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Comparative Study of the Zno and Zno Coated with Sio_2 As Potential Antimicrobial and Anticancer Drugs

Preetha Bhadra[1]*, Biplab Dutta[2], Debopriyo Bhattyacharya[2] and Sampad Mukherjee[2]*

[1]Department of Physics, Indian Institute of Engineering Science and Technology, Shibpur and Centurion University of Technology and Management, Bhubaneswar Odisha India
[2]Department of Physics, Indian Institute of Engineering Science and Technology, Shibpur West Bengal India

ABSTRACT

Zinc, as one of the major trace elements of the human body and co-factor of more than 300 mammalian enzymes, plays an important role in maintaining crucial cellular processes including oxidative stress, DNA replication, DNA repair, cell cycle progression and apoptosis. Thus, it is evident that an alteration in zinc levels in cancer cells can cause a deleterious effect. Research has shown that low zinc concentration in cells leads to the initiation and progression of cancer and high zinc concentration shows toxic effects. Zinc-mediated protein activity disequilibrium and oxidative stress through reactive oxygen species (ROS) may be the probable mechanism of this cytotoxic effect. ZnO has a neutral hydroxyl group attached to its surface, which plays an important role in its surface charge behaviour. Our aim is to show that the effect of Zinc Oxide and Silica coated Zinc Oxide on different microbes and cancer cells. Characterization of Zn nanoparticles have been done by using different analyzing techniques i.e. UV-Vis Spectroscopy, DLS (Dynamic Light Scattering) and SEM (Scanning Electron Microscope). The effect of the Zn nanoparticles on microbes has been measured by the cup disk method where as the effect on cancer cell line (HeLa) (Human Cervical Cancer Cell Line) has been measured by Fluorescence Anisotropy, MTT assay, Reactive Oxygen Species (ROS). The effect on different enzymatic action has also been measured. Regardless of antimicrobial medicinal consideration, dismalness and mortality identified with these microorganism contaminations remain high, somewhat because of the adaptability of those life forms to create protection from almost all anti-infection agents. Our aim is to develop new drugs spot and build up the resulting age of prescription or operators to manage microorganism contaminations.

KEY WORDS: ZINC OXIDE, REACTIVE OXYGEN SPECIES, FLUORESCENCE ANISOTROPY, MTT ASSAY

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INTRODUCTION

Bio nano molecules are those, whose size is comparable with nanoparticles, play an unavoidable important role in regulating various cellular cycles of the body and maintaining crucial cellular homoeostasis.With proper bio engineering, Nanoparticles can be sent in a localized condition in any system of the body and thus it can incorporate the activity of biological components, thus mimicking the biological system of the body according to the need for human benefit. Nanoparticles are highly soluble due to their small size and their solubility can be further increased by proper surface modification and the high surface area to volume ratio of those particle, make them having ample surface area to encapsulate drugs and other materials, thus providing higher therapeutic payload. Another property of these nano particles can be described as the selective targeting nature, thus Nanoparticles can specifically release a therapeutic payload onto the target, reducing the side effects on normal cells, (McNeil, et al, 2009, Wang, et al, 2013, Bisht and Rayamajhi, 2016; Hussain et al, 2019). Marco et.al, 2019).

Research and development in the field of nanotechnology are growing rapidly throughout the world (Vidya *et al.,* 2013). A major contribution of this field is the development of new materials in the nanometer scale (Sivakumar, *et al.,* 2011; Karthikeyan et.al, 2019). These are usually particulate materials with at least one dimension of less than 100 nanometers (nm), even the particles could be zero dimension in the case of quantum dots (Vidya*et al.,* 2013). Metal nanoparticles have been of great interest due to their distinctive features such as catalytic, optical, magnetic and electrical properties (Garima, *et al.,* 2011). Nanoparticles exhibit completely new or improved properties with larger particles of the bulk materials, and these novel properties are derived due to the variation in specific characteristics such as size, distribution, and morphology of the particles (Ravindra, *et al.,* 2011, Ravindran, et.al. 2016). Particularly, nanoparticles (NP) made from metal oxides with sizes less than 100 nm exhibit antimicrobial activities owing to their special characteristics (e.g. small particle size, large surface area), which micro- or macro-sized particles do not possess. Zinc oxide, with its unique physical and chemical properties, such as high chemical stability, high electrochemical coupling coefficient, broad range of radiation absorption and high photo stability, is a multifunctional material (Lou, 1991, Segets, et al. 2009). Recent studies have shown that some NP made of metal oxides, such as ZnO NP, have selective toxicity to bacteria but exhibit minimal effect on human cells (Brayner et al. 2006; Thill et al. 2006; Reddy et al. 2007; Zhang et al. 2007, Sadhukhan et al, 2019).

