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CHEMICAL PRECIPITATION OF ZnO NANOPARTICLES: ANTIMICROBIAL ACTIVITY AND *IN VIVO SUB-ACUTE* NANOTOXICOLOGICAL IMPACT ON THE LIVER AND KIDNEY OF SWISS ALBINO MICE

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ABSTRACT

ZnO nanoparticles can be used in wide range of applications including bionano products, electronics, food additives, sun screens, biosensors, etc. However, the comprehensive toxicological impact posed by ZnO nanoparticles still remains clear. In this research work, we report the preparation and toxicological study of ZnO nanoparticles by simple chemical precipitation method in our laboratory. The prepared ZnO nanoparticles were characterized by using X-ray diffraction, SEM and EADX. The nanoparticles were subjected to antimicrobial assay by agar well diffusion method against both Gram positive *(Staphylococcus aureus and Bacillus subtilis)* and Gram negative bacteria *(Escherichia coli and Klebsiella pneumoniae).* ZnO nanoparticles have maintained considerably multidrug resistance against the clinical pathogens such as *Staphylococcus aureus* and *Bacillus subtilis* (Gram positive bacteria*)* and *Klebsiella pneumoniae and Escherichia coli and* (Gram negative bacteria).The antibacterial efficiency of the nanoparticle mainly dependent on the size of the particle along-with other characteristics. The study was further extended to assess the *in vivo* sub –acute oral toxic effect of ZnO nanoparticles in Swiss Albino Mice. The *invivo* histopathological studies revealed that the mice treated with 500 mg/kgbwt dosage showed moderate toxicity in the central vein of the hepatocytes after 14 days. In conclusion, ZnO nanoparticle was found to show good antibacterial activity but however it causes moderate toxicity in the organs liver and kidney of Swiss Albino Mice after extensive dosage levels.

KEYWORDS:Chemical precipitation, ZnO nanoparticles; characterization; antimicrobial studies; sub acute studies

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INTRODUCTION

Metal oxide nanoparticles and composite materials are widely applied in the field of research and development and they are found to be useful in diverse applications especially in industries including surface coatings, optoelectronics, biosensor, bioengineering, and agriculture[1-3]. Nanomaterials are called a ''wonder of modern medicine''. It is stated that antibiotics kill perhaps a half dozen different disease-causing microorganisms, but nanomaterial's can kill some 650 cells[4]. Over the past few years, various nano-sized antibacterial agents such as metal and metal oxide nanoparticles have been evaluated by researchers. Several types of metal and metal oxide nanoparticles such as silver (Ag), silver oxide (Ag_2O), titanium dioxide (TiO_2), zinc oxide (ZnO), gold (Au), calcium oxide (CaO), silica (SiO_2), copper oxide (CuO), and magnesium oxide (MgO) have been known to show antimicrobial activity[5]. Among transition metal oxides, zinc oxide (ZnO) nanoparticles are of special interest because of its large exciton binding energy of 60 meV with a direct band gap of 3.37 eV[6]. It has been widely used in gas sensors, transparent conductors, piezoelectric application, and as antimicrobial agents[7]. In addition, ZnO nanoparticles have the potential to impact many aspects of food and agricultural systems because of their antimicrobial efficacy especially with the growing need to find alternative methods for formulating new type of safe and cost-effective antibiotics in controlling the spread of resisted pathogens in food processing environment[8]. It has been demonstrated that ZnO nanostructures exhibit antimicrobial activity against on a broad spectrum of bacteria including *staphylococcus aureus*[9]. ZnO nanoparticles seem to have relative toxicity to bacteria but exhibit minimal effect on human cells[10]. We have recently studied that the characteristics of ZnO nanoparticles synthesized by using different precursor materials such as $ZnCl_2$, $ZnSO_4$ and $Zn(NO_3)_2$[11]. In this research work, we report the characteristics of ZnO nanoparticles against both Gram positive *(Staphylococcus aureus and Bacillus subtilis)* and Gram negative *(Escherichia coli and Klebsiella pneumoniae)* bacteria by agar well diffusion method. In continuation to this study, we extended the study to investigate the sub-acute oral toxicity of ZnO nanoparticles in the organs of liver and kidney of Swiss Albino Mice. The results of these experiments are presented and discussed.

