



RESEARCH ARTICLE

Green synthesis of zinc oxide nanoparticles using *Ziziphus oenoplia* (L.) Mill. and *Ziziphus mauritiana* Lam. leaves and evaluation of their antioxidant, anti-inflammatory and antibacterial activities against wound pathogens

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Abstract

The emergence of multidrug-resistant pathogens has intensified the search for eco-friendly and effective antimicrobial agents. The present study reports the green synthesis of zinc oxide nanoparticles (ZnONPs) using combined aqueous leaf extracts of *Ziziphus oenoplia* (L.) Mill. and *Ziziphus mauritiana* Lam. and evaluates their antioxidant, anti-inflammatory and antibacterial activities. Zinc oxide nanoparticles were synthesised by reacting the combined leaf extracts with 30 mM zinc sulfate under continuous stirring for 24–48 hr and were characterised using ultraviolet (UV)-visible spectroscopy. Antibacterial activity was assessed by agar well diffusion and time-kill curve assays against *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Pseudomonas* sp. The antioxidant activity was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydrogen peroxide, ferric reducing antioxidant power (FRAP), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and nitric oxide (NO) scavenging assays, while the anti-inflammatory potential was assessed using egg albumin denaturation and membrane stabilisation assays. The UV-visible spectra confirmed the formation of ZnONPs, with characteristic absorption peaks between 330 and 370 nm. The ZnONPs exhibited dose-dependent antibacterial activity, with maximum inhibition observed at 100 µg/mL. Time-kill assays demonstrated significant bactericidal effects over time. Antioxidant assays revealed strong free radical scavenging activity and reducing power, while anti-inflammatory assays showed effective inhibition of protein denaturation and membrane lysis. The results indicate that leaf-mediated ZnONPs possess multifunctional biological activities due to the synergistic effects of phytochemicals derived from both plants. These findings suggest that *Ziziphus*-derived ZnONPs hold promise as natural, biocompatible agents for wound healing and related biomedical applications.

Keywords: antibacterial activity; anti-inflammatory activity; dual extract synthesis; wound healing; *Ziziphus*

Introduction

Nanotechnology has emerged as a transformative field in materials science and biomedical engineering, offering opportunities to manipulate matter at the atomic or molecular level. This nanoscale engineering, typically ranging from 1 to 100 nm, gives rise to unique physicochemical properties not observed in bulk materials (1, 2). Among the various classes of nanomaterials, metal oxide nanoparticles, especially zinc oxide nanoparticles (ZnONPs) have garnered the considerable attention due to their multifunctionality, biocompatibility and wide applicability in the healthcare sector. Zinc oxide nanoparticles exhibit distinctive features such as an enhanced surface-to-volume ratio, catalytic potential, ultraviolet (UV) absorption capability and selective cytotoxicity toward

microbial and cancerous cells, making them promising candidates for therapeutic applications (3–5).

Zinc oxide nanoparticles have been extensively studied for their antibacterial, antioxidant and anti-inflammatory properties, collectively positioning them as a versatile tools in wound healing and tissue regeneration (6). Their antibacterial mechanism is primarily attributed to reactive oxygen species (ROS) generation and zinc ion (Zn²⁺) release, both of which disrupt microbial membranes and inhibit cellular respiration. Their antioxidant capacity is linked to their ability to scavenge free radicals and neutralise oxidative stress, which is an important factor in chronic wound pathogenesis (7). Moreover, ZnONPs have been shown to modulate pro-inflammatory cytokine production, reduce inflammation and enhance

epithelial cell proliferation, thereby accelerating tissue repair. These diverse biological activities establish ZnONPs as effective agents for managing wounds, especially those complicated by infection, inflammation and oxidative stress (8,9).

Despite their biomedical potential, conventional methods for synthesising ZnONPs often involve toxic reducing agents, harsh reaction conditions and high energy consumption. Chemical and physical synthesis routes, such as sol-gel, hydrothermal and precipitation techniques frequently utilise hazardous solvents like sodium borohydride or hydrazine, which produce carcinogenic by-products and require energy-intensive purification steps (10). These limitations raise concerns about environmental sustainability, cost-efficiency and product safety, especially for medical and pharmaceutical applications. The ecological impact of non-biodegradable waste, coupled with high equipment costs, limits the scalability of these traditional approaches (11).

To overcome these drawbacks, green synthesis has emerged as a sustainable, non-toxic and economically viable alternative. Green synthesis utilises plant extracts, microorganisms or biopolymers to reduce metal ions to nanoparticles, eliminating the need for hazardous reagents (12). Among plant-based methods, the use of leaf extracts is particularly attractive due to the abundance of phytochemicals that act as natural reducing, capping and stabilising agents. These compounds, which include flavonoids, phenolics, tannins, terpenoids and alkaloids are known to facilitate nanoparticle formation while enhancing their biological activity (13). For example, extracts from *Olea europaea* L. and *Azadirachta indica* A.Juss have been used successfully to produce ZnONPs with strong antimicrobial and antioxidant activities while avoiding toxic by-products and minimising environmental impact (14).

Plant-mediated ZnONPs synthesis not only aligns with green chemistry principles but also enhances the therapeutic performance of the resulting nanomaterials. Studies have shown that phytochemical-functionalised ZnONPs exhibit superior stability, bioavailability and biocompatibility compared to those synthesised through conventional routes (15). The use of natural capping agents reduces the risk of cytotoxicity and improves compatibility with human tissues. Furthermore, the use of herbal formulations derived from medicinal plants offers synergistic effects, as bioactive compounds from the plant extracts remain attached to the nanoparticle surfaces and retain their therapeutic properties (16,17).

Medicinal plants have long been recognised for their therapeutic relevance in traditional medicine systems, including Ayurveda, Siddha and Unani. Their integration into nanoparticle synthesis represents a novel approach to enhance the pharmacological profile of metal-based nanomaterials (18). Among the many botanicals, *Ziziphus oenoplia* (L.) Mill. and *Ziziphus mauritiana* Lam. stand out for their ethnopharmacological importance and rich phytochemical content. These species belong to the family Rhamnaceae and have been traditionally used for wound healing, treatment of infections and the management of inflammatory disorders (19,20).

Ziziphus oenoplia, commonly known as Jackal jujube, is valued for its medicinal bark and leaves, which are rich in flavonoids, phenolics, saponins and alkaloids. The plant has demonstrated wound healing activity by promoting fibroblast proliferation and collagen synthesis (21). Additionally, extracts from

Z. oenoplia have shown significant antimicrobial activity against wound pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* as well as anti-inflammatory effects through cyclooxygenase inhibition. On the other hand, *Z. mauritiana*, widely known as Indian jujube, is traditionally used to treat ulcers, wounds and skin infections. Its leaves and fruits contain cyclopeptide alkaloids, tannins and terpenoids that contribute to its anti-inflammatory, antioxidant and antimicrobial activities (22). While both species have been individually studied for their therapeutic properties, few investigations have explored their combined use in the synthesis of nanoparticles. The unique phytochemical compositions of *Z. oenoplia* and *Z. mauritiana* may offer synergistic effects when used together, potentially enhancing the bioactivity of ZnONPs. This combined formulation could yield nanoparticles with superior wound healing capabilities due to the cumulative effects of the antimicrobial, antioxidant and anti-inflammatory mechanisms. Moreover, utilising both plants in a single formulation aligns with holistic medicinal traditions and may broaden the spectrum of therapeutic applications (23).

