Preparation of zinc oxide nanoparticles using *Citrus sinensis* fruit peel aqueous extract and its antimicrobial study

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Abstract: The remarkable properties of nanoparticles within the 1 to 100 nm size range have garnered significant attention due to their unique characteristics arising from their small size and high surface-to-volume ratio. In this research, zinc oxide nanoparticles (ZnO NPs) were synthesized using an eco-friendly approach, employing orange fruit peel aqueous extract as a stabilizer as well as biological reducing agent and zinc nitrate hexahydrate as a zinc precursor. This method not only reduces the need for copious amounts of chemicals but also eliminates the use of hazardous substances in the fabrication process, augmenting the antibacterial attributes of the nanoparticles. As synthesized material underwent characterization through X-ray diffraction (XRD) analysis where the prepared material was single phase crystalline ZnO NPs and particle size was measured to be 31.2 nm using Debye-Scherrer's equation. Energy-dispersive X-ray spectroscopy (EDX) revealed that the sample primarily consisted of 98% zinc with minor impurities such as calcium and potassium in it. Fourier-Transform Infrared (FTIR) analysis exhibited a distinct absorption peak at approximately 550 cm^{-1}, confirming the synthesis of ZnO NPs. The antimicrobial potential of the synthesized ZnO NPs was evaluated against two bacterial strains, *Staphylococcus aureus* and *Escherichia coli*, through the Kirby Bauer method on media conducive to bacterial growth. The prepared nanoparticles exhibited bactericidal activity, effectively inhibiting bacterial growth, and generating discernible zones of inhibition.

Keywords: Antibacterial activity; Characterization; Green synthesis; ZnO NPs; Zone of inhibition.

Introduction

In the past decade, there has been an exponential growth in the field of nanotechnology, driven by its applications in diverse areas such as medicine, chemistry, and biotechnology. This development has led to a whole new world of opportunities for nanoscience, which is especially apparent in fields like drug delivery, optoelectronic, nanomedicine, biosensing, etc. One of the most intriguing attributes of nano-sized particles is their exceptional surface-to-volume ratio[1,2]. This unique feature renders nanoparticles significantly more reactive compared to their bulk counterparts, as there is presence of large surface area for reaction to occur. Thus, nanoparticles have properties different compared to that of bulk counterparts[3].

The synthesis of these nanoparticles is achieved through physical, chemical, or biological means. Techniques like hydrothermal and sol-gel synthesis, laser ablation, microwave-assisted combustion, and lithography, categorized as physical and chemical methods, demand specialized equipment, skilled operators, large amounts of energy and sophisticated instrumentation. These methods also pose health risks due to the toxic byproducts they often generate during synthesis[4,5]. In contrast, the green synthesis approach has gained lots of attention lately[6]. It offers nanoparticles that are not only economically viable but also environmentally favorable and biodegradable. This method stands out as a cost-effective alternative, free from toxic

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substances that could endanger health and provide excellent antimicrobial properties[7].The green synthetic route of nanoparticle synthesis is eco-friendly as a result of utilizing different plants parts, bacteria, fungi, and yeast themselves or their active products as reducing and stabilizing agents. Crude plant extract contains a variety of large organic biomolecules with different types of functional groups bonded to them. Functional groups such as alkenyl (C=C), amide (C=N), alcoholic/phenolic (-OH), amine (C-N), carboxylic (-COOH) are primarily responsible for the reduction and stabilization of prepared nanoparticles[8].

Zinc oxide nanoparticles (ZnO NPs) have gathered interest in the scientific community due to their distinctive optical and chemical characteristics. These properties can be readily achieved by modifying morphology of ZnO NPs. ZnO NPs have been found useful in an array of cutting-edge applications. These include fields such as electronics, communication, sensors, cosmetics, photodetector and solar cells, biology, and the pharmaceutical industry[9-13]. ZnO has been enrolled as one of the safest metal oxides by the U.S. Food and Drug Administration along with anti-inflammatory and anticancer agent[14,15]. Their versatility and unique attributes make them a frontrunner in the field of nanotechnology with application across various disciplines.