Compared with the organic materials, inorganic antibacterial reagents are more stable at high temperatures and pressures (Sawai 2003). Compare to the inorganic antibacterial materials, metal oxides such as zinc oxide (ZnO) have received increasing attention in recent years, not only because they are stable under harsh processing conditions, but also because they are generally regarded as safe materials to human beings and animals (Stoimenov et al. 2002; Fu et al. 2005; Kaushik et.al, 2019).

ZnO has a neutral hydroxyl group attached to its surface, which plays an important role in its surface charge behaviour. At high pH, ZnO exists as ZnO^- due to the transfer of adsorbed protons from its surface towards aqueous solution. At low pH (acidic condition), ZnO exists as $ZnOH_2^+$ due to the transfer of protons from the aqueous environment towards its surface. The isoelectric pH of ZnO nanoparticles is 9-10 (Orel, et.al, 2015, Vinardell et.al, 2015 Roy and Jong,2019).

Thus, ZnO nanoparticles exhibit positive charge under physiological conditions such as blood or tissue fluid (which has pH 7), etc. (Degen et.al,2000, Rasmussen et.al, 2010). On the other hand, cancerous cells usually have high concentration of (negatively charged) anionic phospholipids on their outer membrane (Abercrombie et.al, 1962). The re-emergence of infectious diseases and the continuous development of antibiotic resistance among a variety of disease-causing bacteria pose a serious threat to public health worldwide (Desselberger, 2000, Vandenesch et.al, 2003). Among these pathogenic microorganisms, *Enterococcus, Staphylococcus* and *Streptococcus* are common closely related species that cause a wide variety of infections and diseases (Boyce, 1997; Lowy, 1998; Hancook & Gilmore, 2000, Laura et.al,2019).

Despite antimicrobial therapy, morbidity and mortality associated with these bacterial infections remain high, partially as a result of the ability of these organisms to develop resistance to virtually all antibiotics. New strategies are therefore needed to identify and develop the next generation of drugs or agents to control bacterial infections. CuO nanoparticles are successful in murdering a spread of microorganism. Be that as it may, ZnO nanoparticles with high focus are expected to get the disinfectant effect. The disinfectant property of such nanoparticles relies upon their size, steadiness, and focus extra to the extension medium, that gives bigger maintenance time to microorganism NP association.

MATERIAL AND METHODS

Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (Gibco™, Thermo Fisher Scientific), penicillin (100 U/mL) and streptomycin (100 µg/mL) (Merck, India), TMA-

DPH (1-(4-Trimethylammoniumphenyl)-6-Phenyl-1,3,5-Hexatriene *p*-Toluenesulfonate) (Thermo Fisher Scientific), Dry N,N,dimethylformamide (DMF; Merck, India), nickel chloride hexahydrate ($NiCl_2$, $6H_2O$, Mw = 237.69 g mol−1; Merck, India), sodium hydroxide (NaOH; Merck, India), ethyl alcohol (C_2H_6O; Merck, India), tetraethyl orthosilicate (TEOS; Merck, India), and ammonia solution 25% (NH_4OH; Merck, India) were used in this work. All the materials were used in the experiments without further purification.

Preparation Of Zno@Sio$_2$ Nanoparticle: Hydrothermal method has been used to synthesize the Zinc oxide nanoparticles (Dutta et al., 2017). Details of that hydrothermal procedure for synthesis of metal oxide nanomaterials has been referred from (Dutta et al., 2015). Modified Stóber (Stober et al., 1968) method, a widely used method for synthesis of silica nanoparticles has been used to synthesize the silica coated Zinc oxide. In this typical synthesis procedure, hydrothermally synthesized Zinc oxide nanoparticles (Dutta et al., 2017) were added to the solution of water and ethyl alcohol (in volume ration approximate 4:1). To achieve a well-dispersed mixture, the solution was sonicated for 10 min. After that, ammonia was added to the mixture (in volume ratio 1.4:50) drop by drop to catalyze the Zinc oxide nanoparticles in alcoholic media. The mixture was again sonicated for 40 min after the addition of ammonia, and finally, TEOS was added drop by drop to the mixture (in volume ratio approximately 0.4:50). The final mixture was kept under strong magnetic stirring (500 rpm) for 18 h. The well-mixed colloidal solution was centrifuged at 4000 rpm and washed by ethanol to remove the residuals from the product. The collected product was dried at 80 °C and employed for further characterization.

