

Calcination-Tuned, Plant-Derived ZnO in Chitosan: A Sustainable Dual-Action Antimicrobial and Regenerative Dressing

Saman Mohammed Sulaiman

University of Raparin

Dara M. Aziz

darachem@uor.edu.krd

University of Raparin

Dyari M. Mamand

University of Raparin

Karzan Qurbani

University of Raparin

Sanagar A. Hassan

University of Raparin

Alla Ahmad M. Amin

University of Raparin

Rzgar Farooq Rashid

Knowledge University

Article

Keywords: green synthesis, zinc oxide nanoparticles, chitosan, safe-by-design antimicrobial, One-Health, environmental health, wound dressing

Posted Date: November 28th, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-8004740/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

Safe-by-design antimicrobials that are green to produce and effective against environmentally relevant pathogens are central to One-Health infection control. We report an aqueous, plant-mediated synthesis of zinc oxide nanoparticles (ZnO NPs) using *Anagyris foetida* leaf extract, followed by calcination at 500, 600, and 700°C to tune crystal quality, surface cleanliness, and defect chemistry. The powders were embedded in a biocompatible chitosan (CS) matrix to create a paste-like Phyto-ZnO/CS dressing intended for sustainable wound management and environmental health applications (minimal solvent use, benign reagents, scalable processing). XRD, FTIR, UV–Vis diffuse reflectance (DRS), FESEM, and EDS confirmed phase-pure wurtzite ZnO with temperature-dependent peak sharpening, clean Zn–O lattices with only trace C–O features, quasi-spherical primaries with tightened size distributions (mean $\pm$ SD: 23.15 $\pm$ 3.80 nm at 500°C; 22.75 $\pm$ 3.98 nm at 600°C; 18.86 $\pm$ 2.49 nm at 700°C), and decreasing adventitious light elements after calcination. DRS showed a slight red shift of the absorption edge (band-gap narrowing) consistent with strain/defect re-equilibration. In an infected excisional rat model with once-daily application for 15 days, wound contraction improved from 68% (control) and 86% (CS) to 96% (ZnO-500), 98% (ZnO-600), and 99% (ZnO-700). Histopathology corroborated near-complete re-epithelialization, dense aligned collagen, and minimal residual inflammation for the 700°C formulation, without necrosis. The results link calcination-driven cleaning and defect tuning to enhanced antibacterial and pro-healing performance, positioning Phyto-ZnO/CS-700 as a sustainable, non-toxic, dual-action material aligned with JECE's focus on green/safer novel materials for pathogen control and environmental health.

1. Introduction

Bacterial contamination delays wound closure by prolonging inflammation, degrading provisional matrix, and impeding re-epithelialization; consequently, dressings that both control infection and promote tissue repair are clinically prized[1–3]. Modern wound dressings are designed to provide a moist occlusive environment, absorb exudate, buffer redox/enzymatic stress, and deliver bioactive cues that stimulate angiogenesis and collagen deposition[4, 5]. Nanotechnology offers powerful levers for such multifunctionality: nanoscale carriers and fillers can combine high surface area, tunable defect chemistry, and on-demand release/ROS generation to achieve simultaneous antibacterial action and pro-healing signaling[6]. Among antimicrobial nanomaterials, zinc oxide (ZnO) stands out for broad-spectrum bactericidal activity, chemical/thermal robustness, and biological compatibility. ZnO is a direct, wide-band-gap ($\approx$ 3.3 eV) II–VI semiconductor with large exciton binding energy ($\approx$ 60 meV), properties that underpin efficient near-UV/visible absorption and reactive-oxygen-species (ROS) generation under ambient light—mechanisms central to membrane damage, metabolic arrest, and biofilm inhibition[7–9]. To minimize hazardous solvents and reduce downstream toxicity, green synthesis using plant extracts has emerged as an attractive route to ZnO nanoparticles (NPs), phytochemicals (polyphenols, flavonoids, sugars) act as complexings/capping agents, enabling low-temperature nucleation while limiting environmental burden[10–12]. A critical but underused lever in bio-ZnO optimization is calcination, controlled thermal treatment removes residual organics and hydroxyls, increases

crystallinity, relaxes microstrain, and modulates point-defect populations (e.g., oxygen vacancies), thereby tuning optical band gap and surface reactivity that govern antibacterial potency and tissue compatibility[13–15]. Notably, thermal clean-up can also restructure “soft” agglomerates formed by phytochemical bridges, by burning out organics and reshaping necks, calcination may narrow the apparent primary-particle size distribution and expose cleaner facets-despite coarsening of coherent XRD domains-yielding particles with higher effective contact reactivity[16]. To convert nano powders into a clinically tractable topical, we embed ZnO in chitosan (CS)—a biodegradable, mucoadhesive polysaccharide with intrinsic hemostatic and antimicrobial effects and an excellent safety profile[17, 18], also CS provides a moist, conformal seal that adheres to wound beds, concentrates ZnO locally, and can modulate Zn^{2+} /ROS microflux for durable coverage while limiting systemic exposure[19], and synergy between CS and ZnO has been widely reported, with CS enhancing residence time/adhesion and ZnO contributing potent contact killing and pro-healing Zn^{2+} signaling[20–22].

Here, we report a green, plant-mediated route to ZnO using *Anagyris foetida* leaf extract, followed by calcination at 500°C, 600°C, and 700°C to deliberately tune microstructure and defect chemistry. The resulting nanopowders are incorporated into a Phyto-ZnO/Chitosan bioactive nanocomposite dressing (paste-like ointment) for topical application. We systematically correlate calcination temperature with structure, optics, and bioactivity, showing that higher calcination removes organics, narrows the optical band gap (UV–Vis DRS/Tauc), and tightens particle-size distributions (FESEM), changes that enhance biological performance by increasing light absorption, ROS generation, and Zn^{2+} availability at the interface. Comprehensive characterization (XRD for phase/crystallite size–strain; FTIR for surface chemistry; UV–Vis DRS for band gap; FESEM/EDS for morphology/elemental purity) is coupled to antimicrobial assays against MDR *Staphylococcus aureus* and XDR *Pseudomonas aeruginosa*, as well as an infected excisional wound model in rats to quantify contraction and analyze histopathology. Collectively, our results identify optimally calcined, green ZnO in a CS matrix as a scalable, non-toxic platform for antibacterial wound healing and elucidate how thermal processing governs the structure–property–efficacy nexus in bio-derived ZnO.

2. Materials and methodology

2.1. Materials

Zinc acetate dihydrate ($\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$, $\geq 99\%$), sodium hydroxide (NaOH, pellets, $\geq 98\%$), glacial acetic acid ($\geq 99.7\%$), chitosan (medium molecular weight; degree of deacetylation 75–85%), absolute ethanol ($\geq 99.8\%$), and Whatman No. 1 filter papers were used as received. Spectroscopic-grade KBr (for FTIR pellets) and BaSO_4 (DRS reflectance standard) were employed for spectroscopic sample preparation. Alumina crucibles (calcination), carbon adhesive tabs and aluminum SEM stubs (FESEM/EDS), and sterile glass vials for product storage were used as supplied. For histology, 10% neutral buffered formalin (NBF), hematoxylin, eosin, xylene, and graded ethanol (70–100%) were used according to standard protocols. For animal procedures, ketamine (10%) and xylazine (2%) injectable

solutions (anesthesia), as well as povidone–iodine and 70% ethanol (skin antisepsis), were used as received. Distillated water was used throughout.

2.2. Collection and Authentication of *Anagyris foetida* Plant Material

The green preparation of the *Anagyris foetida* aqueous leaf extract was carried out using fresh aerial parts collected from a natural habitat in Esewa village, Sulaymaniyah Governorate, Kurdistan Region of Iraq (36.066927° N; 45.284518° E). All sampling procedures adhered to institutional, national, and international guidelines for plant collection and research. *A. foetida* is a widely distributed Mediterranean species and is neither endangered nor protected according to the IUCN Red List; therefore, no governmental licenses were required for non-destructive sampling. However, the collection was formally authorised by the Director General of the Research Center, University of Raparin, confirming institutional approval for the scientific use of plant material.

Botanical authentication of the plant material was performed by Dr. Safin Hussein, Department of Biology, College of Science, University of Raparin. A voucher specimen has been prepared and deposited in the Department's publicly accessible teaching herbarium under the registration code BIO_Af13, providing a permanent taxonomic reference for future verification. Harvested leaves were placed in sterile polyethylene bags and transported to the laboratory under cooled conditions to maintain sample integrity prior to extraction.

2.3. Preparation of *Anagyris foetida* Aqueous Leaf Extract

Collected leaves were rinsed with running tap water followed by DW water to remove surface debris, then air-dried at room temperature (24–28°C) in the shade for 48 h and finally oven-dried at 50°C for 12 h. The dried leaves were milled to a coarse powder. Typically, 10.0 g of leaf powder was suspended in 100 mL DW water in a 250 mL Erlenmeyer flask, heated at 80°C for 30 min under magnetic stirring (500 rpm), and allowed to cool to room temperature. The mixture was filtered through muslin cloth and then through Whatman No. 1 paper. The filtrate (plant extract) was stored at 4°C and used within 48 h.

2.4. Green Synthesis of ZnO Precursor Gel

In a 500 mL beaker, 2.195 g zinc nitrate hexahydrate (10 mmol) was dissolved in 100 mL DW water at 60°C under stirring (600 rpm). Subsequently, 20 mL of the freshly prepared *A. foetida* extract was added dropwise ($\approx 1 \text{ mL min}^{-1}$). The pH was adjusted gradually to 10.0–10.5 by adding 1.0 M NaOH solution dropwise under continuous stirring (ice bath optional to control exotherm). The mixture typically turned turbid, indicating formation of a zinc hydroxide/organics gel. Stirring was continued at 60°C for 2 h, followed by an aging period without stirring for 12 h at room temperature. The precipitate was collected by centrifugation at 8000 rpm for 10 min, washed three times with DW water and twice with ethanol to remove residual organics and acetate, and then dried at 60°C overnight ($\approx 12 \text{ h}$) to obtain a xerogel precursor.

2.5. Calcination to Obtain ZnO Nanoparticles

The dried precursor was gently ground in an agate mortar and divided into three equal portions. Each portion was placed in an alumina crucible and calcined in a muffle furnace under air with the following program: heating ramp $5^{\circ}\text{C min}^{-1}$ to the target temperature, dwell 3 h, then natural cooling to room temperature inside the closed furnace sample designed as, sample ZnO-500: 500°C , 3 h, sample ZnO-600: 600°C , 3 h and sample ZnO-700: 700°C , 3 h respectively, and after calcination, the lightly sintered cakes were gently reground to fine powders and stored in airtight glass vials.

2.6. Preparation of Phyto-ZnO/Chitosan Bioactive Nanocomposite Dressings

A 1% (w/v) chitosan stock was first prepared by dispersing chitosan (1.00 g) in deionized water (100 mL) containing glacial acetic acid (1 mL; 1% v/v). The mixture was magnetically stirred at 60°C for 24 h until a clear, homogeneous viscous solution formed, so this stock served as the polymer vehicle for all formulations. For the chitosan control (G1), an aliquot of the stock solution (10 mL) was used without inorganic additive, while for the nanocomposite dressings, separate 10 mL portions of the chitosan stock were transferred to clean beakers, and 0.125 g of the respective ZnO nanopowder was incorporated under continuous stirring at 60°C for 30 min to obtain uniform suspensions/pastes. The three formulations were coded according to the calcination temperature of the nanoparticles used: G2 = Phyto-ZnO/Chitosan (ZnO-500), G3 = Phyto-ZnO/Chitosan (ZnO-600), and G4 = Phyto-ZnO/Chitosan (ZnO-700). On a mass basis, each 10 mL batch contained 0.10 g chitosan and 0.125 g ZnO (nominal loads: chitosan 10 mg mL^{-1} ; ZnO 12.5 mg mL^{-1} ; ZnO:CS $\approx 1.25:1$ w/w), and formulations were allowed to equilibrate at room temperature, transferred to sterile, screw-cap vials, and used for daily topical application (Fig. 1).

