Annals of Biology **39** (2) : 157-163, 2023

Impact Score : 0.32 (Scopus)

Optimization of Phytoconstituent-mediated Synthesis of *Elaeocarpus ganitrus*-derived Zinc Oxide Nanoparticles Possessing Potent Antimicrobial Response against Gram-positive Pathogens

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(Received: November 17, 2022; Accepted: December 21, 2022)

ABSTRACT

The phyto-mediated green methodology using different parts of a plant has been observed to be a simple and novel method, having an edge over the other methods to synthesize nanoparticles. The plant biomolecules act as capping and stabilizing agents for synthesizing nanoparticles with the release of toxic-free by-products and also enhance their antimicrobial ability against a wide range of pathogens. In this direction, the present study involved the green synthesis of zinc oxide nanoparticles by employing *Elaeocarpus ganitrus* plant leaf extract. The various abiotic parameters (concentration of the metal salt, plant leaf extract volume, temperature and pH) were standardized and optimized to obtain the most effective nanoparticles. The synthesis of standardized ZnO nanoparticles was affirmed by UV-Vis spectroscopy, which depicted a sharp absorption peak at 361 nm. The XRD analysis revealed the crystalline nature and hexagonal wurtzite phase of the synthesized ZnO nanoparticles. Upon evaluation of the antibacterial response of these nanoparticles against gram-positive pathogenic bacteria, they were observed to exhibit zone of inhibitions as 27.9±0.63 and 27±0.47 mm against the tested pathogens, thus exhibiting their strong antibacterial ability. Hence, from the present study, it could be concluded that the green synthesis methodology can be used effectively for synthesizing nanoparticles possessing potent antibacterial responses against pathogenic bacterial isolates.

Key words: Antibacterial ability, *Elaeocarpus ganitrus,* nanoparticles, phytoconstituents, XRD

INTRODUCTION

'There's plenty of room at the bottom' entitled lecture, which was delivered by Noble laureate Richard Feynman at the California Institute of Technology in 1959, laid down the foundation stone of a novel and stupendous field of science, namely, nanotechnology, that attracted the scientific community to explore its vast potential (Jamdagni *et al.*, 2018). Nanotechnology can be delineated as the fabrication, exploration, characterization and potential usage of nano-sized materials viz., nanoparticles (NPs) that range from 1-100 nm, for the development of science. It deals with the study of extremely small structures. In recent years, nanotechnology has undergone immense development in various fields of science, including physics, chemistry, environmental science, pharmaceutical industries, biology, medicine, etc. (Vannan *et al.*, 2015).

Several metallic nanoparticles, including gold, silver, copper, iron, magnesium, zinc, platinum, palladium, etc. are being synthesized since the past few years. But zinc oxide (ZnO) has been proposed as a better candidate when compared to the other metal oxides, as it is less toxic, inexpensive and quite easy to prepare and handle. ZnO is biocompatible and safe, and the United States Food and Drug Administration (US FDA) has approved it as a GRAS (generally recognized as safe) metal. Various chemical and physical processes have been employed to manufacture these nanoparticles, but most of these are time-consuming, costly and are not eco-friendly. Hence, there is a dire need to adopt and standardize an eco-friendly approach which is free from toxicity, is cost-effective and involves the one-step synthesis of such NPs (Agarwal *et al.*, 2017; Gupta *et al.*, 2018; Bandeira *et al.*, 2020).

The use of environment-friendly materials like bacteria, plant leaf extract, enzymes and fungi for manufacturing metal NPs offers numerous advantages in terms of being eco-friendly, where hazardous chemicals are not

used, and exhibiting compatibility with pharmaceutical compounds for potential biomedical applications (Kumar and Das 2017; Saeed *et al.*, 2021). With a straight wide gap of 3.37 eV, violet/borderline ultraviolet (UV) absorption at 387 nm, significant and deep exciton-binding energy (60 meV); zinc oxide is a widely desired multifunctional metal oxide with a long list of appealing features (Król *et al.*, 2017).

