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ASPERGILLUS NIGER MEDIATED SYNTHESIS OF ZNO NANOPARTICLES AND THEIR ANTIMICROBIAL AND *IN VITRO* ANTICANCEROUS ACTIVITY

S.Justin Packia Jacob*, R.Bharathkumar and Ashwathram.G

Department of Biotechnology, St.Joseph's College of Engineering, Chennai, India – 600119.

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***Correspondence for Author**
Dr. S.Justin Packia Jacob
Department of Biotechnology, St.Joseph's College of Engineering, Chennai, India.

ABSTRACT

Nanoparticle metal oxides represent a new class of important materials that are increasingly used in research and health-related applications. Highly ionic metal oxides are interesting not only for their wide variety of physical and chemical properties but also for their antibacterial activity. The present study describes the synthesis, characterization, antimicrobial and anticancerous screening of Zinc Oxide (ZnO) nanoparticles. The ZnO nanoparticles were prepared using the fungal filtrate and characterized using UV–visible spectroscopy, FTIR (Fourier-transform IR) spectroscopy, EDX and SEM (scanning electron microscopy) analysis. Agar-well diffusion method was used to evaluate the antimicrobial activity. The sizes of the spherical ZnO nanoparticles were found to be in the range of 39.4-114.6 nm. The results revealed that ZnO nanoparticle is an effective broad spectrum antimicrobial agent and also it exhibit significant cytotoxic effect on HEp-2 cells.

Key Words: Fungal, antimicrobial, anticancerous, ZnO Nanoparticles.

1. INTRODUCTION

Inorganic materials such as metal and metal oxides have attracted increased attention over the past decade due to their ability to withstand harsh process conditions [1, 2]. Of the inorganic materials, metal oxides such as TiO2, ZnO, MgO and CaO are of particular interest as they are not only stable under harsh process conditions but also generally regarded as safe materials to human beings and animals [1, 3]. Progress in utilizing inorganic nanoparticles

for biomedical applications has advanced rapidly due to the extensive amount of work done in the synthesis and modification of these particles [4].

Nanoparticles have been shown to inhibit growth of *Escherichia coli*, *Pseudomonas aeruginosa* and few other microorganisms [5-7]. Zinc oxide has attracted wide interest because of its good photocatalytic activity, high stability, antibacterial property and non-toxicity [8-10]. Zinc oxide and magnesium oxide nanoparticles are reported to disrupt membrane architecture, alter permeability and subsequent accumulation in the cytoplasm [3, 11]. However, the precise nature and mechanism of membrane–nanoparticle interactions are yet to be fully understood. Apart from ZnO nanoparticles, there are many reports about the anticancer activity of silver nanoparticles [12, 13, 14] produced using plant extracts. The effect of nanoparticle on different types of membranes may provide better insight into understanding of membrane–nanoparticle interactions. Moreover ZnO utilizes a multifunctional nanoplatform that bombards malignant cells from the outside, through the external release of reactive oxygen species (ROS) [15].

Various fungal species such as *Fusarium oxysporum* [16], *Fusarium semi- tectum* [17], *Aspergillus fumigates* [18], Pleurotus sojarcaju [19], *Penicillium brevicompactum* [20], *Clostridium versicolor* [21] and *Alternaria alternate* [22] are being used for the synthesis of silver nanoparticles; hence in the present study an attempt is made to synthesize ZnO nanoparticles using *Aspergillus niger*. The nanostructures of the prepared ZnO particles have been confirmed using UV-vis absorbance, EDX and SEM analysis. The antimicrobial and cytotoxic effects of these nanoparticles are also reported.

2.Experimental

All the chemicals and media were purchased from Himedia Ltd., India and were of analytical grade. The *Aspergillus niger* culture was procured from MTCC and *Escherichia coli* (A.T.C.C.8739), *Staphylococcus aureus* (A.T.C.C. 6538) and *Klebsiella pneumoniae* (A.T.C.C. 13883) were procured from ATCC. The antitumor assay was performed on Human laryngeal epithiloma cells (HEp2) obtained from King Institute of Preventive Medicine, Chennai, India.