PHYSICAL CHARACTERIZATION OF MATERIALS AND EXPERIMENTAL DESIGN

Synthesis and physical characterization

The ZnO nanoparticles were synthesized by simple chemical precipitation method using zinc nitrate and sodium hydroxide solutions[11]. The prepared nanoparticles were heat treated at 600^oC for 6 hours. Then, the ZnO nanoparticles were characterized by Shimadzu XRD6000 X-ray diffractometer using CuKα radiation. The surface morphology of the particles was studied by means of JEOL Model JSM-6360 scanning electron microscope. EDAX analysis was also performed with JEOL Model JSM-6360 to find out the atomic weight percentage of elements present in the samples.

Antimicrobial activity

Antimicrobial discs susceptibility were performed by Agar well diffusion method against both Gram positive *(Staphylococcus aureus and Bacillus subtilis)* and Gram negative bacteria *(Escherichia coli and Klebsiella pneumoniae)* with ZnO nanoparticles. Nutrientagar plates were swapped three axes with sterile cotton tipped swab which were dipped in the overnight microorganism. A 8mm hole was bored in agar plate using sterile cork borer. The ZnO nanoparticle suspension was prepared by adding 10mg of dry nanoparticle with 1ml of sterile distilled water in an eppendorf tube. The particle was dispersed well using the ultrasonicator which could speed up the dispersion process after 20 min. sonication. The wells of the agar plates were loaded with 100µl of the ZnO nanoparticle suspension and the plates were allowed to stand 10 min. for perfusion of the nanoparticle and kept incubation at 37°C for 24h. After incubation, the plates were observed for the formation of clear inhibition zone around the well which is an indication for the antibacterial activity of the ZnO nanoparticle. The Zone of inhibition was recorded by measuring the diameter of the inhibition zone around the well. Distilled water was used as a blank or negative control.

Animal Maintenance

The clearance of the ethical committee for experimentation on animals were obtained before starting the experiment (IAEC/KU/BT/14/17). The *in vivo* sub-acute toxicity studies were conducted on male Swiss Albino Mice (with average weight of 25–32 g) and they were obtained from the Kerala Agricultural University, India. The sub-acute toxicity study was performed according to OECD guideline 407. The animals were housed in the animal house of the Department of Biosciences and Technology, Karunya University, India. The experimental mice were maintained under standard conditions of temperature 23 ^oC ± 2 ^oC, with relative humidity of 50 – 70 % and 12-h light-dark cycle. The mice were allowed free access to commercial pellet and water *ad libitum* throughout the experiments. All the experiments were performed according to the "Guidelines for Animal Experimentation" approved by the Institutional Ethical committee (Karunya University, India) The mice were acclimatized to laboratory condition for one week before the commenencement .

Histopathological experimental design

After one week of acclimation, the mice were divided into 2 groups: The maximum dosage taken in our experiments was 500mg/kg bwt as reported earlier[12]. Group 1 is considered as a control group and the mice in Group 2 are orally ingested with 500 mg/kg ZnO nanoparticles for 14 consecutive days. ZnO nanoparticles were suspended in distilled water and administered orally for 14 consecutive days to the animals. At the beginning of the study, the weight of animals was recorded; thereafter the body weight was finally recorded at day before the sacrifice. Animals were monitored frequently at day one and subsequently

twice daily during the course of the treatment. Detailed clinical examination included identification of clinical signs related to: general appearance, body position and posture and behavior pattern. Special attention was given to convulsion, salivation, diarrhea, and lethargy or skin symptoms. At the end of 14th day, the animals were sacrificed via cervical dislocation. The liver and kidney were harvested, weighed and macroscopically examined for lesions and / or abnormalities. Both the organs were kept in 10% buffer formalin for histopathological examination. The above study was carried out to find out the toxicity of the ZnO nanoparticles in Swiss Albino Mice by a repeated oral ingestion.