Synthesis of effective nanoparticles has been reported to be highly dependent upon the standardization and optimization of different parameters involved in the synthesis process (Saranya *et al.*, 2017). Parameters like the concentration of the metal salt, plant extract volume, temperature, and pH play an essential role during the synthesis process and hence need to be optimized. With an increasing concentration of the metal salt after a threshold value, a loss in absorption has been reported to take place (Thakral *et al.*, 2021). With an increase in temperature beyond a threshold point, denaturation of plant biomolecules has been reported, which further results in the loss of absorbance (Jagdish and Nehra, 2022). Similarly, pH is also an important parameter for optimizing nanoparticle synthesis; as an enhancement in pH values exceeding the threshold limit has been found to lead to a loss in absorbance. Therefore, it can be concluded that an optimized and standardized process should be adopted for synthesizing nanoparticles, so that an effective methodology can be prepared and enhanced antibacterial potential can be achieved against a wide range of pathogens (Hoseinpour *et al.*, 2017; Thakral e*t al.*, 2021).

The current study involved the green synthesis of ZnO NPs by employing *Elaeocarpus ganitrus* plant leaf extract. The *E. ganitrus* plant is commonly known as the Rudraksha tree in India. Previous studies had reported that the Rudraksha tree phytochemicals possessed various beneficial activities against arthritis, asthma, epilepsy, coma, leucorrhoea, liver problems, mental illnesses, hysteria, hypertension and also have antioxidant properties (Pandey *et al.*, 2016). Therefore, in the present examination, phytochemicals of this plant have been used as capping agents for synthesizing green metallic oxide nanoparticles. The process of green synthesis of ZnO NPs was standardized and optimized to obtain highly effective nanoparticles having high antimicrobial ability against gram-positive pathogenic bacteria.

MATERIALS AND METHODS

The *E. ganitrus* plant was procured from Yaseen nursery, Rohtak, Haryana (India). Fresh 25-30 g green leaves were gathered and washed numerous times using tap water. Further, the washing was repeated twice using deionized water to remove any remaining dust particles or impurities. The cleaned green leaves were fragmented into fine pieces, placed in a borosil beaker with 500 ml of deionized water, and kept for boiling for 15-20 min on a hot magnetic stirrer. When the colour of the water changed from transparent to pale yellow, the resultant solution was removed from the stirrer and kept at room temperature to cool down. With the aid of Whatman filter paper no 1, the solution was filtered, and the resultant filtrate was kept at 4°C for experimental usage.

0.01 M solution of zinc acetate dehydrate was prepared in an Erlenmeyer flask, and the plant extract was poured drop by drop into it. The pH of the mixture was maintained at 12 by adding 2M NaOH drop-wise, while keeping it at constant stirring on a magnetic stirrer at room temperature for 90 min. The precipitates, which settled down at the bottom of the flask, were purified from the mixture by centrifugation at 7000 rpm for 7 min using distilled water, and a final washing was performed with 80% ethanol. The resultant solution was poured onto a clean glass petri plate and kept for drying at 60°C in a hot air oven for 8-10 h. The complete transformation of metal salt to metal oxide took place during the drying process. The nanopowder so obtained after drying was gathered and preserved in an air-tight vial for further investigation.

The optimization process depicts an optimum threshold value for each parameter (concentration of the metal salt, plant leaf extract volume, temperature and pH) required for the standardization process. By determining an optimal threshold limit, the synthesis process can be generalized. In this study, the parameters employed to optimize the green synthesis of ZnO nanoparticles included: concentration of the metal salt zinc acetate (varying between 0.0025 to 0.02 M), plant leaf

extract volume (from 0.25 to 2 ml), temperature (from 40 to 100°C) and pH (from 8 to 13). While optimizing one parameter for synthesizing the nanoparticles, the rest of the other parameters were kept constant.

