Biosynthesis of Zinc Oxide Nanoparticles from the Deproteinised Leaf Juice and Antibacterial Evaluation

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Abstract

Nanoparticles are established through-processing, consolidating, and deforming materials at the nanoscale. They offer benefits in terms of magnetic properties, catalytic activity, electronic properties, antimicrobial properties and anti-inflammatory effects. Green synthesis of nanoparticle is done through plants. The Medicago sativa L. is a well-known forage crop worldwide. It is rich in secondary metabolites, essential amino acids, and vitamins. This study investigated the synthesis of zinc oxide nanoparticles via a plant mediated approach. Zinc oxide nanoparticles were successfully synthesized with 0.1M zinc acetate dihydrate, reacted with the deproteinized juice (DPJ) of Medicago sativa. The yellow precipitate was centrifuged, dried, and collected for further analysis. The characterization carried out via TEM revealed the presence of spherical zinc oxide nanoparticles. Fourier-transform infrared (FT-IR) spectroscopy revealed an absorption peak for Zn-O bonding between 400 and 600 cm^{-1}. Additional characterization methods, such as X-ray diffraction (XRD), energy dispersive spectroscopy (EDS) and UV-Vis spectroscopy, confirmed the formation of zinc oxide nanoparticles. An antibacterial test was conducted on the synthesized nanoparticles. This study highlights DPJ as an eco-friendly, cost-effective and novel alternative for the biosynthesis of zinc oxide nanoparticles.

Keywords: Green synthesis, Zinc oxide nanoparticles, DPJ, TEM, FTIR.

Introduction

The green synthesis of nanoparticles redefines the boundaries of sustainable nanotechnology. The green synthesis method is environmentally friendly, offering a method that substantially mitigates ecological risk compared to conventional chemical routes (Nath & Banerjee, 2013). Nanomaterials are very minute structures less than 100 nm in size (Singh et al., 2020). Nanomaterials are a part of nanoscience, which is the study of processing, consolidating, and deforming materials at the scale of individual atoms or molecules (Jadoun et al., 2021).
Various types of nanoparticles, such as gold, silver, zinc, copper, and iron, are of interest because of their specific research focus, specifically, metal and metal oxide nanoparticles have attracted significant interest among research groups (Murali et al., 2021). Nanoparticles (NPs) exhibit unique properties, including magnetic, catalytic, electronic, optical, antimicrobial, anti-inflammatory, wound-healing, and antibacterial properties (Droepenu et al., 2021). The rise in antimicrobial resistance has necessitated the exploration of alternative antimicrobial agents, owing to their unique properties, nanoparticles have emerged as promising candidates against pathogens (Parmar et al., 2022). Gold, silver, and zinc nanoparticles offer promising solutions to the growing challenge of antimicrobial resistance (Bakhti et al., 2022; Chaudhary et al., 2019; Rodrigues et al., 2024; Zheng et al., 2017). Nanoparticles can be synthesized through physical, chemical, and biological methods. Physical and chemical methods involve the use of chemicals and reagents; these methods often use toxic reagents, increase environmental hazards, and are costly (Razavi et al., 2015), whereas the biological method is known as green synthesis, which is environmentally friendly, cost-effective, and nontoxic (Kiani et al., 2022a). The green synthesis of nanoparticles is performed using plants and microorganisms, plant-based nanoparticles utilize various parts, such as leaves, roots, calluses, and fruits (Kumar, 2020). In green synthesis, nanoparticles can also be synthesized from fungi. A report investigated the synthesis of silver nanoparticles from Fusarium solani isolated from wheat grain (Abd El-Aziz et al., 2015) and from Trichoderma harzianum. Different types of extracts are used for the synthesis of nanoparticles, including calcium oxide (CaO) nanoparticles synthesized from Papaya leaf extract and Tea extract (Anantharaman et al. 2016). Chandran studied the synthesis of silver nanoparticles using Aloe vera plant extract (Chandran et al., 2006). One study examined the synthesis of Calcium oxide nanoparticles from the fruit extract of Citrullus colocynthis and the fruit peel extract of Red dragon fruit (Hylocereus polyrhizus)
Another study synthesized CuO, CaO, and ZnO from the callus, and another study reported that potassium sulfate (K_2SO_4) nanoparticles were synthesized from Medicago sativa L. (El-Sharkawy et al., 2017). Both of these previous studies reported that they synthesize nanoparticles from callus, zinc oxide nanoparticles have attracted significant interest because of their distinguished properties, including antibacterial, antifungal, wound healing, and photochemical activities (Elumalai & Velmurugan, 2015). Zinc oxide nanoparticles can be processed through the sol-gel method, chemical method, or hydrothermal method, but these methods are toxic, harmful and expensive. The green synthesis of zinc oxide nanoparticles is carried out through various plant extracts, such as Cucurbita pepo, Azadirachta indica L (Bhuyan et al., 2015), Garcinia xanthochymus Hook (Nethravathi et al., 2015), Anisochilus carnosus L. F, (Anbuvannan et al., 2015), Aloe barbadensis miller L (Sangeetha et al., 2011), Eucalyptus globulus Labill (Barzinjy & Azeez, 2020), Cyanometra ramiflora L (Varadavenkatesan et al., 2019), Cayratia pedata L (Jayachandran et al., 2021a), Peltophorum pterocarpum DC (Pai et al., 2019), Kalopanax