2.1. Synthesis of ZnO nanoparticles

The fungal isolate *Aspergillus niger* was cultured in a Potato Dextrose broth incubated at 28±4 °C and at a speed of 150 rpm in an orbital shaker. After 72 h of incubation, the fungal

filtrate was obtained by passing through Whatman No.1filterpaper. The supernatant was separated and stored in a centrifuge tube under refrigerated conditions for further use. 100 ml of 0.1 M solution of zinc sulphate (3mM) was mixed with 10 ml supernatant in a 250mL Erlenmeyer flask and the flask was agitated continuously in a shaker at 150 rpm for 96 h. Conical flasks with either fungal filtrate or zinc sulphate served as positive and negative control respectively.

2.2. UV-vis spectra analysis

ZnO is soluble in distilled water and the colour changes were observed visually. An opaque white colouration was noticed at the synthesis phase. The concentration of ZnO nanoparticles produced was measured using a Systronics UV double-beam spectrophotometer (model 2201), at a resolution of 1 nm, between 200 and 600 nm using 10-mm-optical-path-length quartz cuvettes.

2.3. FTIR (Fourier-transform IR)

Studies on the samples were carried out using Nicolet Impact 400 FTIR spectroscopy to ensure the formation of ZnO nanoparticles.

2.4. EDX analysis

Energy dispersive X-ray (EDX) spectroscopy analysis for the confirmation of elemental ZnO was carried out for the detection of ZnO nanoparticles.

2.5. SEM analysis ZnO nanoparticles

A scanning electron microscope (JEOL 6380A; Tokyo, Japan) was used to record the micrograph images of synthesized ZnO nanoparticles.

2.6. Antimicrobial activity

The study was performed using agar-well diffusion method [23]. The solidified LB agar plates were swabbed with 100 µl of *Escherichia coli* (ATCC-8739) and solidified NB agar plates were swabbed with 100 µl of *Staphylococcus aureus* (ATCC-6538) and *Klebsiella pneumoniae* (ATCC-13883) [10^8 cfu (colony-forming units)]. The wells were prepared on the LB and NB agar plates with the help of a cork borer (10 mm diameter) and the following concentrations of solutions: 50, 100 and 150 µg/ml of ZnO nanoparticle was loaded on to each well. The plates were then incubated overnight at 37°C and the antimicrobial effect was determined by measuring the diameter of the zone of inhibition.

2.7. Cytotoxicity Assay

The cytotoxicity assay of the prepared ZnO nanoparticle was measured using MTT test [24]. The cells were seeded in 24 well tissue culture plates at a density of 1 x 10^{6}, allowed to attach for 24 h and treated with different concentration (3.9 to 500 μl/ml) of ZnO nanoparticles. After the ZnO nanoparticle treatment, the medium was changed and the cells were washed twice with MEM without FCS to remove the dead cells, the cells were incubated with 200 μl (5mg/ml) of MTT for 6-7 h in 5% CO2 incubator for cytotoxicity. Cell viability was marked by the conversion of the tetrazolium salt MTT to a coloured formazan by the mitochondrial dehydrogenases. Colour development was measured photometrically using a spectrophotometer at 595nm after cell lyses in DMSO. The untreated cells absorbance was used as a control reference. The viability was calculated using the following formula.

$$\text{Cell viability (\%)} = \frac{\text{Mean OD}}{\text{Control OD}} \times 100$$

3. RESULTS

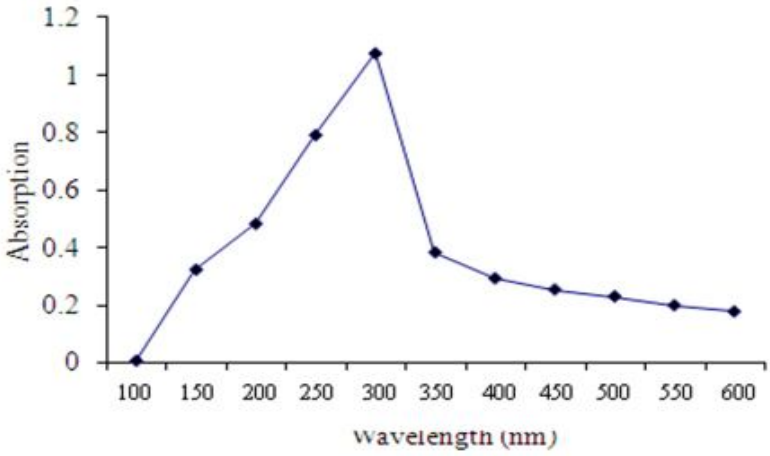

Figure 1. UV-visible spectra of fungal filtrate containing ZnO nanoparticles

The fungal cell filtrate after addition of aqueous zinc sulphate was subjected to optical measurements by UV-vis spectrophotometer. This analysis showed an absorbance peak at 300 nm (Fig.1), which was specific for the ZnO nanoparticles. A cloudy white precipitate was observed and ZnO nanoparticle was harvested as white powder. The sample was stored in a refrigerator and used for further characterization studies. The FTIR spectrum (Fig.2) of ZnO nanoparticles showed distinct peaks at 1615 cm^{-1}, which represent the involvement of