The green synthesis of metal oxide was confirmed with the help of analytical techniques viz., UV-V is spectroscopy and XRD spectroscopy. UV-Vis spectroscopy was carried out using LAB India 3092 UV-Vis, and X-Ray Diffractometer (Rigaku, Ultima IV) was employed to perform XRD scanning.

The biosynthesized ZnO NPs were further subjected to evaluation of their antimicrobial ability and analysis of their minimum inhibitory concentration (MIC) against gram-positive pathogenic bacteria, including *Bacillus licheniformis* and *Staphylococcus sciuri*. These bacterial cultures were procured from Park Nidaan Hospital, Sonipat, Haryana, India.

The disc diffusion method was employed to evaluate the antimicrobial assay (Ahmad and Kalra, 2020). Fresh 24 h bacterial culture of both the strains was spread onto Mueller Hinton agar (MHA) plates. Varying concentrations of ZnO NPs (20, 30, 50 and 70 µg/ml), along with the antibiotic amikacin (30 µg/disc) used as the control were placed onto the plates. The results were evaluated after 24 h of incubation in an incubator.

Minimum Inhibitory Concentration (MIC) can be defined as the least concentration of a sample responsible for evoking a response in opposition to the aimed microbe to hinder its growth. MIC was evaluated by employing the macro broth dilution method, and the results were read using OD600 nm. Both the pathogenic bacteria were cultured in nutrient broth (NB) media overnight, and a final volume was managed at 1×10^5 CFU/ml. MIC was evaluated by seeding the sterile tubes with 2 ml of nutrient broth and adding 2 ml of the nanoparticle solution (256 µg/ml) in the first tube, and a two-fold serial dilution of the nanoparticle suspension was maintained in the consecutive tubes. All the tubes were seeded with 40 µl of the bacterial culture. The tubes were incubated in an incubator for 20-24 h, and the results were evaluated visually by determining the presence and absence of turbidity, and further confirmed by measuring the absorbance at OD600 nm (Sidhu and Nehra, 2020). Both the experiments (evaluation of antimicrobial ability, and MIC of the ZnO NPs) were performed in triplicates, and the interpretation of the results was determined as mean±SD (standard deviation).

RESULTS AND DISCUSSION

A pale white-coloured nanopowder obtained after drying, gave a preliminary confirmation of the synthesis of ZnO nanoparticles. Further confirmation of the synthesis was affirmed by UV-Vis spectrophotometry, which depicted a sharp absorption peak at 361 nm (Fig. 1).

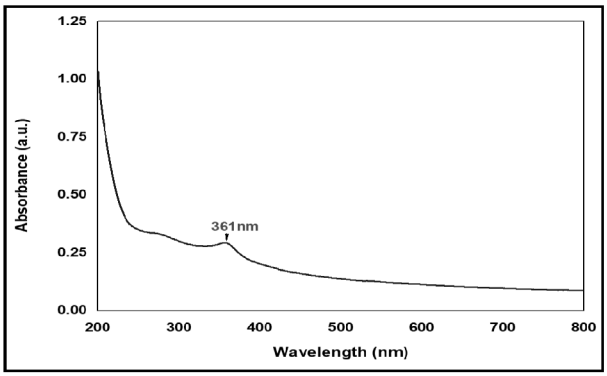

Fig. 1. UV-Vis spectra of synthesized ZnO NPs.

The green synthesis of ZnO NPs was optimized by employing several variables, including the metal salt concentration, plant leaf extract volume, temperature and pH.

In the current study, it was examined that increasing the zinc acetate concentration from 0.0025 to 0.01 M resulted in an increase in absorbance; however, with a further increase in concentration to 0.02 M, widening of the peak with a decrease in absorbance was noted (Fig. 2a). From these results, it could be inferred that a rise in concentration beyond a threshold limit caused a decline in absorption, illustrating a decreasing trend in the production of nanoparticles.

The maximum absorption was discovered to occur at 1 ml of leaf extract when 50 ml of zinc acetate solution was employed. After assessing multiple concentrations of the plant leaf extract (0.25, 0.50, 1 and 2 ml) it was observed that increasing or decreasing the amount of plant leaf extract from 1 ml resulted in reduced absorption and further decreased the synthesis of nanoparticles (Fig. 2b).