septemlobus Thunb (Lu et al., 2019), Lippia adoensis Otto and A. Dietr (Demissie et al., 2020), and Cucurbita pepo (Hu et al., 2019).
Green synthesis offers a more environmentally friendly and cost-effective alternative (Valsan et al., 2023a). The investigation of plant extracts, particularly deproteinized juice (DPJ), as bioresources for nanoparticle synthesis has gained significant attention because of their inherent advantages (Iliyas, 2013). DPJ, a byproduct of green crop fractionation, is abundant in bioactive compounds, enzymes, antioxidants, and reducing agents, making it an ideal candidate for green synthesis (Sayyed IU, 2021). Utilizing DPJ for nanoparticle synthesis not only enhances its value as a byproduct but also reduces environmental issues associated with its disposal, such as bio pollution, nutrient overload, inefficiencies in agricultural processes, soil degradation, and waste management challenges (Iliyas, 2019; Manwatkar & Gogle, 2014). While DPJ has potential as a commercial by-product and for enzyme production, its application in nanoparticle synthesis opens new areas for research, thereby increasing its value (Iliyas, 2019; Sayyed IU, 2021). DPJ is produced from different plants such as Anethum graveolens, Trigonella foenium graecum, Spinacia oleracea, and Medicago sativa. Previous studies have shown that silver nanoparticles are synthesized from the callus of Medicago sativa L. (Akçay et al., 2025; Rabie et al., 2014),
Medicago sativa belongs to the Fabaceae family and is cultivated worldwide as a forage crop (Krol et al. 2019). It is rich in minerals, vitamins, essential amino acids, and important secondary metabolites, which contribute to the synthesis of nanoparticles (Rafińska et al., 2017). Consequently, deproteinized juice (DPJ) derived from Medicago sativa is a suitable candidate for nanoparticle synthesis. Despite the growing interest in green nanotechnology, the synthesis of nanoparticles using DPJ remains largely unexplored. DPJ is rich reservoir of bioactive molecules that serves as crucial reducing and stabilizing agents, effectively preventing the agglomeration of metal ions and ensuring the long-term stability of the resulting nanostructures (Iliyas, 2013; Prasad et al., 2021). In this study, zinc oxide nanoparticles are synthesized from the DPJ of Medicago sativa via an environmentally sustainable and cost-effective waste to wealth methodology. While these nanoparticles have broad applications across agriculture and environmental remediation (Sayyed IU, 2021). The primary aim of this research to organize the green synthesis and physiochemical characterization of ZnO nanoparticles utilizing TEM, energy dispersive X-ray spectroscopy (EDS), ultraviolet-visible spectroscopy (UV-Vis), X-ray diffraction (XRD), and Fourier transform infrared (FTIR) and to systematically evaluate their microbiological efficacy. By determine the Minimum Inhibitory Concentration against diverse bacterial strains, this work assesses the potential of DPJ-mediated nanoparticles to serve as novel, biocompatible alternative to conventional drugs in the face of escalating antibiotic resistance.

Materials and Methods

The experimental work was conducted in the laboratory of department of botany Ramakrishna More ACS college, Akurdi, Pune, India. All the chemical used in experiments are laboratory chemicals.

Collection of plant material

The fresh leaves of Medicago sativa L. (Family: Fabaceae) were collected from farm field of Vadgaon Shinde Village, Lohegaon, Pune, India, in month of August first week 2023. Collection was performed during the rainy season at pre-flowering stage. Plant material authenticated by Botanical Survey of India, Pune. The collected materials were thoroughly washed with distilled water to remove dust. The samples were then shade-dried at room temperature for one hour. The dried material was used for further extraction (Iliyas 2019).

Preparation of Deproteinized Juice

Leaves of Medicago sativa L. were crushed in a mortar and pestle, and homogenized with 20 ml distilled water to obtain a crude aqueous extract. The extract was filtered through blotting paper, and the resulting filtrate was heated at 90°C for 45 min. This boiled solution was allowed to cool at room temperature and then filtered through Whatman filter paper grade 1 (Supertek). was collected and used as deproteinized juice (Iliyas, 2013, 2019).