Hence in this study ZnO NPs was synthesized using *Citrus sinensis* (orange) fruit peel aqueous extract as stabilizing and capping agent along with zinc nitrate hexahydrate as zinc precursor. Although there are literatures synthesizing ZnO NPs using orange peel extract, no further literatures are available describing antibacterial activities and the green synthesis of ZnO NPs using Nepal inhabiting *Citrus sinensis* plant. So, we synthesized ZnO NPs using *Citrus sinensis* plant fruit peel aqueous extract and reported its antibacterial activities for the first time.

Materials and methods

Materials

Citrus sinensis fruit was collected from the local market in Kathmandu. Zinc nitrate hexahydrate used was from HI Media and deionized water for experiment was from Ocean Medico. All reagents used were of analytical grade and were used without further purification. Origin 2018 software was used for data analysis.

Extraction of aqueous *Citrus sinensis* fruit peel extract

The fresh fruit peel of *Citrus sinensis* was washed several times by using deionized water to remove dirt particles. After washing, the peels were left to sun dry and then ground to a fine powder. The fine peel powder (about 2 g) was placed in a 250 ml beaker, mixed with 100 mL of deionized water. The contents were stirred continuously for a duration of 3 hours. Following this maceration process, mixture was subjected to a water bath set at a temperature of 60°C for a period of 60 minutes. Subsequently, the mixture underwent filtration, and the resulting extracts were carefully collected and stored at 4°C in refrigerator for further use.

Figure 1: Flow diagram for preparation of ZnO NPs.

Preparation of zinc oxide nanoparticles

The ZnO NPs were synthesized by mixing 7.05 g of zinc nitrate with fruit peel aqueous extract. These mixtures were then stirred for 60 minutes and then placed in a water bath at 60 °C for 60 minutes. Subsequently, the mixtures were dried at 100°C in oven and then completely dried at 400 °C for 1 hour in furnace. The synthesized ZnO NPs was stored in an eppendorf tube until further requirement. The organic

peel extract contains different organic compounds such as flavonoids, carotenoids etc. which helps in ligation between zinc precursor and organic aromatic rings especially hydroxyl group and forms zinc-ellagate structure[16,17]. This zinc-ellagate structure during calcination decomposes because of which bond between zinc and oxygen can take place. The possible synthesis mechanism is illustrated in figure 2.

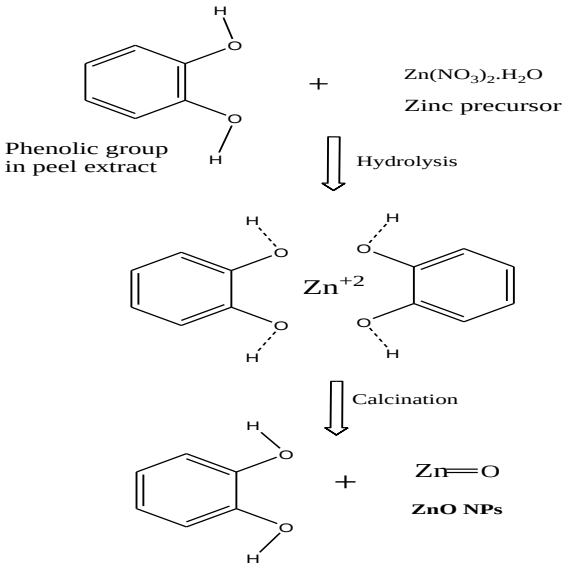

Figure 2: Possible mechanism of ZnO NPs formation using green method[16].

Characterization

The synthesized nanoparticle was analyzed by using different techniques in order to determine their properties. The characterizations of the prepared samples were applied by powder X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR) and UV-Visible Spectroscopy (UV). The crystallite size and structure of ZnO nanoparticles were determined by XRD (Bruker D2 Phaser) employing CuKα radiation (λ = 0.15406 nm) from 2θ ranging from 10° to 80°. The average crystalline size of the ZnO nanoparticles was estimated from the XRD diffraction peaks by using Debye Scherrer'sequation[18].

$$\text{Average crystalline size (D)} = \frac{0.9\,.\,\lambda}{\beta\,.\cos\theta}$$

Where, λ is the wavelength of X-ray, β is full width at half maximum (FWHM) of the most intense XRD peak expressed in radians and θ is Bragg's diffraction angle. The formation of ZnO NPs was also confirmed with the help of FTIR (PerkinElmer Spectrum 2) analysis carried out in the range of 400–4000 cm^{-1}. The elemental detection was performed using EDX technique.