C=N in plane vibrations of aminoacids, the bands from 1099 – 1142 cm^{-1} represent the involvement of C-N in plane vibrations of aliphatic amines. The above bonds commonly occur in proteins indicating the presence of proteins as ligands for ZnO nanoparticles, which increase the stability

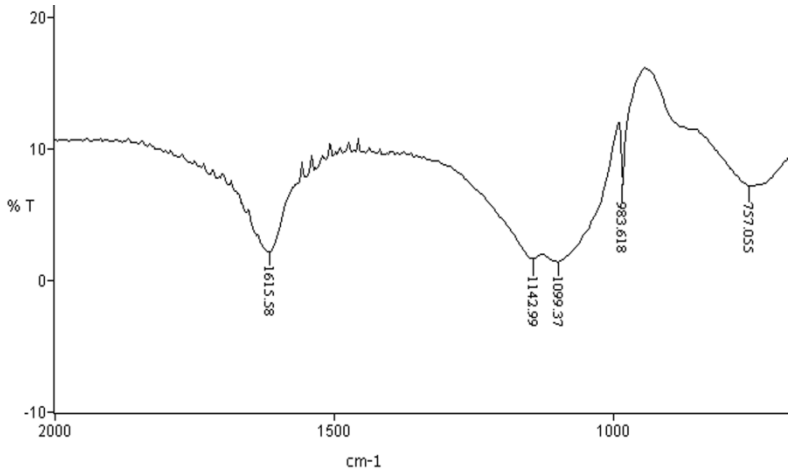

Figure 2. FTIR spectra of ZnO nanoparticles

of nanoparticles synthesized. Rest of the bands showed resemblance to alkenes (983 cm^{-1}) and aromatic (757 cm^{-1}) groups, which is present in the fungal filtrate might have an effect in the synthesis of nanoparticles. The elemental analysis data obtained from EDX is shown in Fig.3.

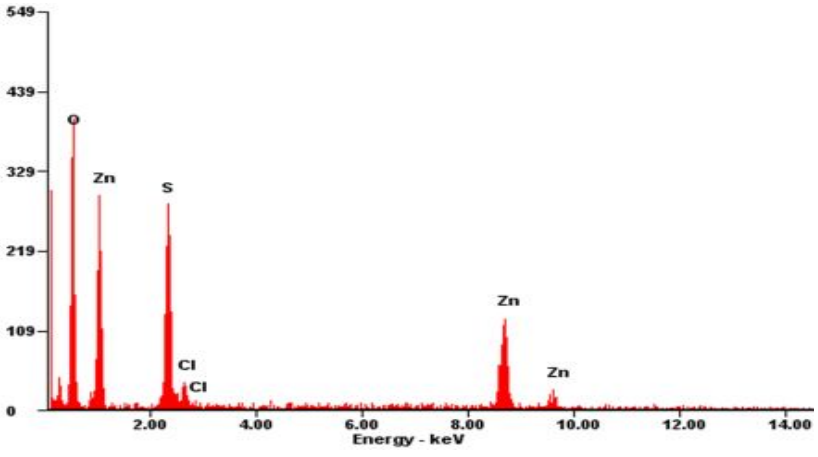

Figure 3. Spectrum of ZnO nanoparticles obtained by EDX spectroscopy

The peaks of Oxygen and Zinc at 0.5, 1.00, 9.00 and 9.8 –eV confirms the formation of ZnO nanoparticles. The size of the spherical ZnO particles was found to be in the range of 39.4-114.6 nm (Fig.4). Significant inhibitory effect was observed against *Klebsiella pneumoniae, Escherichia coli (*gram negative) *and Staphylococcus aureus* (gram positive) bacteria

(Table.1). The cytotoxic effect of ZnO nanoparticle was determined using HEp-2 cell lines by MTT-assay (Fig.5). Significant cytotoxic effect (93%) was observed at 500 µg/ml concentration of ZnO nanoparticle, whereas, at 62.5 µg/ml 51% death (49% viability) was observed (Fig.6).

