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Synthesis of Zinc Oxide Nanoparticles using Peel Extract of Citrus paradisi and its Antibacterial Effects

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ABSTRACT

Introduction: *The interaction of nanoparticles (NPs) with biomolecules and microorganisms is an expanding field of research. Zinc oxide nanoparticles (ZnO-NPs) are known to be one of the multifunctional inorganic nanoparticles with effective antibacterial activity. This study aims to determine the antimicrobial efficacy of biologically synthesized ZnO-NPs.* ***Methods:*** *This quasi experimental study was designed to synthesize of ZnO-NPs from zinc sulphate monohydrate ($ZnSO_4.H_2O$) solution using Citrus paradisi peel extract as reducing agent as well as capping agent which being considered a rapid process that requires no toxic chemicals. The characterization of nanoparticles was done by using (Ultraviolet visible) UV-Vis spectroscopy. This biological synthesis guided ZnO-NPs were then studied on Gram negative bacteria like Escherichia coli (E. coli) using disc diffusion method to evaluate its antibacterial activity.* ***Results:*** *The ZnO-NPs containing solution showed distinctive colour change and a sharp peaked Surface Plasmon Resonance (SPR) appeared at 370 nm which suggested formation of nanoparticles. The antibacterial activity of different concentrations of ZnO-NPs, $ZnSO_4.H_2O$ solution and reference drug ciprofloxacin revealed that ZnO-NPs possessed significant antibacterial effect ($p < 0.001$) compared to $ZnSO_4.H_2O$ solution but relatively less antibacterial effect than that of ciprofloxacin.* ***Conclusion:*** *The results depicted that the biologically synthesized nanoparticles have significant antibacterial property. Wide range of antibacterial effects, safety and detailed mechanisms of ZnO-NPs should be further studied thoroughly.*

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INTRODUCTION

Nanotechnology may be defined as the synthesis, characterization, exploration and application of nano-sized materials for the development of science.[1] It has wide range of applications in nanomedicine, nanoelectronics, energy production and consumer products etc. Nanoparticles (NPs) are the particles having at least one dimension less than 100 nm.[2] Smaller the size of particles greater will be surface area compared to their volume which enhances the reactivity of the nanoparticles.[3] The nanoparticles are synthesized through physical, chemical and biological methods. Conventional physical and chemical methods produce some adverse effects like production of toxic byproduct, requirement of high temperature and pressure, more time consumption and expensive etc.[4] Biological method is one of the most preferred method because it is simple, inexpensive, good stability of nanoparticles, less time consuming, non-toxic byproducts and large scale synthesis etc. Moreover, use of plants or part of plants for the synthesis of nanoparticles is ecofriendly.[3,5] Zinc is an important metal of our body. It acts as a cofactor of enzymes. It supports gonadal activity, growth, wound healing and boost immune system. Zinc oxide nanoparticles (ZnO-NPs) are interesting among all metal oxide nanoparticles due to its antibacterial, antifungal, wound healing and UV filtering properties.[6,7] It has also antioxidant, catalytic and anti-diabetic activity. It has longer durability, higher selectivity and heat resistance capacity.[8]

Bacterial infectious diseases are serious health problems that have drawn the public attention worldwide as a human health threat, which extend to economic and social complications. Increased outbreaks and infections by pathogenic strains, bacterial antibiotic resistance, emergence of new bacterial mutations, lack of suitable vaccine and hospital associated infections are global health hazards to humans. So, innovation of new antibiotics is the immense challenge for modern medical science**.** ZnO-NPs produce antibacterial activity by several mechanisms such as by direct interaction with bacterial cell wall, by liberating antimicrobial ions mainly zinc ions (zn^{2+}) and by forming reactive oxygen species (ROS) etc. Interestingly, ZnO-NPs are reported by several studies as non-toxic to human cells.[9] So, this aspect necessitated their uses as antibacterial agents.

Researchers are using biological method for the synthesis of various nanoparticles due to eco-friendly nature. Many researchers used different kinds of plants for the synthesis of ZnO-NPs such as *Citrus paradisi, Citrus aurantifolia,* Seaweeds, aloe vera, *punica granatum,* neem, tamarind, *Calotropis gigantea,* green tea, lemongrass etc.[10] Among these plants *Citrus paradisi* is cheaper and available in local market of Bangladesh. The local name of *Citrus paradisi* is orange.

The biosynthesis of ZnO-NPs and studies on their antimicrobial effects are still in the infancy stage and limited number of works has been reported. So far, this type of study is new in our country. Therefore, it is thought worthwhile to study the synthesis of ZnO-NPs by biological method using *Citrus paradisi* peel extract as reducing as well as capping agent. Antibacterial activity of ZnO-NPs was also evaluated on Gram negative bacteria like *Escherichia coli* by disc diffusion technique.

