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Biosynthesis of Zinc Oxide Nanoparticles Using Bacillus Species Potentiates Anticancer and Antimicrobial Activity

Hanumitha. S[1], Mahalakshmi. V[2], Abirami. S[3]
[1]Research Scholar, [2]Assistant Professor, [3]Research Scholar and Correponding Author
[1,2]Madras Christian College, [3]V. R. R. Institute of Biomedical Sciences
Chennai, Tamil Nadu, India

ABSTRACT
The biological methods for synthesis of nanoparticles by using various microorganisms, enzymes, plants, and their extracts have been suggested as the probable and promising eco friendly alternatives to the chemical and physical methods of synthesis. The biocompatibility of nanoparticles is more essential for specific biomedical applications and researches. The aim of this study was to evaluate the effect of ZnO nano particles for anticancer and antimicrobial activity. In this study ZnO Nano particles were synthesized by *Bacillussubtilisusing* Zinc acetate dihydrate as substrate and sodium borohydride as reducing agent. The preliminary confirmation of the presence of the nanoparticles was the formation of precipitate and white coloration in the final mixture. The confirmation of the presence of nanoparticles was carried out by SEM analysis. The antimicrobial activity of nanoparticles was determined by well diffusion method and also the synergistic effect of nanoparticles with antibiotics on bacteria was determined by well diffusion method. The anticancer activity of the nanoparticles on MDA MB321 (human breast cancer cells) was determined by MTT assay.

Keywords: *Nanoparticles, anticancer, antimicrobial.*

INTRODUCTION
Nanoparticles (NPs) can be synthesized by chemical and biological methods. NPs synthesized by chemical processes have toxic effects hence there is a growing need to synthesize environmental friendly, cost effective and conveniently reproducible green methods of NP synthesis. (Deepali Sharma, *et. al.,* 2010). When nanoparticles are used for therapeutic purposes, it is mandatory that they exhibit very less or zero toxicity. Therefore biosynthesis, using organic systems such as yeast, fungi, bacteria and plant extracts are developing increasing favour. (Kowshik M*et.al.,* 2002).As most of the bacteria have developed resistance to antibiotics; there is a need of an alternative antibacterial substance. Various types of metals and metal oxide NPs have so far been used based on their antimicrobial activities. However, most metals are toxic for humans with the exception of Ag which is toxic only to prokaryotic cells and viruses. Ag is non-toxic, safe inorganic antibacterial agent being used for centuries and is capable of killing about 650 types of disease-causing organisms in the body (Raffi M, Hussain F *et.al.,*2008).Zinc oxide nanoparticles have received considerable attention due to their unique antibacterial, antifungal, and UV filtering properties, high catalytic and photochemical activity (Duran N *et.al.,*2011). The antibacterial effect of zinc oxide (ZnO) nanoparticles on *Campylobacterjejuni* was investigated for inhibition and inactivation of cell growth. The results showed that *C. jejuni* was extremely sensitive to treatment with ZnO nanoparticles. The action of ZnO nanoparticles against *C. Jejuni* was determined to be bactericidal, not bacteriostatic (Sastry M *et. al.,* 2003). In addition, the current study has clearly demonstrated that the particle size variation and surface area to volume ratio of green ZnO nanoparticle are responsible for significant higher antimicrobial activity. From the results obtained it is suggested that green ZnO NPs could be used effectively in agricultural and food safety applications and also can address future medical concerns.

Aim and Objectives:

To determination the antibacterial and anticancer activity of zinc oxide nanoparticles synthesized from *Bacillus species.*

- Isolation and characterization of *Bacillus species* from soil.
- Biosynthesis of nanoparticles.
- Detection and characterization of nanoparticles by SEM analysis.
- Determination of antibacterial activity of the zinc oxide nanoparticle and its synergistic effect with antibiotics.
- Analysing the anticancer activity of Zinc oxide nanoparticles.