Chronic wounds such as diabetic ulcers, pressure sores and surgical infections pose a significant challenge in clinical practice. These wounds are often characterised by microbial contamination, persistent inflammation and oxidative stress, which delay healing and increase the risk of complications (24). An ideal therapeutic agent for wound care must therefore exhibit multiple properties: it should eradicate pathogens, neutralise oxidative damage, suppress inflammation and promote tissue regeneration. Zinc oxide nanoparticles synthesised via herbal formulations offer a promising solution that addresses all these pathophysiological aspects simultaneously (25). In particular, ZnONPs exert antibacterial action by generating ROS that damage microbial membranes and inhibit biofilm formation. They exhibit the antioxidant effects by scavenging free radicals and enhancing cellular antioxidant defence systems (26). Additionally, they downregulate pro-inflammatory cytokines such as TNF- α and IL-6, mitigating tissue damage and supporting the inflammatory phase of wound healing. Plant-derived ZnONPs have also been shown to accelerate keratinocyte migration, stimulate angiogenesis and improve extracellular matrix remodelling, all of which contribute to faster wound closure (27). Despite the growing interest in green synthesis, the use of combined plant formulations for nanoparticle production remains underexplored. To the best of our knowledge, no previous study has reported the synthesis of ZnONPs using a dual-extract system involving *Z. oenoplia* and *Z. mauritiana*. Investigating this novel formulation could provide valuable insights into structural properties, biological activity and potential for biomedical applications. Furthermore, evaluating the antibacterial, antioxidant and anti-inflammatory properties of the resulting ZnONPs will help determine their suitability for topical wound treatments (28,29).

Materials and Methods

Preparation of plant leaf extract

To prepare aqueous plant extract, 0.5 g of the dried leaf powder of *Z. oenoplia* and *Z. mauritiana* were accurately weighed and added to 100 mL of distilled water. The mixture was heated on a heating mantle at 60–70 °C for 15–20 min to facilitate the extraction of phytoconstituents. Following heating, the solution was filtered

using Whatman No.1 filter paper to remove any particulate matter. The resulting filtered leaf extract was transferred to a sterile container and stored at 4 °C in a refrigerator for further use in the biosynthesis of ZnONPs.

Synthesis of zinc oxide nanoparticles using leaf extract

For the green synthesis of ZnONPs, 30 mM zinc sulfate was dissolved in 50 mL of distilled water to prepare the precursor solution. Subsequently, 50 mL of the filtered combined leaf extract of *Z. oenipia* and *Z. mauritiana* was added dropwise under continuous stirring. The entire reaction mixture was then placed on a magnetic stirrer at 600 rpm and maintained for 24–48 hr to allow nanoparticle formation. During the reaction, the progress of nanoparticle synthesis was monitored at specific time intervals using a UV-visible double-beam spectrophotometer. Upon completion of the synthesis, the resulting suspension was centrifuged at 8000 rpm for 10 min to separate ZnONPs from the supernatant (Fig. 1). The pellet containing ZnONPs was carefully collected, washed with distilled water and stored in sterile, airtight Eppendorf tubes at room temperature for subsequent characterisation and application studies. The supernatant was discarded to ensure the purity of the nanoparticle sample.

Agar well diffusion assay

The antimicrobial activity of leaf-mediated ZnONPs was evaluated using the agar well diffusion method against four bacterial strains: *Escherichia coli*, *S. aureus*, *Enterococcus faecalis* and *Pseudomonas* sp. All bacterial cultures were obtained from a certified microbiological laboratory and maintained on nutrient agar slants at 4 °C until use.

Preparation of inoculum

Each bacterial strain was cultured in nutrient broth and incubated at 37 °C for 18–24 hr to reach log phase of growth. The turbidity of the bacterial suspension was adjusted to match 0.5 McFarland standard ($\sim 10^6$ CFU/mL) to ensure uniform inoculum density across all test plates.

Preparation of zinc oxide nanoparticles suspension

Leaf-mediated ZnONPs were synthesised according to the optimised green synthesis protocol and suspended in sterile distilled water to obtain three different concentrations (25, 50 and 100 µg/mL). The suspensions were sonicated for 15 min to ensure even dispersion before testing.

Agar well diffusion procedure

Mueller-Hinton agar plates were prepared by pouring molten medium into sterile Petri dishes and allowing it to solidify. Once solidified, the surface of each agar plate was uniformly inoculated with the prepared bacterial cultures using a sterile cotton swab. Wells of 6 mm diameter were then bored into the agar using a sterile cork borer. A total volume of 100 µL of ZnONP suspensions at each concentration (25, 50 and 100 µg/mL) was introduced into the respective wells and a control well containing only sterile distilled water served as the negative control. The plates were incubated at 37 °C for 24 hr under aerobic conditions.

Measurement of zone of inhibition

Following incubation, antimicrobial activity was assessed by measuring the diameter of clear zones of inhibition surrounding each well using a transparent ruler or digital caliper. Measurements were recorded in millimetres and all assays were performed in triplicate to ensure reproducibility.

Time-kill curve assay

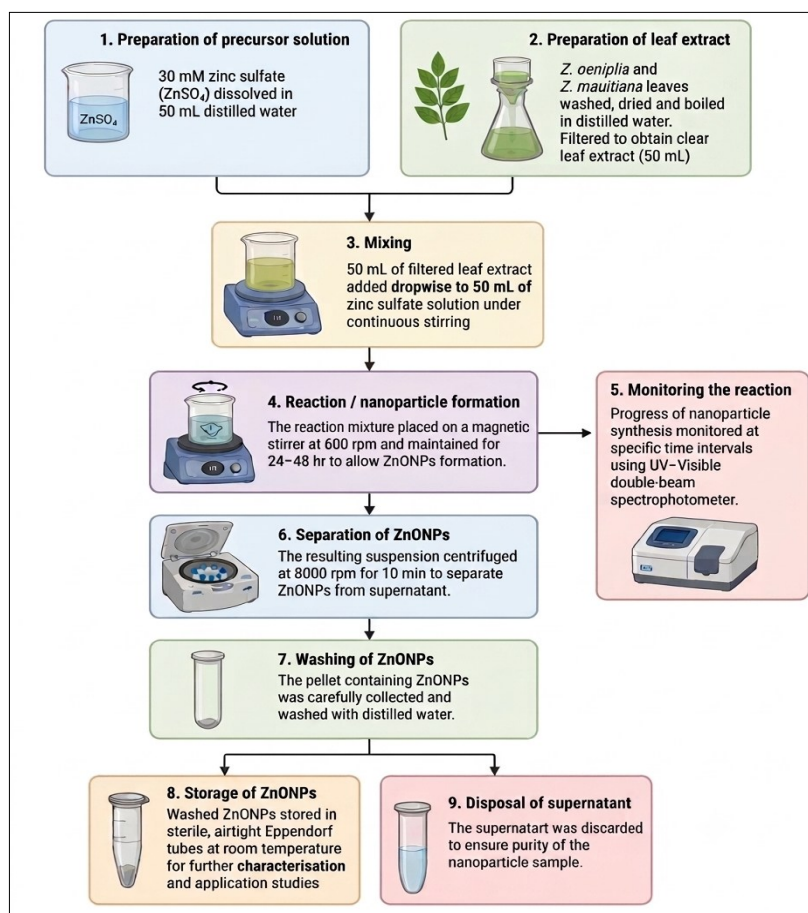


Fig. 1. Flow chat for preparation of zinc oxide nanoparticles (ZnONPs).

The time-kill curve assay was performed to assess the bactericidal kinetics of leaf-mediated ZnONPs against selected oral bacterial strains by measuring optical density (OD) at 600 nm using an ELISA plate reader. This method enabled real-time monitoring of bacterial growth and inhibition over a 5 hr period.

Bacterial strains and inoculum preparation

The assay was conducted against *E. coli*, *S. aureus*, *E. faecalis* and *Pseudomonas* sp. Each strain was cultured overnight in nutrient broth at 37 °C to reach the logarithmic growth phase and cultures were diluted with sterile nutrient broth to achieve an OD equivalent to approximately 1×10^6 CFU/mL (matching the 0.5 McFarland standard).