Biosynthesis of zinc oxide nanoparticles

Initially, 10 ml of DPJ was placed in a beaker on a magnetic stirrer with a hot plate (REMI 5MLH, Q20A). The extract was heated to 50-60°C under constant stirring at 1500 rpm using magnetic bar. Then 40 ml of 0.1 M zinc acetate dihydrate (HIMEDIA GRM692) was added. The reaction mixture was continuously stirred while 0.4 M NaOH (HIMEDIA) solution was added dropwise as a stabilizer until a yellow color and cream precipitate formed, this mixture was subsequently centrifuged at 5000 rpm for 20 minutes. The pellet was washed three times with distilled water and calcined at 400°C for 6 h. A white powder was obtained (Demissie et al., 2020; Jayachandran et al., 2021a). The samples were subjected to various evaluations and measurements, such as UV-vis spectroscopy, Transmission Electron Microscope (TEM), Fourier Transform Infra-red spectroscopy (FTIR), X-ray Diffraction (XRD), and Energy Disperse Spectroscopy (EDS), to confirm the formation, morphology, elemental composition, crystalline structure, and functional groups of synthesized ZnO nanoparticles (Akçay et al., 2025; Valsan et al., 2023b).

Antibacterial activity of the zinc oxide nanoparticles

The antibacterial activity of the synthesized ZnO NPs was tested against Gram-negative and Gram-positive bacteria, namely, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis and positive control Chloramphenicol (SRL) 1mg/ml; all the microorganisms used were available in the microbiology department and they were authenticated and identified by a microbiology expert. The bacterial strains were subsequently grown in nutrient broth (HIMEDIA) at 37°C for 24h. The culture optical density (Systronics Spectrophotometer) was adjusted to 0.5 McFarland standard to get 1x 108 CFU/ml. This activity was carried out via the well diffusion method (Klink et al., 2022a) following previous studies. Muller Hinton Agar (HIMEDIA) plates were prepared. A sterile cotton swab was dipped into inoculum and swab streaked over the entire surface respective MHA plates and then with the help of sterile cork borer wells were made in the plates. Using a cork borer of 6 mm, wells were created on agar plates, cork borer sterilized every time. Different volumes, 20 µl, 40 µl, 60 µl of 1mg/ml of nanoparticles were loaded in the wells and plates were incubated at 37°C for 24 h. Chloramphenicol 1mg/ml used as positive control. After incubation zone of inhibition was measured using metric ruler. The Minimum Inhibitory Concentration (MIC) of the samples against the test organism was determined by using Resazurin (Invitrogen brand) microtiter plate assay (REMA). This assay was performed using flat bottom 96-well clear microtiter plates (IGNEUS Brand).

