Research Article

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Hydrothermal Assisted Green Synthesis of Zinc Oxide Nanostructures using *Azadirachta indica* Flower Extract and their Antibacterial Activity

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ABSTRACT

Nowadays, researchers are focusing on the development of new, effective antibiotics due to the re-emergence and inherent genetic changes of microorganisms. Metal oxide nanoparticles, in particular, have drawn a lot of attention due to their exceptional antimicrobial properties. Among these, ZnO nanomaterials have been extensively explored for biomedical applications due to their non-toxic, bio-safe nature and cost-effectiveness for human and animal use. Recently, researchers have proposed plant-mediated hybrid processes to synthesize nanoparticles. In this study, ZnO nanostructures were synthesized using aqueous extract of *Azadirachta indica* flower as a capping and stabilizing agent. The synthesized ZnO nanostructures were characterized *via* different techniques including X-ray diffractometry, Fourier transform infrared spectroscopy and scanning electron microscopy. Further, the antibacterial activity of ZnO nanostructures was investigated by disc diffusion method against Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli*. The results showed that ZnO nanostructures prepared using fresh neem flowers with the reaction time of 3 hours were highly active against the bacteria.

Keywords: Zinc oxide nanomaterials; *Azadirachta indica* flowers; Antibacterial activity; Biomedical applications

1. INTRODUCTION

The emergence of nosocomial infections and foodborne diseases caused by dangerous microbes poses a significant public threat. According to the World Health Organization, it is more difficult to treat infections like pneumonia, blood poisoning and foodborne illness (Chinemerem *et al.* 2022; Marco-Fuertes *et al.* 2023). Typical multidrug-resistant pathogens, including *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* have evolved by developing defence mechanisms against various antibiotics since the invention of antibiotics in the 20th century. These pathogens adopt a drug-resistant strategy by forming biofilms in which bacterial cells are enclosed in a self-protected polymeric matrix and adhere to living surfaces (Shree *et al.* 2023). Bacterial diseases currently stand as the second-leading cause of death, necessitating the development of new antibiotic agents to combat multidrug resistance (Menichetti *et al.* 2023).

In the past decade, researchers have focused on developing effective antibiotics due to the recurrence and inherent genetic changes in microorganisms (Serwecińska, 2020; Miethke *et al.* 2021). Metal oxide nanoparticles, particularly zinc oxide (ZnO), have gained attention for their exceptional antimicrobial properties, earning them the moniker "nano-antibiotics" (Ahmed *et al.* 2019; Guerrero *et al.* 2020). Among inorganic metal oxide nanomaterials, ZnO nanomaterials, with their non-toxic, bio-safe and cost-effective nature, have gained prominence for biomedical applications (Jha *et al.* 2023). Additionally, ZnO nanoparticles have demonstrated effectiveness in environmental remediation and disinfection (Ma *et al.* 2013). Zinc oxide owing to its significant antibacterial activity at smaller sizes and non-toxic nature to human DNA, is recognized as a safe material by regulatory authorities (Ahmed *et al.* 2019; Singh *et al.* 2012).

Nanotechnology research emphasizes creating nanoparticles of desired sizes and shapes. Various physical and chemical synthesis methods exist, but many involve toxic chemicals, limiting their use in medical and environmental applications. The quest for a facile, rapid and morphology-controllable method to synthesize ZnO nanoparticles suitable for biomedical applications remains a significant challenge. In contrast, the phytofabrication method, such as hydrothermal-biotemplate synthesis using plant extracts, improves stability, biocompatibility and environmental friendliness, making it a promising avenue for nanoparticle synthesis (Zare *et al.* 2019). This study explores the use of *Azadirachta indica* (Neem) flower

extract for the hydrothermal synthesis of ZnO nanostructures, considering their reported ethnomedical benefits and enriched physicochemical properties (Bhuyan *et al.* 2015; Geetha *et al.* 2016; Gnanasangeetha and Saralathambavani, 2013; Madhubala and Kalaivani, 2018; Ahmed *et al.* 2019; Handago *et al.* 2019; Singh *et al.* 2019; Haque *et al.* 2020;).

