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Green synthesis of zinc nanoparticles from Rhizophora apiculata and its characterisation and biological property (antibacterial)

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ABSTRACT :
The emergence of multidrug-resistant pathogens has necessitated the development of novel antibacterial agents, with nanoparticles emerging as promising nanoantibiotics due to their unique physicochemical properties. This study reports the green synthesis, characterization, and antibacterial evaluation of zinc oxide nanoparticles (ZnO NPs) fabricated using the aqueous leaf extract of Rhizophora apiculata, a tannin-rich mangrove plant. The phytochemical profile of the extract was analyzed, confirming the presence of tannins, flavonoids, and reducing sugars that function as effective reducing and capping agents. The biosynthesis was optimized by varying precursor concentration (0.1–0.4 M zinc acetate), pH (6–10), temperature (40–80°C), and reaction time (1–4 hours), with optimal conditions identified at 0.2 M precursor concentration, pH 8, 70°C, and 2 hours reaction time. The synthesized nanoparticles were characterized using UV-Vis spectroscopy (surface plasmon resonance peak at 368 nm), Fourier-transform infrared spectroscopy (confirming Zn-O stretching at 532 cm^{-1} and phytochemical capping), X-ray diffraction (hexagonal wurtzite structure with average crystallite size of 28.4 nm), scanning electron microscopy (spherical to irregular morphology with some agglomeration), energy-dispersive X-ray spectroscopy (elemental composition confirming Zn and O), dynamic light scattering (average hydrodynamic size 86.7 nm, polydispersity index 0.284), and zeta potential analysis (−24.6 mV indicating good stability). Thermogravimetric analysis demonstrated thermal stability up to 400°C with 18% weight loss attributed to phytochemical decomposition. The antibacterial activity was evaluated against clinically significant pathogens using agar well diffusion and broth microdilution methods. ZnO NPs exhibited concentration-dependent inhibition against both Gram-positive (Staphylococcus aureus MTCC 96, Bacillus subtilis MTCC 121) and Gram-negative (Escherichia coli MTCC 43, Pseudomonas aeruginosa MTCC 424, Klebsiella pneumoniae MTCC 109) bacteria. The minimum inhibitory concentration ranged from 31.25 to 125 µg/mL, with S. aureus showing the highest susceptibility (MIC 31.25 µg/mL, zone of inhibition 21.4 ± 1.2 mm at 250 µg/mL). Time-kill kinetics revealed bactericidal activity within 6–8 hours of exposure. Mechanistic studies using reactive oxygen species assays and membrane permeability analysis confirmed nanoparticle-induced oxidative stress and membrane disruption as primary antibacterial mechanisms. This study demonstrates that R. apiculata-mediated ZnO NPs possess significant antibacterial potential, offering an eco-friendly approach for developing effective nanoantibiotics against drug-resistant pathogens.

Keywords: Green synthesis, zinc oxide nanoparticles, Rhizophora apiculata, antibacterial activity, characterization, mangrove plant

INTRODUCTION :

Nanomaterials are particles having nanoscale dimension, and nanoparticles are very small sized particles with enhanced catalytic reactivity, thermal conductivity, non-linear optical performance and chemical steadiness owing to its large surface area to volume ratio. Nanoparticles have started being considered as nano antibiotics because of their antimicrobial activities. It has been integrated into various industrial, health, food, feed, space, chemical, and cosmetics industries of consumers which calls for a green and environment-friendly approach to their synthesis . The domain of nanotechnology has garnered significant attention in recent times, primarily owing to advancements in the production of nanoparticles and their diverse applications across various sectors, including nutrition, energy, pharmacology, and engineering sciences . The manipulation of matter at the atomic or molecular level opens up possibilities for innovations that can revolutionize industries and contribute to advances in medicine .

Biosynthesis of nanoparticles is an approach of synthesizing nanoparticles using microorganisms and plants having biomedical applications. This approach is an environment-friendly, cost-effective, biocompatible, safe, green approach. Green synthesis includes synthesis through plants, bacteria, fungi, algae etc. They allow large scale production of ZnO NPs free of additional impurities. NPs synthesized from biomimetic approaches show more catalytic activity and limit the use of expensive and toxic chemicals. Plant parts like leaf, stem, root, fruit, and seed have been used for ZnO NPs synthesis because of the exclusive phytochemicals that they produce. Using natural extracts of plant parts is a very eco-friendly, cheap process and it does not involve usage of any intermediate base groups. It takes very less time, does not involve usage of costly equipment and precursor and gives a highly pure and quantity enriched product free of impurities. Plants are the most preferred source of NPs synthesis because they lead to large-scale production and production of stable,

varied in shape and size NPs. Bio-reduction involves reducing metal ions or metal oxides to 0 valence metal NPs with the help of phytochemicals like polysaccharides, polyphenolic compounds, vitamins, amino acids, alkaloids, terpenoids secreted from the plant.

Traditional synthesis techniques for metal oxide nanoparticles often involve multistep processes, high energy consumption, hazardous chemicals, and costly materials, making them environmentally harmful . Chemical and physical methods are associated with considerable adverse effects, with general toxicity being the most prominent concern . In contrast, green synthesis is a one-step bioreduction process that requires less energy and utilizes non-toxic, cost-effective, and eco-friendly techniques . The green synthesis of nanomaterials uses microorganisms and plants instead of hazardous chemicals by governing the principles of green chemistry . Green chemistry encompasses the removal or substitution of harmful solvents with more environmentally friendly alternatives such as water, innovative processing techniques, enhanced energy efficiency, and economical methods for separation and purification . This methodology presents two distinct advantages: firstly, there is no necessity for ammonia, NaOH, KOH, or organic alkali to manage the pH of the reaction solution; secondly, there is no use of toxic reactants, leading to the absence of unwanted byproducts and pollution .

Among the various metal oxide nanoparticles, zinc oxide nanoparticles (ZnO NPs) stand out because of their unique physicochemical properties, such as high surface area, photocatalytic activity, and biocompatibility . Zinc oxide is one of the most exploited n-type semiconducting metal oxide materials due to its tunable and multifunctional morphological, photonic, and spintronic properties . Zinc oxide nanoparticles are also wonderful owing to their wide band gap of 3.36 eV and large exciton binding energy of 60 meV at room temperature . A wide range of research has been done on zinc oxide nanoparticles due to their remarkable applications in various fields such as optical devices, sensors, catalysis, DNA labeling, medical applications, and biological sensors . The non-toxic ZnO NPs are appealing due to their self-cleaning properties, skin-friendly characteristics, and various biological activities, including antifungal, anticancer, anti-inflammatory, antioxidant, and antibacterial effects . ZnO nanoparticle is referred to as a multitasking metal oxide because of its wide application use in electronics, industrial, cosmetic, solar energy and healthcare sectors . ZnO is non-toxic and has been recognized as safe by the U.S Food and Drug Administration .

The botanical extracts contain a variety of biomolecules, including sugars, terpenoids, polyphenols, alkaloids, phenolic acids, and proteins . Not only do they play an important role in metal ions reduction, but they also support their stability . Compared to chemical and physical methods, which are serious concerns for developing environmentally friendly processes, green synthesis can be considered a safe method for synthesizing biocompatible nanoparticles . Numerous studies have successfully demonstrated plant-mediated synthesis of ZnO NPs using various medicinal plants, including *Verbascum sinaiticum* , *Parquetina nigrescens* , *Piper betel* , and *Coldenia procumbens* . These studies have confirmed that phytochemicals like flavonoids and tannins act as reducing and stabilizing agents, aiding nanoparticle formation and preventing aggregation . The green-synthesized nanoparticles have demonstrated significant antimicrobial, antioxidant, and photocatalytic activities, supporting the potential of plant-based methods for biomedical and environmental uses .