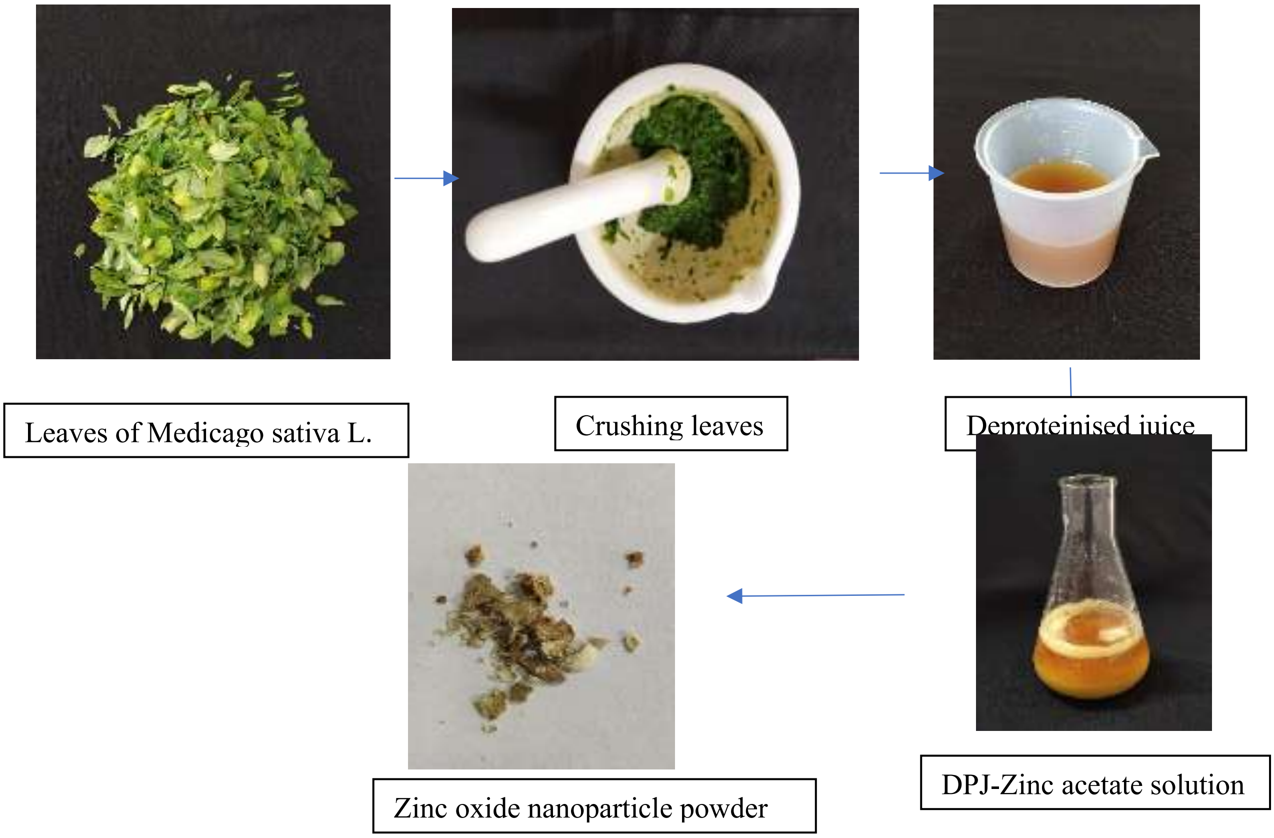

Fig. 1: Step by step synthesis process of zinc oxide nanoparticle.

Characterization of the zinc oxide nanoparticles:

1. Transmission electron microscopy and EDS:

A Nano TEM-analyzer (MODEL JEOL 2100 PLUS MAKE JEOL India pvt. Ltd.) was used to obtain images of the biosynthesized zinc oxide nanoparticles, and EDS (using model Bruker XFlash 6130, 123 eV at Mn Kα and 45 eV at C Kα) confirms the presence of elements particularly Zn and O but does not by itself confirm the ZnO structure. Confirmation of ZnO relies primarily on XRD, UV-Visible, FTIR, and EDS used together (Chen et al., 2019; Ganesh et al., 2019; Kiani et al., 2022b).

2. Fourier transform infrared spectroscopy:

FTIR use by JASCO. The infrared absorption spectrum produced by this characterization technique reveals the chemical bonds in the biosynthesized nanoparticles (Batool et al., 2021a; Kumari et al., 2024; Netala et al., 2015).

3. UV-Vis spectrophotometry:

The optical properties of the synthesized ZnO nanoparticles were observed from the absorption spectra of the nanoparticles. This is characterized via Personal computer (PC) based UV-vis spectrophotometer 119 of Systronics with a wavelength in the range of 200-800 nm (Jayachandran et al., 2021b; Liu et al., 2020).

4. X-ray Diffraction

XRD using by Bruker D8 Venture, analysis of the crystalline material was carried out via X-ray diffraction with a Cu K-alpha radiation source at a wavelength of 1.5402 Å (Fakhari et al., 2019; Pai et al., 2019).

Results and Discussion

The Transmission electron microscopy analysis done using software Espirit 1.9, the methodology involves rigorous sample preparation and statistical image analysis of the synthesized zinc oxide nanoparticles revealed that predominantly spherical morphology. The distribution was varied and polydisperse rather than perfectly uniform, which is common characteristics of green synthesis method using plant extracts. The particles exhibit size distribution in the lower nanometer range, with specific measurements identifying individual nanoparticles with average mean diameter 12.19 nm and, standard deviation was 3.36 nm. Therefore, polydispersity index (PDI) was 0.076 value is below 0.1 it is an indicator of highly uniform particle size distribution, which results that synthesized nanoparticle is monodisperse (Figure 2). While some agglomeration visible, the presence of lighter organic matrix surrounding the dense metallic cores confirms that biomolecules from DPJ are effectively acting as capping as stabilizing agents. These less 20 nm dimensions are particularly significant for microbiological applications. The EDS results revealed that sample contains zinc and oxygen, as shown in Fig. 3.