Materials and methods:

1. Isolation of *Bacillus species*from soil:

Damp rhizosphere soil was collected and heated at 80˚C for 10 minutes to remove unwanted bacteria. The serially diluted soil sample was inoculated onto agar medium by streak plate technique for pure isolated colonies. Based on the colony morphology and Gram's staining the organism was identified as *Bacillus species.*

2. Biosynthesis of Zinc Oxide nanoparticles:

The bacterial culture was allowed to grow in nutrient broth for 24 h. The culture was diluted four times by adding 75ml of sterile nutrient broth. This diluted culture solution was allowed to grow for 24 hours. The bacterial suspension was injected with 50nM zinc acetate dihydride aqueous at a rate of 10ml/min through a burette. The mixture was stirred for 30 min after which 200ml of 50mM $NaBH_4$ aqueous solution as a reducing agent was slowly poured into the mixture at a flow rate of 10ml/min through the burette. Then the final mixture was robustly stirred for 24 h at room temperature. After the mixture has fully reacted, it was centrifuged repeatedly and subsequently the precipitate was collected (Shamsuzzaman*et.al.,* 2014). The powdered form of the nanoparticles was obtained by drying the pellet sample at 50˚C in a hot air oven for about 6 hours. The powdered sample was used for SEM analysis and anticancer studies. (Fig.no. 1)

Figure1. Biosynthesis of Nanoparticles

3. Detection and characterization of nanoparticles SEM Analysis

SEM analysis was carried out to detect the presence of nanoparticles and to characterize them. Images and particle size of the nanoparticles synthesized from bacteria were obtained.

4. Determination of antibacterial activity of the zinc oxide nanoparticles.

Antibacterial activity: Wells were cut in the Mueller Hinton Agar (MHA) and the medium was then swabbed individually with the following organisms namely *E. coli, Pseudomonasaeruginosa, Staphylococcusaureus, Salmonellaparatyphi B, Shigellaboydii, and Proteus vulgaris.* Samples of nanoparticles (100µl) were added into the wells. The plates were incubated at 37˚C for hours.

5. Determination of the synergistic effect of the nanoparticles and antibiotic discs on bacteria

The MHA agar plates were swabbed with the previously mentioned organisms. The antibiotics amikacin (AK), nalidixic acid (NA) and novobiocin (NV) were used against gram negative bacteria and the antibiotics chloramphenicol (C), erythromycin (E) and cefazolin (CZ) were used against Gram positive bacteria. The antibiotic discs were dipped in 20µl of the nanoparticle solution and placed on the medium swabbed with the respective bacteria, they are effective against. Control plates with uncoated antibiotic discs were maintained. The plates were incubated at 37˚C for 24 hours.

6. Analysing the anticancer activity of Zinc oxide nanoparticles

Cell line: MDA MB231 cell line is derived from human breast cancer cells.

MTT assay

The cell proliferation was determined using the 3-(4,5-Dimethylthiazol-2-Yl)-2,5 Diphenyltetrazolium Bromide (MTT) assay (A. Arunkumar.et.al.2006). MDA MB231 cells (1×105 cells/well) were incubated with different concentrations of nanoparticles (10-100ug/ml) for 48 hrs in 96 well plates and were incubated at 37˚C in a humidified mixture of 5% CO2 and 95% air in an incubator. (A. Tsubura et. al 2011). Stock solutions of compounds were initially dissolved in DMSO and further diluted with fresh complete medium. 25 µl of MTT reagent (5 mg/ml in PBS), was added to each well and incubated at 37˚C for 3 hrs. At the end of the incubation period, the supernatants were removed by tilting plate completely without disturbing cell layer and 150µl of DMSO was added to each well. After 15 min of shaking, the

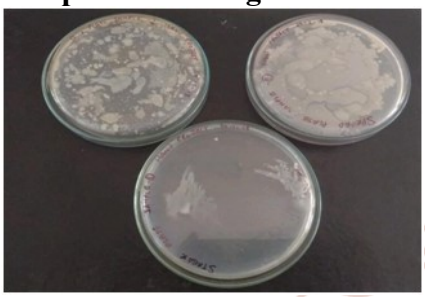
readings were recorded as absorbance at 590 nm on a microplate reader (Biotek, USA).

Results:

1. Isolation of *Bacillus species* from soil.

Following streak plate technique, colonies with dry, flat, and irregular, with lobate margins were observed on nutrient agar plates. (Figure: 2).

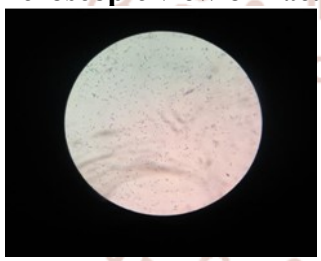
Figure 2: plates showing Bacillus colonies



2. Identification of bacteria

Gram's staining: Gram's staining was carried out to confirm *Bacillus species.* Gram positive long rods were observed under high power objective.(Figure:3). Further confirmation was done by spore staining.