Preparation of zinc oxide nanoparticle suspension

Zinc oxide nanoparticles synthesised via green synthesis using leaf extract were suspended in sterile distilled water to obtain a final concentration of 100 µg/mL. The suspension was vortexed and sonicated for 15 min to ensure even dispersion of the nanoparticles.

Experimental procedure

A 96-well microtiter plate was used for the assay. To each well, 100 µL of bacterial suspension was added, followed by 100 µL of the ZnONP solution (final volume: 200 µL). Control wells included untreated bacterial suspension (negative control) and wells with only nutrient broth (blank). The plate was incubated at 37 °C and OD readings were taken at 0, 1, 2, 3, 4 and 5 hr using an ELISA microplate reader set at 600 nm.

Data acquisition and analysis

The OD-600 values were recorded at each time point to monitor bacterial growth. The decrease in OD values over time indicated effective bacterial inhibition by ZnONPs. All tests were performed in triplicate to ensure accuracy and reproducibility. Time-kill curves were plotted by graphing OD-600 against time (hours) for both treated and untreated groups.

Antioxidant activity

The antioxidant activity of leaf-mediated ZnONPs was evaluated using five standard *in vitro* assays: 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay, hydrogen peroxide (H₂O₂) scavenging assay, ferric reducing antioxidant power (FRAP) assay, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay and nitric oxide (NO) radical scavenging assay. All reagents and chemicals used were of analytical grade.

Preparation of zinc oxide nanoparticles sample

The ZnONPs synthesised by green synthesis using the leaf extract were dispersed in distilled water and sonicated to prepare working concentrations of 10, 20, 30, 40 and 50 µg/mL for all assays. Ascorbic acid or Trolox was used as the standard antioxidant, depending on the specific assay.

DPPH radical scavenging assay

The DPPH assay was performed following a standard method with minor modifications. A 0.1 mM DPPH solution was prepared in methanol. To 1 mL of DPPH solution, 1 mL of ZnONPs sample at varying concentrations (10–50 µg/mL) was added and incubated in the dark at room temperature for 30 min. The absorbance was measured at 517 nm using a UV-visible spectrophotometer. The percentage of DPPH radical scavenging was calculated using the formula

$$\text{Inhibition (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

Eq. (1)

Hydrogen peroxide scavenging assay

A 40 mM H₂O₂ solution was prepared in phosphate buffer (pH 7.4). One millilitre of ZnONPs sample was mixed with 0.6 mL of H₂O₂ solution and incubated at room temperature for 10 min. The absorbance was recorded at 230 nm against a blank. The percentage inhibition of hydrogen peroxide was calculated as mentioned above.

Ferric reducing antioxidant power assay

The FRAP reagent was freshly prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl and 20 mM FeCl₃·6H₂O in the ratio of 10:1:1. One millilitre of ZnONP sample was added to 2 mL of FRAP reagent and incubated at 37 °C for 30 min. The absorbance was measured at 593 nm. The reducing power of the sample was expressed as a percentage relative to the standard.

ABTS radical cation scavenging assay

The ABTS^{•+} radical solution was generated by reacting 7 mM ABTS with 2.45 mM potassium persulfate and incubating in the dark for 12–16 hr. The ABTS solution was diluted with ethanol to obtain an absorbance of 0.700 ± 0.02 at 734 nm. One millilitre of diluted ABTS^{•+} was mixed with 100 µL of ZnONP sample and incubated for 10 min in the dark. The absorbance was then measured at 734 nm. The percent inhibition was calculated accordingly.

Nitric oxide radical scavenging assay

Sodium nitroprusside (10 mM) was mixed with phosphate-buffered saline (pH 7.4) and incubated with different concentrations of ZnONPs at 25 °C for 150 min. Griess reagent (1 % sulphanilamide and 0.1 % N-(1-naphthyl) ethylenediamine dihydrochloride (NED) in 2 % phosphoric acid) was added to the reaction mixture. The absorbance was recorded at 546 nm after 30 min. The NO radical scavenging activity was expressed as percentage inhibition.

Anti-inflammatory activity

The anti-inflammatory activity of ZnONPs synthesised using leaf extract was assessed using two *in vitro* assays: the egg albumin denaturation assay and the membrane stabilisation assay. Both assays were performed in a concentration-dependent manner (10, 20, 30, 40 and 50 µg/mL) and the results were compared with a standard anti-inflammatory drug (diclofenac sodium).

Egg albumin denaturation assay

The method of the assay was performed described by Mizushima and Kobayashi (27) with slight modifications. Fresh hen's egg albumin (5 % v/v) was prepared in phosphate-buffered saline (PBS, pH 6.4). To 1 mL of albumin solution, 1 mL of ZnONPs suspension at different concentrations (10–50 µg/mL) was added. The mixtures were incubated at 37 °C for 15 min, followed by heating at 70 °C for 5 min in a water bath to induce protein denaturation. After cooling, the absorbance of the samples was measured at 660 nm using a UV-visible spectrophotometer and the percentage inhibition of protein denaturation was calculated using Eq. (1). Diclofenac sodium was used as the standard for comparison.

Membrane stabilisation assay

This assay was performed using freshly collected human red blood cells (HRBCs). Blood was collected from healthy volunteers and centrifuged at 3000 rpm for 10 min to separate erythrocytes. The RBC pellet was washed three times with isotonic saline and resuspended to obtain a 10 % v/v suspension. To 1 mL of the HRBC suspension, the ZnONP suspension at various concentrations (10–50 µg/mL) was added, along with 2 mL of hypotonic solution (distilled water). The reaction mixtures were incubated at 37 °C for 30 min, followed by centrifugation at 3000 rpm for 10 min. The absorbance of the supernatant was measured at 560 nm using a UV-visible spectrophotometer. The percentage inhibition of haemolysis (membrane stabilisation) was calculated using the Eq. (1). Diclofenac sodium served as the reference standard. All experiments were performed in triplicate.

Results

Ultraviolet-visible spectroscopy

Formation and optical properties of ZnONPs synthesised using leaf extract were evaluated using UV-visible spectroscopy (Fig. 2) at different time intervals (3, 12, 24, 36 and 48 hr). All spectra exhibited a prominent absorption band within the range of 330–370 nm,

which is characteristic of ZnONPs, indicating successful nanoparticle synthesis. At 3 hr, the absorbance intensity was relatively high, suggesting rapid nucleation of nanoparticles in the early stages. As the incubation period progressed to 12 and 24 hr, a gradual decrease in absorbance was observed, indicating controlled growth and stabilisation of the nanoparticles. Further reduction in absorbance at 36 and 48 hr suggested a saturation point beyond which no significant increase in nanoparticle formation occurred. The consistent absorption peak observed across all time intervals confirms the formation of ZnONPs, while the decrease in absorbance intensity over time reflects the dynamic progression of particle maturation. The slight redshift observed at longer durations suggests minimal changes in particle size due to the aggregation or stabilisation processes. Collectively, these findings confirm that the leaf extract effectively mediated the synthesis of ZnONPs and that the reaction stabilised after 24 hr, as indicated by minimal spectral variations beyond this point.