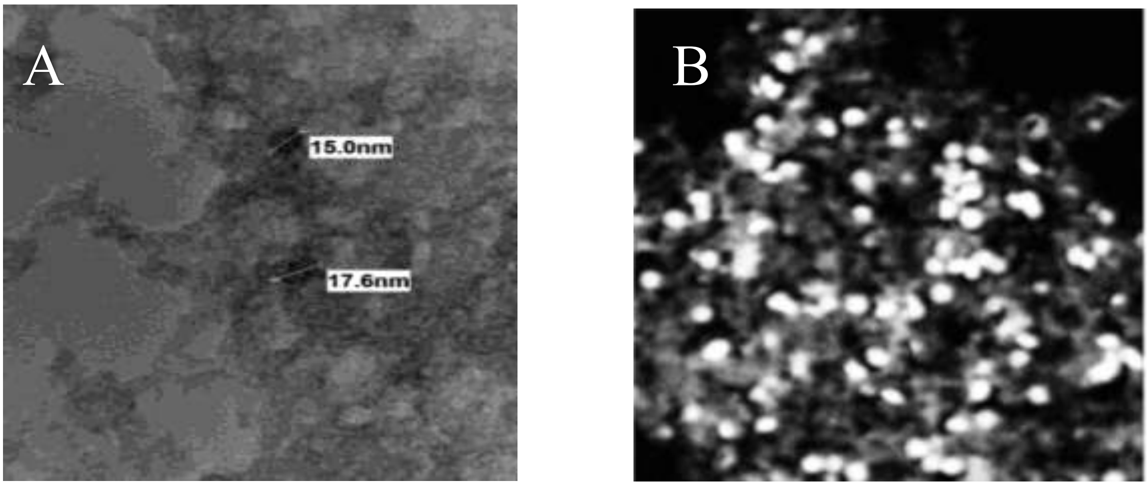

Fig. 2. TEM analysis of synthesized ZnO nanoparticles. A: TEM micrograph showing representative particle size of 15.0 and 17.6 nm. B: TEM micrograph showing the distribution and aggregation pattern of ZnO nanoparticles. Scale bar = 20nm.

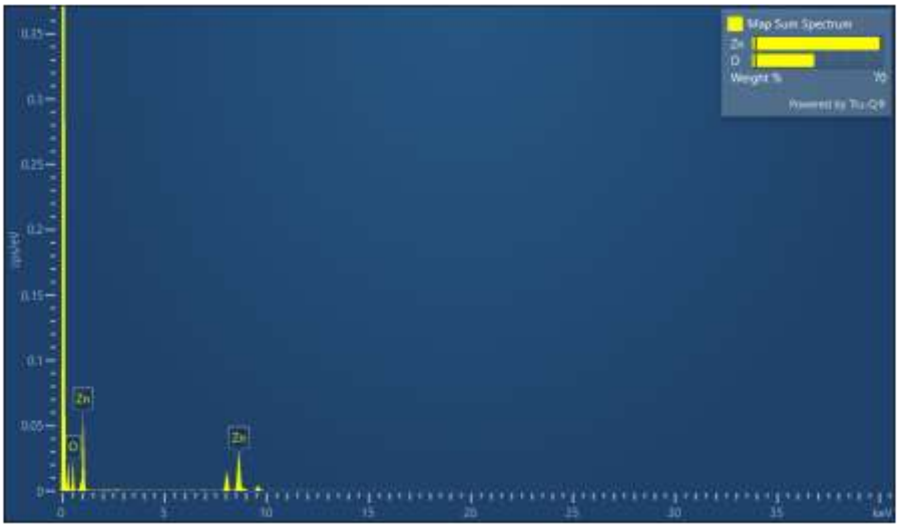

Fig. 3: EDS analysis confirms the presence of zinc and oxygen, which support the formation ZnO nanoparticles.

FTIR analysis provided insights into the composition and functional groups formation in the synthesized ZnO nanoparticles. The FTIR spectra of the synthesized ZnO nanoparticles, ranging from 4000 to 500 cm^{-1}, are shown in Fig. 4. The functional groups responsible for the reduction of Zn ions to ZnO were observed as bands. Characteristic peaks were identified at 1555 cm^{-1}, 2164 cm^{-1}, 3488 cm^{-1}, and 3540 cm^{-1}. The peak at 3488 cm^{-1} corresponds to O-H stretching in carbohydrates and polyphenols (Kumari et al., 2024), whereas the peak at 3540 cm^{-1} is attributed to phenol stretching (Batool et al., 2021b). The 2164 cm^{-1} peak corresponds to the C-C triple bond in the alkyne group (Batool et al., 2021a), and the 1555 cm^{-1} peak indicates the amide II linkage of proteins (Netala et al., 2015).