Gold (Gopalakrishnan and Muniraj 2019) and copper nanoparticles (Gopalakrishnan and Muniraj 2021) were synthesized using the aqueous extract of dried neem flowers and their antibacterial activity against pathogenic organisms in various studies was investigated. To our knowledge, the hydrothermal synthesis of ZnO nanostructures mediated by neem flower extract has not been reported.

The investigation focuses on the neem flower, a traditional medicinal plant and its aqueous extract as a capping and stabilizing agent in ZnO nanostructure synthesis using a hydrothermal method. This study describes, the influence of morphology using aqueous extracts of both fresh and dried neem flowers through a simple extraction method. Structural properties of as-prepared ZnO nanostructures were studied using X-ray diffraction and Fourier transform infrared spectroscopic techniques, while scanning electron microscope images revealed surface morphologies. The antibacterial activity of ZnO against Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* was evaluated using the disc diffusion method, providing insights into the potential applications of these nanostructures in biomedical contexts.

2. EXPERIMENTAL SECTION

Zinc nitrate hexahydrate [$Zn(NO_3)2.6H_2O$] and analytical-grade aqueous ammonium hydroxide [NH_4OH] were obtained from HI-MEDIA. *Azadirachta indica* flowers were collected in Coimbatore, Tamil Nadu, India. Both fresh and dried flower samples were employed for extract preparation. The sample flowers were thoroughly washed with distilled water (DW) and subsequently air-dried at room temperature.

2.1 Preparation of ZnO Nanostructures

Zinc nitrate hexahydrate (0.15 M) was dissolved in 30 mL of distilled water and stirred for 30 minutes. An aqueous ammonium hydroxide solution was slowly added to the zinc solution until the pH reached 12 and the mixture was stirred for an additional 30 minutes. Concurrently, 1.25 g of crushed neem flower was added to 50 mL of DW and stirred magnetically for 30 minutes at 80 ℃. Subsequently, 30 mL of the filtered aqueous solution of neem flower extract was added drop wise to the zinc solution and stirred for another 30 minutes. The resultant solution was transferred to a Teflon autoclave and treated hydrothermally at 180 ℃ for an appropriate duration. The final precipitate was washed and dried at 100 ℃ in a hot air oven for appropriate duration to obtain ZnO nanopowder. A series of reactions was conducted using fresh (F) and dry (D) neem flower extracts, with reaction times of 1 hour and 3 hours. The synthesized ZnO samples were labelled as follows: (a) FNL1, (b) DNL1, (c) FNL3 and (d) DNL3.

2.2 Characterization Techniques

Crystallite nature and phase purity were examined using a Bruker powder X-Ray diffractometer. Shimadzu IR Tracer-100 spectrometer was used to confirm the presence of functional groups in the synthesized ZnO nanostructures. The topological details of ZnO nanostructures synthesized using *A. indica* flower extract were examined using a Bruker scanning electron microscope.

The antibacterial efficacy of synthesized ZnO nanostructures was investigated using the standard disc diffusion technique, targeting both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacterial strains. The experimental procedure involved preparing bacterial suspensions, which were uniformly spread across agar plates and allowed to air-dry at ambient temperature.

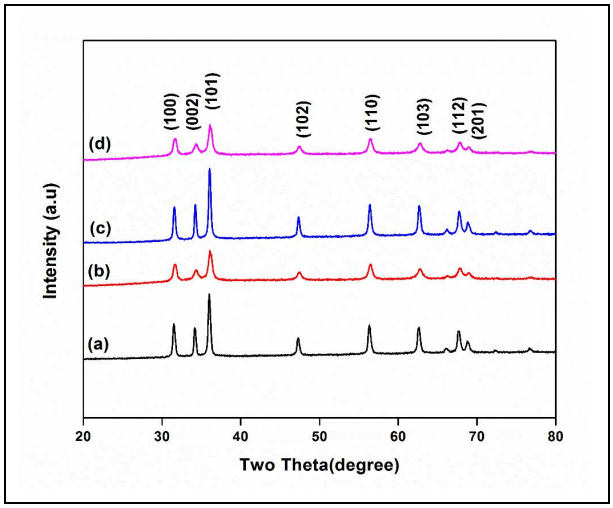


Fig. 1: XRD pattern for (a) FNL1, (b) DNL1 (c) FNL3 and (d) DNL3

3. RESULTS AND DISCUSSION

3.1 XRD Analysis

The XRD patterns of hydrothermal-assisted green synthesized ZnO nanostructures are shown in Fig. 1. The sharp, intense peaks observed at lattice planes (100), (002), (101), (102), (110), (103), (112) and (201), corresponding to 2θ values of 31.51°, 34.16°, 36.18°, 47.31°, 56.30°, 62.54°, 67.72° and 68.88°, respectively, align with the hexagonal wurtzite phase of ZnO (JCPDS