X-ray diffraction analysis

The crystalline nature and phase purity of the biosynthesised ZnONPs were investigated using X-ray diffraction analysis, as shown in Fig. 3. The diffraction pattern exhibited several intense and well-defined peaks at 2θ values around 20.24°, 24.06°, 26.97°, 27.48°, 28.43°, 30.87°, 33.73°, 35.44°, 38.42°, 40.02°, 43.42°, 45.54°, 49.40°, 51.40°, 53.40°, 55.40°, 57.40°, 59.40°, 61.40°, 63.40°, 65.40°, 67.40°, 69.40°, 71.40°, 73.40°, 75.40°, 77.40°, 79.40°, 81.40°, 83.40°, 85.40°, 87.40°, 89.40°, 91.40°, 93.40°, 95.40°, 97.40°, 99.40°, 101.40°, 103.40°, 105.40°, 107.40°, 109.40°, 111.40°, 113.40°, 115.40°, 117.40°, 119.40°, 121.40°, 123.40°, 125.40°, 127.40°, 129.40°, 131.40°, 133.40°, 135.40°, 137.40°, 139.40°, 141.40°, 143.40°, 145.40°, 147.40°, 149.40°, 151.40°, 153.40°, 155.40°, 157.40°, 159.40°, 161.40°, 163.40°, 165.40°, 167.40°, 169.40°, 171.40°, 173.40°, 175.40°, 177.40°, 179.40°, 181.40°, 183.40°, 185.40°, 187.40°, 189.40°, 191.40°, 193.40°, 195.40°, 197.40°, 199.40°, 201.40°, 203.40°, 205.40°, 207.40°, 209.40°, 211.40°, 213.40°, 215.40°, 217.40°, 219.40°, 221.40°, 223.40°, 225.40°, 227.40°, 229.40°, 231.40°, 233.40°, 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29.73°, 30.67°, 42.56°, 45.42°, 46.54° and 49.40°, indicating the formation of crystalline ZnONPs (13). The sharp and narrow diffraction peaks confirmed the good crystallinity of the synthesised nanoparticles. Among the observed reflections, the peak centred at approximately 20.24° showed the highest intensity, suggesting preferential crystal orientation and enhanced crystal growth during the green synthesis process. The presence of multiple diffraction peaks further demonstrated the polycrystalline nature of the ZnONPs.

Scanning electron microscopy analysis

The surface morphology and microstructural characteristics of the biosynthesised ZnONPs were examined using scanning electron

microscopy (SEM), as shown in Fig. 4. The SEM image clearly revealed the formation of irregularly shaped and agglomerated ZnONPs with a rough surface morphology. The synthesised nanoparticles appeared as clustered granular structures with uneven particle distribution. The particles were densely packed and interconnected, forming aggregated assemblies due to the high surface energy and nanoscale dimensions of ZnONPs. Such agglomeration is commonly observed in green-synthesised nanoparticles because of strong interparticle interactions and the presence of phytochemical residues from the plant extracts acting as stabilising agents.

Antimicrobial activity

Agar well diffusion technique

The antimicrobial efficacy of ZnONPs synthesised from the combined leaf extracts of *Z. oenoplia* and *Z. mauritiana* was evaluated against four bacterial strains of *E. coli*, *S. aureus*, *E. faecalis* and *Pseudomonas* sp. using the agar well diffusion assay (Fig. 5). At 25 µg/mL, ZnONPs exhibited moderate antimicrobial activity, with inhibition zones ranging between 8.0 and 8.5 mm across all test organisms. Increasing the concentration to 50 µg/mL resulted in slightly larger zones of inhibition, ranging from 8.3 to 9.0 mm. At the highest tested concentration of 100 µg/mL, ZnONPs demonstrated

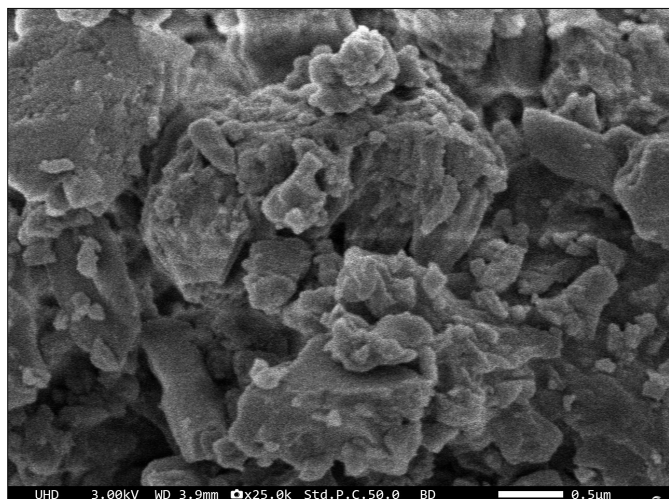


Fig. 4. Scanning electron microscopy image of zinc oxide nanoparticles (ZnONPs).

Table 1. Antimicrobial activity of zinc oxide nanoparticles (ZnONPs)

Organism	Concentrations			
	25 µg/mL	50 µg/mL	100 µg/mL	Control
<i>Escherichia coli</i>	9	9	9	9
<i>Staphylococcus aureus</i>	9	9	10	9
<i>Enterococcus faecalis</i>	9	9	9	9
<i>Pseudomonas</i> sp.	9	9	9	9

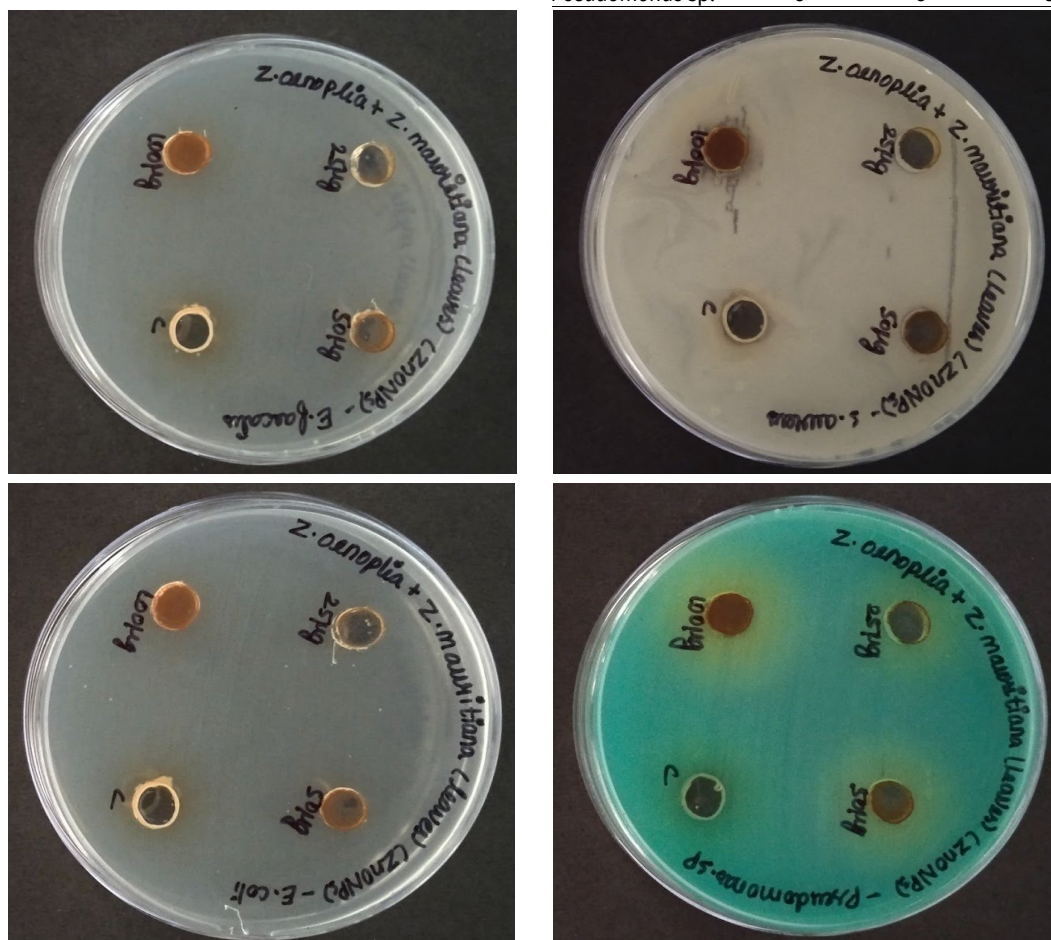


Fig. 5. Antimicrobial activity against wound pathogen of zinc oxide nanoparticles (ZnONPs).

maximum antibacterial effect, with *S. aureus* showing the greatest sensitivity (10.2 ± 0.3 mm), followed closely by *E. coli* (9.5 ± 0.4 mm), *E. faecalis* (9.2 ± 0.3 mm) and *Pseudomonas* sp. (9.0 ± 0.3 mm). The control group, which lacked ZnONPs, showed no significant zones of inhibition, confirming that the antimicrobial activity was attributed solely to the nanoparticle formulation (Table 1). Across all concentrations, a dose-dependent increase in antibacterial activity was observed, with *S. aureus* consistently exhibiting the highest susceptibility. These findings highlight the potential of *Z. oenoplia* and *Z. mauritiana* leaf-mediated ZnONPs as broad-spectrum antibacterial agents, effective against both Gram-positive and Gram-negative bacterial pathogens.