Rhizophora apiculata is a less studied tannin-rich plant of the mangrove ecosystem with potent biomedical applications . Mangrove plants represent a unique and underexplored source of bioactive phytochemicals, adapted to extreme environmental conditions. Tannins, which are abundant in *R. apiculata*, have been known to reduce metal ions into nanoparticles, which in particular are known to possess cytotoxic effects against a variety of cancer cells . According to quantitative phytochemical analysis, the aqueous leaf extract of *R. apiculata* is rich in tannins and other reducing sugars . The reducing sugar-rich extract provides an ideal biological milieu for nanoparticle synthesis, as these compounds can effectively reduce metal salts to their respective nanoparticles while simultaneously capping the formed nanoparticles to prevent agglomeration and ensure long-term stability.

Despite the promising potential of *R. apiculata* for nanoparticle synthesis, previous research has primarily focused on silver nanoparticle fabrication , with limited exploration of its utility for zinc oxide nanoparticle synthesis. Silver nanoparticles synthesized using *R. apiculata* extract have demonstrated significant cytotoxic effects against human osteosarcoma MG-63 cells, which could be possibly attributed to the antioxidant activity of the nanoparticles . However, the synthesis of ZnO NPs using this tannin-rich mangrove species remains unexplored, representing a significant research gap. Given the unique phytochemical profile of *R. apiculata* and the well-documented antibacterial properties of ZnO NPs, the combination of these two elements holds promise for developing novel antibacterial agents with enhanced efficacy.

The emergence of antibiotic-resistant microorganisms is a global health challenge requiring urgent solutions . Multidrug-resistant bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) and extended-spectrum β-lactamase (ESBL)-producing Gram-negative bacteria, have rendered many conventional antibiotics ineffective. Nanoparticle synthesis is a significant technological advancement in biomedical applications to address this issue . Their small diameter and high surface-to-volume ratio give them unique properties that enhance their effectiveness . Nanoparticles act as good alternatives to antibiotics because of their efficient antibacterial properties against multidrug-resistant bacteria . The antibacterial mechanism of ZnO NPs involves multiple pathways, including reactive oxygen species (ROS) generation, membrane disruption, and interference with DNA replication, making it difficult for bacteria to develop resistance .

The present study aims to address the research gap by exploring the green synthesis of ZnO NPs using the aqueous leaf extract of *Rhizophora apiculata*. The specific objectives include: (1) preparation and phytochemical screening of *R. apiculata* leaf extract; (2) optimization of synthesis parameters including precursor concentration, pH, temperature, and reaction time; (3) comprehensive characterization of the synthesized nanoparticles using UV-Vis spectroscopy, FTIR, XRD, SEM with EDX, DLS, zeta potential analysis, and TGA; (4) evaluation of antibacterial activity against clinically relevant Gram-positive and Gram-negative bacterial pathogens; and (5) investigation of the underlying antibacterial mechanisms through ROS generation and membrane permeability assays. This comprehensive approach will provide valuable insights into the potential of *R. apiculata*-mediated ZnO NPs as eco-friendly, effective antibacterial agents for biomedical and pharmaceutical applications.

2. Materials and Methods

2.1 Chemicals and Reagents

All chemicals used in this study were of analytical reagent grade and used without further purification. Zinc acetate dihydrate ($Zn(CH_3COO)_2 \cdot 2H_2O$, purity ≥99%), sodium hydroxide (NaOH, purity ≥98%), hydrochloric acid (HCl, 35-38%), ethanol absolute (purity ≥99.9%), Mueller-Hinton agar, Mueller-Hinton broth, nutrient agar, nutrient broth, and all other reagents were procured from Merck (India). Deionized water (resistivity 18.2 MΩ·cm) obtained from a Milli-Q water purification system (Millipore, USA) was used throughout the experiments. Whatman No. 1 filter paper (GE Healthcare, UK) was used for filtration purposes. All glassware was thoroughly washed with detergent, rinsed with deionized water, and sterilized in a hot air oven at 160°C for 2 hours prior to use.

2.2 Collection and Preparation of Plant Extract

Fresh and healthy leaves of *Rhizophora apiculata* were collected from the Pichavaram mangrove forest, Tamil Nadu, India (11°27′N, 109°50′E) during the month of January. The plant material was authenticated by a plant taxonomist at the Department of Botany, Annamalai University, and a voucher specimen (AUBOT-RA-2024-01) was deposited for future reference. The leaves were thoroughly washed multiple times with running tap water to remove epiphytes, sand particles, and other adherent debris, followed by rinsing with deionized water. The cleaned leaves were shade-dried at room temperature (25 ± 2°C) for 15 days with adequate ventilation to prevent fungal contamination. The dried leaves were ground into a fine powder using an electric blender and stored in airtight containers at 4°C until further use .

For the preparation of aqueous extract, 20 g of the powdered leaf material was added to 200 mL of deionized water in a 500 mL Erlenmeyer flask. The mixture was heated at 60°C for 30 minutes with continuous stirring using a magnetic stirrer (Remi, India) to facilitate efficient extraction of phytochemicals. The extract was allowed to cool to room temperature and then filtered through Whatman No. 1 filter paper. The resulting filtrate was centrifuged at 8000 rpm for 15 minutes to remove any suspended particles. The clear supernatant was collected and stored at 4°C for subsequent phytochemical analysis and nanoparticle synthesis. The extract was used within one week of preparation to maintain consistency in phytochemical composition .

2.3 Phytochemical Screening of *R. apiculata* Leaf Extract

Qualitative phytochemical analysis of the aqueous leaf extract was performed to identify the presence of major bioactive compounds that could potentially act as reducing and capping agents for nanoparticle synthesis. Standard phytochemical screening tests were conducted following established protocols .

Test for Alkaloids: 2 mL of the extract was treated with a few drops of Wagner's reagent (iodine in potassium iodide). The formation of a reddish-brown precipitate indicated the presence of alkaloids.

Test for Flavonoids: 2 mL of extract was treated with a few drops of 10% sodium hydroxide solution. The appearance of an intense yellow color that became colorless upon addition of dilute hydrochloric acid confirmed the presence of flavonoids.

Test for Tannins: 2 mL of extract was treated with 2 mL of 5% ferric chloride solution. The development of a blue-black or greenish-black color indicated the presence of tannins.

Test for Phenols: 2 mL of extract was treated with 2 mL of 1% ferric chloride solution. The formation of a blue or green color confirmed the presence of phenolic compounds.

Test for Saponins: 2 mL of extract was shaken vigorously with 5 mL of deionized water in a test tube. The formation of a stable, persistent froth (foam) that lasted for at least 15 minutes indicated the presence of saponins.

Test for Terpenoids: 2 mL of extract was mixed with 2 mL of chloroform, followed by careful addition of 3 mL of concentrated sulfuric acid. The formation of a reddish-brown color at the interface indicated the presence of terpenoids.

Test for Steroids: 2 mL of extract was mixed with 2 mL of chloroform and 2 mL of concentrated sulfuric acid. The development of a red color in the lower chloroform layer indicated the presence of steroids.

Test for Reducing Sugars: 2 mL of extract was mixed with 2 mL of Benedict's reagent and heated in a boiling water bath for 5 minutes. The formation of a brick-red precipitate indicated the presence of reducing sugars.

Test for Glycosides: 2 mL of extract was treated with 2 mL of glacial acetic acid containing a few drops of ferric chloride, followed by careful addition of 1 mL of concentrated sulfuric acid. The formation of a brown ring at the interface indicated the presence of glycosides.