Anti Microbial Activity: Cup-Disc Method: The number of the zone of inhibition has been deduced from three parallel studies and those are taken as the mean value of those. These studies were compared with the known drugs available in the market. The ZnO and ZnO@SiO2 showed an average value of the zone of inhibition where the combination of these two nanoparticles showed a maximum zone of inhibition. The lower concentration of the mixed drug has an effect on the bacterial and the fungal growth which has been measured by calculating the zone of inhibition and the values are (+/-) SD of three parallel measurements.

Cell Culture: Human cervical epithelial malignant carcinoma cell lines (HeLa) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (Gibco™, Thermo Fisher Scientific), penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37°C in a humidified atmosphere containing 5% CO2. HeLa cells at a concentration of 1.5×105 cells/mL were grown in a 25 cm^3 flask of complete culture medium. At 85 % confluency HeLa cells were and trypsinized, and seeded on a 96 well tissue culture plate for overnight according to the selection of experiments.

Mtt Assay: Approximately 1 × 10^5 mL^{-1} HeLa cells in their exponential growth phase were seeded in a flat-bottomed 96-well Tissue culture plate for 24h at 37°C in a 5% CO_2 incubator. Series of concentrations (5, 25, 50, 100, and 250 µg/mL) of ZnO and ZnO@SiO_2 nanoparticles in the medium were added to the plate in a triplicate manner. Cytotoxicity evaluation of NiO and NiO@SiO_2 nanoparticles was performed using MTT assay and MTT was added to each well and the plates incubated for 3 h in a dark chamber. 100 µl of DMSO was added to dissolve the formazan crystals and the absorbance read at 540 nm using ELISA reader (EPOCH, BIOTEK) (Zhu et al., 2001).) The % of survival was calculated using untreated cells as 100 %.

$$\% \text{ Cell Viability} = \frac{\text{(A) Control} - \text{(B) test}}{\text{(A) Control X 100}}$$

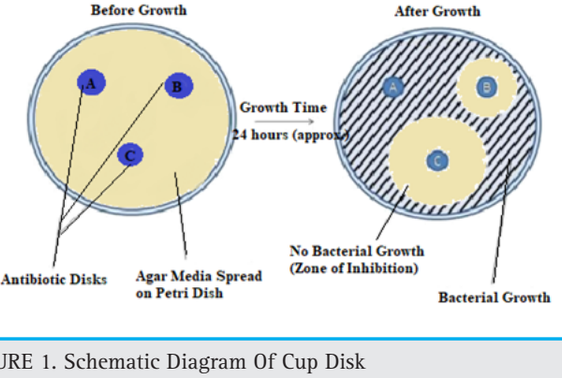

FIGURE 1. Schematic Diagram Of Cup Disk

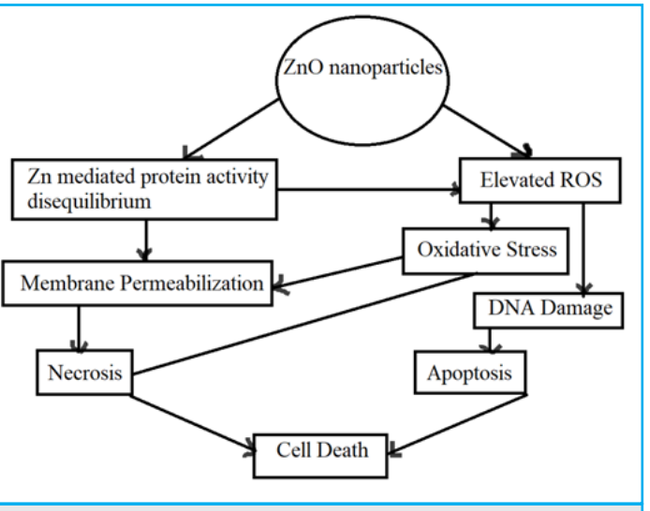

FIGURE 2. Schematic Diagram of the action of ZnO nanoparticles

Where B test is the absorbance of the test sample and A control is the absorbance of the control sample. Non-treated cells were used as the control, and the samples were imaged using an inverted photomicroscope. The Values of MTT assay correspond to mean and standard deviations of three independent experiments.