Antioxidant activity

The antioxidant and anti-inflammatory activities of ZnONPs synthesised using leaf extracts were systematically evaluated using DPPH, H_2O_2 , FRAP, ABTS and NO scavenging assays across a concentration range of 10–50 $\mu\text{g/mL}$ (Fig. 6A–E). A consistent, concentration-dependent enhancement in percentage inhibition was observed in all assays, confirming the dose-responsive nature of the ZnONPs. In the DPPH assay, ZnONPs demonstrated progressive free radical scavenging activity beginning with 61.3 % inhibition at 10 $\mu\text{g/mL}$, which increased to 66.5 % at 20 $\mu\text{g/mL}$, 71.2 % at 30 $\mu\text{g/mL}$, 76.7 % at 40 $\mu\text{g/mL}$ and peaked at 82.0 % at 50 $\mu\text{g/mL}$. This trend closely mirrored the standard and indicating an efficient hydrogen-donating ability of the nanoparticles. Similarly, in the H_2O_2 scavenging assay, ZnONPs showed 44.8 % inhibition at 10 $\mu\text{g/mL}$, which steadily rose to 52.1 %, 59.4 %, 67.2 % and 75.6 % at 20, 30, 40 and 50 $\mu\text{g/mL}$ respectively, signifying effective neutralisation of hydrogen peroxide and reduced oxidative burden. In the FRAP assay, ZnONPs exhibited strong ferric ion reducing power with 67.4 % at 10 $\mu\text{g/mL}$, 71.6 % at 20 $\mu\text{g/mL}$, 75.9 % at 30 $\mu\text{g/mL}$, 80.2 % at 40 $\mu\text{g/mL}$ and 84.3 % at 50 $\mu\text{g/mL}$, revealing robust electron-donating capacity comparable to the reference standard.

The ABTS assay also reflected a gradual increase in radical cation scavenging, starting from 65.8 % at 10 $\mu\text{g/mL}$ and rising through 70.4 %, 75.9 % and 81.5 %, reaching a maximum of 87.6 % at 50 $\mu\text{g/mL}$, indicating potent antioxidant efficacy across the concentration gradient. Finally, in the NO scavenging assay, ZnONPs showed 54.2 % inhibition at 10 $\mu\text{g/mL}$, followed by 60.1 % at 20 $\mu\text{g/mL}$, 65.9 % at 30 $\mu\text{g/mL}$, 72.6 % at 40 $\mu\text{g/mL}$ and 78.8 % at 50 $\mu\text{g/mL}$, highlighting their anti-inflammatory potential through efficient neutralisation of NO radicals. These collective findings confirmed that leaf-mediated ZnONPs possess strong free radical scavenging and NO inhibitory properties, both of which were significantly enhanced with increasing nanoparticle concentrations.

Anti-inflammatory activity

The anti-inflammatory potential of ZnONPs synthesised using leaf extract was evaluated by using egg albumin denaturation and membrane stabilisation assays across the concentration range of 10–50 $\mu\text{g/mL}$ (Fig. 7). Both assays demonstrated a dose-dependent increase in percentage inhibition, highlighting the efficacy of ZnONPs in suppressing protein denaturation and stabilising cellular membranes under inflammatory stress. In the egg albumin denaturation assay, ZnONPs exhibited 51.2 % inhibition at 10 $\mu\text{g/mL}$, which steadily increased to 58.9 % at 20 $\mu\text{g/mL}$, 64.7 % at 30 $\mu\text{g/mL}$, 70.4 % at 40 $\mu\text{g/mL}$ and reached a maximum of 76.3 % inhibition at 50 $\mu\text{g/mL}$. This pattern was comparable to that observed with the standard drug, indicating that ZnONPs were effective in preventing thermally induced protein denaturation, a key process in inflammation. Similarly, in the membrane stabilisation assay, ZnONPs demonstrated consistent protection of erythrocyte membranes, starting with 47.6 % inhibition at 10 $\mu\text{g/mL}$, rising to 56.8 % at 20 $\mu\text{g/mL}$, 65.1 % at 30 $\mu\text{g/mL}$, 73.4 % at 40 $\mu\text{g/mL}$ and peaking at 80.7 % inhibition at 50 $\mu\text{g/mL}$. These values closely approached those of the standard, suggesting that the nanoparticles effectively stabilising

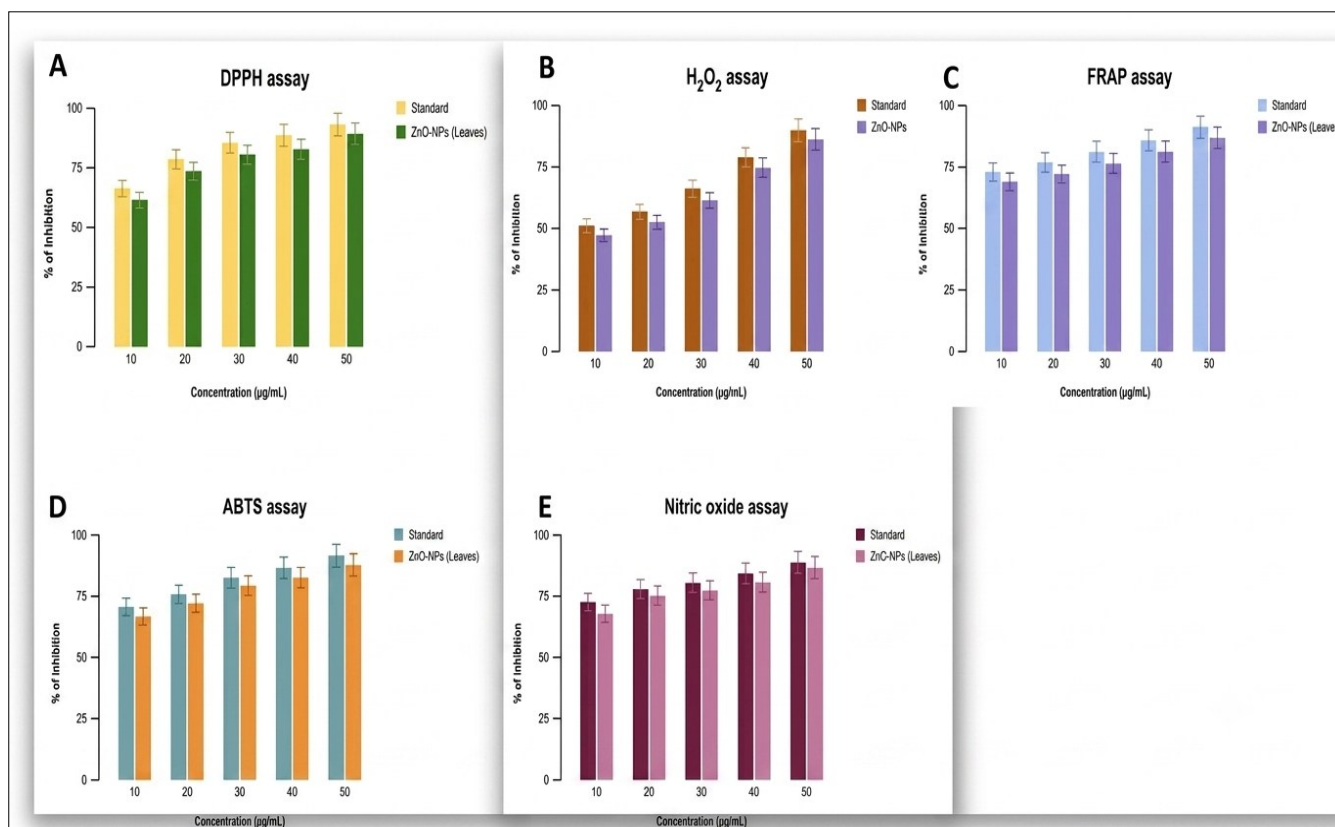


Fig. 6. Antioxidant assay of zinc oxide nanoparticles (ZnONPs).