2.7. Characterization techniques

A Powder X-ray Diffractometer (XRD) identifies the crystal phase and overall crystallinity of ZnO and enables crystallite-size estimation from the broadening of characteristic diffraction peaks. An FTIR Spectrometer records infrared spectra to verify the Zn–O lattice vibration and to confirm removal of organic and acetate residues originating from precursors or plant extracts. A UV–Vis Diffuse Reflectance Spectrophotometer with an integrating sphere measures diffuse reflectance in the 200–800 nm range, allowing optical band-gap estimation via Kubelka–Munk and Tauc analyses. A Field-Emission Scanning Electron Microscope (FESEM) provides high-resolution images that reveal particle size, shape, and surface morphology of the ZnO nanopowders. An Energy-Dispersive X-ray Spectrometer (EDS/EDX) attached to the FESEM performs elemental analysis and mapping to quantify Zn and O and to screen for residual Na, C, and N.

2.8. Ethical concerns

All procedures in this study were reviewed and approved by the Institutional Review Board of the University of Raparin (Approval Code: 2866/28-5-2023). All experiments were carried out in strict accordance with national regulations, international standards for the care and use of laboratory animals,

and the guidelines of the AVMA (2020) and NIH (2011). Furthermore, all methods are reported in accordance with ARRIVE guidelines (<https://arriveguidelines.org>).

2.9. Animal and Housing

Fifteen adult male albino rats (*Rattus norvegicus*), weighing 150 ± 20 g and aged 8–10 weeks, were obtained from the animal facility of the College of Science, University of Raparin (Raniyah District, Kurdistan Region of Iraq). Animals were housed in plastic cages containing wood-chip bedding and maintained under a 12:12 h light/dark cycle at $25 \pm 2^\circ\text{C}$. The light cycle was controlled using an automated system. All rats were provided with standard laboratory pellets and had ad libitum access to food and water.

2.10. Experimental design

The rats were randomly divided into five groups, each of which including three rats and divided as follows: group 1: control (untreated open wounds); group 2: creation of an infected wound treated with regular chitosan application; group 3: creation of an infected wound treated by zinc nanoparticles prepared at 500°C and Group 4: creation of an infected wound treated with zinc nanoparticles prepared at 600°C ; and Group 5: creation of an infected wound treated with prepared zinc nanoparticles at 700°C . Wounds in different groups were treated on a daily basis for 15 days.

2.11. Anesthesia and Wound Creation

Anesthesia was induced by intraperitoneal injection of ketamine hydrochloride (10%; 50 mg/kg) and xylazine hydrochloride (2%; 5 mg/kg). After full anesthesia, each rat was positioned in ventral decubitus, and the dorsal area was shaved and disinfected. A full-thickness circular wound (10 mm diameter) was created using a biopsy punch and scalpel, removing the epidermis, dermis, and hypodermis. The wound surface was then inoculated with a standardized suspension of multidrug-resistant *S. aureus* to establish infection. Animals received their assigned treatments daily until the end of the 15-day study period.

2.12. Euthanasia, Tissue Sampling, and Histopathological Examination

On the 15th postoperative day, two animals from each experimental group were randomly selected for histopathological evaluation, as this time point corresponds to the late proliferative and early remodeling phases of wound healing. Animals were first subjected to deep anesthesia by intraperitoneal injection of ketamine hydrochloride (10% solution; 100 mg/kg) combined with xylazine hydrochloride (2% solution; 10 mg/kg). After 5–7 minutes, the depth of anesthesia was confirmed by the absence of pedal and corneal reflexes. Humane euthanasia was then performed by cervical dislocation in accordance with AVMA (2020) and NIH (2011) guidelines, and death was verified by loss of respiration and heartbeat.

Immediately after euthanasia, full-thickness skin specimens were excised from each rat. Each sample included the wound bed, wound margins, and approximately 2 mm of adjacent unwounded skin to

ensure comprehensive representation of both injured and healthy tissue. The specimens were promptly fixed in 10% neutral buffered formalin, processed using standard paraffin-embedding procedures, and stained with hematoxylin and eosin (H&E). Microscopic evaluation was conducted at 10× and 40× magnifications to assess tissue architecture, inflammation, granulation tissue formation, re-epithelialisation, and collagen deposition.

2.13. Hematoxylin and Eosin (H&E) Staining Procedure

The staining process began with deparaffinization, in which paraffin was removed by immersing the tissue sections in xylene three times, each for 2 minutes. This was followed by rehydration through a descending series of alcohol concentrations (absolute, 95%, 90%, 80%, and 70%), with each step lasting 5 minutes, and then rinsing under running water for 10 minutes. The sections were subsequently stained with hematoxylin for 5 minutes and washed again under running water for 10 minutes to remove excess stain. Next, eosin was applied for 2 minutes to counterstain the tissue, after which the slides were dehydrated in a graded series of alcohol, cleared with xylene, and finally mounted with a cover slip.

2.14. Macroscopic Evaluation of Wound Healing

Full-thickness excisional wounds (10 mm in diameter) were created on the dorsal skin of albino rats under aseptic conditions. Animals were randomly assigned into five groups. C (Control) as untreated group, G1 (Chitosan-treated) group. G2 (ZnO NPs at 500°C) – G3 (ZnO NPs at 600°C) and G4 (ZnO NPs at 700°C) as they treated with green synthesized ZnO nanoparticles prepared from *Anagyris foetida* aqueous leaf extract and calcined at 500°C, 600°C and 700°C respectively. Wound healing progression was monitored macroscopically on Days 0, 1, 3, 5, 7, 9, 11, 13, and 15. Standardized digital images were taken under identical conditions, and wound diameters were measured in millimeters.

Wound contraction (%) was calculated using the formula:

$$\text{wound contraction (\%)} = \frac{\text{Initial wound size} - \text{wound size at specific day}}{\text{Initial wound size}} \times 100$$

2.15. Antibacterial Activity Assessment

2.15.1. Disc Diffusion Assay

The antibacterial activity of the biosynthesized ZnO nanoparticles was evaluated against clinical isolates of multidrug-resistant *Staphylococcus aureus* (MDR) and extensively drug-resistant *Pseudomonas aeruginosa* (XDR) obtained from routine diagnostic specimens collected at the Microbiology Laboratory of Shar Hospital, Sulaymaniyah Governorate, Kurdistan Region of Iraq. Antimicrobial susceptibility testing was performed using the standard Kirby–Bauer disc diffusion method on Mueller–Hinton agar, in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines. Clinical isolates were subcultured in nutrient broth and incubated at $37 \pm 0.5^\circ\text{C}$ for 24 h. The resulting bacterial suspensions were adjusted to the 0.5 McFarland turbidity standard ($\approx 1.5 \times 10^8$ CFU/mL). For each assay, 100 μL of

the standardized inoculum was uniformly spread across the surface of Mueller–Hinton agar plates under aseptic conditions. Sterile Whatman No. 1 filter paper discs (6 mm diameter) were impregnated with 10 mg of ZnO nanoparticles and placed onto the inoculated agar. The plates were incubated at 37°C for 24 h, after which the diameters of the inhibition zones were measured in millimeters [23]. Norfloxacin discs (30 µg/disc) served as positive controls to validate assay performance and ensure methodological reliability.

2.15.2. Minimum Inhibitory and Bactericidal Concentration Determination

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the ZnO nanoparticles were determined against both MDR *S. aureus* and XDR *P. aeruginosa*. Stock suspensions of ZnO nanoparticles were serially diluted to final test concentrations of 0, 0.5, 1, 2, 4, 6, 8, 10, 20, 40, 60, and 80 µg/mL. In sterile 96-well microplates, 50 µL of standardized bacterial suspension (1×10^8 CFU/mL) was combined with nanoparticle suspensions at the specified concentrations, and the final reaction volume was adjusted to 200 µL using Luria–Bertani (LB) broth. Plates were incubated at 37°C for 24 h, and bacterial growth was quantified by measuring optical density at 600 nm (OD_{600}) using a microplate spectrophotometer[24].

2.15.3. Anti-biofilm Activity Assessment

The anti-biofilm activity of ZnO nanoparticles was examined using a modified crystal violet microtiter plate assay. Standardized bacterial suspensions of MDR *S. aureus* and XDR *P. aeruginosa* (1×10^8 CFU/mL) were prepared in LB broth. In 96-well plates, 50 µL of each suspension was mixed with nanoparticle solutions at concentrations ranging from 0 to 80 µg/mL (0, 0.5, 1, 2, 4, 6, 8, 10, 20, 40, 60, 80 µg/mL). The final volume in each well was adjusted to 200 µL with LB broth. Plates were incubated at 37°C for 48 h to facilitate biofilm formation. After incubation, planktonic cells were gently removed by washing with phosphate-buffered saline (PBS). The remaining biofilms were stained with 200 µL of 0.1% crystal violet solution for 15 min at room temperature. Excess stain was removed through PBS washing, and bound crystal violet was subsequently solubilized with 200 µL of 95% ethanol[25]. Biofilm biomass was quantified spectrophotometrically by measuring the absorbance of the extracted dye at 600 nm.

3. Result and discussion

3.1. Structural analysis and crystallite size–microstrain evaluation

The XRD patterns of ZnO-500, ZnO-600, and ZnO-700 (Fig. 2) exhibit the characteristic reflections of hexagonal wurtzite ZnO (space group $P6_3mc$), notably (100), (002), (101), (102), (110), (103), and (112)/(202), without detectable impurity phases, confirming phase purity after calcination of the bio-derived precursor[26, 27]. The persistence and progressive sharpening or intensification of these peaks with temperature indicate improved crystallinity and ordering of the Zn–O lattice, in line with thermal

decomposition of residual organics and densification of the oxide network[28, 29]. Close inspection of the dominant (101) reflection (Table 1) shows a subtle but systematic shift of 2θ from 36.438° (ZnO-500; $d = 2.4658 \text{ \AA}$) to 36.649° (ZnO-600; $d = 2.4521 \text{ \AA}$), followed by slight relaxation to 36.600° (ZnO-700; $d = 2.4552 \text{ \AA}$). According to Bragg's law, the shift to higher angles corresponds to a decrease in lattice spacing and is commonly associated with compressive residual stress and small stoichiometric deviations (e.g., oxygen deficiency) that contract the lattice[30, 31]. The partial back-shift at 700°C suggests stress relaxation as the crystallites coarsen and defect concentrations equilibrate under prolonged thermal exposure in air[32]. Relative to the reference JCPDS card for wurtzite ZnO ($2\theta \approx 36.25^\circ$, $d \approx 2.476 \text{ \AA}$ for (101)), the measured positions imply a net contraction at $500\text{--}700^\circ\text{C}$, consistent with the removal of intercalated organics and the formation of compact ZnO domains from the plant-mediated precursor[33, 34]. Crystallite sizes were estimated from the (101) peak using the Scherrer relation $D = K\lambda/(\beta\cos\theta)$ after correcting the full width at half maximum (FWHM) for instrumental broadening with a standard[35], while Scherrer values provide a first approximation (domain size along the scattering vector), size broadening and lattice strain often coexist in nanocrystalline ZnO synthesized via green routes due to rapid nucleation, defect incorporation, and subsequent thermal coalescence[36, 37]. To decouple these effects, a Williamson–Hall (W–H) analysis was performed using multiple reflections, fitting $\beta \cos \theta = K\lambda/D + 4\varepsilon \sin \theta$, where the intercept yields the coherent domain size D and the slope gives the microstrain ε [38, 39]. The W–H trends (intercept increase and slope decrease with calcination temperature) indicate progressive crystallite growth accompanied by a reduction in microstrain from ZnO-500 to ZnO-700, which is consistent with peak sharpening, the stress relaxation inferred from the (101) 2θ evolution, and the improved long-range order observed in Fig. 2. The microstructural evolution deduced from XRD is physically reasonable for phytochemical-mediated ZnO, at lower temperatures, incomplete burnout of ligands and by-products and a high density of point defects (e.g., V_o , Zn_i) impose lattice distortions and compressive and tensile components that broaden reflections and slightly shift peak positions[40, 41], with higher calcination, removal of residual organics and surface diffusion-driven coalescence (Ostwald ripening) promote larger coherent domains and partial defect annealing, thereby lowering ε and narrowing the FWHM[42, 43]. The modest residual shift relative to the powder standard can be ascribed to a small, persistent non-stoichiometry and crystallite-shape anisotropy typical of wurtzite ZnO nanocrystals.