METHODS

This Quasi Experimental Study was conducted in the Department of Pharmacology and Therapeutics, Rajshahi Medical College, Rajshahi, in collaboration with the Department of Microbiology, Rajshahi Medical College, and Rajshahi during the period of July 2015 to June 2016. Protocol of this study was approved by Institutional Review Board (IRB) of Rajshahi Medical College, Rajshahi.

Preparation of peel extract: At first, within a glass beaker (500 ml) *Citrus paradisi* peel extract was prepared by boiling 100 gm peel in 400 ml of deionized water at 80^0C for 30 minutes. The extract was filtered through Whatman filter paper and stored in refrigerator at 4^0C for further experiments.[11]

Synthesis of ZnO-NPs: The mixture of 90 ml aqueous peel extract of *Citrus paradisi* and 300 ml of 3 mM $ZnSO_4.H_2O$ solution was made in a beaker and stirred for 3 hours at 75-80^0C by Magnetic stirrer with hot plate (Model no. MS-300, Qingdao Tlead International Co., Ltd. China) and kept for observation at room temperature up

to change in colour. The change of colour from yellowish green to light yellow proved for the formation of ZnO-NPs.[11]

UV-Vis Spectral Analysis: The presence of ZnO-NPs **in** the prepared solution was analyzed by UV-Vis spectroscopy using spectrophotometer (Model 340, Sequoia-Turner Corporation, Germany). Absorbance of prepared solution was measured repeatedly after 01 hour, 24 hours, 07 days and 21 days to see the stability of ZnO-NPs. The scanning range for the samples was 350-440 nm.[12] Baseline correction of the spectrophotometer was carried out by using a blank reference.

Antibacterial Activity of ZnO-NPs: The antibacterial activity of ZnO-NPs, $ZnSO_4.H_2O$ solution and Ciprofloxacin were studied by Disc diffusion method on *Escherichia coli* in Mueller-Hinton Agar media (Himedia, Mumbai, India). Twenty four hours fresh cultures were prepared and the standardized (McFarland No. 0.5) inoculum was made and used for the antibacterial study.[3] By using micropipette 10 µg, 20 µg, 30 µg, 40 µg and 50 µg of ZnO-NPs and $ZnSO_4$. H_2O solution was added in sterile paper discs (5mm) made of Whatman filter paper. For 01 µg of $ZnSO_4$, 02 µL of $ZnSO_4$ solution and for the 01 µg of ZnO-NPs, 01 µL of ZnO nanosolution were put into Whatman paper disc**.** Disc containing ZnO-NPs, $ZnSO_4.H_2O$ solution and Ciprofloxacin were placed on solidified agar plates with the help of a sterile forceps.The cultured agar plates were incubated at 37°C for 24 hours. The diameter of the Zones of inhibition including the diameter of discs were measured after 24 hours of incubation with transparent millimeter ruler.[7]

Statistical analysis:

The results were calculated as mean±standard deviation (SD). Data of zone of inhibition of *E. coli* created by ZnO-NPs and $ZnSO_4.H_2O$ (aq) solution were compared using paired *t*-test. A *p*-value less than 0.05 were considered statistically significant.

RESULTS

Synthesis of ZnO-NPs:

Establishment of ZnO-NPs was proved by the change of colour from yellowish green to light yellow.

UV-Vis Spectral Analysis

Absorption spectra of ZnO-NPs had a sharp peak and absorbance maxima at 370 nm. There was no significant change observed in peak position for 1st and 21st day (Figure 1).