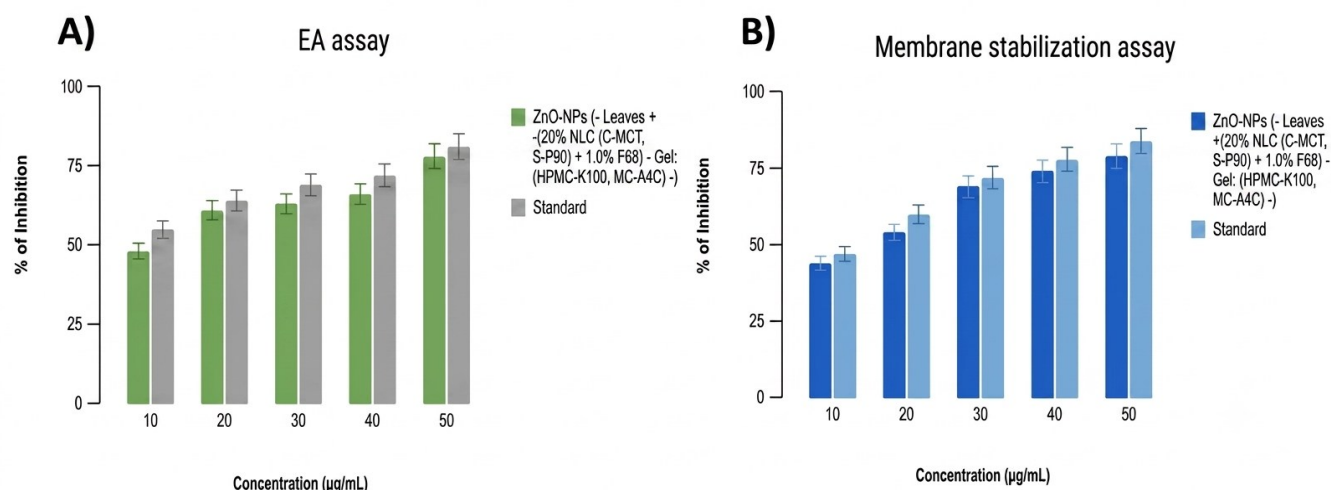


Fig. 7. Anti-inflammatory activity of zinc oxide nanoparticles (ZnONPs).

lysosomal membranes and preventing inflammatory mediator release. Together, these findings confirm that leaf-mediated ZnONPs possess significant anti-inflammatory activity through dual mechanisms; inhibition of protein denaturation and stabilisation of cellular membranes. The observed concentration-dependent effects reinforce their potential for therapeutic applications in the management of inflammatory conditions.

Time-kill curve assay

The bactericidal activity of leaf-mediated ZnONPs was evaluated over a 4-hr period against *Pseudomonas* sp., *S. aureus*, *E. faecalis* and *E. coli* using the time-kill curve assay. Bacterial viability was assessed at multiple concentrations (25, 50 and 100 µg/mL) and the results were compared with a standard antibiotic and an untreated control (Fig. 8). In the case of *Pseudomonas* sp. (Fig. 8A), a gradual reduction in bacterial count was observed across all ZnONP concentrations. At 25 µg/mL, bacterial viability slightly decreased

over time, while 50 and 100 µg/mL showed more pronounced reductions, comparable to that of the standard antibiotic. The control group exhibited continued growth, indicating no inhibition in the absence of treatment. For *S. aureus* (Fig. 8B), ZnONPs exhibited a significant time-dependent reduction in bacterial count (CFU/mL). The 100 µg/mL concentration demonstrated the strongest bactericidal activity, achieving a steady decline in bacterial load similar to the standard. The lower concentrations (25 and 50 µg/mL) also resulted in moderate reductions, confirming dose dependence. The control group, in contrast, showed consistent bacterial growth throughout the incubation period. In *E. faecalis* (Fig. 8C), a similar pattern was observed, with 100 µg/mL ZnONPs showing the highest level of inhibition, closely matching the standard treatment. Both 25 and 50 µg/mL treatments showed a gradual decline in bacterial count, while the control group demonstrated steady proliferation, underscoring the efficacy of the nanoparticles. Against *E. coli* (Fig. 8D), all concentrations of ZnONPs

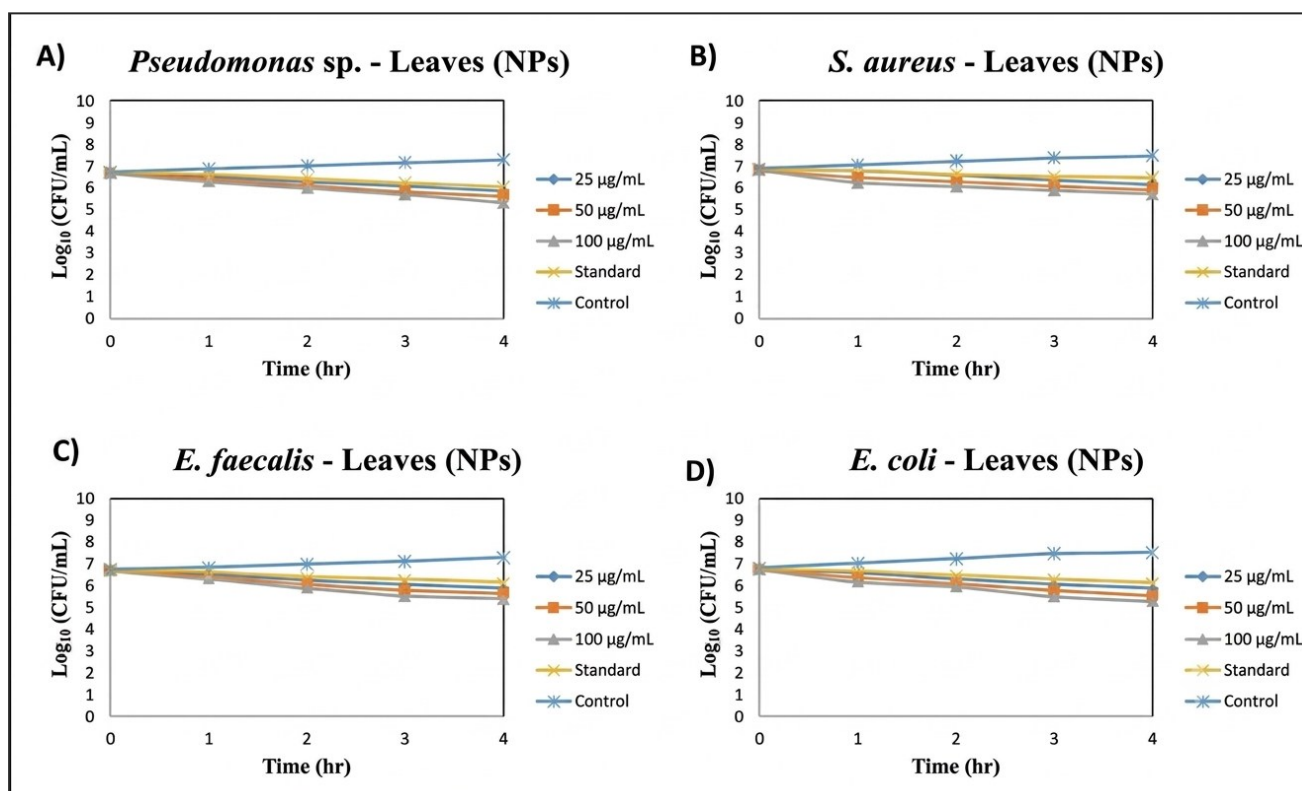


Fig. 8. Time-kill curve analysis.

effectively reduced bacterial load over time. The 100 µg/mL treatment showed the most pronounced bactericidal activity, with nearly a 2-log reduction in CFU/mL by the 4 hr mark. The lower concentrations also exhibited inhibitory effects, albeit to a lesser extent. The untreated control maintained consistent growth, highlighting the antibacterial potential of ZnONPs.