Figure: 3 Microscopic view of *Bacillus species*



Synthesis of nanoparticles

After incubation there was formation of precipitate and white coloration in the final mixture, which was a preliminary confirmation for the presence of nanoparticles. (Figure: 4a and 4b).

Figure 4a: Precipitate formation

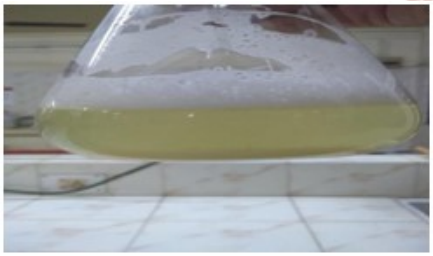


Figure 4b: Final mixture

Detection and characterization of nanoparticles by SEM analysis

Scanning electron microscopy analysis was carried out to determine the size and shape of the nanoparticles. The size of the nanoparticles was found to be in the range of 100 – 200 nm and the shape appeared to be spherical (Figure:5)

SEM ANALYSIS

Figuress 5a (Magnification 500X)

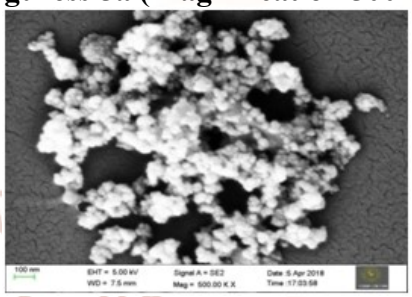
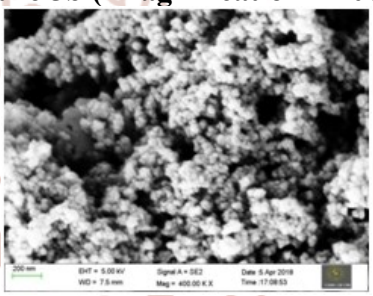


Figure 5b (Magnification – 400X)



Antibacterial activity of the zinc oxide nanoparticles.

Zones of inhibitions were seen in plates of *Klebsiellapneumoniae, Escherichiacoli.*

Plates showing the antibacterial activity of ZnO NPs (Fig: 6a and 6b)

Fig 6a: Antibacterial activity against <u>*E.coli*</u>

Fig 6b: Antibacterial activity against <u>*K. pneumoniae*</u>

Synergistic effect of the nanoparticles with the antibiotic discs against pathogenic bacteria – *diameter of* zones of inhibition.

Graphical representation of synergisitc effect of ZnO NPs and antibiotic discs on the test bacteria (Figure: 7, 8)

Figure 7:

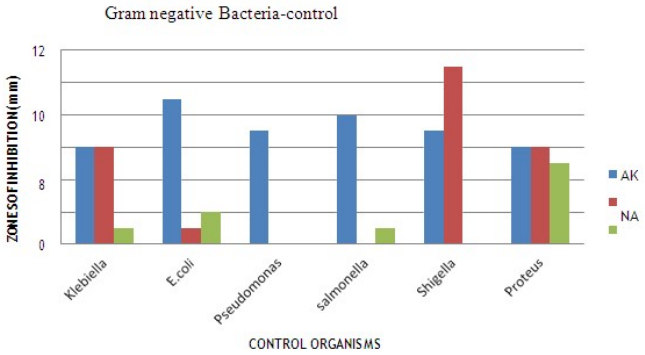
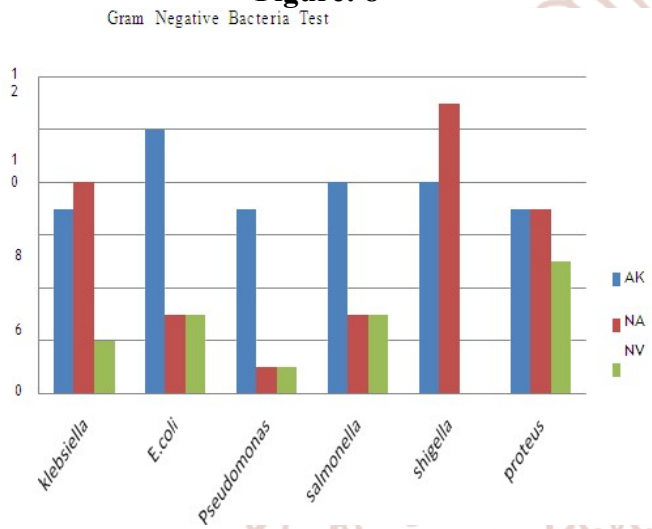


