

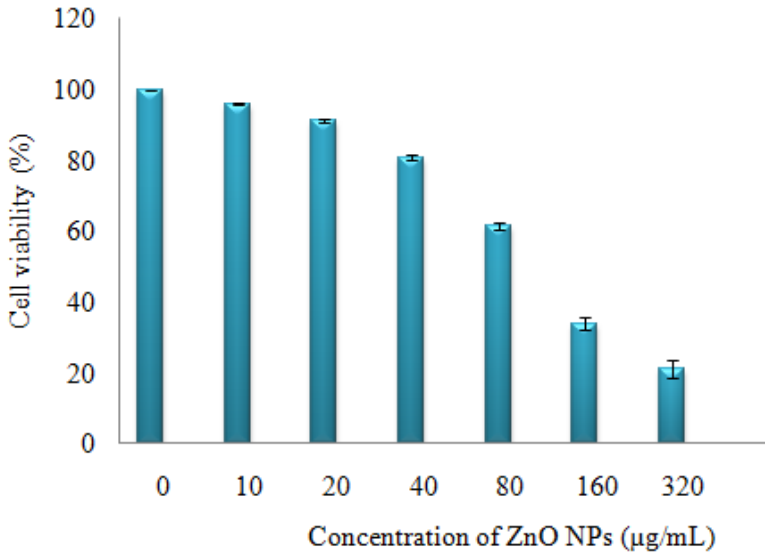

Fig. 5 Cytotoxic effect of ZnO NPs in HeLa cell lines. Cells were treated with various concentrations (0, 10, 20, 40, 80, 160 and 320 µg/mL) of ZnO NPs for 24 h grown in a serum free media. The percentage of cell death induced was determined using the MTT assay

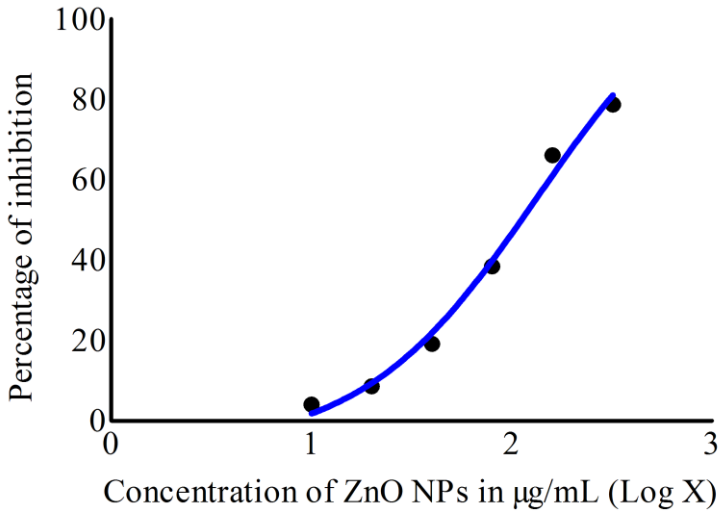

Fig. 6 Determination of IC_{50} value of ZNPs in HeLa

CONCLUSION

In this study, we focused on the antibacterial and anticancer activity of ZnO NPs prepared by simple and cost effective SCS using a fruit extract as bio fuel. This work confirmed the antibacterial efficacy of ZnO NPs against both gram-positive and gram-negative bacteria namely *C. perfringens* and *S. enterica*. MTT assay results indicate that when the concentration of ZnO NPs was 320 µg/mL, the viability of HeLa cells dropped to around 21 %. ZnO NPs, with their unique properties, are showing increasing application in cancer research and therapy. With their selective targeting property and usefulness as a carrier agent, ZnO NPs can be good substitutes for traditional cancer therapy. The results suggest that with the aid of oxide based NPs conditional chemotherapeutic agents may have even broader range of applications in the treatment of cancer cells. The dosage particles size dependent activity against cancer cells and the variation in toxicity need to be further investigated to establish optimum standard.

ACKNOWLEDGEMENTS

The author G. K. Prashanth thanks the Management of Sri KET and Dr. V R Manjunath, Principal of Sir M. Visvesvaraya Institute of Technology, Bengaluru for their constant encouragement. The authors acknowledge SAIF, IITB for TEM and CIF, Pondicherry University for SEM measurements.

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