RESULTS AND DISCUSSION

Table 1
Antibacterial potential of ZnO nanoparticles

Gram Positive bacteria	Zone of inhibition in mm (mean ± S.D.)	Gram Negative bacteria	Zone of inhibition in mm (mean ± S.D.)
Staphylococcus aureus	22.33±0.19	*Klebsiella pneumoniae*	26.6±0.69
Bacillus subtilis	23.66±0.69	*Escherichia coli*	17±0.57

Notes: values are mean of three triplicates

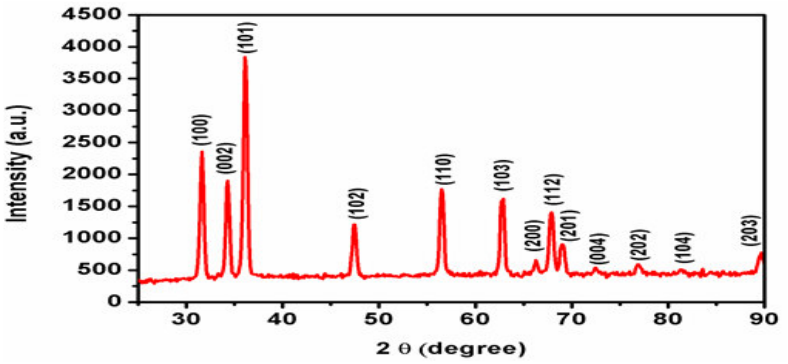

Figure 1
X-ray diffraction of the ZnO nanoparticles prepared by chemical precipitation method

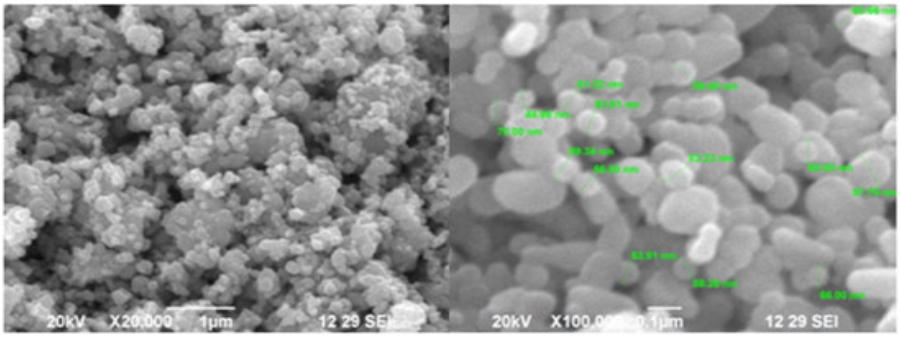

Figure 2
SEM photographs obtained on the ZnO nanoparticles prepared by chemical precipitation method

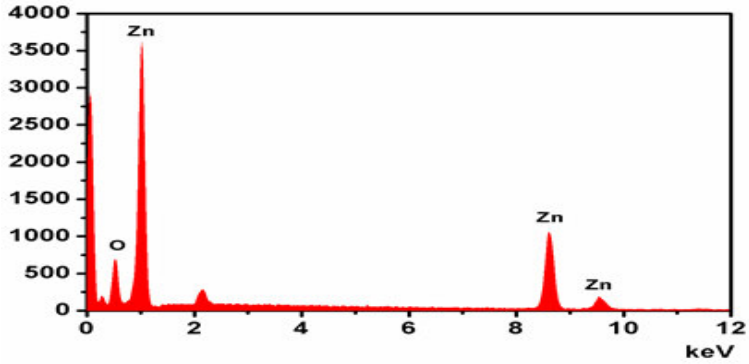

Figure 3
EADX spectra obtained on the ZnO nanoparticles prepared by chemical precipitation method

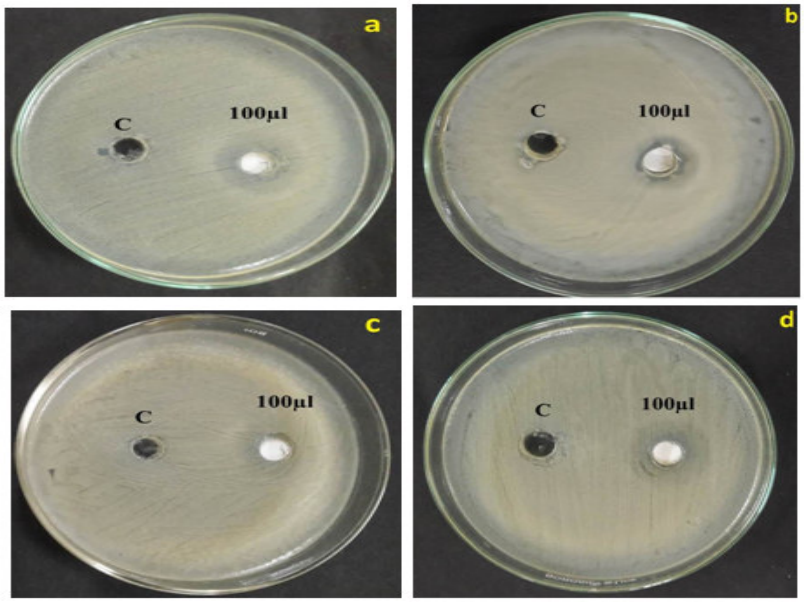

Figure 4
Evaluation of antibacterial activity of ZnO nanoparticles against a) Staphylococcus aureus (b) Bacillus subtilis (c) Escherichia coli (d) Klebsiella pneumoniae C- control (water)