Table 1

Peak parameters of the ZnO (101) reflection extracted from XRD patterns for samples calcined at 500–700°C (2θ position, d -spacing, and FWHM)

Samples	FWHM Left [$^\circ 2\theta$.]	d -spacing [$\text{\AA}$]	2θ (deg)	Plane (hkl)
ZnO-500	0.4920	2.46579	36.4384	101
ZnO-600	0.8856	2.45209	36.6493	101
ZnO-700	0.8856	2.45523	36.6006	101

3.2. FESEM morphology and EDS analysis of ZnO-500, ZnO-600, and ZnO-700

Representative FESEM micrographs (Fig. 3 **(a,c,e)**) reveal that all powders consist of quasi-spherical ZnO nanoparticles arranged in cauliflower-like secondary aggregates—a morphology frequently observed for wurtzite ZnO obtained by plant-mediated routes due to rapid nucleation followed by diffusion-limited clustering[44–46]. Individual primary particles display smooth surfaces and locally well-defined necks, consistent with solid-state sintering of adjacent crystallites during calcination[47], with increasing calcination temperature, the particle outlines become sharper and the interparticle porosity within aggregates appears reduced, indicating enhanced densification and removal of surface-bound organics originating from the *Anagyris foetida* extract[48, 49]. Size-distribution analysis derived from the FESEM images (Fig. 2 (a-c) ≥ 200 particles per sample) shows narrow, near-Gaussian distributions with mean diameters of 23.15 ± 3.80 nm (ZnO-500), 22.75 ± 3.98 nm (ZnO-600), and 18.86 ± 2.49 nm (ZnO-700). The modest decrease in the apparent particle diameter at 700°C, despite improved crystallinity, can be rationalized by the burnout of phytochemical capping species and acetate residues that promote “soft” agglomeration at lower temperatures, their removal, together with surface diffusion and neck reshaping, yields denser primary particles whose projected areas are smaller even as coherent domain sizes increase (as indicated by XRD)[50]. Similar trends—peak sharpening in XRD alongside reduced FESEM-measured particle diameters—have been attributed to a transition from multi-crystallite, organics-bridged agglomerates to more compact, fewer-crystallite particles after high-temperature annealing[51]. The reduced standard deviation for ZnO-700 further supports a temperature-driven narrowing of the size distribution, consistent with ripening-limited growth and partial strain relaxation[52]. From a biomedical perspective aligned with our wound-healing and antibacterial aims, the temperature-driven removal of organics and the formation of compact ~ 19 – 23 nm ZnO primaries are beneficial, also cleaner surfaces and narrower size distributions enhance bactericidal performance by maximizing nanoparticle–bacteria contact, enabling controlled Zn^{2+} release at physiological pH, supporting ROS generation that induces oxidative stress, and introducing nanoscale asperities capable of membrane disruption[53]. The reduced residual C/N in EDS and the higher crystallinity, most evident for ZnO-700—are expected to improve cytocompatibility by minimizing leachables while sustaining bioactive Zn^{2+} delivery that promotes keratinocyte migration/proliferation, collagen deposition, and pro-angiogenic signaling required for orderly closure[54], moreover, the observed necked aggregates are well suited for immobilization within biopolymer dressings (e.g., chitosan/alginate/gelatin), enabling sustained local ion/ROS release and durable antimicrobial coverage without compromising vapor permeability—key criteria for advanced wound dressings and antibacterial coatings.

EDS spectra for ZnO-500, ZnO-600, and ZnO-700 as shown in Fig. 4. **(a–c)** exhibit the expected Zn K α (~ 8.64 keV) and K β (~ 9.57 keV) lines together with the O K α signal (~ 0.52 keV), with no extraneous peaks attributable to precursor-derived contaminants, occasional C K α (~ 0.28 keV) from the conductive tape was excluded from quantification. Standardless ZAF-corrected quantification yielded the following weight-percent compositions: ZnO-500: Zn = 78.95 wt%, O = 21.05 wt%; ZnO-600: Zn = 81.78 wt%, O = 18.22 wt%; ZnO-700: Zn = 86.54 wt%, O = 13.46 wt%. Converted to atomic percent, these correspond to Zn/O atomic ratios of ~ 0.92 (47.9 at% Zn / 52.1 at% O), ~ 1.10 (52.3 at% Zn / 47.7 at% O), and ~ 1.57 (61.1 at% Zn / 38.9 at% O) for ZnO-500, ZnO-600, and ZnO-700, respectively (stoichiometric ZnO ≈ 1.00).

Two factors, acting in the same direction, rationalize the systematic increase of Zn signal with calcination temperature. First, progressive thermal burnout of adsorbed water, hydroxyls, and residual phytochemicals reduces the contribution of surface oxygen-containing species, shifting the apparent composition toward Zn-rich values even if the lattice remains close to ZnO[55, 56], this interpretation is consistent with FTIR evidence of dehydroxylation/organic removal after high-T calcination, Secondly, at the measurement conditions used, EDS tends to under-report light elements like oxygen in heavy matrices because of strong absorption and fluorescence effects in the Zn-rich interaction volume (limitations well documented for O quantification in metal oxides)[57]. A genuine increase in lattice oxygen deficiency (formation of V_o) at higher temperature can also contribute, particularly as surface hydroxyls desorb and Zn–O networks densify, oxygen vacancies are well known in annealed ZnO and are often reflected by reduced O signals in EDS together with XRD/DRS signatures of defect evolution[58] [6, 7]. Taken together, the EDS results corroborate the microstructural picture drawn from FESEM/XRD: calcination cleans and densifies the nanoparticles, minimizes residual non-ZnO species, and may modestly increase Zn-rich defects (or, at minimum, the apparent Zn fraction by EDS), changes that are relevant for the intended antibacterial and wound-healing applications where clean surfaces and controlled Zn^{2+} availability are beneficial[59].

3.3. FTIR analysis

Figure 5a presents the overview FTIR spectra of ZnO-500, ZnO-600, and ZnO-700. Across all three samples the spectra are largely featureless in the mid-IR, consistent with efficient removal of acetate and most phytochemical residues upon calcination, with only two diagnostically important vibrations persisting. The first is a weak–moderate band centered at $\sim 1038\text{ cm}^{-1}$ (marked 1037.99 cm^{-1} for all samples), so this feature is commonly assigned to C–O/C–O–C stretching of residual carbohydrate/phenolic fragments originating from the leaf extract or to acetate-derived C–O stretching that can remain at trace levels even after high-temperature treatment; in pressed pellets, a contribution from surface carbonate/bicarbonate species formed by atmospheric CO_2 adsorption on basic ZnO sites can also appear in the $1000\text{--}1100\text{ cm}^{-1}$ region[60, 61]. Its low intensity and the simultaneous absence of strong $\nu(C=O)/\nu_{as}(COO^-)$ features in the $1700\text{--}1300\text{ cm}^{-1}$ window indicate that only minor organic/oxy-carbon species remain after calcination, in agreement with the dehydroxylation/cleanup expected at $500\text{--}700^\circ\text{C}$ [62][2, 4]. Any broad OH stretching of physisorbed water ($\sim 3200\text{--}3600\text{ cm}^{-1}$) and H–O–H bending ($\sim 1630\text{ cm}^{-1}$) are strongly suppressed, further supporting a predominantly inorganic surface after annealing. The second key vibration is the Zn–O lattice stretching mode of wurtzite ZnO resolved in the low-wavenumber magnifications (Fig. 5b–d). The band maxima occur at $\sim 418.0\text{ cm}^{-1}$ (ZnO-500), $\sim 410.3\text{ cm}^{-1}$ (ZnO-600), and $\sim 406.4\text{ cm}^{-1}$ (ZnO-700). Two trends are noteworthy. (i) The band progressively sharpens with calcination temperature, reflecting enhanced crystallinity and reduced phonon damping as microstrain and grain-boundary disorder diminish—fully consistent with the peak narrowing observed in XRD[63, 64]. (ii) The band exhibits a small but systematic red shift ($418 \rightarrow 406\text{ cm}^{-1}$). Within a simple harmonic picture $\nu = (2\pi c)^{-1} \sqrt{\frac{k}{u}}$, a lower frequency implies a reduction in the effective force constant k of the Zn–O bond and changes in the local dielectric environment[65]. Two

complementary mechanisms can account for this: (a) defect evolution, particularly a modest increase in oxygen-vacancy (V_o) concentration at higher annealing temperatures as surface hydroxyls desorb and the lattice equilibrates under air; V_o soften the Zn–O bond and downshift IR-active phonons[66], and (b) strain relaxation/particle coarsening, which reduces phonon confinement and internal stress, slightly lowering the vibrational frequency while narrowing the bandwidth[67]. The trend dovetails with our EDS results that show an increasingly Zn-rich apparent composition with temperature, a hallmark of V_o formation and light-element under-counting that nevertheless tracks the same direction as the FTIR red shift. Taken together, the presence of only a faint $\sim 1038\text{ cm}^{-1}$ C–O/ CO_3 -related feature and the well-defined, temperature-sensitive Zn–O band in the $420\text{--}400\text{ cm}^{-1}$ window confirm that the products are phase-pure ZnO with largely clean surfaces, while the subtle phonon red shift and linewidth evolution provide a sensitive spectroscopic signature of crystallite growth, strain relaxation, and defect re-equilibration across ZnO-500 $\rightarrow$ ZnO-700, so these insights complement the XRD/FESEM findings and are particularly relevant for applications where surface cleanliness and controlled defect populations govern ion release and bio-interactions.

3.4. Optical analysis

3.4.1. UV-visible absorbance analysis

The UV–Vis spectra of the ZnO nanoparticles calcined at 500°C , 600°C , and 700°C show a slight redshift in the main absorption edge, with the characteristic wavelengths shifting from 356 nm (ZnO500) to 364 nm (ZnO600) and 367 nm (ZnO700). This progressive redshift corresponds to a small decrease in the optical bandgap as the calcination temperature increases. The UV–Vis spectra of ZnO nanoparticles calcined at different temperatures show a clear redshift of the main absorption maximum from 356 nm for ZnO500 to 364 nm for ZnO600 and 367 nm for ZnO700, as shown in Fig. 6. The XRD analysis revealed that the crystallinity improved with higher calcination temperature, while the average crystallite size actually decreased a common effect of enhanced thermal energy driving better crystal ordering and reducing lattice strain, which limits particle agglomeration. Energy-dispersive X-ray (EDX) results also showed a gradual reduction in oxygen content with temperature, indicating the formation of more oxygen vacancies. These oxygen vacancies introduce shallow donor levels just below the conduction band, narrowing the effective bandgap and producing the observed redshift. In addition, improved crystallinity reduces structural defects that can scatter light, making the band-edge transition more defined and allowing slight bandgap narrowing due to increased carrier concentration (Burstein–Moss and defect-state effects acting together).