Fluorescence Anisotropy: The fluorescence anisotropy of HeLa was assessed by the determination of TMA-DPH steady-state fluorescence polarization after the cell membrane exterior phospholipid layer permeation of the probe (Dowell, 2002; Pearson,1996; Pearson, et.al,2001, Shrivastava, et.al. 2007; Katona,2004; Lakowicz,2004; Hollan, 1996).

For the measurement of the changes in the TMA-DPH fluorescent properties following the membrane permeation, we added 2.5µM TMA-DPH to a 2 ml of cell in the measuring cuvette. The cell suspension with the fluorescent probe was incubated for 30 min at 37°C. The measurement has been done between excitation and emission state, 360 nm and 430 nm respectively.

ROS Analysis: Membrane fluidity of cancer cells was shown to have a decisive role in the direct cell to cell contact and the modulation of the activity of membrane enzymes are to be affected by the increased release of reactive oxygen species (ROS) (Garden, 2001).

For the measurement of the intracellular ROS, DCF-DA was added to a 2 ml of HeLa suspensions. The cell suspension with DCF-DA was incubated for 60 min at 37^0 C in a dark condition. Cells without nanoparticles were used as control. Fluorescence intensity was measured in

Table 1. Zone of Inhibition of Bacteria: (Values are mean +/- SD of three parallel measurements = Number of zones of inhibition.) (Concentration in µg/ml and Zone of inhibition in mm)

Drug Name	Drug Concentration (µg/ml)	*E. coli*	*S. typhi*	*S. aureus*	*S.pyrogenes*
ZnO	5	-	-	-	-
	25	13	12	14	12
	50	15	14	16	13
	100	17	15	19	17
	250	20	17	20	20
$ZnO@SiO_2$	5	-	-	-	-
	25	8	9	7	6
	50	10	12	11	10
	100	13	13	14	12
	250	14	13	15	13

Table 2. Known drugs used for bacteria

Drug Name	Drug Conc. (µg/m)	*E.coli*	*S.typhi*	*S.aureus*	*S.pyrogens*
Ampicillin	5	13	14	11	10
	25	15	19	14	13
	50	17	15	15	16
	100	18	17	19	18
	250	20	21	22	20
Norfloxacin	5	23	19	18	20
	25	25	20	20	22
	50	26	22	23	26
	100	28	25	24	28
	250	30	26	24	32
Amoxiline	5	21	20	18	19
	25	23	23	20	22
	50	25	24	23	23
	100	27	26	24	25
	250	28	29	26	28
Cifroflloxacin	5	20	21	19	17
	25	22	23	21	20
	50	26	24	23	20
	100	28	27	25	22
	250	30	29	28	25

a fluorescence spectrophotometer (model Hitachi, USA) at excitation and emission wavelengths of 504 and 529 nm, respectively.

Antioxidant Enzymes Activities: Superoxide dismutase (SOD) and Catalase (CAT) activities were measured by commercially available kits. The cells were seeded into 12- well plates at a concentration of 7×10^5 cells/well and all measurements were performed according to supplier's recommendations.

RESULTS AND DISCUSSION

The Zinc oxide Nano Particles have shown better effect than the Silica coated nano Particles. In some cases the

Table 3. Anti Fungal Activity: Zone of Inhibition of Fungi: (Values are mean +/- SD of three parallel measurements = Number of zones of inhibition). (Concentration in µg/ml and Zone of inhibition in mm)

Drug Name	Drug Concentration (µg/ml)	*A. nigar*	*C. clavus*	*C. albicans*
ZnO	5	-	-	-
	25	14	17	15
	50	17	19	17
	100	19	20	18
	250	20	22	20
$ZnO@SiO_2$	5	-	-	-
	25	8	7	5
	50	10	9	8
	100	11	10	10
	250	12	11	12

Table 4. Known Drug Concentration

Drug Name	Drug Concentration (µg/ml)	*A. nigar*	*C. clavus*	*C. albicans*
Greseofluvin	5	17	18	19
	25	21	20	20
	50	22	23	21
	100	23	24	24
	250	27	28	26
Nystitin	5	19	17	18
	25	20	21	20
	50	20	22	21
	100	23	24	23
	250	28	27	25

Zinc oxide nanoparticles have shown good results in comparison to the known drugs which is a positive indication of using these nanoparticles as a potential antimicrobial drugs. We also checked their activity on the fungal growth and these particles have also shown a great effect on reducing the fungal growth. These nanoparticles can also be used to reduce the fungal growth and the contamination occurs from it. The results of the zone of inhibition in bacteria and the antibiotics have shown in table 1 and table 2 and the zone of inhibitions for fungi and available anti-fungal have shown in table 3 and table 4.