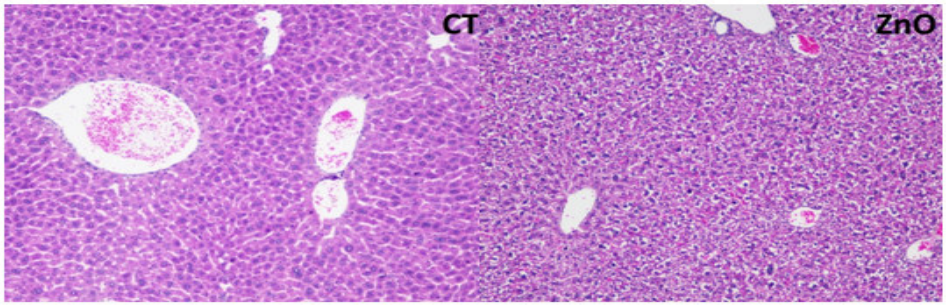

Figure 5
Sub-acute histopathological examination of liver in mice after oral administration of ZnO nanoparticles (CT) with control i.e., with no dosage of nanoparticles (shows normal morphology of hepatocytes); (ZnO) ZnO nanoparticle treated liver shows cytoplasmic vacuolation mild congestion in the central vein

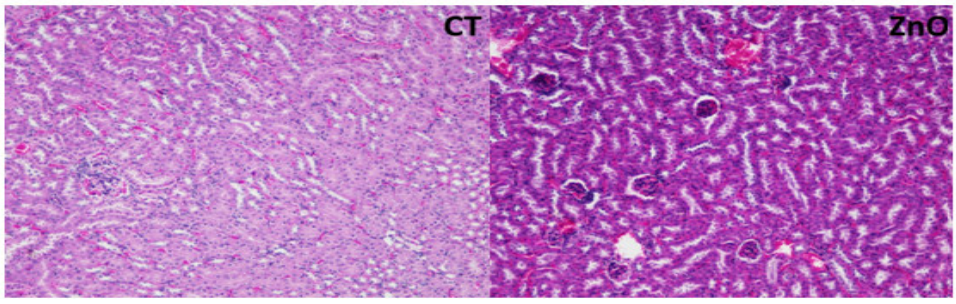

Figure 6
Sub- acute histopathological examination of mice kidney after oral administration of ZnO nanoparticles (CT) with control i.e., with no dosage of nanoparticles (shows normal cortex and medulla); (ZnO) ZnO with 500mg/kg body weight (b.w.) (shows mild mesangial hyper cellularity)

XRD studies

Figure 1 illustrates the X-ray diffraction pattern of the synthesized ZnO nanoparticles. The detected peaks are indeed indexed as ZnO phase with hexagonal structure which is in agreement with standard JCPDS (card No: 89-1397) card for ZnO. No other characteristic peaks of any impurities were detected in the sample. The result suggested that high-purity ZnO was formed in the synthesis. The intensity of the peaks is very sharp and narrow, indicating the materials are of high quality and good crystallinity.

SEM measurements

Figure 2 exhibits the SEM images of the ZnO nanoparticles. From the SEM results, it was found that the particles are spherical shaped and agglomerated

with each other. The grain size of the samples was found in between the range of 50-100 nm. The agglomeration of particles found in the samples may be due to the high temperature treatment.

EDAX analysis

Figure 3 shows the energy dispersive X-ray microanalysis (EDAX) spectra of the ZnO nanoparticles. The strongest peaks observed in the spectrum related to Zinc and Oxygen. The elemental constitution of ZnO nanoparticles with major peaks found to have a weight percentage of 46.38 of zinc and 53.62 of oxygen . The study confirmed the formation of ZnO nanoparticle in the chemical precipitation method.