The slight redshift of the ZnO nanoparticles' absorption edge—from 356 nm at 500°C to 364 nm at 600°C and 367 nm at 700°C —has meaningful biological implications. A longer-wavelength (lower-bandgap) ZnO can absorb a broader portion of near-UV and visible light, which enhances the generation of reactive oxygen species (ROS) such as $\cdot\text{OH}$ and $\text{O}_2\cdot^-$ when the particles are illuminated. These ROS are well known for their strong antibacterial and antifungal activity, leading to more effective inactivation of pathogens and biofilm suppression. In addition, the improved crystallinity and smaller crystallite size

obtained at higher calcination temperatures increase surface area and provide more active sites for interaction with microbial cell walls, boosting photocatalytic sterilization. The moderate oxygen-vacancy concentration observed at higher temperatures also contributes to ROS formation, further strengthening antimicrobial action while maintaining biocompatibility for applications like wound dressings, antibacterial coatings, and environmental disinfection. Thus, the temperature-induced redshift and associated structural changes enhance ZnO's usefulness in biological and biomedical applications that rely on light-activated antimicrobial or photocatalytic effects.

3.4.2. 3. ASF method for calculate bandgap energy

The Absorption Spectrum Fitting (ASF) method is a widely used optical technique to determine the bandgap energy (E_g) of semiconductors directly from their UV–Vis absorption data without the need to calculate an explicit absorption coefficient. In this method the absorbance A is related to the photon energy $h\nu$ by the Tauc-type expression [68].

$$\left(\frac{A}{\lambda}\right)^n \propto (h\nu - E_g)$$

1

,

where λ is the wavelength of incident light, $h\nu = 1240/\lambda$ (eV when λ is in nm), and n depends on the type of electronic transition. From the linear fit region of Fig. 7, between $\left(\frac{A}{\lambda}\right)^n$ versus $1240/\lambda$ is made and the linear portion of the curve is extrapolated to intercept the energy axis $\left(\frac{A}{\lambda}\right)^n = 0$; the intercept gives the optical bandgap. For ZnO, the fundamental optical transition is a direct allowed transition, because electrons move directly from the valence band maximum to the conduction band minimum without a change in momentum. For direct allowed transitions, the exponent $n = 2$ is appropriate, which is why in ASF analysis of ZnO the graph is plotted as $\left(\frac{A}{\lambda}\right)^2$ versus $1240/\lambda$. ZnO nanoparticles calcined at 500°C, 600°C, and 700°C show a bandgap energy decrease from 3.37 eV to 3.14 eV and 3.05 eV, respectively.

This gradual narrowing arises from improved crystallinity, crystallite size, and the creation of oxygen vacancies at higher temperatures, which introduce shallow donor states and slightly lower the optical bandgap, thereby enhancing visible-light absorption and photocatalytic activity. The bandgap energy of ZnO nanoparticles is significantly influenced by the calcination temperature during synthesis. Research indicates that as the calcination temperature increases, the bandgap energy tends to decrease slightly, which is attributed to changes in particle size and defect density. This literature review synthesizes findings from various studies to elucidate this relationship. Studies show that the bandgap energy of ZnO nanoparticles decreases from 3.29 eV to 3.23 eV when the calcination temperature is raised from 350°C to 600°C [69]. Similarly, another study reports a decrease from 3.91 eV to 3.83 eV at higher

temperatures [70]. Increased calcination temperature leads to larger particle sizes, which can reduce the quantum confinement effect, thereby lowering the bandgap energy [71]. The presence of defects such as oxygen vacancies and interstitial zinc contributes to the observed optical properties and bandgap reduction [69].

3.5. Biological studies

3.5.1. Macroscopic Evaluation and Quantitative Wound Contraction

Macroscopic follow-up over 15 days demonstrated a clear treatment-dependent acceleration of excisional wound closure in the infected rat model (Fig. 8). In the untreated control group, wounds contracted slowly and remained partially open at the study endpoint, reaching 68% closure on Day 15 with persistent scab material and visible scar tissue, which is typical of infected, unmanaged rodent excisions where prolonged inflammation and microbial burden delay granulation and re-epithelialization[72, 73]. Daily topical chitosan (G1) improved the clinical course, achieving 86% contraction by Day 15 and visibly earlier size reduction and partial epithelial bridging compared with control, so this behavior aligns with the known mucoadhesive and hemostatic properties of chitosan, which support provisional matrix formation, moisture retention, and recruitment of reparative cells while conferring moderate intrinsic antimicrobial activity through polycationic membrane interactions[74–76]. Treatment with the green-synthesized ZnO nanoparticles produced a stepwise enhancement in wound resolution that tracked the calcination temperature of the nanomaterial. ZnO-500 (G2) yielded 96% contraction by Day 15 and earlier scab shedding with smoother wound edges relative to chitosan, while ZnO-600 (G3) further improved the response to 98% contraction with near-complete closure observed by Days 13–15. The most pronounced effect was recorded for ZnO-700 (G4), which reached 99% contraction by Day 15, with almost complete epithelialization and minimal residual scarring. The quantitative trends in Fig. 9 mirror the serial photographs in Fig. 8 and indicate that the ZnO dressings shortened the inflammatory phase and hastened the transition to proliferative and remodeling phases, consistent with literature showing that nanoparticulate ZnO promotes keratinocyte and fibroblast migration/proliferation, enhances collagen deposition, and stimulates angiogenic signaling while concurrently lowering the bacterial load at the wound bed[77–79].

The temperature dependence of efficacy can be rationalized by the coupled changes in ZnO surface chemistry and microstructure described earlier. Calcination from 500 → 700°C removes a larger fraction of phytochemical residues and surface hydroxyls, yielding cleaner, more crystalline nanoparticles with controlled oxygen-vacancy populations and a narrower size distribution (FESEM)[80, 81], also cleaner surfaces improve direct contact-killing and reduce release of organic leachable that could impede cell attachment, while nanometric dimensions (~ 19–23 nm) increase effective surface area and curvature-driven interactions with bacterial envelopes[82]. Point defects such as V_o can mediate low-flux reactive oxygen species (ROS) generation under ambient conditions and modulate Zn^{2+} release kinetics; both mechanisms are implicated in broad-spectrum antibacterial action and in signaling pathways that

upregulate re-epithelialization and matrix remodeling when exposure is locally constrained within a dressing[83], so the progressive improvement from G2 to G4 is therefore consistent with a synergy of higher crystallinity, reduced organic capping, and defect-tuned surface reactivity that together enhance antimicrobial clearance and foster a pro-healing microenvironment.

Consistent with standard practice, wound contraction was quantified as $\% \text{contraction} = ((A_0 - A_t) / A_0) \times 100\%$, where A_0 and A_t denote the initial and time- t wound areas, respectively, providing an integrated metric of epithelial advance and granulation tissue formation[84, 85], although the present study focused on macroscopic outcomes, the morphological evolution—faster scab detachment, smoother margins, and earlier flattening of the wound bed in G3–G4—suggests more efficient resolution of infection and inflammation with ZnO at higher calcination temperatures, in agreement with prior reports of ZnO-based dressings that combine bactericidal activity with Zn^{2+} -mediated support of keratinocyte proliferation and collagen synthesis[18, 86, 87], altogether, the photographic series and contraction analysis substantiate that green-synthesized ZnO nanoparticles, particularly the 700°C-calcined formulation, significantly improve wound closure dynamics relative to chitosan alone and untreated controls in this infected excision model.

3.5.2. Histopathology (H&E): epidermal repair, collagen remodeling, and inflammation resolution

Representative H&E sections collected on Day 15 (late proliferative–early remodeling window) show a clear treatment-dependent gradient in tissue repair that mirrors the macroscopic contraction data. The reference panel of normal rat skin (Fig. 10a) displays an intact orthokeratinized epidermis overlying a collagen-rich dermis with adnexal structures (hair follicles, sebaceous units) and subjacent hypodermis/muscle (annotation provided for orientation), establishing the microanatomic benchmarks used in our comparisons. In untreated controls (C)(Fig. 10b), the wound bed remains partially epithelialized with discontinuous, thinned neo epidermis and areas of surface ulceration covered by fibrinous crust. The underlying granulation tissue is immature, characterized by edema, prominent dilated capillaries, and a dense mixed inflammatory infiltrate (neutrophils > macrophages/lymphocytes), with loosely arranged, pale collagen and conspicuous fibroblast reactivity; adnexal structures are absent at the margin, and the dermal–epidermal junction remains flattened, so these features indicate prolonged inflammation and delayed transition to remodeling—a typical consequence of bacterial contamination and persistently high protease/ROS loads that degrade provisional matrix and impair keratinocyte migration[88, 89]. Chitosan treatment (G1)(Fig. 10c) yields partial histologic rescue relative to C. Sections show nearly continuous re-epithelialization with a thicker, more uniform neo epidermis and early stratum corneum formation, also the granulation zone is more organized, with reduced edema, increased fibroblast density, and capillary sprouts indicative of active angiogenesis. Collagen deposition is greater than in C but remains immature—short, wavy fibers with mixed orientation—and a moderate chronic inflammatory infiltrate persists, so these findings align with chitosan’s known actions as a hemostatic, mucoadhesive scaffold that maintains moisture balance, limits microbial proliferation via

polycationic membrane disruption, and recruits macrophages/fibroblasts to support granulation and partial epithelial bridging[90–92].

Striking improvements emerge with ZnO nanoparticle dressings, with efficacy increasing with calcination temperature. In G2 (ZnO-500) (Fig. 10d), the epidermis fully spans the defect and shows stratification with developing rete ridges; the dermis contains more compact collagen bundles interspersed with numerous fibroblasts and maturing neo vessels, while leukocyte density is clearly diminished versus C and G1. Occasional adnexal buds appear at the margins, suggesting initiation of appendage regeneration. In G3 (ZnO-600) (Fig. 10e), re-epithelialization is near-normal in thickness and architecture, with a continuous keratin layer and a well-defined basal layer, also the dermis displays densely packed, better-aligned collagen with fewer cellular elements, consistent with progression into early remodeling; inflammatory foci are sparse and capillaries exhibit a more mature luminal profile. G4 (ZnO-700) (Fig. 10f) presents the most advanced repair phenotype, a uniformly stratified keratinized epidermis closely resembling uninjured skin, restoration of a papillary/reticular dermal gradient with thick, eosinophilic collagen bundles oriented parallel to the surface, minimal residual inflammation, and scattered adnexal structures at the interface- hallmarks of near-complete remodeling, notably, across ZnO groups we found no histologic evidence of necrosis or excessive foreign-body response, supporting local biocompatibility at the applied dose. The temperature-dependent hierarchy ($G4 \geq G3 > G2 > G1 > C$) can be mechanistically explained by the microstructural and surface-chemical evolution of ZnO with calcination described earlier, higher-temperature calcination cleans phytochemical residues and increases crystallinity while tuning oxygen-vacancy populations, thereby producing nanoparticles with cleaner surfaces, narrower size distributions ($\sim 19\text{--}23\text{ nm}$), and controlled Zn^{2+} /ROS microflux[93, 94], so these attributes are known to (i) reduce bioburden by contact-mediated membrane damage and oxidative stress to bacteria, lowering inflammatory cytokines and protease burden in the wound bed; (ii) stimulate keratinocyte and fibroblast activity, with Zn^{2+} acting as a cofactor for DNA synthesis and enzymes involved in matrix remodeling; (iii) enhance collagen deposition and cross-linking (improved type I/III balance, lysyl-oxidase-dependent maturation), and (iv) promote angiogenesis via upregulation of VEGF and related pathways—collectively accelerating the shift from inflammation to proliferation and ultimately to remodeling with reduced scarring[95]. The superior histologic organization in G3–G4, including well-stratified epithelium and mature collagen architecture, is therefore consistent with a synergy of antimicrobial clearance and pro-repair signaling unique to optimally calcined ZnO. In sum, Day-15 histology corroborates the macroscopic contraction data, controls remain trapped in an inflammatory/immature granulation state; chitosan advances re-epithelialization but with incomplete dermal maturation; ZnO at 500–700°C delivers progressive gains culminating in near-normal skin architecture for ZnO-700, marked by minimal inflammation, organized collagen, and restored epidermal continuity, so these observations support the therapeutic rationale for green-synthesized, optimally calcined ZnO nanoparticles as active components of antimicrobial wound dressings.