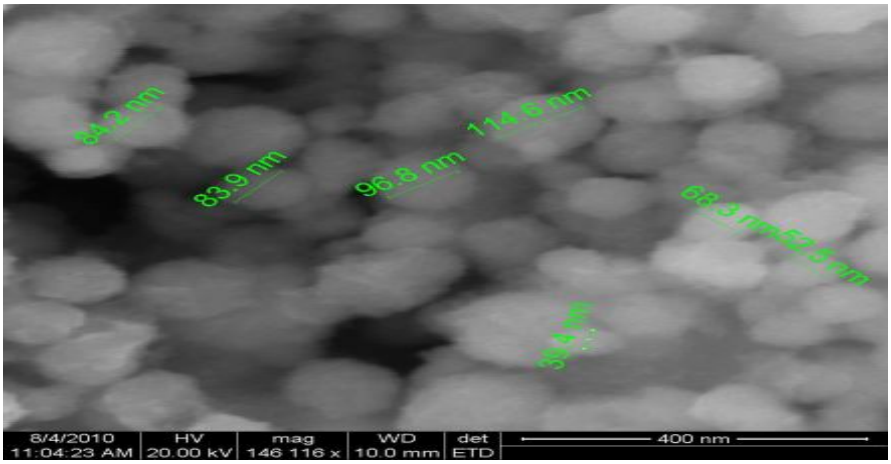

Figure 4. SEM image of ZnO nanoparticles

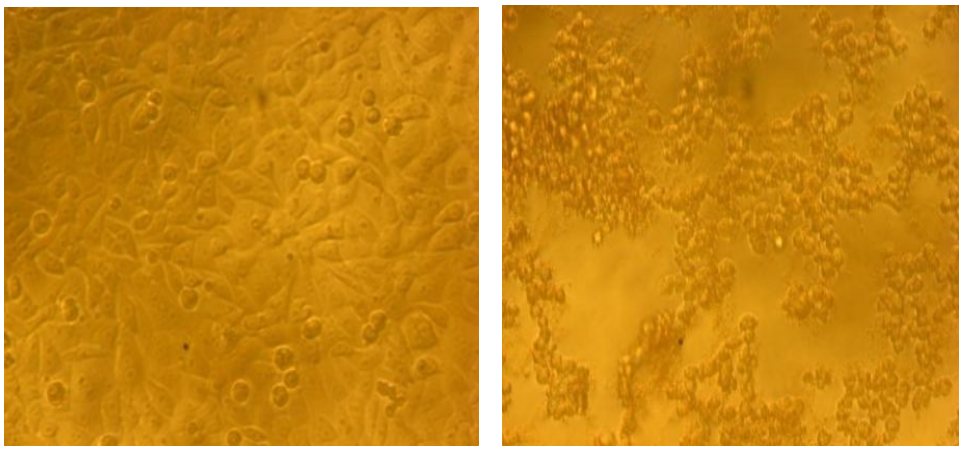

Figure 5. Image of Hep-2 cell line a).Control b). ZnO nanoparticle treated cells

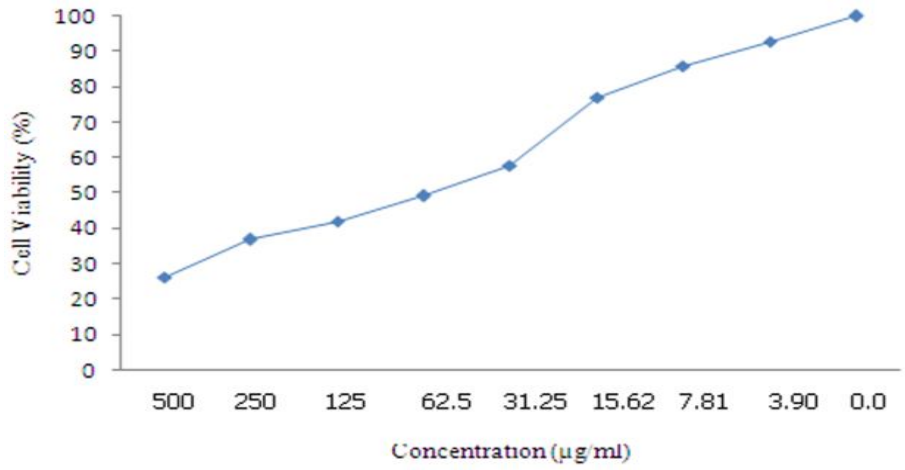

Figure 6. Cytotoxicity studies of ZnO nanoparticles on Hep-2 cell line.