Antibacterial Study

The antimicrobial activity of green synthesized zinc oxide nanoparticles was performed by the agar diffusion method and the corresponding value of the zone of inhibition was evaluated. The antibacterial activity of the nanoparticle was tested against bacteria *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 3292). For this purpose, Muller Hinton agar media was used for bacterial growth. Antibacterial properties were tested using two solvents, normal saline (0.9% NaCl) and DMSO at different concentrations. The suspension of ZnO NPs having different concentration were put in agar well made media which were inoculated by bacterial suspension in normal saline. Then it was incubated at 37°C for 24 hours and zone of inhibition (ZOI) was measured using ruler.

Results and discussion

Size and phase analysis using XRD pattern

Figure 3(A) shows the XRD results of the synthesized ZnO NPs where we can observe the presence of several distinct diffraction peaks at 32.23°, 34.84°, 36.23°, 47.86°, 57.07°, 63.28° and 68.29° and these peaks can be indexed to the (100), (002), (101), (102), (110), (103) and (112) crystal planes of ZnO respectively. All peaks are nearly matched with the hexagonal wurtzite crystalline phase (JCPDS card No. 36-1451) of ZnO indicating that the prepared sample contained only ZnO particles without other impurities. Further, the particle size of the prepared ZnO was calculated using the data obtained from Lorentzian fitting of the XRD spectrum as shown in figure 3(B). With the help of Lorentzian fitting, FWHM was calculated and by using the Debye-Scherer's equation and the particle size was calculated to be 31.2 nm. The obtained XRD pattern and average crystalline size are comparable to previous studies[19,20].

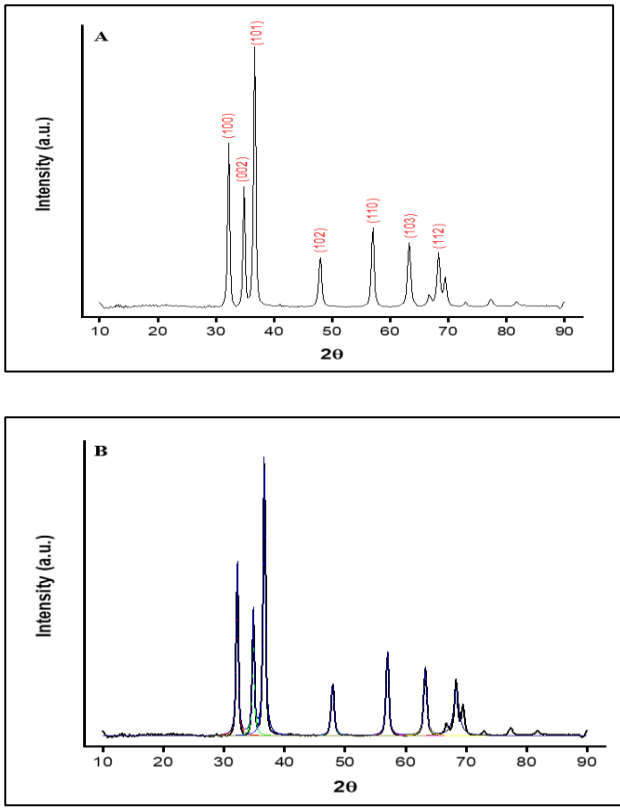

Figure 3: (A) XRD pattern of synthesized ZnO NPs (B) Lorentzian fitting plot of the experimental XRD plot.

FT-IR analysis

The FT-IR spectrum of the prepared sample is shown in figure 4. Various bands can be seen in the 1600–800 cm^{-1} regions which corresponds to the organic content from plant extract and CO_2 and H_2O absorbed from atmosphere within the sample of ZnO NPs. The sharp peak present at 530 cm^{-1}, corresponds to the characteristic bond vibration of Zn–O, which confirms that the synthesized material is zinc oxide. The result of the present study is in agreement to previous work[21].