3.5.3. In Vitro Antibacterial Activity

The antibacterial performance of green-synthesized ZnO nanoparticles calcined at 500°C, 600°C, and 700°C was systematically investigated against MDR *Staphylococcus aureus* and XDR *Pseudomonas aeruginosa* using disc diffusion, MIC, and MBC assays. A clear pathogen-dependent trend was observed, with Gram-positive *S. aureus* showing significantly higher susceptibility to ZnO treatment compared with the Gram-negative *P. aeruginosa*.

3.5.3.1. Disc Diffusion Assay

The disc diffusion results (Fig. 11) revealed that ZnO calcined at 500°C (ZnO-500) produced the largest inhibition zone against MDR *S. aureus* (13.5 ± 0.4 mm), which was markedly higher than those obtained for ZnO-600 (9.6 ± 0.33 mm) and ZnO-700 (10.23 ± 0.2 mm). Importantly, all ZnO samples exhibited significantly stronger inhibition than the Norfloxacin control (6.33 ± 0.2 mm). In contrast, inhibition zones for XDR *P. aeruginosa* were comparatively limited, with ZnO-500, ZnO-600, and ZnO-700 producing zones of 8.6 ± 0.23 mm, 8.2 ± 0.2 mm, and 6.6 ± 0.4 mm, respectively—values only marginally exceeding the Norfloxacin control (6 mm). These results reinforce the intrinsic antibacterial potency of ZnO against Gram-positive pathogens and the inherent tolerance of Gram-negative bacteria conferred by their outer membrane barrier[96].

3.5.3.2. MIC and MBC Determination

Consistent with the disc diffusion data, MIC and MBC analyses highlighted the superior efficacy of ZnO-500 against *S. aureus*(Fig. 12a). ZnO-500 exhibited the lowest MIC (8 µg/mL) and MBC (40 µg/mL), followed by ZnO-600 (MIC: 10 µg/mL; MBC: 60 µg/mL). ZnO-700 showed a similar MIC (10 µg/mL) but failed to achieve bactericidal activity even at > 80 µg/mL, underscoring reduced potency at higher calcination temperatures. Against *P. aeruginosa*(Fig. 12b), MIC values were consistently higher (≥ 40 µg/mL), with MBC values > 80 µg/mL for all calcination conditions, so these findings confirm that ZnO nanoparticles are far more effective against MDR *S. aureus* than against XDR *P. aeruginosa*.

The pronounced antibacterial activity of ZnO-500 can be rationalized in terms of its physicochemical characteristics. XRD and FESEM analyses revealed that calcination at 500°C yields nanoparticles with smaller crystallite domains, moderate crystallinity, and higher defect density compared with samples calcined at 600°C and 700°C. Such features are well-established drivers of enhanced antibacterial action, as they facilitate higher surface reactivity, increased Zn²⁺ ion dissolution, and greater reactive oxygen species (ROS) generation[97]. In addition, the residual surface-associated phytochemicals and structural imperfections in ZnO-500 may promote closer interactions with bacterial membranes, further amplifying its antibacterial effect. By contrast, prolonged calcination at 600–700°C enhances crystallinity, reduces defect concentrations, and yields denser particle surfaces, thereby diminishing Zn²⁺ release and ROS production and attenuating antibacterial activity[98].

The differential response between Gram-positive and Gram-negative bacteria is largely attributable to cell envelope architecture. In *S. aureus*, the thick peptidoglycan layer provides a favorable surface for

ZnO adsorption and facilitates ROS penetration, leading to oxidative stress and cell wall disruption. Conversely, in *P. aeruginosa*, the outer membrane enriched with lipopolysaccharides acts as a permeability barrier, impeding nanoparticle adhesion and ROS entry, and contributing to the reduced antibacterial efficacy observed[99].

These observations underscore the critical role of calcination temperature in dictating ZnO bioactivity. Interestingly, while prior studies on other oxide nanostructures have demonstrated that higher calcination temperatures and improved crystallinity correlate with superior antibacterial activity[100], the present findings show the opposite trend for ZnO. Here, intermediate calcination (500°C) optimises antibacterial performance by balancing crystallinity with a high density of reactive surface defects. This highlights that the relationship between thermal processing and biological efficacy is highly material-specific and must be tailored accordingly.

Collectively, these results establish that ZnO nanoparticles exert potent antibacterial effects against MDR *S. aureus* but show limited activity against XDR *P. aeruginosa*. Furthermore, calcination at 500°C provides the optimal balance of structural and surface features for antibacterial efficacy, underscoring the importance of defect engineering and controlled thermal processing in designing ZnO nanomaterials for antimicrobial applications.

3.5.3.3. Antibiofilm Activity of ZnO nanoparticles

The antibiofilm potential of ZnO nanoparticles calcinated at 500°C, 600°C, and 700°C was evaluated against *Staphylococcus aureus* (Fig. 13a) and *Pseudomonas aeruginosa* (Fig. 13b). At a concentration of 6 µg/mL, all calcination temperatures resulted in approximately 50% inhibition of *S. aureus* biofilm formation, with reductions of 57%, 49.92%, and 50.04% observed for 500°C, 600°C, and 700°C, respectively. Complete (100%) eradication of *S. aureus* biofilm required 40 µg/mL of ZnO calcinated at 500°C, whereas higher concentrations (80 µg/mL) were necessary for nanoparticles calcinated at 600°C and 700°C. These findings indicate that lower calcination temperature preserved structural features conducive to stronger antibiofilm activity against Gram-positive bacteria.

In contrast, *P. aeruginosa* displayed greater resistance to ZnO nanoparticles. At 10 µg/mL, ZnO calcinated at 500°C achieved 64.82% biofilm inhibition, while at 20 µg/mL, nanoparticles calcinated at 600°C and 700°C inhibited biofilm formation by only 54.95% and 52.35%, respectively. Even at the maximum tested concentration (80 µg/mL), the highest reduction achieved was 88.73% with 500°C calcinated ZnO, with no complete biofilm eradication observed. The enhanced tolerance of *P. aeruginosa* biofilms may be attributed to its complex extracellular polymeric substance (EPS) matrix and efflux systems, which limit nanoparticle penetration and oxidative stress-induced damage[100]. The observed differences between *S. aureus* and *P. aeruginosa* reflect the distinct structural and physiological adaptations of Gram-positive and Gram-negative bacteria. ZnO nanoparticles likely disrupt biofilms through the generation of reactive oxygen species (ROS), release of Zn²⁺ ions, and direct interactions with bacterial cell surfaces, which compromise membrane integrity and inhibit quorum-sensing pathways critical for biofilm maintenance[101]. The superior performance of ZnO calcinated at 500°C

can be linked to its smaller crystallite size, higher surface area, and enhanced defect density, all of which increase ROS generation and Zn²⁺ release. These findings demonstrate that ZnO nanoparticles exhibit significant antibiofilm efficacy, particularly against *S. aureus*, while higher resistance in *P. aeruginosa* underscores the clinical challenge posed by Gram-negative biofilms. The results also suggest that calcination temperature critically influences nanoparticle performance, with 500°C producing the most potent antibiofilm activity.

4. Conclusion

We developed a green Phyto-ZnO/Chitosan dressing by embedding *Anagyris foetida*-derived ZnO nanoparticles in a biocompatible CS matrix and established a clear structure–property–efficacy link. Controlled calcination (500–700 °C) removed phytochemical residues, increased crystallinity, tuned defect chemistry, and slightly narrowed the optical band gap, as confirmed by XRD/FTIR/DRS/FESEM/EDS. When translated into paste dressings, therapeutic performance improved with temperature: Day-15 wound contraction rose from 68% (control) and 86% (CS) to 96% (ZnO-500), 98% (ZnO-600), and 99% (ZnO-700). Histology mirrored these outcomes—progressing from incomplete epithelial bridging and edematous granulation (control) to a uniformly stratified epidermis, well-aligned collagen, and minimal inflammation with ZnO-700, without necrosis or foreign-body reaction. Mechanistically, cleaner ZnO facets and defect-tuned surfaces enhance contact-killing, ROS generation, and regulated Zn²⁺ release, while CS provides adhesion, hemostasis, moisture balance, and localizes the nanoparticles—yielding a synergistic pro-healing milieu. Practically, the process is water-based, scalable, and compatible with simple topical application. Limitations include modest cohort size, single ZnO loading, and the absence of quantitative bacterial and Zn²⁺/ROS kinetics. Future work should map dose–response and safety windows, quantify antimicrobial/biofilm clearance, and evaluate device-level properties (adhesion, WVTR, sterilization stability). Overall, Phyto-ZnO/CS-700 emerges as a promising, non-toxic candidate for managing infected wounds.

Declarations

CRedit authorship contribution statement

Saman Mohammed Sulaiman (a): Conceptualization; Methodology; Investigation; Data curation; Visualization; Project administration; Writing – original draft; Writing – review & editing. **Dara M. Aziz (b):** Conceptualization; Methodology; Resources; Supervision; Project administration; Writing – review & editing. **Dyari M. Mamand (c):** Methodology; Formal analysis; Validation; Visualization; Writing – review & editing. **Karzan Qurbani (d):** Methodology (in vivo); Investigation (animal study and histology); Resources; Validation; Writing – review & editing. **Sangar A. Hassan (b):** Formal analysis; Data curation; Validation; Writing – review & editing. **Alla Ahmad M. Amin (b):** Investigation; Data curation; Resources. **Rzgar Farooq Rashid (e):** Investigation (microbiology); Validation; Visualization; Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors gratefully acknowledge the College of Science, University of Raparin (Ranya, Kurdistan Region, Iraq) for access to laboratory facilities and the animal care unit that supported the in-vivo experiments. We thank colleagues in the materials characterization laboratories for assistance with XRD, FTIR, UV–Vis DRS, FESEM, and EDS measurements, and the veterinary staff for their guidance during surgical procedures and postoperative care. This research received no specific grant from funding agencies in the public, commercial, or not-for-profit sectors

Data availability

All data generated or analyzed during this study are included in this published article. No additional datasets or code were generated

Funding

This research received no external funding.

References

1. Sood, A. et al. *Bioengineered nanoparticles for targeted topical delivery in wound healing: advances and future outlook*. Nanomedicine, : pp. 1–32. (2025).
2. Renuka, K., Priyanka, S. & Rose, J. R. *Curcumin-Powered Nanohydrogel Systems for Diabetic Foot Ulcers: Next-Generation Phytomedicine Approaches in Wound Care* p. 1–15 (Biomedical Materials & Devices, 2025).
3. Sinoliya, P., Niraj, R. R. K. & Sharma, V. *Cracking the Code: How Nano-Informatics is Crafting Intelligent Nano-Weapons to Outsmart Multiple Drug Resistance (MDR)*.
4. Sikka, M. P., Bargir, J. A. & Garg, S. *Modern developments in burn wound dressing* (Research Journal of Textile and Apparel, 2024).
5. Gürsoy, E. N. et al. *Modern Strategies in Wound Healing: The Rise of Bacterial Cellulose Dressings*. Advanced Therapeutics, : p. e00072. (2025).
6. Duan, Y. et al. Engineering Gas-Releasing Nanomaterials for Efficient Wound Healing. *ChemBioChem* **26** (4), e202400790 (2025).
7. Phogat, P., Jha, R. & Singh, S. Synthesis of novel ZnO nanoparticles with optimized band gap of 1.4 eV for high-sensitivity photo electrochemical detection. *Mater. Today Sustain.* **27**, 100823 (2024).