4. DISCUSSION

In this paper, we have shown for the first time the use of *Aspergillus niger* filtrate in the extracellular synthesis of ZnO nanoparticles. In the biosynthesis of metal oxide nanoparticle by a fungus, enzymes are produced which reduce a salt to its metallic solid nanoparticles through the catalytic effect. The fungal cell filtrate mediated synthesis of ZnO nanoparticle was confirmed

Table1. Antimicrobial activity of ZnO nanoparticle (average of three replicates)

Organisms	Concentration (µg/ml)	Zone of Inhibition(mm)
Staphylococcus aureus	50	12
	100	15
	150	17
Klebsiella pneumoniae	50	12
	100	16
	150	18
Escherichia coli	50	12
	100	16
	150	21
Pseudomonas aeruginosa	No activity	

by UV-vis spectrophotometer. This analysis showed an absorbance peak at 300 nm, which was specific for the zinc oxide nanoparticles. The exact mechanism of the synthesis of ZnO nanoparticles was not known, but in the earlier report, it was confirmed that the presence of NADP- dependent nitrate reductase enzyme in extracellular cell filtrate of the fungus was responsible for the synthesis of silver nanoparticles [25]. The distinct peaks at 1615 cm^{-1}, which represent the involvement of C=N in plane vibrations of aminoacids confirmed proteins as ligands for ZnO nanoparticles, which increases the stability of nanoparticles synthesized. In the earlier study, Quercetin which belongs to a group of plant pigments called flavonoids have been used for silver nanoparticle synthesis [26]. Since *A.niger* is used in the industrial production of vitamin C [27], a band at 757 cm^{-1} (aromatic groups) might have appeared due to the involvement of vitamin C in the formation of ZnO nanoparticles.

The sizes of the spherical ZnO nanoparticles were found to be in the range of 39.4-114.6 nm. It has already been proved that both nano-sized and micron-sized ZnO suspensions are active in inhibiting the bacterial growth; the nano-sized ZnO suspension clearly has a much higher activity than the micron-sized ZnO suspension [28]. In par with the above, in the present study ZnO nanoparticle was found to have a broad spectrum of antibacterial activity.

Significant inhibitory effect was observed against *Klebsiella pneumoniae, Escherichia coli (*gram negative) and *Staphylococcus aureus* (gram positive) bacteria. It seems that active oxygen species generated by ZnO nanoparticles could be responsible for the antimicrobial activity, the presence of active oxygen species has been detected by Yamamoto *et al* [29].

Significant cytotoxic effect of ZnO nanoparticle was also determined using HEp-2 cell lines by MTT-assay. In the earlier investigation, it was observed that ZnO nanoparticles are selective in exerting cytotoxic effect on cancerous cells but no cytotoxic effect was observed on normal cells [6]. This kind of selective cytotoxic effect was identified due to the generation of ROS in the glioma cells, whereas normal astrocytes exhibited lower levels of ROS in response to the ZnO nanoparticles [30]. Reactive Oxygen Species (ROS) typically include the superoxide radical, hydrogen peroxide and the hydroxyl radical, which cause damage to cellular components such as lipids, DNA and proteins and eventually death [31]. The toxicity of ZnO nanoparticles against rapidly dividing HEp-2 cells raises exciting opportunities for their potential use as anti-cancer agents. Further studies are needed to check the cytotoxic effect of ZnO nanoparticles on normal human epithelial cells. Toxicity results of ZnO nanoparticles in Chinese hamster ovary cells indicated a NOAEC and IC50 at 54 and 340 g/ml, respectively [32]. The preferential toxicity of ZnO nanoparticles towards cancerous T-cells in a substantial magnitude (28-35 times) was also reported [33]. Therefore, accurately assessing the toxicity and safety of these nanomaterials to human health is of utmost importance. Further studies are needed to clarify the possible anticancer application of these nanoparticles for human use and to analyze the molecular mechanisms behind the effects observed.

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