card # 36-1451). The calculated average crystallite sizes using the Scherrer formula were determined to be 23 nm, 16 nm, 28 nm and 11 nm for FNL1, DNL1, FNL3 and DNL3 samples, respectively. The observed variations in peak intensity and grain size suggest the influence of the flower extract on the crystalline structure of ZnO, potentially attributed to the chemical structure of the flower extract under different reaction conditions (Pillai *et al.* 2020). The increase in crystallite size for fresh neem flower extract can be attributed to the agglomeration of crystallites, which in turn is attributed to the phytochemicals present in the fresh extract. In contrast, for dry flower extract, a decrease in the crystallite size was observed which may be due to effective capping ability, which may prevent the crystallites from attaching and agglomerating (Ben *et al.* 2022; Supin *et al.* 2023).

3.2 FTIR Analysis

The FTIR spectra of all ZnO samples are illustrated in Fig. 2. The neem flower extract-assisted ZnO nanostructures exhibited distinct bands at various wavenumbers.

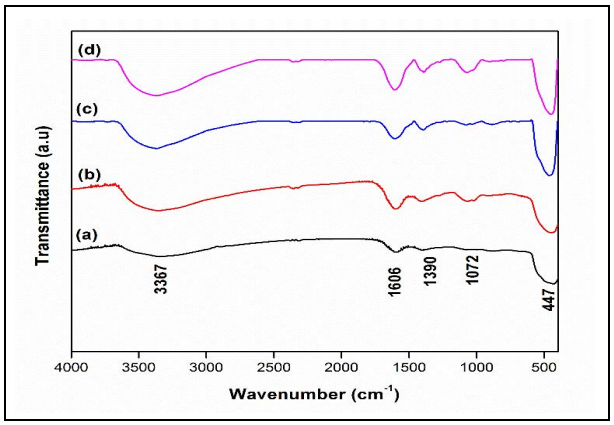


Fig. 2: FTIR spectra for (a) FNL1, (b) DNL1 (c) FNL3 and (d) DNL3

The peak observed at 3367 cm^{-1} is assigned to the stretching vibration of the hydroxyl functional group (Rehana *et al.* 2017). The band at 1606 cm^{-1} is attributed to C=C stretching of the aromatic ring (Elumalai and Velmurugan, 2015). The O-H stretching vibration of polyols is evident at 1390 cm^{-1} (Rehana *et al.* 2017), while the band at 1072 cm^{-1} is due to the C-N stretching mode of aromatic or aliphatic amines (Sharmila *et al.* 2018). The characteristic Zn-O bending vibration is observed at 447 cm^{-1} for all the samples (Ahmed *et al.* 2019).

The FTIR results unequivocally indicate the abundance of phenol and flavonoid compounds in the synthesized ZnO nanostructures. These functional groups present in the neem flower extract are likely capped on the surface of ZnO nanostructures, contributing to their stabilization (Alamdari *et al.* 2020; Zahoor *et al.* 2023). The surface structure of the biosynthesized ZnO is a crucial factor influencing the properties of ZnO nanostructures (Rehana *et al.* 2017). This insight emphasizes the importance of understanding the surface chemistry in modifying the properties of ZnO nanostructures for specific applications.