Figure: 8



Plates showing the synergistc effect of ZnO NPs on bacteria (Figure: 9a, 9b, 10a&10b)

Fig 9a:E.coli control and test

Fig 9b: Klebsiella control and test

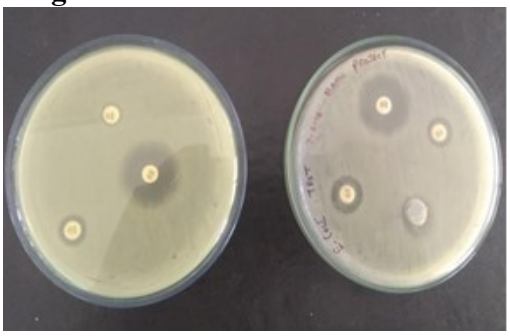


Figure 10a: Shigella control and test

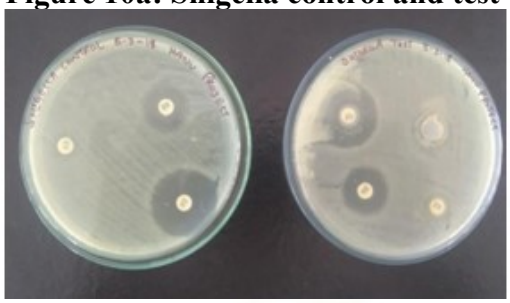
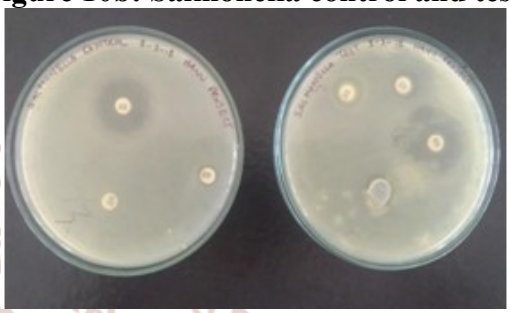


Figure 10b: Salmonella control and test



Anticancer activity of ZnO nanoparticles

The cytotoxic effects of different concentrations of ZnO NPs on human breast cancer cell lines (MDA MB231) were studied. Results showed that at higher concentrations there is significant mortality. A minimum concentration of 60µg induces 50% of cell mortality (Table: 3) (Figure: 11)

Table 3: Table showing anticancer activity of nanoparticles against human breast cancer cell

S. No	Concentration (µg/ml)	MDA MB231 cell lines
1.	0	100
2.	10	80
3.	20	71
4.	40	59
5.	60	50
6.	80	49.3
7.	100	44.5

Graph representing the anticancer activity of ZnO NPs on human breast cancer cells (Figure: 11)

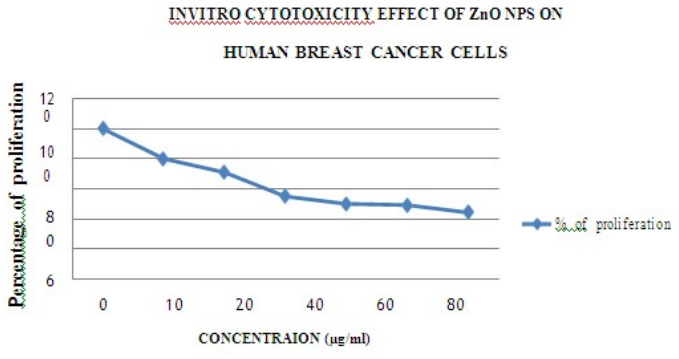


Figure: 12 - MICROSCOPIC IMAGES OF THE FFECT OF ZnO NPs ON HUMAN BREAST CANCER CELLS.