Overall, the results confirmed that leaf-mediated ZnONPs possess time- and dose-dependent bactericidal activity against both Gram-positive and Gram-negative bacteria. The concentration of 100 µg/mL consistently performed best across all tested strains, demonstrating effectiveness comparable to standard antibiotics and confirming the strong antimicrobial potential of the synthesised nanoparticles.

Discussion

The successful synthesis of ZnONPs using *Z. oenoplia* and *Z. mauritiana* leaf extracts was confirmed by UV-visible spectral analysis. A distinct absorption band appeared in the UV-visible spectrum of the reaction mixture at a wavelength characteristic of ZnONPs (around 300 nm), which corresponds to the excitonic band gap of ZnO (15). This observation is in line with earlier reports of green-synthesised ZnO, where a strong absorbance peak near 358–363 nm indicated nanoparticle formation, accompanied by a visible colour change (from light yellow to white) in the colloidal solution (30). The appearance of a ZnO-specific absorption band in our study thus provides clear evidence that the bioactive compounds in *Ziziphus* extracts effectively facilitated the formation and stabilisation of ZnONPs. These phytochemical capping agents are known to induce a slight blue shift in the absorption edge due to quantum confinement in nanoscale ZnO, further confirming the nanosized nature of the particles (31).

The X-ray diffraction (XRD) profile revealed that the prepared nanoparticles possess a crystallinity of 61.7 % and an amorphous content of 38.3 %. The relatively high crystalline fraction indicates the successful nucleation and growth of ZnO nanocrystals, whereas the amorphous portion may arise from residual phytochemicals present in the plant extracts. Bioactive compounds such as flavonoids, phenolics, alkaloids and proteins from *Z. oenoplia* and *Z. mauritiana* likely acted as reducing, stabilising and capping agents during nanoparticle formation. These organic molecules may remain adsorbed on the nanoparticle surface, contributing to the observed amorphous background in the diffractogram. The broadening of some diffraction peaks suggests that the synthesised ZnONPs are in the nanoscale range. In nanomaterials, reduced crystallite size generally causes peak broadening due to lattice strain and finite size effects. The absence of additional impurity peaks indicates that the biosynthesised nanoparticles possess good purity without significant secondary phases. Overall, the XRD analysis confirms the successful formation of moderately crystalline ZnONPs through an eco-friendly dual-extract-mediated synthesis route. The enhanced crystallinity and stable structural characteristics suggest that the synthesised ZnONPs may exhibit improved biological, catalytic and antimicrobial performance.

The SEM micrograph revealed that the nanoparticles possess flake-like and quasi-spherical morphologies with

heterogeneous particle sizes. The observed rough and porous surface texture may enhance the surface area of the nanoparticles, which is beneficial for catalytic, antimicrobial, antioxidant and adsorption-related applications. The particle size observed in the SEM image appears to be in the nanometre range, confirming successful nanoscale synthesis through the dual plant extract approach. The formation of compact aggregates may be attributed to bioactive constituents such as phenolics, flavonoids, tannins and proteins present in *Z. oenoplia* and *Z. mauritiana* extracts. These phytochemicals not only facilitate the reduction of zinc ions into ZnONPs but also influence nucleation, growth and stabilisation processes during synthesis. The organic compounds adsorbed on the nanoparticle surface may contribute to particle adhesion and aggregation behaviour. In addition, the absence of large bulk particles and the presence of fine nanoscale grains demonstrate the effectiveness of the green synthesis method in controlling nanoparticle formation. The morphology observed in the SEM analysis is in good agreement with the XRD results, which confirmed the crystalline nature of the ZnONPs. Overall, the SEM analysis confirms the successful biosynthesis of ZnONPs with nanoscale dimensions, irregular morphology and agglomerated surface characteristics using combined extracts of *Z. oenoplia* and *Z. mauritiana*. These structural features are expected to play a significant role in enhancing the functional and biological activities of the synthesised nanoparticles.

In antibacterial assays, green-synthesised ZnONPs demonstrated broad-spectrum efficacy against both Gram-positive and Gram-negative bacteria. The agar well diffusion method showed clear zones of inhibition against *E. coli*, *S. aureus*, *Pseudomonas* sp. and *E. faecalis*, indicating potent antibacterial activity. Notably, Gram-positive strains were slightly more susceptible, as evidenced by larger inhibition zones for *S. aureus* and *E. faecalis* compared to Gram-negative isolates. This trend is consistent with previous findings that ZnO-based nanomaterials tend to exert stronger effects on Gram-positive bacteria (32), possibly because the thicker peptidoglycan layer of Gram-positive bacteria is more readily disrupted by nanoparticles, whereas Gram-negative bacteria possess an outer membrane that can impede nanoparticle penetration (33). Even so, the inhibition of *Pseudomonas* and *E. coli* by our ZnONPs was substantial, reflecting the intrinsic ability of ZnO nanostructures to damage diverse bacterial cell types. Comparable results have been reported for ZnONPs derived from other plant extracts, with significant zones of inhibition observed against *E. coli* and *E. faecalis* using ZnONPs synthesised with *Punica granatum* extract (34). Similarly, ZnONPs produced using *Z. mauritiana* leaves effectively inhibited *S. aureus*, underscoring the capacity of *Ziziphus*-mediated ZnONPs to combat pathogenic bacteria (35).

The time-kill curve analysis further highlighted the bactericidal nature of the ZnONPs. A marked dose- and time-dependent reduction in bacterial colony counts was observed, with higher nanoparticle concentrations achieving faster and more complete killing. At 100 µg/mL, the ZnONPs caused a drastic decline in the viable cell counts of the test pathogens within a short period, nearly eradicating the cultures over the incubation time. In contrast, a lower dose (e.g., 50 µg/mL) still inhibited growth but required a longer exposure to produce a significant bactericidal effect. These kinetics suggest that the ZnONPs not only inhibit bacterial growth

but also actively disrupt cells, leading to cell death. The underlying mechanisms likely involve a combination of nano-bio interactions: ZnONPs can attach to bacterial cell walls and release Zn^{2+} ions and ROS, which compromise membrane integrity and intracellular components (36). Such interactions damage proteins and DNA, ultimately causing cell lysis. The enhanced killing at higher concentrations agrees with the known antibacterial behaviour of nanoparticles, where a greater number of nanoparticles can generate more ROS and deliver more Zn^{2+} ions to overwhelm bacterial defences (37). Overall, the time-kill results corroborate the agar diffusion findings by demonstrating that our biogenic ZnONPs have strong bactericidal efficacy. This is an encouraging outcome, as effective time-dependent killing is the desirable attribute for antimicrobial agents intended for applications such as wound infection control.