8. Manisekaran, R. et al. Copper, Zinc, and Titanium-Based Semiconductor Nanomaterials for Antimicrobial Coatings and Their Mechanisms. *Nano Select.* **6** (5), e202400155 (2025).
9. Alkhafaji, A. A. et al. Pharmaceutical properties for green fabricated ZnO and Ag nanoparticle-mediated *Borago officinalis*: In silico predications study. *Green. Process. Synthesis.* **14** (1), 20240237 (2025).
10. Angle, A. S. *Green Synthesis Methods for Metallic Zinc Oxide Nanoparticles* 63 (Synthesizing and Characterizing Plant-Mediated Biocompatible Metal Nanoparticles, 2024).
11. Jain, A. & Bhise, K. *Green Synthesis Methods for Metallic Zinc Oxide Nanoparticles: A Sustainable Angle*, in *Synthesizing and Characterizing Plant-Mediated Biocompatible Metal Nanoparticles*. IGI Global. pp. 63–90. (2025).
12. Dubey, S. et al. Breaking barriers in eco-friendly synthesis of plant-mediated metal/metal oxide/bimetallic nanoparticles: antibacterial, anticancer, mechanism elucidation, and versatile utilizations. *J. Nanomaterials.* **2024** (1), 9914079 (2024).
13. Mahajan, M. et al. Green synthesis of ZnO nanoparticles using *Justicia adhatoda* for photocatalytic degradation of malachite green and reduction of 4-nitrophenol. *RSC Adv.* **15** (4), 2958–2980 (2025).
14. Ananthakrishnan, R. & Goswami, K. Deciphering the effect of calcination temperature on crystallinity, morphology, oxygen vacancy and photocatalytic activity of bi-phasic ZnO/ZnCo₂O₄ heterostructured nanomaterials. *Mater. Chem. Phys.* **327**, 129792 (2024).
15. Arifin, N. M. et al. Enhancing the properties of nanostructure TiO₂ thin film via calcination temperature for solar cell application. *Energies* **17** (14), 3415 (2024).
16. Roy, A. et al. An updated review on carbon nanomaterials: Types, synthesis, functionalization and applications, degradation and toxicity. *Green. Process. Synthesis.* **13** (1), 20240150 (2024).
17. Yadav, S. et al. Chitosan-based nanoformulations: preclinical investigations, theranostic advancements, and clinical trial prospects for targeting diverse pathologies. *AAPS PharmSciTech.* **25** (8), 263 (2024).
18. Wafi, A. & Khan, M. M. Green synthesized ZnO and ZnO-based composites for wound healing applications. *Bioprocess Biosyst. Eng.* **48** (4), 521–542 (2025).
19. Rathna, R. P. & Kulandhaivel, M. Advancements in wound healing: integrating biomolecules, drug delivery carriers, and targeted therapeutics for enhanced tissue repair. *Arch. Microbiol.* **206** (4), 199 (2024).
20. Ali, S. A. et al. Enhancing physical characteristics and antibacterial efficacy of chitosan through investigation of microwave-assisted chemically formulated chitosan-coated ZnO and chitosan/ZnO physical composite. *Sci. Rep.* **14** (1), 9348 (2024).
21. Aouadi, A. et al. Introducing the antibacterial and photocatalytic degradation potentials of biosynthesized chitosan, chitosan–ZnO, and chitosan–ZnO/PVP nanoparticles. *Sci. Rep.* **14** (1), 14753 (2024).
22. Scolari, I. R., Pérez, P. L. & Granero, G. E. *Synergistic bactericidal combinations between gentamicin and chitosan capped ZnO nanoparticles: A promising strategy for repositioning this first-line*

- antibiotic. Heliyon*, **10**(3). (2024).
23. Aazam, E. S. & Thomas, R. Synthesis, characterization, and electronic structure of bioactive vanillin based fluorescent Schiff bases. *J. Mol. Liq.* **395**, 123820 (2024).
 24. Hussein, S. et al. Synthesis of silver nanoparticles from aeromonas caviae for antibacterial activity and in vivo effects in rats. *Biol. Trace Elem. Res.* **202** (6), 2764–2775 (2024).
 25. Smail, A. M. et al. Antimicrobial efficacy of CdFe₂O₄/Fe₂O₃ nanocomposite against extensively drug-resistant *Pseudomonas aeruginosa*: influence of calcination temperature and crystalline phases. *BioNanoScience* **15** (2), 213 (2025).
 26. Snedeker, L. P. et al. Organic phase conversion of bulk (wurtzite) ZnO to nanophase (wurtzite and zinc blende) ZnO. *Solid State Sci.* **7** (12), 1500–1505 (2005).
 27. Kim, H. S. et al. Facile synthesis and electrochemical properties of carbon-coated ZnO nanotubes for high-rate lithium storage. *Ceram. Int.* **44** (15), 18222–18226 (2018).
 28. Kumar, S. G. & Rao, K. K. Zinc oxide based photocatalysis: tailoring surface-bulk structure and related interfacial charge carrier dynamics for better environmental applications. *RSC Adv.* **5** (5), 3306–3351 (2015).
 29. Kayani, Z. N., Saleemi, F. & Batool, I. Effect of calcination temperature on the properties of ZnO nanoparticles. *Appl. Phys. A.* **119** (2), 713–720 (2015).
 30. Humphreys, C. The significance of Bragg's law in electron diffraction and microscopy, and Bragg's second law. *Found. Crystallogr.* **69** (1), 45–50 (2013).
 31. Cao, S. G. et al. Stress-induced cubic-to-hexagonal phase transformation in perovskite nanosheet films. *Nano Lett.* **17** (8), 5148–5155 (2017).
 32. Dubois, J. et al. Thermal stability of nanocomposite metals: In situ observation of anomalous residual stress relaxation during annealing under synchrotron radiation. *Acta Mater.* **58** (19), 6504–6512 (2010).
 33. Rodríguez-Barajas, N. et al. Plant-mediated synthesis and interaction of ZnO against breast and prostate cancer. *Results Chem.* **9**, 101654 (2024).
 34. Mutukwa, D., Taziwa, R. T. & Khotseng, L. A review of plant-mediated ZnO nanoparticles for photodegradation and antibacterial applications. *Nanomaterials* **14** (14), 1182 (2024).
 35. Zak, A. K. et al. Synthesis, magnetic properties and X-ray analysis of ZnO. $97\text{X}0.030$ nanoparticles (X = Mn, Ni, and Co) using Scherrer and size–strain plot methods. *Solid State Sci.* **14** (4), 488–494 (2012).
 36. Lim, K. et al. Temperature-driven structural and morphological evolution of zinc oxide nano-coalesced microstructures and its defect-related photoluminescence properties. *Materials* **9** (4), 300 (2016).
 37. Camarda, P. et al. Luminescence mechanisms of defective ZnO nanoparticles. *Phys. Chem. Chem. Phys.* **18** (24), 16237–16244 (2016).

38. Olasanmi, O. & Mukolu, A. *Williamson-hall analysis and absorption spectrum fitting in the estimation of crystallite size and band gap energy of CdZnS thin films. Recent. Adv. Nat. Sci.*, : pp. 81–81. (2024).
39. Kawsar, M. et al. *Synthesis of nano-crystallite hydroxyapatites in different media and a comparative study for estimation of crystallite size using Scherrer method, Halder-Wagner method size-strain plot, and Williamson-Hall model. Heliyon*, **10**(3). (2024).
40. Guan, C., Yue, X. & Xiang, Q. *The Role of Lattice Distortion in Catalysis: Functionality and Distinctions from Strain. Adv. Mater.*, : p. 2501209. (2025).
41. Warwick, J. et al. Lattice strain evolution during tensile and compressive loading of CP Ti. *Acta Mater.* **60** (19), 6720–6731 (2012).
42. Reichenberger, S. et al. Perspective of surfactant-free colloidal nanoparticles in heterogeneous catalysis. *ChemCatChem* **11** (18), 4489–4518 (2019).
43. De Coster, V. et al. Designing nanoparticles and nanoalloys for gas-phase catalysis with controlled surface reactivity using colloidal synthesis and atomic layer deposition. *Molecules* **25** (16), 3735 (2020).
44. Tiwari, A. & Dhoble, S. Critical analysis of phase evolution, morphological control, growth mechanism and photophysical applications of ZnS nanostructures (zero-dimensional to three-dimensional): a review. *Cryst. Growth. Des.* **17** (1), 381–407 (2017).
45. Malik, M. A., Ramasamy, K. & Revaprasadu, N. *The recent developments in nanoparticle synthesis.* (2016).
46. Farhadi, A., Hosseinkhani, O. & Behbahani, R. M. *Design, Synthesis, and Application of Enhanced (Ph3p) 3rhi and (Et3p) 3rhi Catalysts for the Monsanto Acetic Acid Process: Experimental and Theoretical Insights.*
47. Lenka, R., Patro, P. & Mahata, T. Shape forming and sintering of ceramics, in *Handbook on Synthesis Strategies for Advanced Materials: Volume-II: Processing and Functionalization of Materials.* Springer. 1–54. (2022).
48. Ezzat, S. E. *Autotoxic effect of Anagyris foetida L. alkaloids content.* (2021).
49. Scalera, F. et al. Influence of the calcination temperature on morphological and mechanical properties of highly porous hydroxyapatite scaffolds. *Ceram. Int.* **39** (5), 4839–4846 (2013).
50. Abuzeid, H. M. et al. Green synthesis of nanoparticles and their energy storage, environmental, and biomedical applications. *Crystals* **13** (11), 1576 (2023).
51. He, L. et al. Effects of calcination temperature and heating rate on the photocatalytic properties of ZnO prepared by pyrolysis. *J. Colloid Interface Sci.* **509**, 448–456 (2018).
52. Schaadt, D., Hu, D. & Ploog, K. Stress evolution during ripening of self-assembled InAs/ GaAs quantum dots. *J. Vacuum Sci. Technol. B: Microelectron. Nanometer Struct. Process. Meas. Phenom.* **24** (4), 2069–2074 (2006).