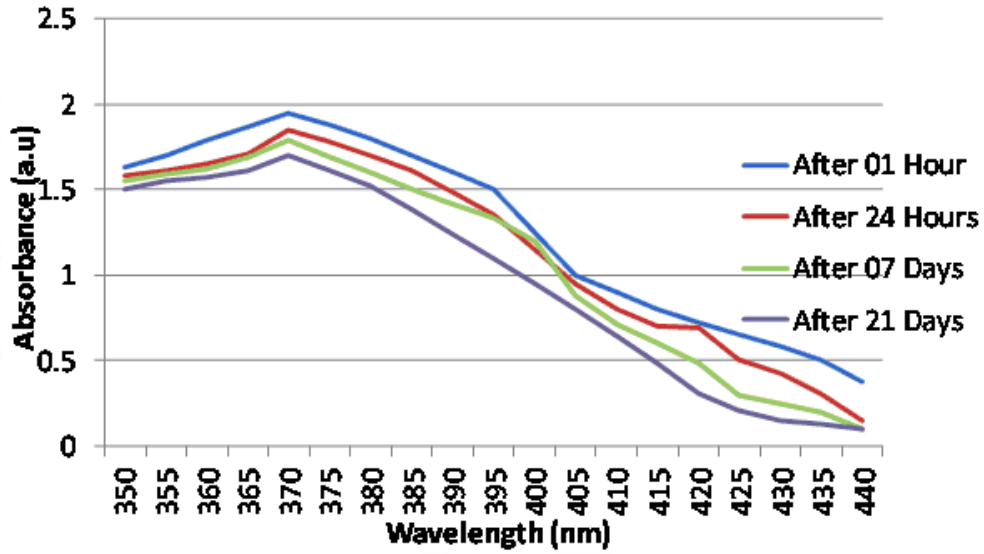

Figure 1. Showing UV-Vis spectra of synthesized ZnO-NPs using *C. paradisi* peel extract

Antibacterial Activity of ZnO-NPs

Zone of inhibition produced by ZnO-NPs, $ZnSO_4.H_2O$ and Ciprofloxacin are shown in Table I. Results were expressed as Mean±SD.

Ciprofloxacin (5 mcg/disc) produces Zone of inhibition 20.83±2.14 mm, which was greater than ZnO-NPs and $ZnSO_4.H_2O$ solution. On the other hand, ZnO-NPs exhibited greater Zones of inhibition than $ZnSO_4.H_2O$ at different strengths. Highest activity of ZnO-NPs was found at the concentration of 50 µg (10.26±0.65 mm). No Zone of inhibition was found in control (Peel extract/ Deionized water) solution. Statistical analysis proved that ZnO-NPs have got significant antibacterial activity ($p< 0.001$) as compared to $ZnSO_4.H_2O$ at all strength.

Table I. Showing Zones of inhibition found in *E. coli* cultures

Concentrations (µg/disc)	Zones of inhibition (mm) (Mean±SD)			
	$ZnSO_4.H2O$	ZnO-NPs	Ciprofloxacin (5 µg/disc)	Control
10 µg	00±00	5.1±0.17		
20 µg	5.15±0.12	6.8±0.78		
30 µg	5.67±0.37	7.53±0.7	20.83±2.14	00
40 µg	6.00±0.63	8.55±0.67		
50 µg	6.62±0.43	10.26±0.65		

$p- < 0.001$

DISCUSSION

Green synthesis of ZnO-NPs from plant extract is better than others.[5] Plants are easily available, safe and nontoxic, in most cases have a broad variety of metabolites. That can aid in reduction of metallic ions quicker than others. Nanoparticles exhibit different colour in aqueous solution due to excitation of surface plasmon vibration.[1,13] In the ZnO-NPs, electrons oscillate collectively. These oscillations affect how light interacts with the nanoparticles. The specific oscillations depend on the particles size and shape. So particles of different sizes have different colours in different surface plasmon absorption peak. Mishra et al.[5] found brownish yellow coloured ZnO-NPs with surface plasmon absorption peak at 364 nm. They used peel extract of *Punica granatum* for the synthesis of ZnO-NPs. Pale-white coloured ZnO-NPs with surface plasmon absorption peak at 325 nm was observed by Senthilkumar et al.[3] They used green tea for the synthesis of ZnO-NPs. Another study done by Ramesh et al.[6] were found light white coloured ZnO-NPs with surface plasmon absorption peak at 208 nm and 215 nm. They used *Citrus aurantifolia* for the synthesis of ZnO-NPs. Rajamanickam et al.[1] stated brown coloured ZnO-NPs with surface plasmon absorption peak at 310 nm. They used Actinomycetes for the synthesis of ZnO-NPs. No peak was observed due to presence of impurities in NPs. Widening of peak indicates very small sized particles.[14] We have found light yellow coloured ZnO-NPs with surface plasmon absorption peak at 370 nm wavelength. Our observation is in agreement with the other study reported by Kumar et al.[11]

UV-vis spectroscopy is one of the most widely used techniques for characterization of nanoparticles. The absorption phenomenon shown by the nanoparticles is due to surface plasmon resonance. The position and shape of plasmon absorption of nanoparticles were strongly dependent on the particle size, dielectric constant and surface

absorbed species. Nanoparticles of various shapes and sizes, from approximately 40 to 120 nm, having colours ranging from violet ~400 nm to red ~700 nm wavelength, were characteristics.[15] Surface plasmons are essentially the light waves that are trapped on the surface because of their interaction with the free electrons on metal. When metal nanoparticles are embedded in dielectric media and specimens re-exposed to electromagnetic radiation, Surface Plasmon Resonance (SPR) absorption band is observed at a specific wavelength depending upon the nature of metal, size of the particles and distribution.