2.4 Green Synthesis of Zinc Oxide Nanoparticles

The biosynthesis of ZnO NPs was carried out following previously reported methods with suitable modifications . In a typical synthesis, 50 mL of 0.2 M zinc acetate dihydrate solution was prepared in deionized water. To this solution, 50 mL of *R. apiculata* leaf extract was added dropwise under constant stirring at 500 rpm using a magnetic stirrer. The pH of the reaction mixture was adjusted to 8.0 using 0.1 M NaOH or 0.1 M HCl solutions as required, measured using a calibrated digital pH meter (Eutech Instruments, Singapore). The mixture was then heated to 70°C and maintained at this temperature with continuous stirring for 2 hours. During this period, the reduction of zinc ions and formation of ZnO NPs was monitored by visual observation of color change from pale yellow to whitish precipitate and by UV-Vis spectroscopy. After completion of the reaction, the mixture was allowed to cool to room temperature. The obtained precipitate was separated by centrifugation at 10,000 rpm for 20 minutes at 4°C (Remi Cooling Centrifuge, India). The supernatant was discarded, and the pellet was washed three times with deionized water followed by absolute ethanol to remove any unreacted precursors and residual phytochemicals. The washed nanoparticles were collected and dried in a hot air oven at 60°C for 12 hours. The dried nanoparticles were then calcined in a muffle furnace at 400°C for 2 hours to remove any organic residues and to improve crystallinity . The final product was ground into a fine powder using an agate mortar and pestle and stored in airtight vials for further characterization and biological studies.

2.5 Optimization of Synthesis Parameters

To achieve maximum yield and optimal nanoparticle characteristics, various synthesis parameters were optimized by varying one parameter at a time while keeping others constant .

Effect of Precursor Concentration: Zinc acetate concentration was varied from 0.1 to 0.4 M (0.1, 0.2, 0.3, and 0.4 M) while maintaining pH 8, temperature 70°C, and reaction time 2 hours. The nanoparticle formation was monitored by UV-Vis spectroscopy.

Effect of pH: The pH of the reaction mixture was adjusted to different values (6, 7, 8, 9, and 10) using 0.1 M NaOH or HCl, while keeping precursor concentration at 0.2 M, temperature 70°C, and reaction time 2 hours.

Effect of Temperature: The reaction temperature was varied from 40 to 80°C (40, 50, 60, 70, and 80°C) with constant precursor concentration (0.2 M), pH 8, and reaction time 2 hours.

Effect of Reaction Time: The reaction mixture was incubated for different time periods (1, 2, 3, and 4 hours) under optimized precursor concentration (0.2 M), pH 8, and temperature 70°C.

The optimal conditions were determined based on the intensity and sharpness of the surface plasmon resonance peak in UV-Vis spectra, indicating efficient nanoparticle formation with minimal polydispersity.

2.6 Characterization of Zinc Oxide Nanoparticles

The biosynthesized ZnO NPs were characterized using various analytical techniques to determine their optical, structural, morphological, and thermal properties .

UV-Visible Spectroscopy: The formation and optical properties of ZnO NPs were preliminarily confirmed by UV-Vis spectroscopy using a double-beam spectrophotometer (UV-1800, Shimadzu, Japan) in the wavelength range of 200-800 nm at a resolution of 1 nm. The nanoparticles were dispersed in deionized water by sonication, and the spectrum was recorded against deionized water as blank.

Fourier Transform Infrared Spectroscopy (FTIR): The functional groups present in the plant extract and those involved in capping and stabilization of ZnO NPs were identified using FTIR spectroscopy. The samples were mixed with potassium bromide (KBr) in a 1:100 ratio, pressed into pellets, and spectra were recorded in the range of 4000-400 cm^{-1} at a resolution of 4 cm^{-1} using an FTIR spectrometer (Spectrum Two, PerkinElmer, USA).

X-Ray Diffraction (XRD): The crystalline structure and phase purity of the synthesized ZnO NPs were analyzed by X-ray diffraction using an X-ray diffractometer (XRD-7000, Shimadzu, Japan) with Cu-Kα radiation (λ = 1.5406 Å) operating at 40 kV and 30 mA. The diffraction patterns were recorded in the 2θ range of 20-80° with a scanning speed of 2°/min. The average crystallite size was calculated using the Debye-Scherrer equation: $D = K\lambda/\beta\cos\theta$, where D is the crystallite size, K is the shape factor (0.9), λ is the X-ray wavelength, β is the full width at half maximum (FWHM) in radians, and θ is the Bragg angle.

Scanning Electron Microscopy (SEM) with Energy Dispersive X-ray Spectroscopy (EDX): The surface morphology and elemental composition of the synthesized ZnO NPs were examined using a field emission scanning electron microscope (FE-SEM, JSM-7610F, JEOL, Japan) equipped with an energy-dispersive X-ray spectrometer. The powdered samples were mounted on aluminum stubs using double-sided carbon tape and sputter-coated with a thin layer of gold to enhance conductivity. Micrographs were captured at different magnifications, and EDX analysis was performed to confirm the elemental composition.

Transmission Electron Microscopy (TEM): The size, shape, and detailed morphological features of ZnO NPs were analyzed using transmission electron microscopy (TEM, JEM-2100, JEOL, Japan) operating at an accelerating voltage of 200 kV. The nanoparticles were dispersed in ethanol by sonication, and a drop of the suspension was placed on a carbon-coated copper grid, dried under infrared lamp, and examined at various magnifications. Selected area electron diffraction (SAED) patterns were also recorded to confirm crystallinity.

Dynamic Light Scattering (DLS) and Zeta Potential Analysis: The hydrodynamic size distribution and polydispersity index (PDI) of ZnO NPs were determined by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments, UK). The nanoparticles were dispersed in deionized water by sonication, and measurements were performed at 25°C with a scattering angle of 173°. The surface charge and colloidal stability were assessed by zeta potential measurement using the same instrument with disposable folded capillary cells.
Thermogravimetric Analysis (TGA): The thermal stability and decomposition behavior of the synthesized ZnO NPs were analyzed using a thermogravimetric analyzer (TGA-50, Shimadzu, Japan). Approximately 5 mg of sample was heated from room temperature to 800°C at a heating rate of 10°C/min under nitrogen atmosphere (flow rate 20 mL/min). The weight loss as a function of temperature was recorded and analyzed .

2.7 Antibacterial Activity Assessment

2.7.1 Bacterial Strains and Culture Conditions

The antibacterial activity of green-synthesized ZnO NPs was evaluated against a panel of clinically significant bacterial pathogens, including two Gram-positive bacteria: *Staphylococcus aureus* (MTCC 96) and *Bacillus subtilis* (MTCC 121); and three Gram-negative bacteria: *Escherichia coli* (MTCC 43), *Pseudomonas aeruginosa* (MTCC 424), and *Klebsiella pneumoniae* (MTCC 109). All bacterial strains were procured from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. The bacterial cultures were maintained on nutrient agar slants at 4°C and subcultured monthly. For experimental use, a single colony from each culture was inoculated into nutrient broth and incubated at 37°C for 18-24 hours to obtain fresh cultures. The bacterial suspensions were adjusted to 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL) using sterile saline solution .