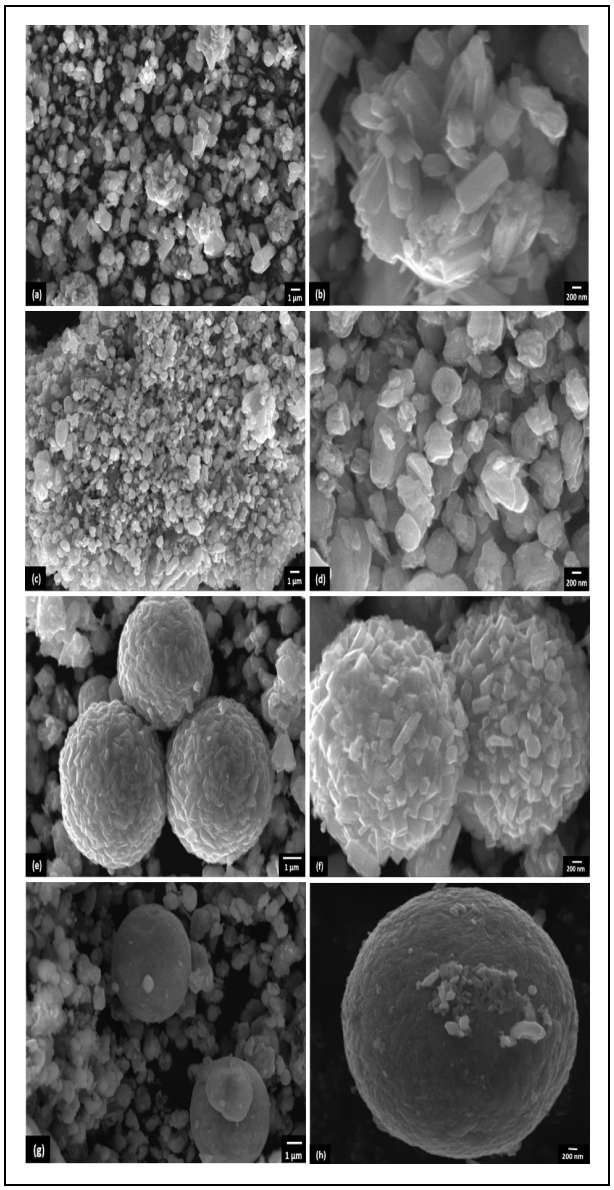


Fig. 3: SEM images for (a,b) FNL1 (c,d) DNL1 (e,f) FNL3 and (g,h) DNL3

3.3 SEM Analysis

The surface morphology is depicted in Fig. 3. Upon a 1-hour reaction time, agglomerated polygonal granules and spherical shapes were observed for the fresh and dry flower extracts, respectively. The agglomeration of ZnO nanostructures is attributed to the high surface energy of nanoparticles that increase agglomeration and formation of asymmetrical clusters (Tripathy *et al.* 2014; Barzinjy and Azeez, 2020).

Extending the reaction time to 3 hours revealed the formation of microsphere structures for both fresh and dry flower extracts. Notably, microspheres with a rough surface were observed in the case of fresh flower extract, while those derived from dry flowers exhibited a smoother surface. The formation of rougher microspheres can be explained on the basis of nucleation, growth and agglomeration. The higher moisture content in fresh neem flower extract might lead to slower heterogeneous nucleation and growth rates compared to samples synthesized using dry flower extract. Due to their large surface area and high surface energy, ZnO nanoparticles tend to form on secondary microparticles. This process is caused by Ostwald ripening, in which adjacent small nanoparticles spontaneously combine to form irregular or rougher microsphere structures (Thanh *et al.* 2014; Abdelbaky *et al.* 2023). The organic compounds present in *A. indica* flower extract act as both templates and surfactants for the synthesis of ZnO micro-sphere-like structures (Ramimoghadam *et al.* 2013). The surface roughness of the ZnO microspheres might be due to the organic molecules present in fresh neem flower extract. In contrast, the smooth surface of ZnO obtained using dry *A. indica* flower extract might be due to the capping ability of dry flower extract (Maher *et al.* 2023). These results revealed that the biomolecules in the aqueous flower extract act as both linker molecules in the self-assembly and stabilizing agents during the growth process (Singh *et al.* 2016).

The spherical shape of ZnO nanostrurures might be due to the presence of different functional groups such as ketones, alcohols, amines and carboxylic acids (Droepenu *et al.* 2021). These variations in morphology of prepared ZnO nanostructures indicate the influence of reaction time and nature of the flower (fresh and dry). Further, it can be seen that the size and shape of the nanostructures depend on the biomolecules that act as reducing, capping and stabilizing agents present in *A. indica* flowers (Pillai *et al.* 2020; Gopalakrishnan and Muniraj, 2021).