In present study, ZnO-NPs exhibited a single and well defined peak in the absorbance spectrum with maximum absorbance at 370 nm which corresponds to characteristic SPR of ZnO-NPs. Several studies[1,3,5] have observed only single absorption peak like us. On the other hand, Ramesh et al.[6] and Meruvu et al.[14] have founded double and twelve surface plasmon absorption peak respectively.

The synthesized ZnO-NPs were stable without shifting the surface plasmon absorbance band observed at 1st, 7th and 21st day. But after 21 days stability was lost. Stable SPR peak indicates that new particles do not aggregate.[16] Mishra et al.[5] reported synthesis of ZnO-NPs by peel extract of *Punica granatum* was more stable than that of us. Their NPs were stable more than six months.

Therefore, the overall findings concluded that synthesis of ZnO-NPs using peel extract of *Citrus paradisi* were roughly spherical in shape and having size about 70 nm and stable for 21 days. Further study should be done to evaluate the size and shape of NPs.

In this study, the antibacterial activity of ZnO-NPs was tested against common pathogenic organism like Gram negative bacteria *Escherichia coli.* Zone of inhibition is the only criterion which has been used to compare the activity. The anti-bacterial activity of ZnO-NPs showed concentration dependent activity. Though ZnO-NPs exhibited lower zone of inhibition than Ciprofloxacin, ZnO-NPs created significantly greater bacterial zone of inhibition and appeared sensitive compared to $ZnSO_4.H_2O$. The control solution of peel extract and deionized water did not show any antibacterial activity in *E. coli* cultures. The diameters of zone of inhibition in the agar plate were measured in mm and summarized in Table I.

In a similar study done by Senthilkumar et al.[3] evaluated concentration dependent activity of ZnO-NPs on Gram positive and Gram negative bacteria prepared from *Camellia sinensis*. They obtained no zone of inhibition in *E. coli* cultures using 10 µg/disc and 20 µg/disc ZnO nano solution. But we have found 5.1 mm and 6.8 mm zone of inhibition with the concentrations of 10 µg/disc and 20 µg/disc respectively. It was may be either due to structural differences or due to resistant organism. Another study conducted by Prasad et al.[17] evaluated concentration dependent activity of ZnO-NPs on Gram negative bacteria like *E. coli* prepared by combustion method. They showed that 18 mm zone of inhibition in *E. coli* cultures was found at a dose of ZnO-NPs (50 µg/disc), which was possibly due to very small size (30 nm) and highly pure nanoparticles. But we have found 10.26 mm zone of inhibition at same concentration may be due to crude compound and different methods of synthesis. Namasivayam et al.[18] have found greater zone of inhibition (30 mm) with the dose of 50 µg/disc than us and Prasad et al.[17] It might be due to different method of synthesis (chemical method) and smaller particle size than us. The small size of metallic nanoparticles ensures that a significantly large surface area of the particles is in contact with the bacterial effluent. Considering a hypothetical case with spherical particles of uniform size, a reduction in the particle size from 10 m to 10 nm will increase the contact surface area by 10^9.

The exact antibacterial mechanism of ZnO-NPs is not clearly known. The smaller size of NPs facilitates easy entry into the microbial cell mem-

brane, produce broad contact with microorganism and enables inhibition mechanisms inside the cell. ZnO-NPs generate hydrogen peroxides which chemically interact with membrane protein and lipid bilayers. The antimicrobial activities of these NPs may involve both the production of reactive oxygen species (ROS) and the accumulation of NPs in the cytoplasm on the outer membranes. ROS causes membrane dysfunction and cell death by oxidizing the membrane lipids.[3,19] Xia et al.[20] have suggested that smaller sized NPs can enter the mitochondria of cells through various pathways and thereby induce oxidative stress and cell death via apoptosis.

CONCLUSION

In the present study, zinc oxide nanoparticles were successfully obtained by *Citrus paradisi* peel extract assisted synthesis. Significant colour change and UV-Vis spectroscopy suggested the formation of nanoparticles. Those zinc oxide nanoparticles showed significant antibacterial activity in compared to zinc sulphate monohydrate. Characterization, wide range of antimicrobial activity, mechanism of actions and safety profile of zinc oxide nanoparticles may be recommended.

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Conflict of interest: There is no conflict of interest.