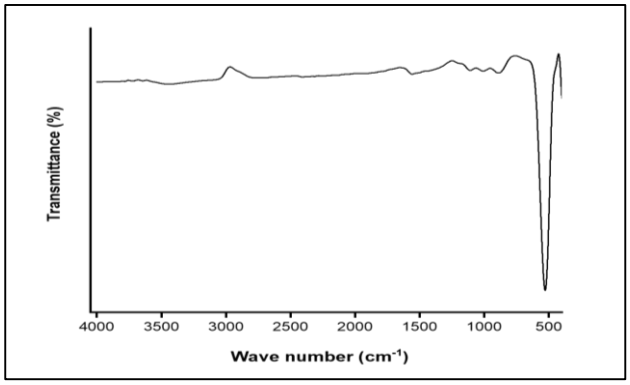

Figure 4: FT-IR spectrum of synthesized ZnO NPs.

EDX analysis

The result of EDX analysis is given in table 1 and in figure 5. This revealed a high signal for zinc, observed at 8.6 keV[22]. The EDX instrumentation used could not analyze the oxygen content. The constitution of zinc in sample was found to be 98.16%. This showed that the sample was highly pure and trace impurities present were metals such as K, Ca, Cr, Fe and S etc.

Table 1: Elemental composition of ZnO NPs sample.

Analyte	Result (%)
Zinc (Zn)	98.16
Potassium (K)	0.997
Calcium (Ca)	0.496
Sulphur (S)	0.188
Copper (Cu)	0.08
Chromium (Cr)	0.051
Iron (Fe)	0.029

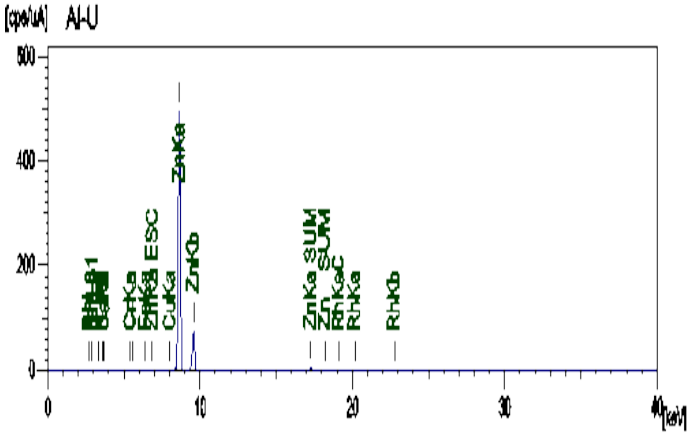

Figure 5: EDX spectrum of prepared ZnO NPs.

Antibacterial test

Plant based ZnO NPs are used extensively in the field of medicine, health, and environment. In this study the antibacterial potential of synthesized ZnO NPs was examined by measuring the zone of inhibition against both gram-positive (*S. aureus*) and gram-negative (*E. coli*) strain. Suspension of ZnO NPs were prepared in saline water and DMSO. The result of the test is shown below in figure 6.