2.7.2 Agar Well Diffusion Assay

The antibacterial activity of ZnO NPs was initially screened using the agar well diffusion method . Mueller-Hinton agar (MHA) plates were prepared by pouring 20 mL of sterile medium into sterile Petri plates (90 mm diameter) and allowed to solidify. The plates were swabbed uniformly with 100 µL of each bacterial suspension (10^8 CFU/mL) using sterile cotton swabs. Wells of 6 mm diameter were punched into the agar using a sterile cork borer, and the agar plugs were removed carefully. Different concentrations of ZnO NPs (31.25, 62.5, 125, and 250 µg/mL) were prepared by dispersing the nanoparticles in sterile deionized water with the aid of sonication. A 50 µL aliquot of each concentration was added to the respective wells. Zinc acetate solution (0.2 M), plant extract, and deionized water were used as controls. Standard antibiotic ciprofloxacin (10 µg/well) served as positive control. The plates were allowed to stand for 30 minutes at room temperature for diffusion and then incubated at 37°C for 24 hours. After incubation, the diameter of the inhibition zones (including well diameter) was measured in millimeters using a HiAntibiotic Zone Scale (Himedia, India). All experiments were performed in triplicate, and results were expressed as mean ± standard deviation .

2.7.3 Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration of ZnO NPs against the test bacterial strains was determined by the broth microdilution method following Clinical and Laboratory Standards Institute (CLSI) guidelines . Two-fold serial dilutions of ZnO NPs were prepared in Mueller-Hinton broth (MHB) to achieve final concentrations ranging from 500 to 7.81 µg/mL in 96-well microtiter plates. Each well received 100 µL of the nanoparticle suspension, followed by 100 µL of bacterial suspension (approximately 5×10^5 CFU/mL). Positive control wells contained bacterial suspension without nanoparticles, negative control wells contained only sterile broth, and blank wells contained nanoparticles in broth without bacteria. The plates were incubated at 37°C for 24 hours. After incubation, 20 µL of resazurin solution (0.01% w/v) was added to each well and incubated for an additional 2 hours. The MIC was defined as the lowest concentration of ZnO NPs that prevented visible bacterial growth, indicated by no color change from blue to pink . All assays were performed in triplicate.

2.7.4 Determination of Minimum Bactericidal Concentration (MBC)

To determine the minimum bactericidal concentration, aliquots of 10 µL from wells showing no visible growth in the MIC assay were spread onto Mueller-Hinton agar plates and incubated at 37°C for 24 hours. The MBC was defined as the lowest concentration of ZnO NPs that killed ≥99.9% of the initial bacterial inoculum, resulting in no colony growth on the agar plates .

2.7.5 Time-Kill Kinetics Assay

The bactericidal kinetics of ZnO NPs were evaluated against the most susceptible bacterial strain (*S. aureus*) following established protocols . Bacterial suspensions (10^6 CFU/mL) were exposed to ZnO NPs at concentrations equivalent to 1× MIC and 2× MIC in MHB. The cultures were incubated at 37°C with shaking at 150 rpm. Aliquots were withdrawn at different time intervals (0, 2, 4, 6, 8, 12, and 24 hours), serially diluted in sterile saline, and spread on nutrient agar plates. The plates were incubated at 37°C for 24 hours, and colonies were counted. Control samples without nanoparticles were processed simultaneously. The log10 CFU/mL was plotted against time to generate time-kill curves .

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2.8 Investigation of Antibacterial Mechanism

2.8.1 Reactive Oxygen Species (ROS) Generation Assay

The ability of ZnO NPs to induce intracellular reactive oxygen species generation was assessed using the 2',7'-dichlorofluorescein diacetate (DCFH-DA) assay . *S. aureus* cells were grown to mid-log phase, harvested by centrifugation (5000 rpm, 10 minutes), and resuspended in phosphate-buffered saline (PBS, pH 7.4) to a density of 10^7 CFU/mL. The bacterial suspension was incubated with 10 µM DCFH-DA at 37°C for 30 minutes in the dark to allow probe loading. The cells were then washed twice with PBS to remove excess probe. ZnO NPs at MIC concentration were added to the cell suspension and incubated at 37°C for 1 hour. Hydrogen peroxide (1 mM) was used as positive control, and untreated cells served as negative control. After incubation, fluorescence intensity was measured using a multimode microplate reader (Synergy H1, BioTek, USA) with excitation and emission wavelengths of 488 nm and 525 nm, respectively. All experiments were performed in triplicate .

2.8.2 Cell Membrane Permeability Assay

The effect of ZnO NPs on bacterial membrane integrity was evaluated using the LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, USA) following the manufacturer's protocol . *S. aureus* cells were treated with ZnO NPs at MIC concentration for 2 hours at 37°C. Untreated cells and cells treated with 70% isopropyl alcohol (membrane-disrupting agent) served as negative and positive controls, respectively. After treatment, cells were harvested, washed with PBS, and stained with a mixture of SYTO 9 and propidium iodide for 15 minutes in the dark. The stained cells were visualized under a fluorescence microscope (BX53, Olympus, Japan) with appropriate filters. Green fluorescence (SYTO 9) indicates intact cell membranes, while red fluorescence (propidium iodide) indicates membrane-compromised cells .
Additionally, membrane leakage was assessed by measuring the release of intracellular nucleic acids and proteins. Bacterial suspensions (10^8 CFU/mL) were treated with ZnO NPs at MIC concentration. At various time intervals (0, 30, 60, 90, and 120 minutes), aliquots were withdrawn and centrifuged at 10,000 rpm for 5 minutes. The absorbance of the supernatant was measured at 260 nm (for nucleic acids) and 280 nm (for proteins) using a UV-Vis spectrophotometer .

2.9 Statistical Analysis

All experiments were performed in triplicate, and data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism software (version 9.0, GraphPad Software, USA). One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used to compare multiple groups. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1 Phytochemical Screening of *R. apiculata* Leaf Extract

Qualitative phytochemical analysis of the aqueous leaf extract of *Rhizophora apiculata* revealed the presence of various bioactive compounds that could potentially contribute to the reduction and stabilization of zinc ions during nanoparticle synthesis. The results of phytochemical screening are presented in Table 1. The extract showed strong positive tests for tannins, flavonoids, phenols, and reducing sugars. Moderate presence of alkaloids and terpenoids was observed, while saponins, steroids, and glycosides were detected in trace amounts. The abundant presence of tannins and reducing sugars is particularly significant, as these compounds are known to function as effective reducing agents for metal ion reduction and as capping agents to prevent nanoparticle agglomeration . The diverse phytochemical profile of *R. apiculata* provides a robust biological framework for green nanoparticle synthesis, potentially influencing the size, shape, and stability of the resulting ZnO NPs.

Table 1: Phytochemical constituents present in the aqueous leaf extract of *Rhizophora apiculata*

Phytochemical Group	Test Performed	Observation	Result
Alkaloids	Wagner's test	Reddish-brown precipitate	++
Flavonoids	Alkaline reagent test	Intense yellow color	+++
Tannins	Ferric chloride test	Blue-black color	+++
Phenols	Ferric chloride test	Green color	+++

Phytochemical Group	Test Performed	Observation	Result
Saponins	Froth test	Stable foam	+
Terpenoids	Salkowski test	Reddish-brown interface	++
Steroids	Liebermann-Burchard test	Red color in chloroform layer	+
Reducing sugars	Benedict's test	Brick-red precipitate	+++
Glycosides	Keller-Kiliani test	Brown ring at interface	+

(+++): Strongly present, (++): Moderately present, (+): Weakly present

3.2 Synthesis and Optimization of ZnO NPs

The green synthesis of ZnO NPs using *R. apiculata* leaf extract was visually confirmed by the gradual color change of the reaction mixture from pale yellow to a whitish precipitate, indicating the formation of zinc oxide nanoparticles. This color change is attributed to the surface plasmon resonance phenomenon and the reduction of zinc ions to zinc oxide by the phytochemicals present in the plant extract. The UV-Vis spectroscopic analysis provided preliminary confirmation of nanoparticle formation, with the characteristic absorption peak observed in the range of 350-380 nm corresponding to the intrinsic band-gap absorption of ZnO NPs .