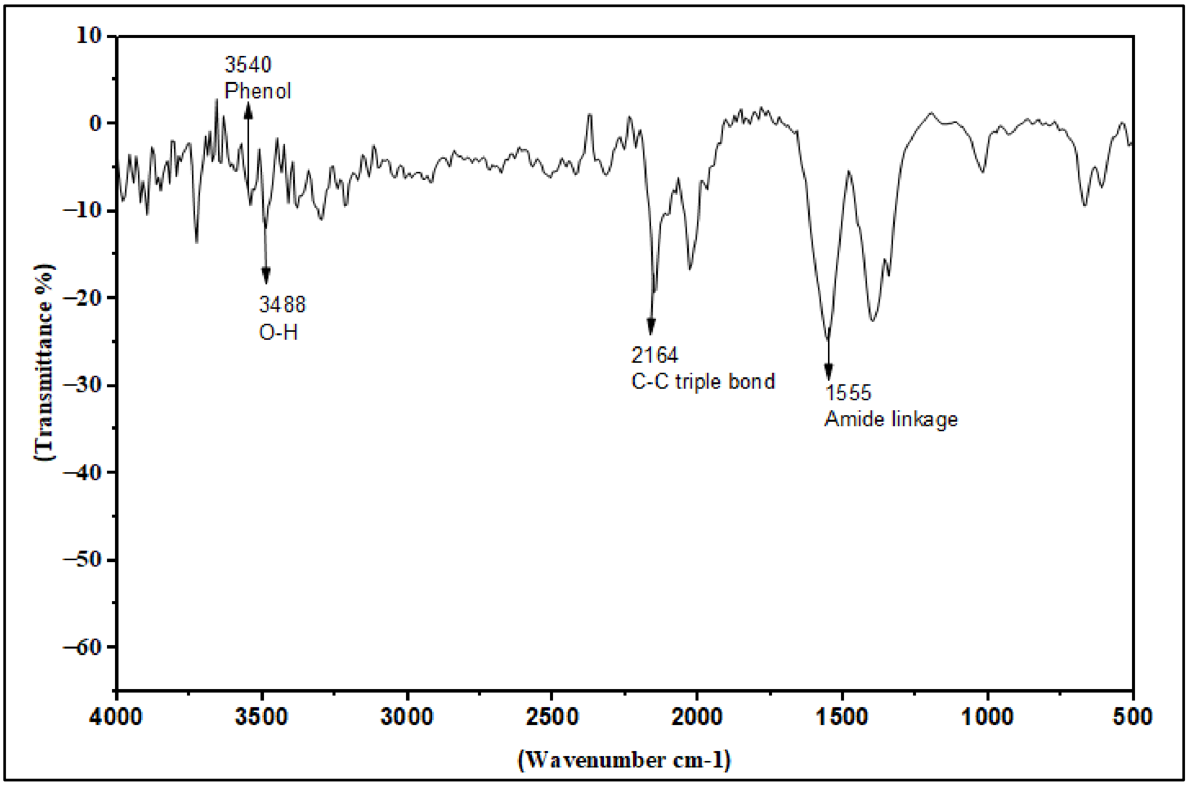

Fig 4: FTIR spectrum peaks at 1555 cm^{-1}, 2164 cm^{-1}, 3488 cm^{-1}, and 3540 cm^{-1} of synthesized zinc oxide nanoparticles.

The XRD data shown in Figure 5 reveal the phase identification of metal oxides and nanomaterials, which often show broadened peaks due to their small crystallite sizes. The resulting characteristic peaks are 36.70°, 47.96°, and 69.3°. The structure of the resulting Nano-ZnO is hexagonal wurtzite, which matches with JCPD card no. 36-1451 (Król et al., 2019). Nano crystallinity was confirmed by estimating the crystallite size via the Scherrer formula (Fakhari et al. 2019).