REFERENCES

1. Rajamanickam U, Mylsami P, Viswanath S, Muthusamy P. Biosyntheis of Zinc Nanoparticles Using Actinomycetes for Antibacterial Food Packaging. ICNFS. 2012; 39: 195-199.
2. Rasmussen JW, Martinez E, Louka P, Wingett DG. Zinc oxide nanoparticles for selective destruction of tumor cells and potential for drug delivery applications. Expert Opin Drug Deliv. 2010; 7(9): 1063- 1077.
3. Senthilkumar SR, Sivakumar T. Green tea (*Camellia sinensis)* mediated synthesis of zinc oxide nanoparticles and studies on their antimicrobial activities. Int J Pharm Pharm Sci. 2014; 6(6): 461-465.
4. Cauerhff A, Casro GR. Bionanoparticles, a green nano chemistry approach. Electron J Biotechnol. 2013; 16(6): 1-10.
5. Mishra V, Sharma R. Green synthesis of zinc oxide nanoparticles using fresh peels extract of *Punica granatum* and its antimicrobial activities. Int J Pharm Res Health Sci. 2015; 3(3): 694-699.
6. Ramesh P, Rajendran A, Subramanian A. Synthesis of zinc oxide nanoparticles from fruit of *Citrus auratifolia* by chemical and green method. AJPCR. 2014; 2(4): 189-195.
7. Gunalan S, Rejendran V, Sivaraj R. Green synthesis of zinc oxide nanoparticles against bacterial and fungal pathogens. Prog Nat Sci. 2012; 22(6): 693-700.
8. Padmavathy N,Vijayaraghavan R. Enhanced bioactivity of ZnO Nanoparticles- an anti icrobial study. Sci Technol Adv Mater. 2008; 9(3): 1-7.
9. Sirelkhatim A, Mahmud S, Seeni A, Kaus NHM, Ann LC, Bakhori SKM, et al. Review on zinc oxide Nanoparticles: Antibacterial Activity and toxicity mechanism. Nano-Micro Lett. 2015; 7(3): 219-242.
10. Samat NA, Nor RM. Sol-gel synthesis of zinc oxide nanoparticles using *Citrus aurantifolia* extracts. Ceram Int. 2013; 39: 545-548.
11. Kumar B, Smita K, Cumbal L, Debut A. Green approach for fabrication and applications of zinc oxide nanoparticles. Bioinorg Chem Appl. 2014; 2014: 1-7. DOI: 10.1155/ 2014/523869.
12. Navale GR, Thripuranthaka M, Late DJ, Shinde SS. Antimicrobial activity of ZnO nanoparticles against pathogenic bacteria and fungi. JSM Nanotechnol Nanomed. 2015; 3(1): 1-9.

13. Saini J, Kashyap D, Batra B, Kumar S, Kumar R, Malik DK. Green synthesis of silver nanoparticles by using neem (*Azadirachta Indica*) and amla (*Phyllanthus Emblica*) leaf extract. Indian J Appl Res. 2013; 3(5): 209-210.
14. Meruvu H, Vangalapati M, Chippada SC, Bammidi SR. Synthesis and characterization of zinc oxide nanoparticles and its antimicrobial activity against *Bacillus Subtilis* and *Escherichia Coli*. Rasayan J Chem. 2011; 4(1): 217-222.
15. Mock JJ, Barbic M, Smith DR, Schultz DA, Schultz S. Shape effects in plasmon resonance of individual colloidal silver nanoparticles. J Chem Phys. 2002; 116(15): 6755-6759.
16. Sileikate A, Prosycevas I, Puiso J, Juraitis A, Guobiene A. Analysis of silver nanoparticles produced by chemical reduction of silver salt solution. Int J Mater Sci. 2006; 12(4): 287-291.
17. Prasad D, Girija CR, Reddy AJ, Nagabhushana H, Nagabhushana BM, Venkatesha TV, et al. A study on the Antibacterial Activity of ZnO Nanoparticles prepared By Combustion Method against *E. Coli*. Int J Eng Res Appl. 2014; 4(6): 84-89.
18. Namasivayam SKR, Prasanna M, Subathra S. Synergistic antibacterial activity of zinc oxide nanoparticles with antibiotics against the human pathogenic bacteria. J Chem Pharm Res. 2015; 7(3): 133-138.
19. Pal S, Tak YK, Song JM. Does the antibacterial activity of silver nanoparticles depend on the shape of the nanoparticle? A study of the Gram-negative bacterium *Escherichia coli*. Appl Environ Microbiol**.** 2007; 73(6): 1712-1720.
20. Xia T, Kovochich M, Nel AE. Impairment of mitochondrial function by particulate matter (PM) and Their toxic components: implications for PM-induced cardiovascular and lung disease. Front Biosci**.** 2007; 12: 1238-1246.