Temperature and pH are other crucial parameters in the biogenesis of ZnO miniature particles. In the current study, the synthesis of green ZnO NPs was optimized at a range of

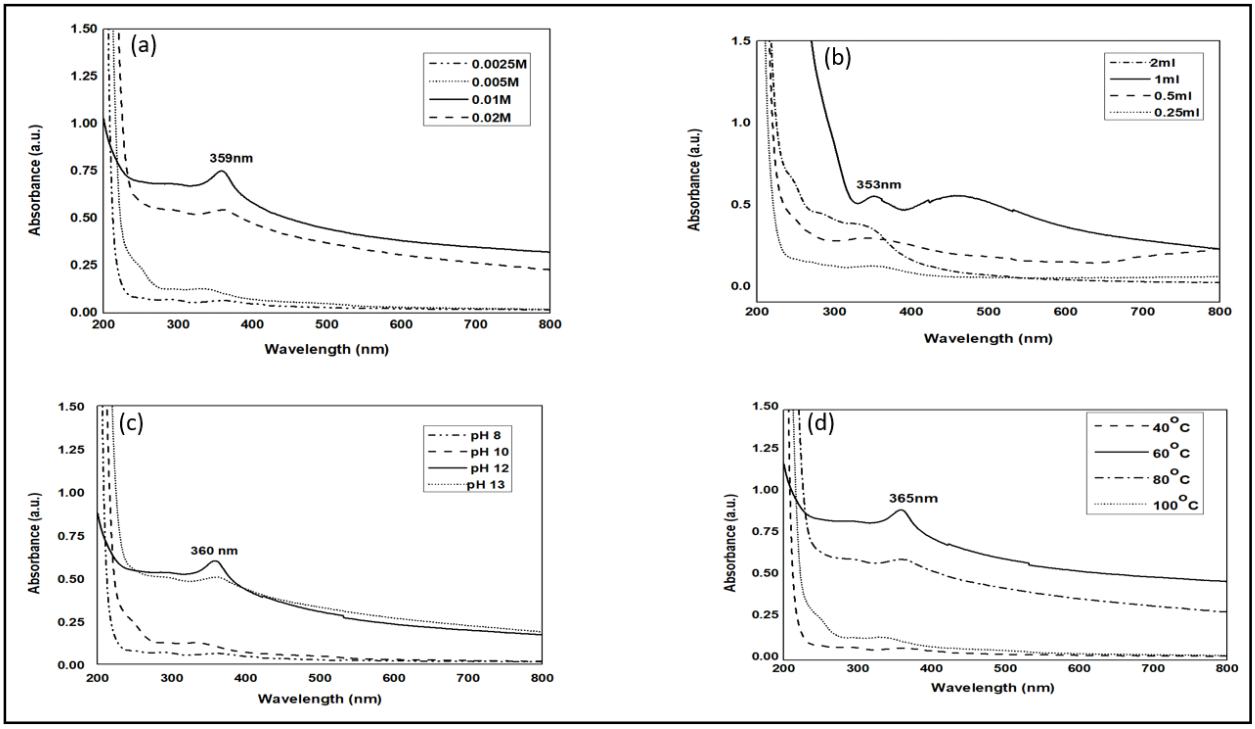

Fig. 2. Standardization and optimization of parameters for green synthesis of ZnO NPs: (a) Molarity (concentration) of zinc acetate; (b) Plant leaf extract volume; (c) pH and (d) temperature.

temperatures (from 40 to 100°C). A straight absorption line showed that at 40°C, no noticeable synthesis occurred. At 60°C, the absorbance was at its maxima with a peak at 365 nm, but at 80°C, the absorbance decreased, and the peak widened. With no sign of the expected significant peak at 100 °C, the absorbance dropped dramatically with an increase in temperature. This may have happened, since, with an increase in temperature, the crucial plant biomolecules responsible for reducing and stabilizing the synthesized nanoparticles got denatured.