53. Xin, H. et al. Design and nanoengineering of photoactive antimicrobials for bioapplications: from fundamentals to advanced strategies. *Adv. Funct. Mater.* **34** (38), 2402607 (2024).
54. Linju, M. & Rekha, M. Role of inorganic ions in wound healing: an insight into the various approaches for localized delivery. *Therapeutic Delivery.* **14** (10), 649–667 (2023).
55. Yu, X. et al. Thermal Generation Behavior, Key Groups and Disaster-Causing Mechanism of Unloaded Bulk Coal Under High Ground Temperature Conditions. *Nat. Resour. Res.* **34** (2), 1123–1145 (2025).
56. Wang, Z. et al. Synthesis of zinc oxide nanoparticles and their applications in enhancing plant stress resistance: A review. *Agronomy* **13** (12), 3060 (2023).
57. Kuisma-Kursula, P. Accuracy, precision and detection limits of SEM–WDS, SEM–EDS and PIXE in the multi-elemental analysis of medieval glass. *X-Ray Spectrometry: Int. J.* **29** (1), 111–118 (2000).
58. Hsieh, P. T. et al. Structural and luminescent characteristics of non-stoichiometric ZnO films by various sputtering and annealing temperatures. *Phys. B: Condens. Matter.* **403** (1), 178–183 (2008).
59. Shi, L. E. et al. Synthesis, antibacterial activity, antibacterial mechanism and food applications of ZnO nanoparticles: a review. *Food Addit. Contaminants: Part. A.* **31** (2), 173–186 (2014).
60. Jindo, K. et al. Physical and chemical characterization of biochars derived from different agricultural residues. *Biogeosciences* **11** (23), 6613–6621 (2014).
61. Saikia, I., Hazarika, M. & Tamuly, C. Synthesis, characterization of bio-derived ZnO nanoparticles and its catalytic activity. *Mater. Lett.* **161**, 29–32 (2015).
62. Kumar, A. et al. *Acidic microhydrated Portlandite $\text{Ca}^{2+}(\text{OH})_2(\text{H}_2\text{O})_2$: Temperature driven cyclic to acyclic hydrogen-bonding networks.* *Polyhedron*, : p. 117665. (2025).
63. Schipporeit, S. *Decomposition, Quantitative and Qualitative Evaluation of TiO_2 Thin Film Raman Spectra* (Universitaet Duisburg-Essen (Germany), 2020).
64. Bjørheim, T. S. & Kotomin, E. Ab initio thermodynamics of oxygen vacancies and zinc interstitials in ZnO. *J. Phys. Chem. Lett.* **5** (24), 4238–4242 (2014).
65. Collins, A. *Ultrashort pulse laser scribing of thin flexible glass.* (2016).
66. Ding, H. et al. Oxygen vacancy: an electron–phonon interaction decoupler to modulate the near-band-edge emission of ZnO nanorods. *J. Phys. Chem. C.* **116** (32), 17294–17299 (2012).
67. Hanus, R., Garg, A. & Snyder, G. J. Phonon diffraction and dimensionality crossover in phonon-interface scattering. *Commun. Phys.* **1** (1), 78 (2018).
68. Mamand, D. M. et al. Optical band gap modulation in functionalized chitosan biopolymer hybrids using absorption and derivative spectrum fitting methods: A spectroscopic analysis. *Sci. Rep.* **15** (1), 3162 (2025).
69. Zandi, S. et al. Microstructure and optical properties of ZnO nanoparticles prepared by a simple method. *Phys. B: Condens. Matter.* **406** (17), 3215–3218 (2011).
70. Hassan, F. et al. Synthesis of ZnO nanoparticles by a hybrid electrochemical-thermal method: influence of calcination temperature. *Bangladesh J. Sci. industrial Res.* **50** (1), 21–28 (2015).

71. Ayon, S. A., Billah, M. M. & Hossain, M. N. Effect of annealing temperature on structural, optical, and photocatalytic properties of modified sol–gel-driven ZnO nanoparticles. *Surf. Interface Anal.* **55** (6–7), 443–449 (2023).
72. Ansell, D. M. et al. A statistical analysis of murine incisional and excisional acute wound models. *Wound Repair. Regeneration.* **22** (2), 281–287 (2014).
73. Galiano, R. D. et al. Quantitative and reproducible murine model of excisional wound healing. *Wound repair. regeneration.* **12** (4), 485–492 (2004).
74. Song, Y. et al. Chitosan-based multifunctional hydrogel with bio-adhesion and antioxidant properties for efficient wound hemostasis. *Colloids Surf., B.* **234**, 113697 (2024).
75. Liu, C. et al. Injectable thermogelling bioadhesive chitosan-based hydrogels for efficient hemostasis. *Int. J. Biol. Macromol.* **224**, 1091–1100 (2023).
76. Geisberger, G. et al. Chitosan-thioglycolic acid as a versatile antimicrobial agent. *Biomacromolecules* **14** (4), 1010–1017 (2013).
77. Vitasovic, T. et al. Antibacterial action of Zn²⁺ ions driven by the in vivo formed ZnO nanoparticles. *ACS Appl. Mater. Interfaces.* **16** (24), 30847–30859 (2024).
78. Naqvi, Q. A. et al. Size-dependent inhibition of bacterial growth by chemically engineered spherical ZnO nanoparticles. *J. Biol. Phys.* **45** (2), 147–159 (2019).
79. Yuan, Z. et al. The ZnO-based fibrous poly (L-lactide-co-glycolide)/gelatin dressings enable rapid hemostasis and skin regeneration in an infectious wound model. *J. Drug Deliv. Sci. Technol.* **89**, 105072 (2023).
80. Kabagambe, M. et al. Temperature dependent microstructural defects and surface charge effects on antioxidant activity of green synthesized nanoceria. *Sci. Rep.* **15** (1), 29110 (2025).
81. Abduh, N. A., Algarni, T. S. & Al-Odayni, A. B. Green synthesis of Zn-doped TiO₂ nanomaterials for photocatalytic degradation of crystal violet and methylene blue dyes under sunlight. *Biomass Convers. Biorefinery.* **15** (3), 4849–4865 (2025).
82. Sayed, F. A. Z. et al. Morphologic design of nanostructures for enhanced antimicrobial activity. *J. Nanobiotechnol.* **20** (1), 536 (2022).
83. Huang, X. et al. Sustained zinc release in cooperation with CaP scaffold promoted bone regeneration via directing stem cell fate and triggering a pro-healing immune stimuli. *J. Nanobiotechnol.* **19** (1), 207 (2021).
84. Jørgensen, L. B. et al. Methods to assess area and volume of wounds—a systematic review. *Int. Wound J.* **13** (4), 540–553 (2016).
85. Foltynski, P., Ciechanowska, A. & Ladyzynski, P. Wound surface area measurement methods. *Biocybernetics Biomedical Eng.* **41** (4), 1454–1465 (2021).
86. Vagena, I. A. et al. Functionalized ZnO-based nanocomposites for diverse biological applications: current trends and future perspectives. *Nanomaterials* **14** (5), 397 (2024).

87. Tettey-Engmann, F. A. *Innovative Hybrid Scaffold Design Using 3D Bioprinting and Electrospinning for Accelerated Wound Healing Applications* (North Carolina Agricultural and Technical State University, 2024).
88. Rhea, L. & Dunnwald, M. Murine excisional wound healing model and histological morphometric wound analysis. *J. visualized experiments: JoVE*. (162). p. 10.3791/61616 (2020).
89. Lipsky, Z. W. et al. Mechanisms and implications of bacterial invasion across the human skin barrier. *Microbiol. Spectr.* **10** (3), e02744–e02721 (2022).
90. Pourshahrestani, S. et al. Polymeric hydrogel systems as emerging biomaterial platforms to enable hemostasis and wound healing. *Adv. Healthc. Mater.* **9** (20), 2000905 (2020).
91. Nascimento, A. B. *Wound Healing Strategies-Hyaluronic Acid (HA) and Chitosan (CS) as Wound Dressing Materials*. Universidade de Lisboa (Portugal). (2020).
92. Liu, H. et al. A functional chitosan-based hydrogel as a wound dressing and drug delivery system in the treatment of wound healing. *RSC Adv.* **8** (14), 7533–7549 (2018).
93. Nandi, P. & Das, D. Photocatalytic degradation of Rhodamine-B dye by stable ZnO nanostructures with different calcination temperature induced defects. *Appl. Surf. Sci.* **465**, 546–556 (2019).
94. Vijayaraghavan, R. Chemical manipulation of oxygen vacancy and antibacterial activity in ZnO. *Mater. Sci. Engineering: C.* **77**, 1027–1034 (2017).
95. Balaram, G. *Histomorphological Analysis of Cutaneous Granulomatous Lesions* (Rajiv Gandhi University of Health Sciences (India), 2019).
96. Abd El-Mageed, M. The investigation for eradication and inhibition of Gram-positive and Gram-negative bacteria by metal zinc oxide nanoparticles. *Al-Azhar J. Agricultural Res.* **48** (1), 54–66 (2023).
97. Venu Gopal, V. & Kamila, S. Effect of temperature on the morphology of ZnO nanoparticles: a comparative study. *Appl. Nanosci.* **7** (3), 75–82 (2017).
98. Sirelkhatim, A. et al. Review on zinc oxide nanoparticles: antibacterial activity and toxicity mechanism. *Nano-micro Lett.* **7** (3), 219–242 (2015).
99. Ghai, I. A barrier to entry: examining the bacterial outer membrane and antibiotic resistance. *Appl. Sci.* **13** (7), 4238 (2023).
100. Chen, Y. et al. Moxifloxacin-Loaded Polymeric Nanoparticles for Overcoming Multidrug Resistance in Chronic Pulmonary Infections Caused by *Pseudomonas aeruginosa*. *ACS Appl. Mater. Interfaces.* **17** (4), 5695–5709 (2025).
101. Husain, F. M. et al. Biosynthesized zinc oxide nanoparticles disrupt established biofilms of pathogenic bacteria. *Appl. Sci.* **12** (2), 710 (2022).