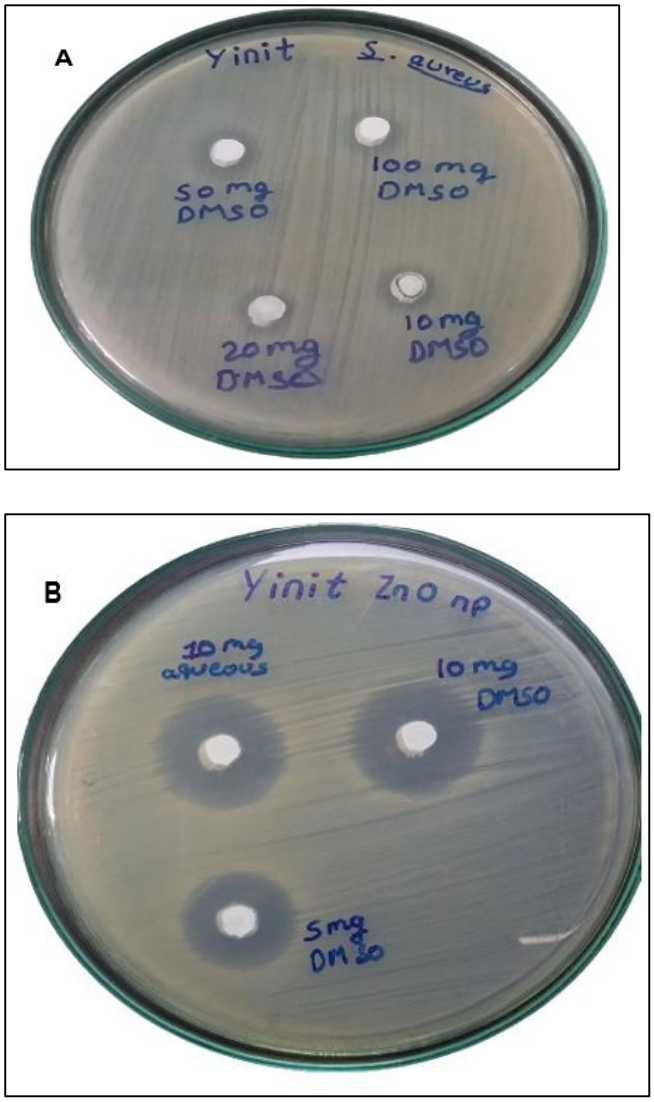

Figure 6: Antibacterial activity of ZnO NPs against *S. aureus* (A) and *E. coli* (B).

For gram negative bacteria *Escherichia coli*, the clear zone of inhibition (ZOI) was observed. The observed zone of inhibition was found to be 14 mm using 5mg/mL, 16 mm using 10 mg/mL ZnO NPs in DMSO while 18 mm using 10 mg/mL ZnO NPs in saline respectively. This finding was similar to that of Naiel*et al.*[23]. Similarly, in case of gram positive bacteria, *Staphylococcus aureus*, the clear zone of inhibition was found to be 7 mm at 10 mg/mL, 8 mm at 20 mg/mL and 10 mm at 50 mg/mL DMSO solution respectively. Results from antibacterial assay supported that *Escherichia coli* was more susceptible to ZnO NPs than *Staphylococcus aureus* which is in line with the works of Applerotet *al.*[24]. Observed ZOI due to synthesized ZnO NPs are tabulated in Table 2.

The possible mechanism behind observed antibacterial activity of ZnO NPs may be due to the production of Reactive Oxygen Species (ROS) when metal oxide nanoparticles encounter bacterial cells. These ROS molecules are harmful to bacteria as they disrupt the normal functioning of the respiratory chain and inhibit certain enzymes. Consequently, this disruption results in the creation and buildup of various ROS, including singlet oxygen, hydroxyl radicals, hydrogen peroxide, and superoxide anions, among others. These ROS can inflict damage on the internal components of bacteria, such as their proteins and DNA[25-27]

Table 2: Zone of inhibition of synthesized ZnO NPs against different microorganisms.

Solvent	Concentration (mg/ml)	Zone of Inhibition (mm)	
		S. aureus	E. coli
Aqueous (0.9 % saline) solution)	10	-	18
DMSO	5	-	14
	10	7	16
	20	8	N.D
	50	10	N.D

*** ND means not determined and (-) means not shown ZOI.**

Conclusions

This study demonstrated the efficiency of the aqueous extract of *Citrus sinensis* fruit peel as a reducing, capping and stabilizing agent leading to the successful synthesis of wurtzite hexagonal close packing ZnO NPs. As prepared ZnO NPs was characterized by different scientific tools such as FT-IR, XRD, EDX analysis. XRD analysis showed that the synthesized material was singe phase ZnO NPs with average grain size of 31.2 nm using Debye Scherrer's equation. FT-IR study showed a sharp peak at 530 cm^{-1} which was characteristic absorption region for Zn-O bond vibration. Antibacterial test of the prepared ZnO NPs showed its antibacterial efficiency towards both gram positive and negative strain but it showed higher efficiency against *E coli*, a gram negative bacteria. Hence, as

synthesized ZnO NPs can be applied as a potential antibacterial agent.

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