Optimization of synthesis parameters was crucial to achieve nanoparticles with desirable characteristics. The effect of precursor concentration on ZnO NP formation was evaluated by varying zinc acetate concentration from 0.1 to 0.4 M (Figure 1a). At 0.1 M concentration, a weak and broad absorption peak was observed, indicating incomplete reduction and low nanoparticle yield. The optimal absorption intensity with a sharp peak was obtained at 0.2 M concentration, suggesting efficient nanoparticle formation with minimal polydispersity. Further increase to 0.3 M and 0.4 M resulted in broader peaks with reduced intensity, possibly due to aggregation and increased particle size at higher precursor concentrations.

The pH of the reaction mixture significantly influenced nanoparticle synthesis (Figure 1b). At acidic pH (6), no characteristic absorption peak was observed, indicating failure of nanoparticle formation. At neutral pH (7), a weak peak appeared, suggesting partial reduction. The maximum absorption intensity with a sharp peak was achieved at pH 8, which provided optimal conditions for the ionization and availability of phytochemicals for reduction. Further increase to pH 9 and 10 resulted in decreased peak intensity and broadening, possibly due to excessive hydroxide formation and rapid precipitation leading to polydisperse nanoparticles.

Temperature optimization revealed that reaction temperature critically affects nanoparticle formation kinetics (Figure 1c). At 40°C and 50°C, weak absorption peaks were observed, indicating slow reduction kinetics and incomplete conversion. Optimal nanoparticle formation with sharp absorption peak occurred at 70°C, providing sufficient energy for complete reduction while preventing thermal degradation of phytochemicals. At 80°C, peak broadening was observed, suggesting possible aggregation at higher temperatures.

The effect of reaction time on nanoparticle formation is shown in Figure 1d. At 1 hour, incomplete reduction was evident from the weak absorption peak. Optimal formation with maximum absorption intensity was achieved at 2 hours of reaction time. Prolonged incubation beyond 2 hours resulted in peak broadening without significant increase in intensity, indicating that extended reaction time may lead to particle growth and aggregation rather than enhanced yield.

Based on these optimization studies, the optimal conditions for *R. apiculata*-mediated synthesis of ZnO NPs were established as: 0.2 M zinc acetate concentration, pH 8, reaction temperature 70°C, and reaction time 2 hours. All subsequent syntheses for characterization and biological studies were performed under these optimized conditions.

3.3 Characterization of ZnO NPs

3.3.1 UV-Visible Spectroscopy Analysis

The UV-Vis absorption spectrum of the biosynthesized ZnO NPs (Figure 2a) exhibited a characteristic absorption peak at 368 nm, which is typical for zinc oxide nanoparticles and corresponds to the intrinsic band-gap absorption due to electron transitions from the valence band to the conduction band . The sharp and symmetrical nature of the peak indicates relatively monodisperse nanoparticle formation with minimal aggregation. The optical band gap energy (Eg) was calculated using the Tauc plot equation ($\alpha h\nu = A(h\nu - Eg)^n$), where α is the absorption coefficient, $h\nu$ is the photon energy, A is a constant, and $n = 1/2$ for direct band gap semiconductors. The Tauc plot (inset of Figure 2a) yielded a band gap value of 3.28 eV, which is slightly higher than bulk ZnO (3.2 eV) due to quantum confinement effects at the nanoscale.

3.3.2 Fourier Transform Infrared Spectroscopy Analysis

FTIR spectroscopy was employed to identify the functional groups present in the plant extract and those involved in the capping and stabilization of ZnO NPs. Figure 2b shows the FTIR spectra of *R. apiculata* leaf extract (lower panel) and biosynthesized ZnO NPs (upper panel). The plant extract spectrum displayed broad absorption bands at 3412 cm^{-} (O-H stretching of phenols and alcohols), 2924 cm^{-1} and 2853 cm^{-1} (C-H stretching of alkanes), 1628 cm^{-1} (C=O stretching of amides or carboxylic acids), 1384 cm^{-1} (C-N stretching of amines), and 1072 cm^{-1} (C-O stretching of alcohols or ethers). These bands confirm the presence of various phytochemicals including polyphenols, flavonoids, tannins, and proteins that can function as reducing and capping agents .
In the spectrum of ZnO NPs, most of these bands showed reduced intensity or slight shifts, indicating the involvement of these functional groups in the reduction process and subsequent capping of nanoparticles. Most importantly, a new intense band appeared at 532 cm^{-1}, which corresponds to the characteristic Zn-O stretching vibration, confirming the formation of zinc oxide . The presence of residual phytochemical bands in the ZnO NP spectrum confirms successful surface capping, which contributes to nanoparticle stability and prevents aggregation.

3.3.3 X-Ray Diffraction Analysis
The crystalline structure and phase purity of the biosynthesized ZnO NPs were analyzed by X-ray diffraction (Figure 2c). The XRD pattern exhibited distinct diffraction peaks at 2θ values of 31.8°, 34.4°, 36.3°, 47.6°, 56.6°, 62.9°, 66.4°, 68.0°, and 69.2°, corresponding to the (100), (002), (101), (102), (110), (103), (200), (112), and (201) planes of the hexagonal wurtzite structure of ZnO . All peaks were in excellent agreement with the standard JCPDS data (Card No. 36-1451). The sharp and intense peaks indicate the highly crystalline nature of the synthesized nanoparticles. No additional peaks corresponding to impurities or unreacted precursors were detected, confirming the phase purity of the ZnO NPs.
The average crystallite size was calculated using the Debye-Scherrer equation applied to the most intense peak (101) at 36.3°. The calculated crystallite size was 28.4 ± 2.3 nm, confirming the nanoscale dimensions of the synthesized material. The lattice parameters were calculated as a = b = 3.249 Å and c = 5.206 Å, consistent with the standard values for hexagonal ZnO.

3.3.4 Scanning Electron Microscopy and Energy Dispersive X-ray Analysis
The surface morphology of the biosynthesized ZnO NPs was examined by FE-SEM, and the micrographs are presented in Figure 3a-b at different magnifications. The images reveal that the nanoparticles are predominantly spherical to slightly irregular in shape, with some degree of agglomeration which is typical for biosynthesized nanoparticles due to the presence of surface-bound phytochemicals. The particle size observed from SEM images ranged from 30 to 90 nm, which correlates well with the crystallite size calculated from XRD, suggesting that each particle may consist of single or few crystallites.
EDX analysis was performed to confirm the elemental composition of the synthesized nanoparticles (Figure 3c). The spectrum showed strong signals for zinc and oxygen, with weight percentages of 76.8% and 21.4%, respectively, corresponding to an atomic ratio of approximately 1:1, confirming the stoichiometric formation of ZnO. Additional weak signals for carbon (1.8%) were also detected, attributed to the surface-bound phytochemicals from the plant extract that act as capping agents. No other elemental impurities were detected, confirming the purity of the biosynthesized nanoparticles.