Antimicrobial studies

Antibacterial potential of ZnO nanoparticles was carried out using agar well diffusion method and the results are shown in Figure 4 and Table 1. It reveals that ZnO nanoparticles are effective potential antibacterial agents on both Gram positive *(Staphylococcus aureus* and *Bacillus subtilis)* and Gram negative bacteria *(Escherichia coli* and *Klebsiella pneumoniae)* bacteria. However, the zone of inhibition in bacterial growth was found to be good against S*taphylococcus aureus, Bacillus subtilis* and *Klebsiella pneumoniae* than *Escherichia coli.* Low performance found in *Escherichia coli.* It was reported that the bacteria wall in *Escherichia coli* is composed of two cell membranes, an outer membrane and a plasma membrane with a thin layer of peptidoglycan with a thickness 7 – 8 nm. Nanoparticles within such ranges can readily pass through the peptidoglycan and hence are highly susceptible to damage[13]. Those nanoparticles are not within the above range may not be active for the above category of bacteria. Hence, this study suggested that the antibacterial efficiency of the nanoparticle mainly dependent on the size of the particle along-with other characteristics.

Sub-acute histopathological studies

As indicated in the experimental section, the mice were administered with ZnO nanoparticles in order to study the nanotoxicological effect in a systematic manner. After oral administration, their clinical signs and mortality were monitored on daily basis. There was no lethality and abnormal signs of clinical symptoms in the control and zinc oxide nanoparticle treated groups. The treated mice did not show any significant difference in the body weight similarly. No obvious differences were observed in the organ weight or behavioral change compared to the control group until 14 consecutive days of oral injection. From the sub-acute studies, it was found that there was no significant sign of convulsion, death, salivation, diarrhea, and lethargy or skin symptoms in the treated mice. On the 15th day, the mice were sacrificed and their organs such as liver and kidney were taken from the mice by cervical dislocation process for histopathological assessment. Then, representative blocks of liver and kidney tissues from each lob were taken and possessed for paraffin embedding using the standard microtechnique. Sections of (~5 µm) of livers and kidneys stained with hemotoxylin and eosin were observed microscopically for the evaluation of acute histopathological changes. From Figure 5, the following observations were made. The sub-acute histopathological results of control group (CT) (with no dosage of nanoparticles) of mice have shown normal lobular architecture. The mice treated with ZnO nanoparticles at higher dosage (500mg/kg) level have accumulated with the NPs in the liver and further they caused oxidative stress leading to DNA damage apoptosis. Also, the liver treated with 500mg/kg of ZnO dosage level has shown altered hepatocytes and binucleation in the central vein when compared with control. From Figure 6, the following inferences were made. The sub-acute histopathological examination of control group (CT) of mice (without no dosage of nanoparticles) has shown normal morphology of cortex and medulla and no interstitium inflammation along-with normal tubular architecture. The kidney treated with 500 mg/kg of ZnO dosage level has shown mild mesangial hyper cellularity when compared with control.From the sub-acute histopathological studies[14-16], it was found that the mice treated with 500 mg/kg body weight (b.w.) caused moderate liver and kidney injury by gastrointestinal ingestion. Also, there was non-significant mortality and abnormal behavior or no symptoms such as decrease in food and water intake, diarrhea, loss of movement and change in size of eyes were observed.

CONCLUSIONS

The present study revealed that high crystalline and pure ZnO nanoparticle was successfully synthesized by chemical precipitation method. The prepared nanostructures were characterized by Powder XRD, SEM and EDAX. The powder XRD analysis confirmed the hexagonal structure of the ZnO nanoparticle and sharp exitonic peaks suggested the high crystallinity of the ZnO nanocrystals. SEM morphology confirmed the presence of grains in the sample in the range of 50 to 100 nm.The EDAX spectra exhibited the presence of elements (Zn and O) as per the stoichiometric composition in the samples. ZnO nanoparticles have maintained considerably multidrug resistance against the clinical pathogens such as *Staphylococcus aureus* and *Bacillus subtilis* (Gram positive bacteria*)* and *Klebsiella pneumoniae and Escherichia coli and* (Gram negative bacteria). The *invivo* sub-acute studies carried out with ZnO nanoparticles in Swiss Albino Mice revealed that the organs such as liver and kidney are moderately injured after 14 consecutive days of continuous oral ingestion of nanoparticles. However, there is a need for further toxicity study, emphasizing on chronic and gene toxicity assessment and focusing more on possible liver and kidney damage.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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