Figure 16a: Untreated cells

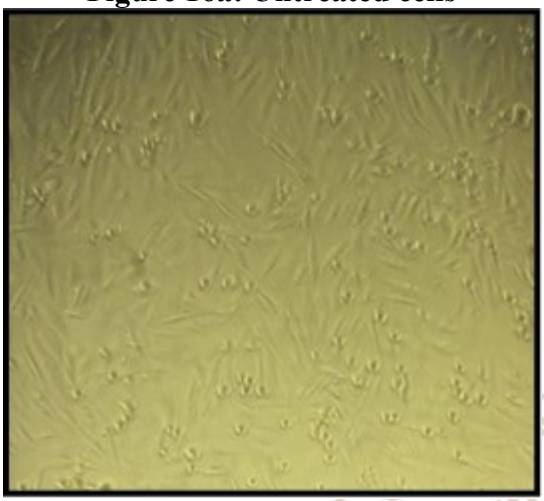
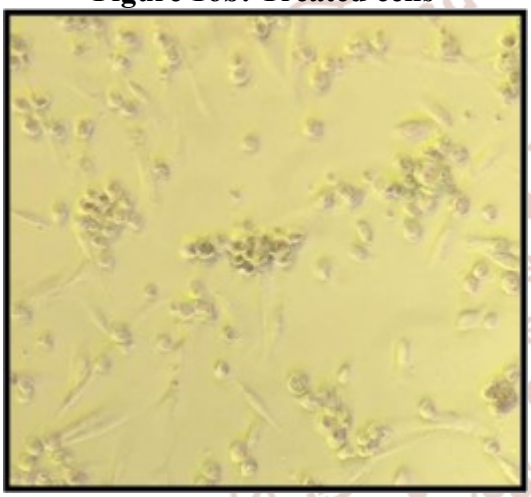


Figure 16b: Treated cells



DISCUSSION:

Zinc (II) ion solution is reduced into ZnO nanoparticles using microbes like *Bacillus thuringienesis* and *Bacillus species*. Characterization of Zinc nanoparticles was done by UV-Visible spectrophotometer for its Surface Plasmon resonance, SEM for morphologies, XRD and FTIR for its crystalline nature (A. K. Jha, et. al, 2010). Since bacteria belonging to the genus *Bacillus* are good candidates for the synthesis of nanoparticles, Bacillus was chosen for the study. In the isolation step, the sample was subjected to heat at 80˚C for *Bacilli* are thermo tolerent organisms, which will eliminate all the other organisms in the soil. The preliminary confirmation of the presence of the nanoparticles was the formation of precipitate and white coloration in the final mixture.This is in conformity with the procedure adapted by Shamasuzzman *et.al in the year*2017 which says while synthesizing the nanoparticles, both the zinc acetate solution and sodium borohydride solution were added in drops through a burette, set at a fixed flow rate, so that there is proper mixing of all the components to enable high yield. A low cost and simple procedure for synthesis of zinc oxide NPs using reproducible bacterium, *Aeromonashydrophila,* was reported. X-ray diffraction (XRD) confirmed the crystalline nature of the NPs, and atomic force microscopy (AFM) showed the morphology of the nanoparticle to be spherical, oval with an average size of 57.72 nm. The antibacterial and antifungal activity was tested with corresponding well diffusion and minimum inhibitory concentration. The maximum zone of inhibition was observed in the ZnO NPs against *Pseudomonas aeruginosa* (~ 22 ±1.8 mm) and *Aspergillusflavus* (~ 19 ±1.0 mm) (Jayaseelana C *et.al.,* 2014).In this study, the confirmation of the presence of nanoparticles was carried out by SEM analysis.SEM results confirmed the presence of the nanoparticles. The nanoparticles were spherical in shape and its size was about 100 to 200 nm. The antimicrobial activity of nanoparticles was determined by well diffusion method and the synergistic effect of nanoparticles with antibiotics on bacteria was determined by well diffusion method. The nanoparticles had antibacterial activity on the bacteria *E.coli, K. pneumoniae*and *P. aeruginosa*. In the presence of nanoparticles, the efficiency of the antibiotics was increased. Selvakumari D *et. al.,in the year* 2015 reported that the anticancer activity on MCF7 (Breast cancer cell) and A549 (Lung Cancer cell) were determined by the MTT (Methylthiazolydiphenyltetrazolium bromide) assay. A549 and MCF-7 cells were exposed to ZnO-NPs and it exhibited 50% reduction at a very low concentration 31.2 µg/ml. Thus, the reduction in cell viability with NPs induces cytotoxicity in cancerous cells. There is a size dependent effectiveness of ZnO nanoparticles in the removal of cancer cells and also a positive correlation with reduced toxicity. Similarly the anticancer activity of the nanoparticles on MDA MB321 (human breast cancer cells) was determined by MTT assay. ZnO nanoparticles had anticancer activity against human breast cancer cell line (MDA MB321). The cells showed 50% decrease in their number at the nanoparticle concentration of 60µg/ml.

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