MTT assay was undertaken in order to evaluate the cell viability in cells stressed by Zinc oxide nanoparticles and Silica coated Zinc oxide nanoparticles. First we evaluated the effects of NiO Nanoparticles on HeLa cells viability. Incubation with 5µg/ml, 25µg/ml, 50µg/ml, 100µg/ml, 250µg/ml for 3, 6, 12 and 24 h resulted in a concentration dependent decrease in cell viability, the LC50 was 79.83 ± 0.856 µg/ml. Based on these results Zinc oxide nanoparticles and Silica coated Zinc Oxide nano particles at submaximal concentrations after 12 h, 50 and 100 µg/ml were selected in this study. The main findings of this assay are the LC50 of Zinc oxide nanoparticles and Silica coated Zinc Oxide nanoparticles was 79.83 µg/ml and submaximal concentrations of 80 and 100 µg/ml were selected in this study. Similar results were obtained in previous findings demonstrated a dose dependent reduction of MTT-value in HeLa cells treated with Zinc oxide nano particles and Silica coated Zinc oxide nanoparticles, though cells were different (Ahamed 2011 and Capasso, et al. 2014).

According to the National Cancer Institute (USA), vegetables crude extracts are cytotoxic considered when their IC50 values are less than 30 µg/ml (Da, et al. 2013). After a large screening, Zinc oxide nanoparticles and

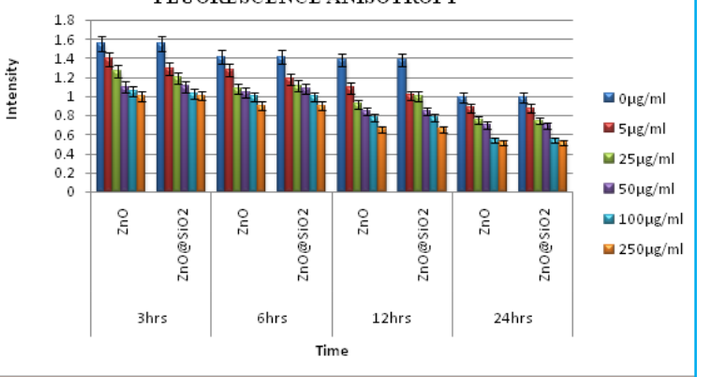

FIGURE 3. Fluorescence Anisotropy of HeLa Cells treated with Zinc oxide-nanoparticles and Silica coated Zinc oxidenanoparticles. Data represented are mean ± SD of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 treatments compared to control.

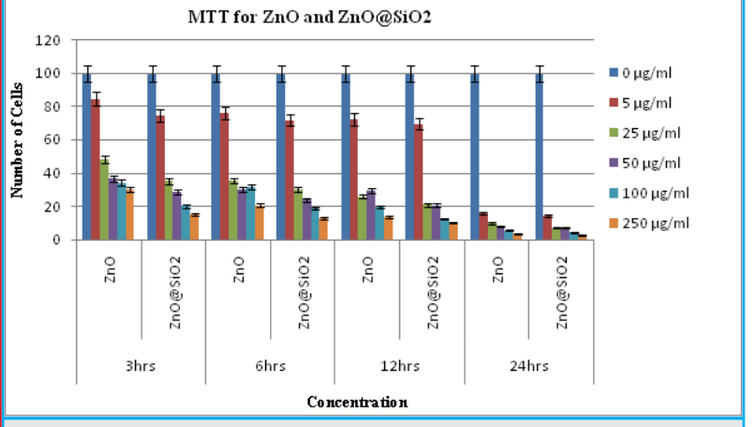

FIGURE 4. MTT Assay of HeLa Cells treated with Zinc oxidenanoparticles and Silica coated Zinc oxidenanoparticles. Data represented are mean ± SD of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 treatments compared to control.