Additionally, pH is thought to play a significant part in the biogenesis of ZnO NPs. At pH 8, the absorption in the current study was essentially linear, with no discernible peak, but from pH 10 to 12, the absorption increased and improved, with a strong peak at 366 nm being seen at pH 12. A further decrease in absorption was seen when the pH was raised to 13 (Figs. 2c and 2d).

This led to the conclusion that the formation of a solution of 0.01 M metal salt zinc acetate and 1 ml of leaf extract at a temperature of 60°C and pH of 12, were the ideal settings for manufacturing ZnO nanoparticles. Thus, from the present investigation, it could be inferred that it was crucial to standardize and optimize the process of NP synthesis with respect to various parameters before using them as a potent source of antimicrobial agents. The process of standardization and optimization of NPs further paves the way to utilize them in an *in vivo* system. Several other researchers have also reported the significance of standardization and optimization of green synthesized ZnO NPs for several parameters as used in the present investigation (Hoseinpour *et al.*, 2017; Saranya *et al.*, 2017; Thakral *et al.*, 2021). In an earlier study, it has been shown that a rise in the concentration (up to 0.01 M) of the metal salt (zinc acetate) led to a stable absorption and notable synthesis of ZnO NPs. However, the study also showed that increasing the concentration beyond 0.01 M led to a fall in ZnO NPs synthesis and a reduction in absorption, as observed in our present study also.

XRD was carried out to examine the crystallinity and structure of the ZnO NPs (Fig. 3). The diffraction peaks of the synthesized ZnO NPs at 2θ values corresponded to 31.69°, 33.89°, 36.32°, 45.43°, 56.45°. The Bragg's reflections were attributed at (100), (002), (101), (102) and (110) crystal planes, and were examined to be wurtzite hexagonal phase of ZnO, as examined

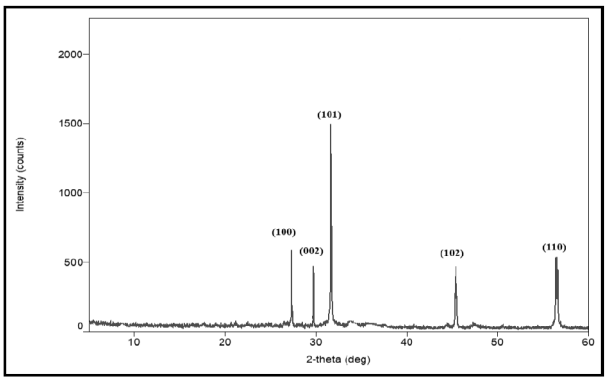

Fig. 3. XRD spectra of green synthesized ZnO NPs.

to be in harmony with Joint Committee on Powder Diffraction Standards (JCPDS file 36-1451). Other scientists have also demonstrated similar results in their studies (Ali *et al.*, 2016; Umavathi *et al.*, 2020).

The interplanar spacing of nanoparticles was calculated by employing Bragg's Law equation:

$$n\lambda = 2\, d\sin\theta$$

Where n = 1, λ= X-Ray wavelength and θ = Bragg's angle of diffraction.

With the help of the Debye-Scherrer equation, the crystallite size was calculated:

$$D = 0.9\lambda/\beta\cos\theta$$

Where 0.9 = Scherrer's constant, β = Full width at half maximum (FWHM) located at peak 2θ = 36.09°, and θ = Bragg's angle of diffraction examined at an intense peak comparably to (101) plane. Crystallite size was examined to be 21.28 nm.