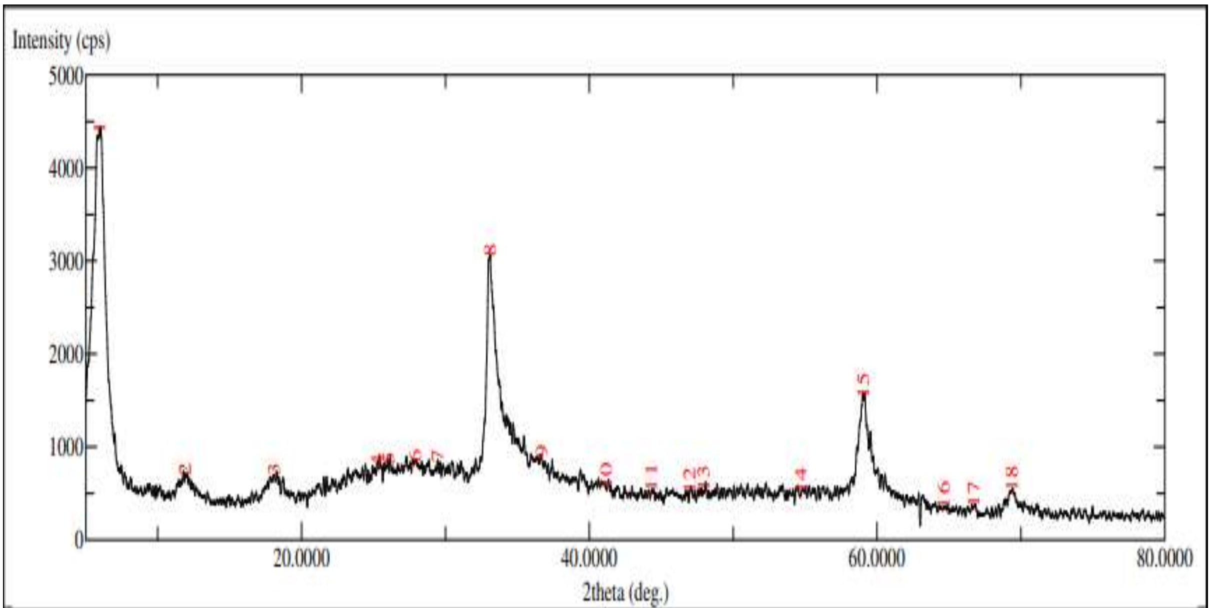

Fig. 5: XRD graph of synthesized zinc oxide nanoparticles

The Scherrer equation (Fakhari et al. 2019)-

$$D = \frac{k\lambda}{\beta \cos(\theta)}$$

where

- D = crystallite size (nm)
- K = shape of the factor (constant 0.9)
- λ = X-ray wavelength (Cu Kα = 1.5406 A° = 0.15406 nm)

- β = FWHM of the peak (in radians)
- θ = Bragg angle = ½ × 2θ (in radians)

The resulting ZnO contains nanocrystalline metal oxide phases with an average crystallite size of 13.85 nm, confirming that it is a nanomaterial, as shown in Table 1.

Table 1: Crystallite size calculation for peaks using the Scherrer equation of zinc oxide nanoparticles.

Theta	d (Å)	FWHM (°)	Crystallite Size (nm)
36.7	2.45	0.635	13.18
47.9	1.89	0.612	14.21
69.3	1.35	0.682	14.17

The results of UV-Visible used to determine formation and stability, it is the interaction between light with electron of nanomaterials. This was confirmed by UV spectrum analysis in the range of 200 nm to 800 nm. The absorbance peak was reported 324 nm, as shown in Fig. 6.

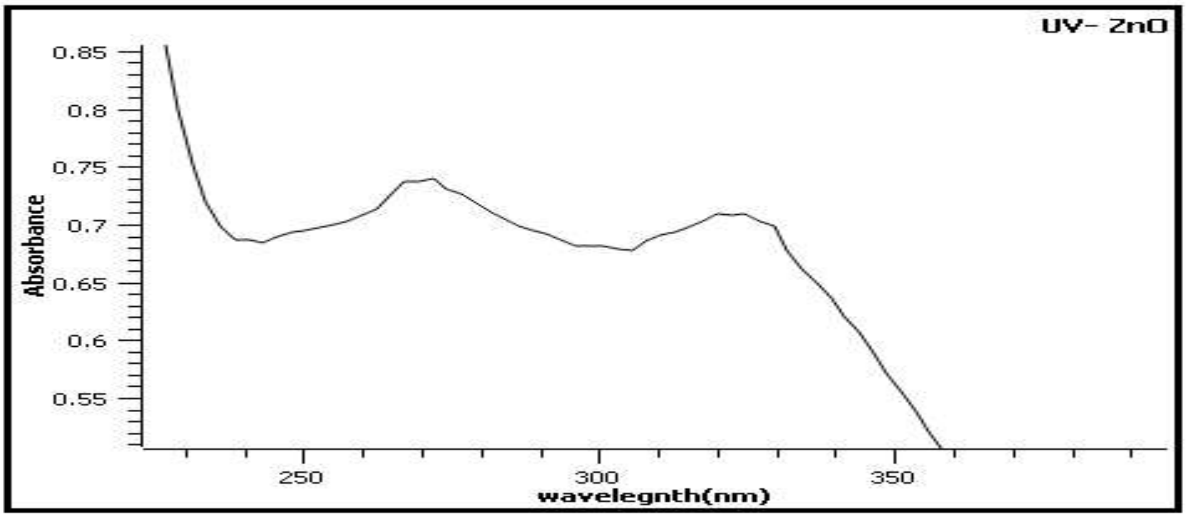

Fig. 6: UV spectrum of synthesized zinc oxide nanoparticles

The antibacterial activity results indicate that Gram-negative and Gram-positive bacteria can inhibit bacterial growth, as shown in Figure 7. compare with standard chloramphenicol, both Gram-negative and Gram-positive bacteria presented greater results. The increase in the concentration zone of inhibition also increased. As shown in Table 2. ZnO NPs are more effective against positive bacteria than against negative bacteria (Klink et al., 2022a) and positive control (standard) chloramphenicol. These results agreed with those of previous reports (Klink et al., 2022b). The results revealed that the highest inhibition zone diameter was achieved for Staphylococcus aureus, 10.2 mm, and MIC was 0.666 mg/ml, and the lowest was achieved for Pseudomonas aeruginosa, approximately 7.5 mm in diameter, MIC was 0.166mg/ml.