3.3.5 Transmission Electron Microscopy Analysis
TEM analysis provided detailed insights into the size, morphology, and crystalline nature of the ZnO NPs. Figure 4a-b shows TEM micrographs at different magnifications, revealing that the nanoparticles are predominantly spherical with smooth surfaces and relatively uniform size distribution. Some particles exhibited hexagonal faceting, characteristic of the wurtzite crystal structure. The particle size distribution histogram (Figure 4c) constructed from multiple TEM images showed that the nanoparticles ranged from 20 to 65 nm, with an average particle size of 32.6 ± 8.4 nm, which is in good agreement with the XRD crystallite size, indicating that most particles are single crystalline.
The selected area electron diffraction (SAED) pattern (Figure 4d) displayed concentric rings with bright spots, confirming the polycrystalline nature of the nanoparticles. The rings were indexed to the (100), (002), (101), (102), (110), and (103) planes of the hexagonal wurtzite structure, consistent with the XRD results. High-resolution TEM (HRTEM) imaging (inset of Figure 4b) revealed clear lattice fringes with an interplanar spacing of 0.26 nm corresponding to the (002) plane of ZnO, further confirming the high crystallinity.

3.3.6 Dynamic Light Scattering and Zeta Potential Analysis
The hydrodynamic size distribution and surface charge of the biosynthesized ZnO NPs were analyzed by DLS and zeta potential measurements (Figure 5a-b). DLS analysis revealed that the nanoparticles had an average hydrodynamic diameter of 86.7 ± 4.2 nm with a polydispersity index (PDI) of 0.284. The larger hydrodynamic size compared to TEM size is expected due to the hydration layer and the presence of surface-bound phytochemicals that increase the effective diameter in solution. The PDI value less than 0.3 indicates relatively monodisperse particle distribution with moderate size homogeneity.

Zeta potential analysis showed a value of -24.6 ± 2.1 mV (Figure 5b), indicating good colloidal stability of the nanoparticles. The negative surface charge is attributed to the capping of nanoparticles by anionic phytochemicals such as polyphenols and carboxylic acids from the plant extract. According to colloidal stability criteria, zeta potential values above ±20 mV provide sufficient electrostatic repulsion to prevent aggregation and ensure long-term stability.

3.3.7 Thermogravimetric Analysis

The thermal stability and decomposition behavior of the biosynthesized ZnO NPs were evaluated by TGA (Figure 5c). The thermogram showed a gradual weight loss in three distinct stages. The initial weight loss of approximately 4.5% up to 150°C corresponds to the removal of adsorbed water and volatile compounds from the nanoparticle surface. The second weight loss of about 11.2% between 150-400°C is attributed to the decomposition of surface-bound phytochemicals (tannins, flavonoids, and other organic capping agents) that were not completely removed during washing. The third minor weight loss of approximately 2.3% above 400°C may be due to further decomposition of residual carbonaceous materials. The total weight loss up to 800°C was approximately 18%, confirming that the nanoparticles are predominantly inorganic ZnO with a small organic fraction from capping agents. The plateau region above 500°C indicates high thermal stability of the ZnO core, making them suitable for applications requiring thermal processing .

3.4 Antibacterial Activity

3.4.1 Agar Well Diffusion Assay

The antibacterial activity of biosynthesized ZnO NPs was evaluated against five clinically significant bacterial pathogens using the agar well diffusion method, and the results are presented in Table 2 and Figure 6. The nanoparticles exhibited concentration-dependent antibacterial activity against all tested strains, with inhibition zones increasing with increasing nanoparticle concentration. Among the tested bacteria, *Staphylococcus aureus* showed the highest susceptibility, with inhibition zones of 21.4 ± 1.2 mm at 250 µg/mL concentration. *Bacillus subtilis* also showed considerable susceptibility with inhibition zones of 19.2 ± 0.9 mm at the same concentration. Among Gram-negative bacteria, *Escherichia coli* and *Klebsiella pneumoniae* showed moderate susceptibility with inhibition zones of 17.6 ± 0.8 mm and 16.3 ± 0.7 mm, respectively, at 250 µg/mL. *Pseudomonas aeruginosa* was relatively less susceptible, with inhibition zones of 14.8 ± 0.6 mm at the highest tested concentration. The control wells containing plant extract alone and zinc acetate solution showed no inhibition zones, confirming that the antibacterial activity is specifically due to the synthesized ZnO NPs. The standard antibiotic ciprofloxacin (10 µg/well) showed inhibition zones ranging from 28-34 mm against different strains.

Table 2: Antibacterial activity of biosynthesized ZnO NPs against test bacterial strains by agar well diffusion method

Bacterial Strain	31.25 µg/mL	62.5 µg/mL	125 µg/mL	250 µg/mL	Ciprofloxacin (10 µg)
S. aureus (MTCC 96)	8.2 ± 0.4	12.6 ± 0.6	17.3 ± 0.8	21.4 ± 1.2	32.4 ± 1.4
B. subtilis (MTCC 121)	7.4 ± 0.3	11.2 ± 0.5	15.8 ± 0.7	19.2 ± 0.9	30.6 ± 1.2
E. coli (MTCC 43)	6.8 ± 0.3	9.4 ± 0.4	13.6 ± 0.6	17.6 ± 0.8	31.2 ± 1.3
P. aeruginosa (MTCC 424)	—	7.2 ± 0.3	10.4 ± 0.5	14.8 ± 0.6	28.6 ± 1.1
K. pneumoniae (MTCC 109)	6.2 ± 0.2	8.6 ± 0.4	12.2 ± 0.5	16.3 ± 0.7	29.8 ± 1.2

3.4.2 Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The MIC and MBC values of ZnO NPs against the test bacterial strains are presented in Table 3. The MIC values ranged from 31.25 to 125 µg/mL, with the lowest MIC (31.25 µg/mL) observed against S. aureus, confirming its highest susceptibility. B. subtilis showed an MIC of 62.5 µg/mL. Among Gram-negative bacteria, E. coli and K. pneumoniae exhibited MIC values of 62.5 µg/mL and 125 µg/mL, respectively, while P. aeruginosa showed the highest MIC of 125 µg/mL. The MBC values ranged from 62.5 to 250 µg/mL, with the MBC/MIC ratio ≤ 2 for all tested strains, indicating that the ZnO NPs exhibit bactericidal rather than bacteriostatic activity. The lowest MBC (62.5 µg/mL) was observed against S. aureus, while the highest MBC (250 µg/mL) was required for P. aeruginosa.

Table 3: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of ZnO NPs against test bacterial strains

Bacterial Strain	MIC (µg/mL)	MBC (µg/mL)	MBC/MIC Ratio
S. aureus (MTCC 96)	31.25	62.5	2
B. subtilis (MTCC 121)	62.5	125	2
E. coli (MTCC 43)	62.5	125	2
P. aeruginosa (MTCC 424)	125	250	2
K. pneumoniae (MTCC 109)	125	250	2

3.4.3 Time-Kill Kinetics

Time-kill kinetics assay was performed against S. aureus (the most susceptible strain) at 1× MIC and 2× MIC concentrations to evaluate the bactericidal dynamics of ZnO NPs (Figure 7). In the control group (without nanoparticles), bacterial growth increased steadily over 24 hours, reaching approximately 9 log10 CFU/mL. Treatment with ZnO NPs at 1× MIC (31.25 μg/mL) resulted in a gradual reduction in viable counts, with complete killing (no detectable colonies) observed after 12 hours of exposure. At 2× MIC (62.5 μg/mL), more rapid killing was observed, with a reduction of 3 log10 CFU/mL within 4 hours and complete eradication within 8 hours of exposure. These results demonstrate the concentration-dependent bactericidal activity of the biosynthesized ZnO NPs and confirm their effectiveness in eliminating S. aureus within a relatively short time frame.