The antimicrobial ability of the ZnO nanoparticles was examined against gram-positive pathogenic bacteria *S. sciuri* and *B. licheniformis* by using the disc diffusion method. It was noticed that the ZnO nanoparticles were bestowed with a strong antibacterial response against the tested pathogens with a strong zone of inhibition of the size 27.9±0.63 mm against *B. licheniformis* and 27±0.47 mm against *S. sciuri* (Table 1 and Fig. 4). The antibiotic amikacin used as a positive control did not show any zone of inhibition. Therefore, it could be concluded that both pathogens were 100% resistant to the amikacin antibiotic, whereas showing susceptibility to the synthesized ZnO NPs. A similar study was reported wherein ZnO NPs were manufactured using *Berberis aristata* leaf extract, and the zones of inhibition for *Bacillus cereus* and *Bacillus subtilis* were observed to be 19.0±0.5 and 26.0±0.8 mm and for *Staphylococcus aureus* as 18.0±0.5 mm (Chandra *et al.*, 2019). In another study, wherein ZnO NPs were synthesized using *Phoenix dactylifera*, they were found to exhibit a 15.9 mm zone of inhibition against a high concentration (100 µg/ml) of ZnO NPs (Rambabu *et al.*, 2021). In both these earlier reported studies, the antimicrobial behaviour of the green-manufactured ZnO nanoparticles was observed to be lesser than that obtained in the present study.

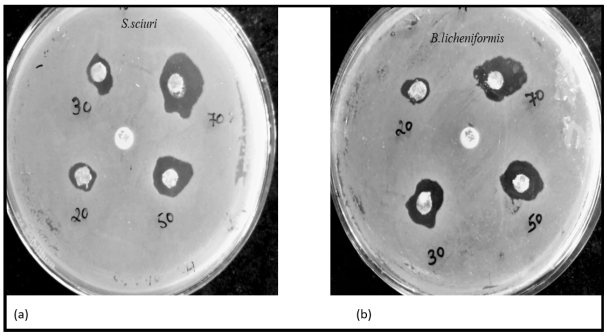

Fig. 4. Antibacterial ability of ZnO NPs against Gram-positive pathogenic bacteria (a) *S. sciuri* and (b) *B. licheniformis*. *Amikacin antibiotic* (30 µg/disc) has been used as a positive control, and ZnO NPs have been used in varying concentrations (20, 30, 50 and 70 µg/ml).

MIC values for the gram-positive bacteria were analyzed by observing the presence and absence of turbidity, and by measuring turbidity using OD600 nm. The MIC value for *B. licheniformis* was observed to be 32 µg/ml, whereas, for *S.sciuri*, it was found to be 64 µg/

Table 1. Antibacterial activity and MIC of green synthesized ZnO NPs against Gram-positive bacteria

S. No.	Bacterial isolate	Inhibition zone (mm±SD)				Amikacin (µg/disc)	MIC of ZnO NPs (µg/ml)
		Concentration of synthesized ZnO NPs (µg/ml)					
		20	30	50	70	30	
1.	*Bacillus licheniformis*	10±0.14	16.4±0.53	22.3±0.29	27.9±0.63	No zone	32
2.	*Staphylococcus sciuri*	7.3±0.33	11.5±0.86	18.8±0.84	27±0.47	No zone	64

ml (Table 1). In a similar study, using *Berberis aristate* to synthesize ZnO NPs, the MIC value for *B. cereus* was observed to be 128.0 µg/ml, for *B. subtilis* 64.0 µg/ml and for *S. aureus*, it was recorded as 128.0 µg/ml (Chandra *et al.*, 2019), which is comparatively higher than that reported in the present study. Thus, from the current investigation, it could be deduced that ZnO NPs synthesized in a green manner at optimal standardized conditions exhibited stronger antimicrobial response than reported earlier.

CONCLUSION

The current study was emphasized upon the green chemistry for the manufacturing of ZnO NPs at optimized and standardized conditions. The optimized nanoparticles were further exploited for evaluating their antibacterial ability against gram-positive pathogenic bacteria. And it could be concluded from the results that the green chemistry-mediated synthesis of ZnO NPs was endowed with more vital antibacterial behaviour against the tested pathogenic bacterial isolates; thus, paving the way for the use of an alternative therapeutic means for dealing with several bacterial pathogens.

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