3.4 Antibacterial Activity

The antibacterial activity of hydrothermal-assisted green synthesized ZnO nanostructures was evaluated using the disc diffusion method. The corresponding zones of inhibition for *Staphylococcus aureus* and *Escherichia coli* are presented in Fig. 4 (i) and (ii), respectively. The zone of inhibition values for samples a, b, c and d against *S. aureus* and *E. coli* were measured at 13 mm, 11 mm, 15 mm and 14 mm, respectively. A graphical representation of inhibition values is illustrated in Fig. 4 (iii).

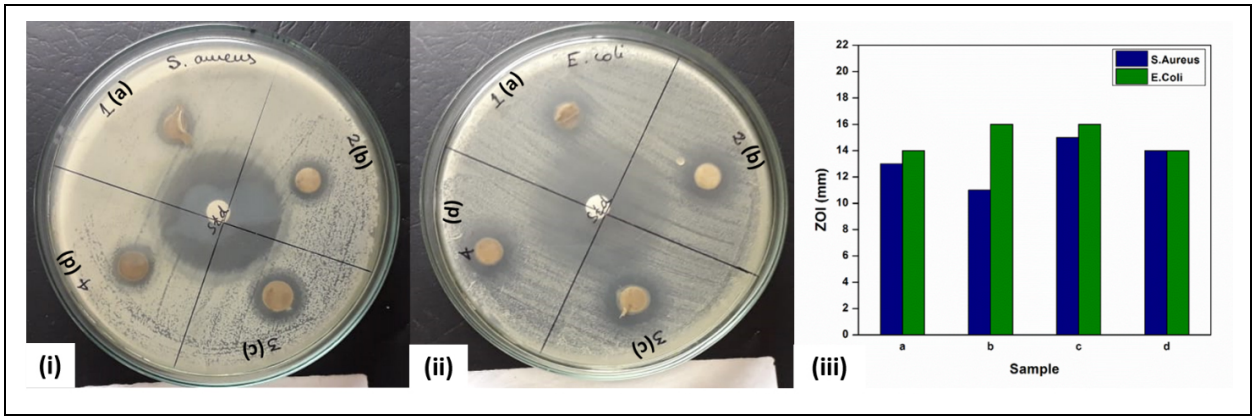


Fig. 4: (i, ii, iii) Antibacterial activity for (a) FNL1, (b) DNL1 (c) FNL3 and (d) DNL3

A significant antibacterial activity against both Gram-positive and Gram-negative bacteria was observed. The efficacy of nanomaterials in antibacterial action is attributed to various mechanisms, including the generation of reactive oxygen species (ROS), interaction between the nanomaterial and the bacterial cell wall, internalization of the nanomaterial and the release of Zn^{2+} ions.

Sample c demonstrated superior antibacterial activity compared to the other samples, potentially due to its microsphere morphology. The microsphere structure of ZnO in sample c allowed for effective interaction with bacterial cells, causing harm through the generation of ROS and inducing ion diffusion. Notably, the size and morphology of sample c facilitated interaction with the bacterial cell surface rather than internalization, contributing to its heightened antibacterial effect (Babayevska *et al.* 2022).

Further, the ROS generated on the surface of ZnO cause fatal damage to microbes through direct contact and penetration into the cell membrane (Padmavathy and Vijayaraghavan, 2008; Abebe *et al.* 2020). Moreover, the increased antibacterial activity of ZnO nanostructures was due to the abrasive surface which consisted of edges and corners that cause membrane injury (Sirelkhatim *et al.* 2015). The diverse antibacterial mechanism of prepared ZnO nanostructures emphasizes the potential of antibacterial resistance towards both Gram-positive and Gram-negative bacteria.

4. CONCLUSION

In this study, zinc oxide nanostructures were synthesized *via* a facile hydrothermal-assisted green synthesis method employing fresh and dry neem flowers as effective capping and stabilizing agents by varying the reaction time. The crystallinity and purity of biosynthesized ZnO nanostructures were confirmed by XRD and FTIR analyses. Surface morphology was characterized by SEM. Furthermore, the antibacterial activity was evaluated against both Gram-positive and Gram-negative bacteria and it was found that FNL3 sample displayed superior antibacterial activity, compared to other samples. These results indicate that the antibacterial activity is dependent upon the distinct surface morphology of ZnO nanostructures.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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