Silica coated Zinc Oxide nanoparticles (60 and 80 µg/ml) concentrations were selected due to their best actions. The present study agree with the results of Remila et al. (2015) who have demonstrated that pre-treatment of THP-1 cells with *P. lentiscus* extracts for 24 h strongly inhibited H_2O_2 damage, with maximum protection at 100 µg/ml (Remila, et al. 2015). The triplicate study of the cell culture has shown that the number of cells is decreasing by the increment of time and the concentration of the drugs respectively.

In order to investigate the effect of Zinc oxide nanoparticles and Silica coated Zinc Oxide nanoparticles induced cytotoxicity mediated through ROS generation, HeLa cells were treated with the two selected concentrations of the Zinc oxide nanoparticles and Silica coated Zinc Oxide nanoparticles. We detected a significant decrease of ROS level in cells treated with Zinc oxide nanoparticles and Silica coated Zinc Oxide nanoparticles (Figs. 4 and 5). Oxidative stress, which is an imbalance between ROS production and the antioxidant systems favouring a ROS excess, has been identified as a common mechanism for cell damage. During oxidative stress, ROS are produced mainly from the mitochondrial electron transport chain. To minimize the damage induced by ROS, free radicals can be transformed to other less toxic molecules, for example, the superoxide anion is enzymatically converted into hydrogen peroxide by superoxide dismutase (SOD) and hydrogen peroxide may be enzymatically converted into water by catalase or glutathione peroxidase enzymes (Huerta-García et.al, 2014). Nanoparticles have been demonstrated to generate more free radicals and ROS than larger particles, likely due to their higher surface area (Sioutas, et al, 2005). NiO Nanoparticles have been reported to reduce

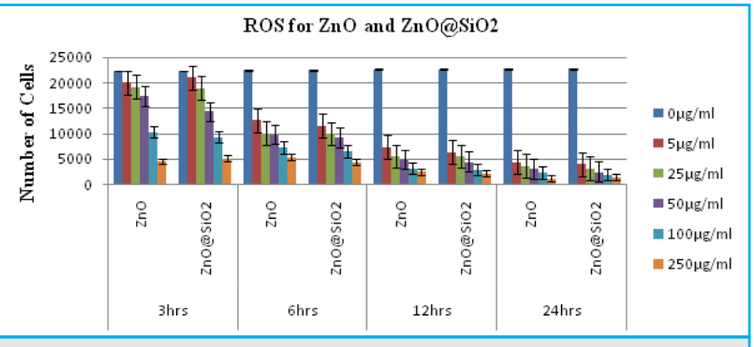

FIGURE 4. ROS of HeLa cells treated with Zinc oxidenanoparticles and Silica coated Zinc oxidenanoparticles. Data represented are mean ± SD of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 treatments compared to control.

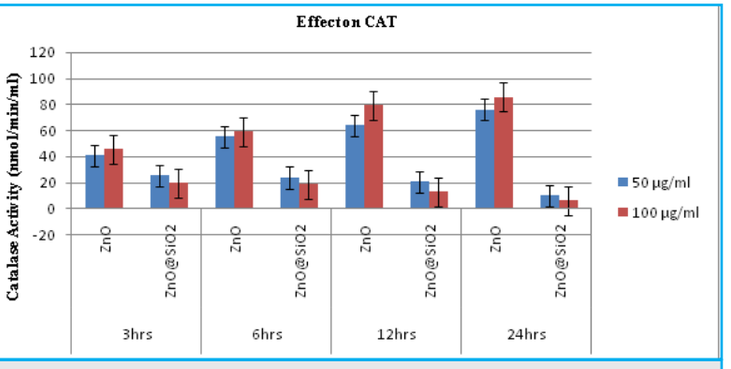

FIGURE 6. Effects of Zinc oxide nanoparticles and Silica coated Zinc Oxide nanoparticles on CAT activity in HeLa cells. Cells were treated with different concentrations for 3, 6. 12 and 24 hours. Data represented are mean ± SD of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 treatments compared to control.

cell viability and to induce oxidative stress by depletion of glutathione and induction of reactive oxygen species in HEp-2 and MCF-7 cells (Siddiqui, et al, 2013), cell death via apoptotic pathway and ROS generation in HepG2 cells in dose-dependent manner (Ahamed, et al, 2012), Zinc oxide nano particles also increased intracellular ROS, apoptosis and necrosis in BEAS-2B and A549 cells (Capasso, et al, 2014).