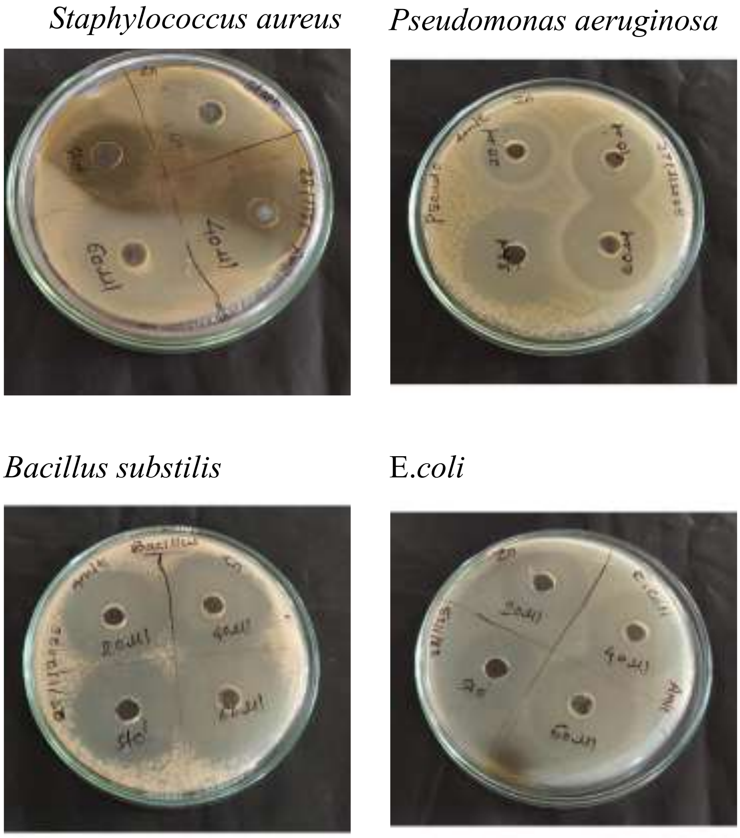

Fig 7: Evaluation of antibacterial efficacy of ZnO via agar well diffusion method. Varying volumes (20µl, 40µl, 60µl) of 1mg/ml of nanoparticle suspension into agar wells. Followed 48 hrs. incubation period at room temperature, replicated three times and data validated using SPSS software.

Table 2: zone of inhibition (mm) of Antibacterial activity

Pathogens	20 µl	40 µl	60 µl	Standard
Pseudomonas aeruginosa	5.1	6.6	7.5	10.0
Escherichia coli	4.9	7.6	8.4	11.0
Staphylococcus aureus	7.2	8.8	10.2	9.0
Bacillus substilis	4.5	5.6	8.7	9.0

These findings indicate that ZnO has greater antimicrobial activity against Staphylococcus aureus than against Pseudomonas aeruginosa. The antibacterial assessment revealed that the biosynthesized ZnO nanoparticles possesses potent inhibitory activity. Comparative analysis indicated that the ZnO nanoparticles yielded significantly larger zone of inhibition against the targeted pathogens than the commercial antibiotic Chloramphenicol. In the future, these ZnO nanoparticles could be better alternatives against drug-resistant bacteria.

Conclusion

The study confirmed that the Deproteinized juice from Medicago sativa L. is an effective reducing and stabilizing agent for the green synthesis of Zinc oxide nanoparticles. The structural and optical characterization including UV-Vis, XRD, and TEM confirmed the formation of crystalline ZnO nanostructures with characteristics absorption peak at 324 nm and narrow size range of 15-17 nm. TEM analysis indicated an agglomerated morphology, the resulting nanoparticles exhibited potent, broad spectrum antibacterial activity against Gram-positive and Gram-negative bacterial strains. The result suggests that DPJ is an excellent candidate for the large scale, biocompatible synthesis of nanoparticles, effectively bridging the gap between agricultural waste utilization and nanotechnology. Given their significant inhibitory potential which, in surpassed the efficacy of standard antibiotics like Chloramphenicol. Future research should focus on optimizing the synthesis parameters to control agglomeration and evaluating the long-term cytotoxicity of these nanoparticles to ensure their safety in clinical and environmental remediation.

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Novelty statement:
This study presents new insights into an innovative, sustainable strategy that transforms agricultural by-products into value-added nanomaterials.

Author contribution:

Amit Potbhare: Writing original draft, Methodology, Formal analysis, Data curation, **Sayyed Ilyas:** Supervision, Writing- review and editing, Conceptualization, Methodology, Visualization.
Ethical Approval:
No ethical approval is required.
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Conflicts of interest:
The authors have declared no conflict of interest.

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