3.5 Antibacterial Mechanism Studies

3.5.1 Reactive Oxygen Species Generation

To elucidate the mechanism of antibacterial action, intracellular ROS generation in S. aureus treated with ZnO NPs was assessed using the DCFH-DA fluorescent probe. As shown in Figure 8a, treatment with ZnO NPs at MIC concentration for 1 hour resulted in a significant increase in fluorescence intensity (approximately 3.8-fold) compared to untreated control cells ($p < 0.001$). The ROS level induced by ZnO NPs was comparable to that of the positive control (1 mM H_2O_2), which showed approximately 4.2-fold increase. This substantial increase in ROS production indicates that oxidative stress is a major mechanism contributing to the antibacterial activity of the synthesized ZnO NPs. The generated ROS, including superoxide anions (O_2^-), hydroxyl radicals (•OH), and hydrogen peroxide (H_2O_2), can cause oxidative damage to cellular components including lipids, proteins, and DNA, ultimately leading to bacterial cell death .

3.5.2 Cell Membrane Permeability and Integrity

The effect of ZnO NPs on bacterial membrane integrity was evaluated using the LIVE/DEAD BacLight staining method (Figure 8b-e). In untreated control cells, predominantly green fluorescence was observed (Figure 8b), indicating intact cell membranes. Cells treated with 70% isopropyl alcohol (positive control) showed intense red fluorescence (Figure 8c), confirming complete membrane disruption. Treatment with ZnO NPs at MIC concentration resulted in a mixture of green and red fluorescent cells (Figure 8d-e), with approximately 65% of cells exhibiting red fluorescence after 2 hours of exposure, indicating significant membrane damage.

The membrane damage was further confirmed by measuring the leakage of intracellular nucleic acids and proteins, monitored by absorbance at 260 nm and 280 nm, respectively (Figure 8f). Treatment with ZnO NPs resulted in a time-dependent increase in absorbance at both wavelengths, with significant leakage observed as early as 30 minutes and continuing to increase up to 120 minutes. After 120 minutes of exposure, the A260 and A280 values were approximately 4.2-fold and 3.8-fold higher than untreated controls, respectively ($p < 0.001$). This leakage of intracellular contents confirms that membrane disruption is a key mechanism of ZnO NP-mediated bacterial killing, and it correlates well with the observed ROS generation, as oxidative stress can lead to lipid peroxidation and subsequent membrane damage.

4. Discussion

The present study successfully demonstrates the green synthesis of zinc oxide nanoparticles using the aqueous leaf extract of Rhizophora apiculata, a tannin-rich mangrove plant, and provides comprehensive characterization along with evaluation of antibacterial properties. The phytochemical screening revealed abundant presence of tannins, flavonoids, phenols, and reducing sugars in the extract, which aligns with previous reports on R. apiculata . These phytochemicals play dual roles in nanoparticle synthesis: they act as reducing agents that convert zinc ions to zinc oxide, and as capping agents that stabilize the formed nanoparticles and prevent agglomeration . Tannins, being polyphenolic compounds with multiple hydroxyl groups, are particularly effective in metal ion reduction through electron donation mechanisms. The hydroxyl groups undergo oxidation to quinone forms while simultaneously reducing Zn^{2+} to ZnO . Similarly, flavonoids with their keto groups and reducing sugars with aldehyde groups contribute to the bioreduction process. This multi-component reducing system from the plant extract provides an advantage over chemical synthesis methods that rely on single reducing agents, potentially leading to better-controlled nanoparticle characteristics.

The optimization of synthesis parameters revealed that precursor concentration, pH, temperature, and reaction time critically influence nanoparticle formation. The optimal conditions identified (0.2 M zinc acetate, pH 8, 70°C, 2 hours) are comparable to those reported for other plant-mediated ZnO NP syntheses . The pH-dependent synthesis is particularly noteworthy, as pH 8 provided optimal conditions for deprotonation of phenolic hydroxyl groups, enhancing their reducing capacity. At lower pH, protonation of these groups reduces their electron-donating ability, while at higher pH (>9), rapid precipitation of zinc hydroxide may occur, leading to polydisperse and aggregated nanoparticles . The temperature of 70°C provided sufficient thermal energy to overcome activation barriers for reduction while preventing thermal degradation of heat-labile phytochemicals, which could occur at higher temperatures.

The UV-Vis absorption peak at 368 nm with a calculated band gap of 3.28 eV confirms the quantum confinement effect in the synthesized nanoparticles, as this value is slightly higher than bulk ZnO (3.2 eV) . Similar absorption peaks have

been reported for ZnO NPs synthesized using various plant extracts, including Coldenia procumbens (368 nm) , Piper betel (355 nm) , and Verbascum sinaiticum (400 nm) . The variations in peak positions may be attributed to differences in particle size, morphology, and surface modifications by different phytochemicals from different plant sources.

FTIR analysis provided crucial evidence for the involvement of phytochemicals in nanoparticle synthesis and stabilization. The appearance of the characteristic Zn-O stretching vibration at 532 cm^{-1} confirmed successful ZnO formation, while the presence of phytochemical-derived bands in the nanoparticle spectrum confirmed surface capping . This capping is essential for nanoparticle stability, as the adsorbed phytochemicals provide electrostatic and steric stabilization, preventing aggregation. The zeta potential value of -24.6 mV supports this interpretation, as values above ±20 mV indicate good colloidal stability due to sufficient electrostatic repulsion between particles. Similar zeta potential values have been reported for other green-synthesized ZnO NPs, such as -22.2 mV for Piper betel-mediated nanoparticles . The XRD analysis confirmed the formation of phase-pure hexagonal wurtzite ZnO with an average crystallite size of 28.4 nm. This crystallite size is within the range reported for other plant-mediated ZnO NPs: 52.9 nm for Verbascum sinaiticum , 15.6 nm for Parquetina nigrescens , and 46-84 nm for Coldenia procumbens . The variations in crystallite size likely reflect differences in the reducing and capping efficiencies of phytochemicals from different plant sources. The relatively small crystallite size obtained in this study is advantageous for antibacterial applications, as smaller nanoparticles have higher surface area-to-volume ratios, enabling greater interaction with bacterial cells and enhanced ROS generation .

SEM and TEM analyses revealed predominantly spherical nanoparticles with some hexagonal faceting, ranging from 20-65 nm in size. The morphology is consistent with other green-synthesized ZnO NPs, where spherical or quasi-spherical shapes are commonly observed . The slight discrepancy between TEM size (32.6 nm) and DLS hydrodynamic size (86.7 nm) is expected and well-documented, as DLS measures the hydrodynamic diameter including the hydration layer and surface-bound phytochemicals, while TEM measures the actual physical size of dried nanoparticles. The PDI value of 0.284 indicates relatively narrow size distribution, which is desirable for reproducible biological applications.

Thermogravimetric analysis revealed a total weight loss of 18% up to 800°C, with the major loss between 150-400°C corresponding to decomposition of surface-bound organic capping agents. This confirms that the synthesized nanoparticles consist of a crystalline ZnO core (~82%) coated with a thin layer of phytochemicals (~18%). The thermal stability up to 400°C makes these nanoparticles suitable for applications requiring moderate thermal processing .

The antibacterial evaluation demonstrated that R. apiculata-mediated ZnO NPs possess potent, concentration-dependent antibacterial activity against both Gram-positive and Gram-negative bacteria, with MIC values ranging from 31.25 to 125 µg/mL. These values are comparable to or better than many previously reported green-synthesized ZnO NPs. For example, Verbascum sinaiticum-mediated ZnO NPs showed MIC values of 125-250 µg/mL against similar strains , while Coldenia procumbens-mediated nanoparticles showed zones of inhibition of 13.96 mm against S. aureus at 100 µg/mL , compared to 17.3 mm at 125 µg/mL in the present study. The superior activity observed in our study may be attributed to the unique phytochemical profile of R. apiculata, which provides effective capping and potentially synergistic antibacterial effects with the phytochemicals themselves.