Our results confirmed that Zinc oxide nanoparticles and Silica coated Zinc Oxide nanoparticlesare toxic to HeLa cells. In the Fig 6, ROS analysis has been shown in triplicate studies. These analyses showed that the requirement of the oxygen got low with the increase of time and concentration of the drug. These need of oxygen lead the cells to the apoptosis and thus the cell dies due to the treatment of the drugs. These have also coincided with the result of the MTT assay. With the increase of the concentration of the nanoparticles, the number of viable cells decreased. The nanoparticles showed the better result as the variation of the valance electron was increased as a result those reacted with the protein particles of the cells and dissociated it which leads the cells to destroy.

Pre-incubation of cells with both concentrations 50 and 100 µg/ml of Zinc oxide nanoparticles and Silica coated Zinc Oxide nanoparticles led to enhance the antioxidant enzymes, SOD and CAT, activities shown in Figs. 5 and 6. Similarly, the Zinc oxide nanoparticles and Silica coated Zinc Oxide nanoparticles also induce a significant depletion of antioxidants. The accumulation of ROS, e.g. superoxide radicals (O2%) and hydroxyl free radicals (%OH) decrease the defensive effects of cellular antioxidant enzymes, e.g. SOD, CAT (Li, et al. 2012). Exposure of HT22 hippocampal cells to CuO Nanoparticles resulted decrease in the activity of SOD and the other detoxification enzymes which has been founded in this work (Niska, et al. 2015).

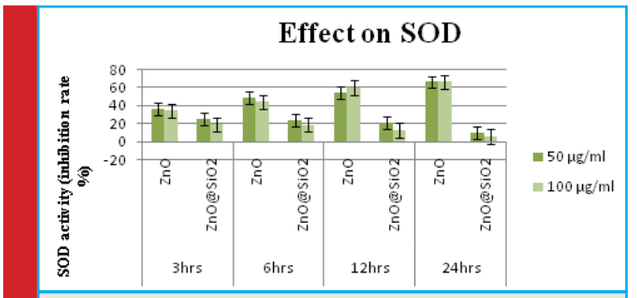

FIGURE 5. Effects of Zinc oxide nanoparticles and Silica coated Zinc Oxide nanoparticleson SOD activity in HeLa cells. Cells were treated with different concentrations for 3, 6. 12 and 24 hours. Data represented are mean ± SD of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 treatments compared to control.

DISCUSSION

As per our research is concerned, we have found much more promising result on both anti microbial and anti cancer effect. We observed that the growth of both Gram-positive and Gram-negative bacteria was inhibited by increasing concentrations of ZnO NPs. We further explored the effect of the ZnO NPs on the cellular morphology (Hussain et al, 2019).

The recent data of Karthikeyan et al, (2019) have showed that the REM doped ZnO which is being costly but the procedure making of our doped particle is both cost effective and easy. Different review (Sadhukhan et al, 2019; Kaushik et al, 2019; Roy and Jong, 2019; Laura et al 2019; Marco et al, 2019; Xiuting et al, 2019) of the articles lead us to do the experiments with different gram positive and gram negative bacteria and our material has shown effect in much more lower concentration both in the bacteria and cancer cells. Our Spectroscopic data analysis also confirmed the lower concentration effects on the both. This work will completely open the new era of personalized drug for each.

CONCLUSION

Nanoparticles in medicine are a new and emerging topic of interest for researchers. With all their promising characteristics, the in vivo application of nanoparticles is still rare and there is currently a serious lack of in vivo research into nanoparticles. Hence, a much better collaboration between clinicians, biologists and material scientists is required for the in-depth understanding of cancer biology and intelligent design of NPs for their better clinical use. This is in fact an achievable aim, considering the highly promising characteristics of ZnO nanoparticles and their inherent nature of selectivity and toxicity towards cancer cells, making them unequivocally a key tool for next-generation cancer treatment. ZnO NP exhibited impressive antibacterial properties against different food borne pathogens as well as fungi and the inhibitory effects increased as the concentrations of ZnO nanoparticles increased. ZnO NP could distort bacterial cell membrane, leading to loss of intracellular components, and ultimately the death of cells. These results demonstrate that ZnO NP could be potentially considered as an effective antibacterial agent for protecting agricultural and food safety. Thus we have found that the ZnO can be a potential anti cancerous and antimicrobial drug for next generation of treatment.

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