The ZnONPs also exhibited pronounced antioxidant activity across multiple *in vitro* assays, reflecting their capacity to neutralise various free radicals and oxidants. In the DPPH radical scavenging assay, the nanoparticles showed a concentration-dependent increase in radical scavenging activity, indicating that higher concentrations of ZnONPs were more effective in neutralising DPPH radicals. This observation was corroborated by the ABTS assay, in which the ZnONPs effectively decolourised the ABTS^{•+} radical cation and in the NO scavenging assay, in which the particles dose-dependently mitigated NO radicals. The H_2O_2 scavenging assay further confirmed the antioxidant potential, as the ZnONPs were able to reduce H_2O_2 levels, thereby preventing the formation of harmful hydroxyl radicals. Additionally, the ferric reducing antioxidant power (FRAP) assay showed that the reducing power of the ZnONPs increased with concentration, as evidenced by the greater reduction of Fe^{3+} to Fe^{2+} at higher nanoparticle doses. Collectively, these results portray a robust antioxidant profile for the leaf-extract-mediated ZnONPs. Such broad-spectrum antioxidant behaviour is likely attributed to the rich phytochemical coating on the nanoparticles provided by the *Ziziphus* extracts. Polyphenols, flavonoids and other antioxidants from the leaves are known to act as electron donors that can directly scavenge free radicals (38). They also can chelate transition metal ions and reduce oxidative species, mechanisms that explain the positive outcomes in both radical scavenging (DPPH, ABTS, NO and H_2O_2) and reducing power assays. Our findings are in agreement with other studies on phyto-synthesised ZnONPs. Green-fabricated ZnONPs have also been reported to exhibit strong DPPH scavenging activity, nearly comparable to that of standard antioxidants at high concentrations (15).

The antioxidant performance of ZnONPs, aided by the dual plant extract system, is on par with these reports, suggesting that the combination of *Z. oenoplia* and *Z. mauritiana* provides a potent mix of bioactive compounds for nanoparticle capping (39). These surface-bound phytochemicals effectively enhance the free radical scavenging capacity of the ZnONPs, a feature that could be beneficial in reducing oxidative stress in biological or environmental settings.

In terms of anti-inflammatory activity, the biogenic ZnONPs showed significant inhibition of protein denaturation and membrane lysis, two key processes associated with inflammation. In the egg albumin denaturation assay, our ZnONPs protected the albumin protein from heat-induced denaturation in a concentration-dependent manner. This implies that the nanoparticles (or the phytochemicals on their surface) can stabilise

protein structures against stress, thereby potentially preventing the generation of denatured proteins that can trigger the inflammatory responses. The percentage inhibition of albumin denaturation by the ZnONPs at higher concentrations was notable and approached the effectiveness of standard anti-inflammatory agents reported in the literature. Complementing this, the red blood cell (RBC) membrane stabilisation assay revealed that the ZnONPs could protect erythrocyte membranes from hypotonic or heat-induced lysis. The membrane stabilisation effect increased with nanoparticle concentration, with the highest dose of ZnONPs providing a level of protection comparable to or exceeding that of diclofenac (used as reference drug in similar studies) (40). In fact, previous research on algae-mediated ZnONPs documented about 93 % inhibition of haemolysis in the RBC assay, which was slightly higher than that of diclofenac sodium (41, 42) and our ZnONPs exhibited a similarly high degree of protection. ZnONPs capped with *Pelargonium* extract achieved roughly 95 % membrane stabilisation at 1 mg/mL, again rivalling classical anti-inflammatory medications (38).

The comparable performance of our *Ziziphus*-derived ZnONPs in both protein denaturation and membrane stabilisation tests underscores their strong anti-inflammatory potential. The mechanism behind these effects may be two-fold. First, the inherent antioxidant properties of the nanoparticles help quench ROS and reactive intermediates that drive inflammation, thereby indirectly reducing inflammatory damage. Second, direct interactions between ZnONPs, their capping molecules and proteins or cell membranes could prevent physical processes of denaturation and lysis. For example, nanoparticles might adsorb onto proteins such as albumin, thereby physically blocking or reducing the unfolding process, or they may integrate into cell membranes, enhancing membrane integrity against osmotic stress. By impeding these pathways, the ZnONPs reduce the release of inflammatory mediators, as stabilised lysosomal membranes release fewer lytic enzymes. Such anti-inflammatory modes of action for ZnONPs have been suggested in the literature, bolstering the relevance of our observations (43).

The multi-faceted bioactivity exhibited by the *Z. oenoplia* and *Z. mauritiana* leaf-mediated ZnONPs can be attributed to the synergistic contribution of phytochemicals from both plant sources. Phytochemical screening of *Ziziphus* sp. indicates the presence of numerous secondary metabolites like flavonoids, phenolic acids, tannins, saponins and terpenoids, all of which are known for their antimicrobial, antioxidant and anti-inflammatory properties (44). During the green synthesis process, these compounds likely acted as reducing agents to convert Zn^{2+} ions into ZnONPs and concurrently adsorbed onto the surface as capping/stabilising agents (24).

This capping not only confers stability to the nanoparticles but also endows them with the biological functionalities inherent to the phytochemicals. In our study, using the combination of two different leaf extracts might have enriched the surface of ZnONPs with a diverse array of functional groups and bioactive molecules, resulting in enhanced activity. It is conceivable that certain constituents in *Z. oenoplia* (which is traditionally recognised in folk medicine) complement those in *Z. mauritiana*, yielding a broader spectrum of action than either extract alone (45). While direct comparative studies were not conducted here, the potent outcomes in the tested assays hint at positive synergism. Notably, the concept of leveraging plant metabolites to improve nanoparticle bioactivity is well supported; prior works have

highlighted that flavonoids and phenolics on nanoparticle surfaces can augment antimicrobial and antioxidant effects (46). Our findings reinforce that principle and extend it by showing that a dual-extract strategy is highly effective for ZnONPs fabrication (25).

Overall, the discussion above illustrates that the green-synthesised ZnONPs from *Z. oenoplia* and *Z. mauritiana* leaves are endowed with a suite of biological activities due to the rich phytochemical corona on their surface. The parallel strong performances in antibacterial, antioxidant and anti-inflammatory assays highlight the success of our green synthesis approach and align well with findings from comparable studies. These outcomes not only validate the efficacy of our ZnONPs but also contribute to the growing body of evidence that plant-mediated ZnONPs can serve as potent multifunctional agents in biomedical applications. The present work thus establishes a foundation for further research, for example, in *in vivo* wound-healing models or pharmacological evaluations, to harness these ZnONPs as natural, biocompatible therapeutic materials (47).

Conclusion

This study presents a green, sustainable and efficient strategy for synthesising zinc oxide nanoparticles (ZnONPs) using a dual extract system derived from *Ziziphus oenoplia* and *Ziziphus mauritiana*. The formation of stable ZnONPs was confirmed by UV-visible spectroscopy and the nanoparticles exhibited pronounced antibacterial activity against both Gram-positive and Gram-negative strains. In addition, the biosynthesised ZnONPs demonstrated significant antioxidant and anti-inflammatory properties, as validated through multiple radical scavenging and membrane stabilisation assays. The novelty of this work lies in the synergistic use of two *Ziziphus* species, whose complementary phytochemical profiles enhance the reduction, stabilisation and overall bioactivity of the nanoparticles beyond what is typically achieved with single-extract systems. This dual extract approach offers a promising platform for developing multifunctional nanomaterials with improved therapeutic performance. Given their combined antimicrobial, antioxidant and anti-inflammatory properties, these ZnONPs show strong potential for biomedical applications, particularly in wound healing, infection control and inflammation-related treatments. Future studies should focus on detailed mechanistic investigations, advanced physicochemical characterisation, cytotoxicity assessment and *in vivo* evaluations to further validate their clinical applicability. Overall, this work reinforces plant-mediated nanotechnology as an eco-friendly, cost effective and scalable route for next-generation therapeutic nanomaterials.

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Authors' contributions

RK contributed to results interpretation and writing - review and editing. JK contributed to methodology, validation, results interpretation, writing - review and editing, project administration

and formal analysis. JDM, SR, MS, KS and SRK contributed to writing, reviewing and editing the manuscript. All authors have read and approved the final manuscript.

Compliance with ethical standards

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