The differential susceptibility between Gram-positive and Gram-negative bacteria observed in this study (Gram-positive being more susceptible) is consistent with previous reports . This difference is attributed to structural variations in their cell envelopes. Gram-positive bacteria have a thick peptidoglycan layer that is relatively porous and allows nanoparticle penetration, while Gram-negative bacteria possess an additional outer membrane with lipopolysaccharides that acts as an effective permeability barrier . Among the tested strains, S. aureus showed the highest susceptibility, which is clinically significant given the prevalence of methicillin-resistant S. aureus (MRSA) as a major nosocomial pathogen. The MBC/MIC ratio ≤ 2 for all strains confirms the bactericidal nature of the nanoparticles, which is advantageous over bacteriostatic agents as it ensures complete eradication of pathogens and reduces the risk of resistance development.

The time-kill kinetics revealed rapid bactericidal activity, with complete killing of S. aureus within 8 hours at 2× MIC concentration. This rapid action is characteristic of nanoparticle-mediated killing and contrasts with conventional antibiotics that often require longer exposure times. The rapid killing is beneficial for preventing bacterial proliferation and minimizing the window for resistance development .

The mechanistic studies provide compelling evidence that ROS generation and membrane disruption are primary mechanisms of antibacterial action. The 3.8-fold increase in ROS production upon nanoparticle treatment indicates significant oxidative stress induction. ZnO NPs can generate various ROS species through multiple pathways: (1) photocatalytic generation under visible light (which was present during incubation), (2) release of Zn^{2+} ions that interfere with electron transport chains, and (3) direct interaction with bacterial membranes leading to electron leakage . The

generated ROS cause oxidative damage to membrane lipids (lipid peroxidation), proteins (protein carbonylation), and DNA (strand breaks), ultimately leading to cell death .

The membrane damage observed through LIVE/DEAD staining and leakage assays correlates well with ROS generation, as lipid peroxidation directly compromises membrane integrity. The leakage of intracellular nucleic acids and proteins indicates catastrophic membrane failure, leading to loss of essential cellular components and collapse of membrane potential. This dual mechanism of ROS generation and membrane disruption is advantageous because it targets multiple cellular components simultaneously, making it difficult for bacteria to develop resistance through single mutations .

The presence of surface-bound phytochemicals from R. apiculata may contribute to the antibacterial activity in several ways. First, these phytochemicals themselves may possess intrinsic antibacterial properties, providing additive or synergistic effects with ZnO NPs. Second, the phytochemical capping may enhance nanoparticle-bacteria interactions through specific binding to bacterial surface structures. Third, the capping layer may modulate the release of Zn^{2+} ions, providing sustained antibacterial activity. Studies on R. apiculata-mediated silver nanoparticles have demonstrated significant biological activities including cytotoxicity against cancer cells , and it is plausible that similar bioactive phytochemicals contribute to the enhanced antibacterial activity observed in this study.

The environmental significance of this work lies in the utilization of a mangrove plant for nanoparticle synthesis, contributing to the principles of green chemistry. Mangrove ecosystems are unique and productive, and R. apiculata is widely available in coastal regions. The use of plant extracts eliminates the need for toxic reducing agents (such as sodium borohydride or hydrazine) and organic solvents, making the process environmentally benign. Furthermore, the synthesis is energy-efficient, requiring only moderate temperatures (70°C) compared to high-temperature physical methods.

However, this study has certain limitations that should be acknowledged. First, the exact phytochemicals responsible for reduction and capping were not isolated and identified individually; the study relied on qualitative phytochemical screening. Future studies should focus on identifying specific bioactive compounds through chromatographic techniques and evaluating their individual contributions to nanoparticle synthesis and antibacterial activity. Second, the in vitro antibacterial activity, while promising, needs validation through in vivo studies using animal infection models to assess efficacy, toxicity, pharmacokinetics, and biocompatibility. Third, the potential for resistance development against ZnO NPs should be investigated through long-term exposure studies. Fourth, the cytotoxicity of these nanoparticles against human cell lines needs thorough evaluation to establish safety for potential therapeutic applications. Some studies have reported that green-synthesized ZnO NPs show selective toxicity towards bacterial cells while maintaining high viability of mammalian cells , but this needs confirmation for R. apiculata-mediated nanoparticles.

Future perspectives include exploring the potential of these nanoparticles for various biomedical applications such as wound dressings, antibacterial coatings for medical devices, and incorporation into formulations for topical applications. The demonstrated antibacterial activity against clinically significant pathogens suggests potential utility in combating hospital-acquired infections. Additionally, the nanoparticles could be explored for synergistic combinations with conventional antibiotics to overcome resistance in multidrug-resistant strains.

5. Conclusion

This study successfully demonstrates an eco-friendly, cost-effective green synthesis approach for zinc oxide nanoparticles using the aqueous leaf extract of Rhizophora apiculata, a tannin-rich mangrove plant. The phytochemical-rich extract, containing abundant tannins, flavonoids, phenols, and reducing sugars, effectively reduced zinc acetate to crystalline ZnO nanoparticles while simultaneously providing surface capping that ensures colloidal stability. Systematic optimization of synthesis parameters identified optimal conditions of 0.2 M precursor concentration, pH 8, 70°C temperature, and 2 hours reaction time, which yielded nanoparticles with desirable characteristics. Comprehensive characterization confirmed the formation of phase-pure hexagonal wurtzite ZnO nanoparticles with an average crystallite size of 28.4 nm, spherical to irregular morphology, good colloidal stability (zeta potential -24.6 mV), and thermal stability up to 400°C. The surface plasmon resonance peak at 368 nm with a band gap of 3.28 eV confirmed quantum confinement effects at the nanoscale.

The biosynthesized ZnO NPs exhibited potent, concentration-dependent antibacterial activity against clinically significant pathogens, with MIC values ranging from 31.25 to 125 µg/mL. Staphylococcus aureus showed the highest susceptibility (MIC 31.25 µg/mL, zone of inhibition 21.4 mm at 250 µg/mL), while Gram-negative bacteria, particularly Pseudomonas aeruginosa, were relatively less susceptible. The bactericidal nature of the nanoparticles (MBC/MIC ≤ 2) and rapid killing kinetics (complete eradication within 8 hours at 2× MIC) demonstrate their therapeutic potential. Mechanistic investigations revealed that the antibacterial activity is primarily mediated through enhanced reactive oxygen species generation (3.8-fold increase) and subsequent bacterial membrane disruption, leading to leakage of intracellular contents.

The green synthesis approach utilizing R. apiculata offers several advantages over conventional chemical methods, including environmental sustainability, cost-effectiveness, elimination of toxic reagents, and the added benefit of phytochemical capping that enhances biocompatibility. The study establishes R. apiculata as a promising biological resource for nanoparticle synthesis and contributes to the growing field of plant-based nanotechnology. However, further in vivo studies are warranted to evaluate the therapeutic efficacy, toxicity profile, and pharmacokinetic behavior of these nanoparticles in animal models before clinical translation. Additionally, identification of specific phytochemicals responsible for nanoparticle synthesis and their individual contributions to antibacterial activity would provide deeper insights for optimizing synthesis protocols. Despite these limitations, the findings of this study position R. apiculata-mediated ZnO nanoparticles as promising candidates for developing novel antibacterial agents to address the growing challenge of multidrug-resistant bacterial infections, aligning with the global need for sustainable and effective nanoantibiotics.

6. References

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