Figures

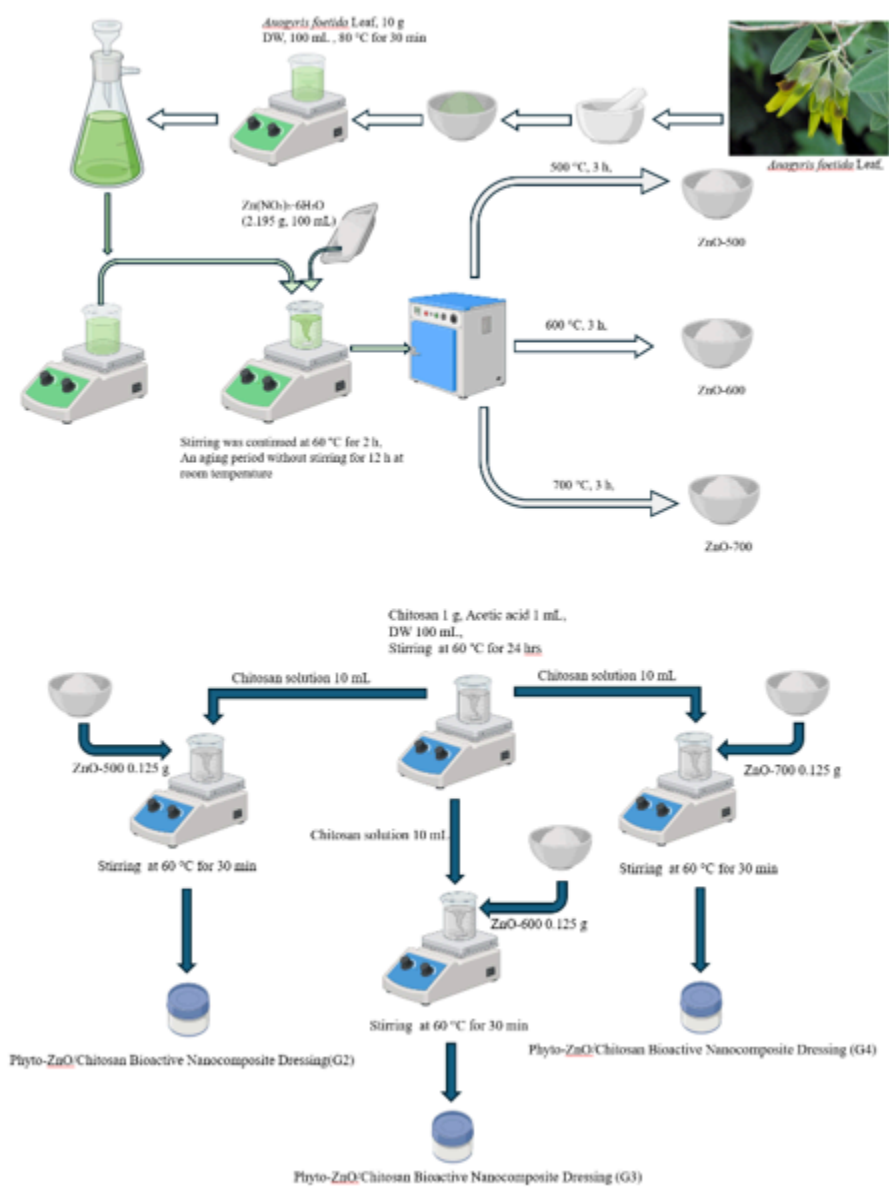


Figure 1

Diagram of the green synthesized ZnO NPs and paste-like Phyto-ZnO/CS

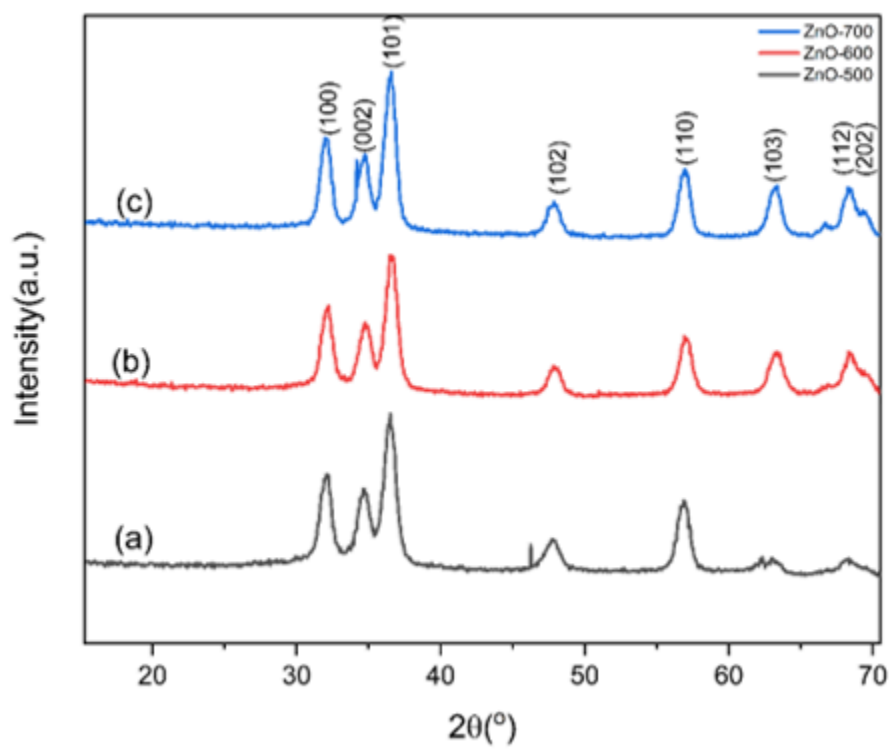


Figure 2

XRD spectra of (a) ZnO-500, (b) ZnO-600 and (c) ZnO-700

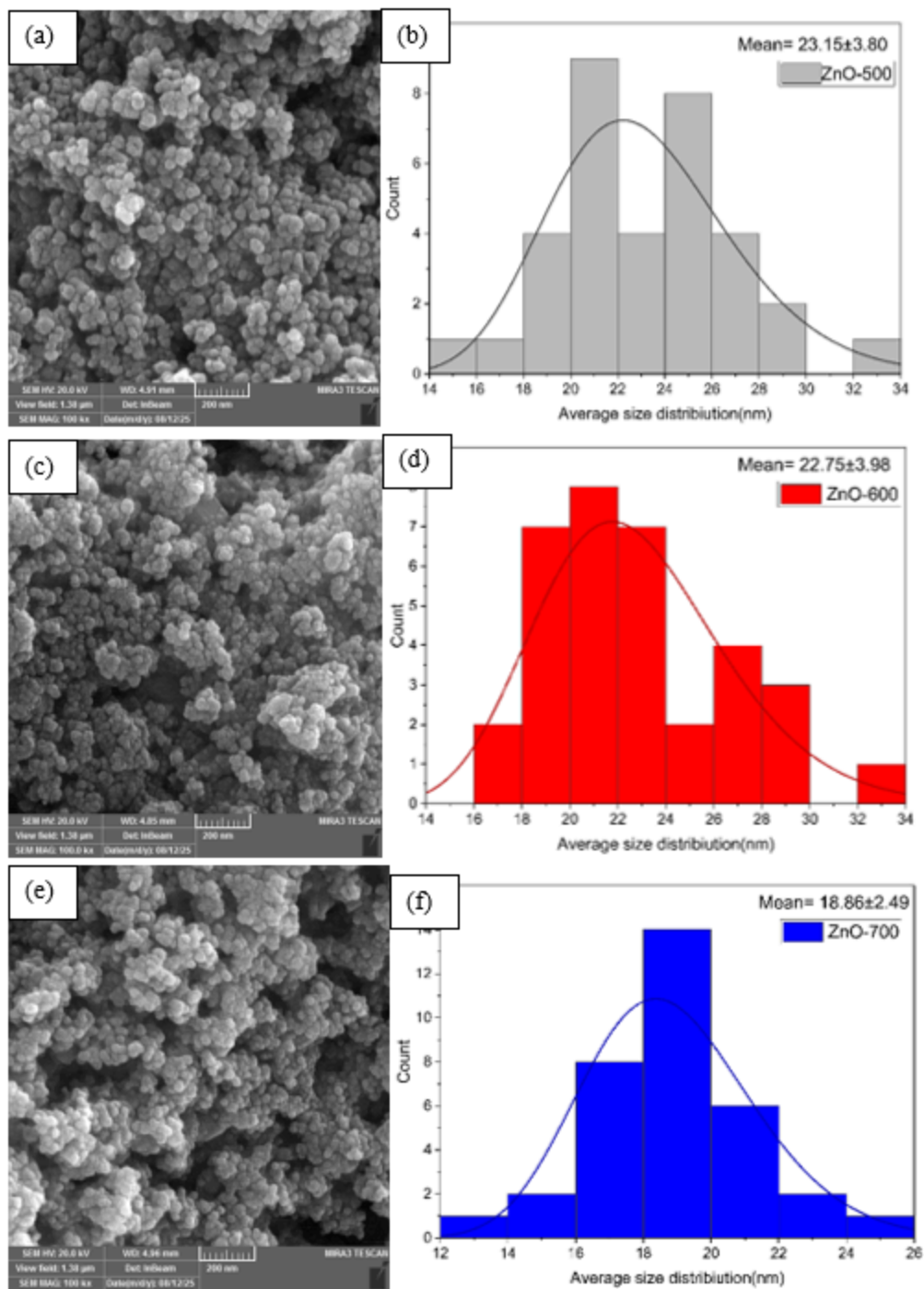


Figure 3

FESEM morphology and size distribution of ZnO nanoparticles: (a,b) ZnO-500; (c,d) ZnO-600; (e,f) ZnO-700

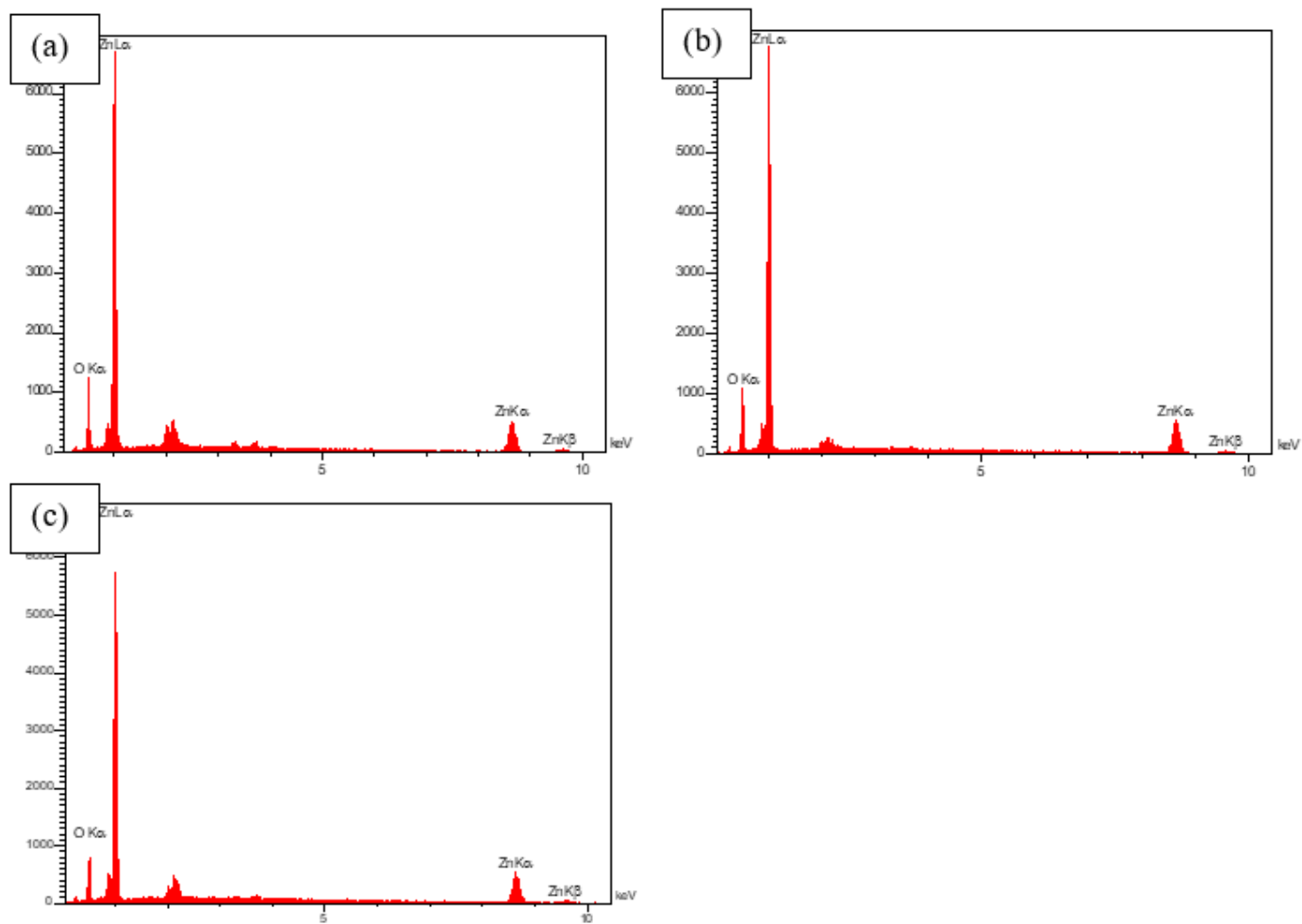


Figure 4

SEM-EDS spectra of ZnO nanoparticles: (a) ZnO-500, (b) ZnO-600, and (c) ZnO-700

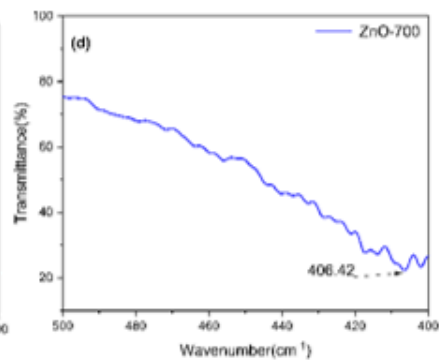
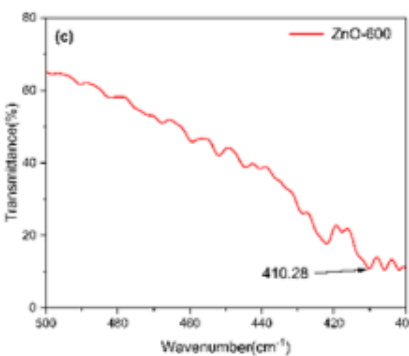
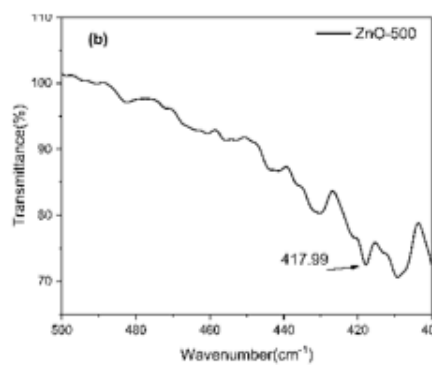
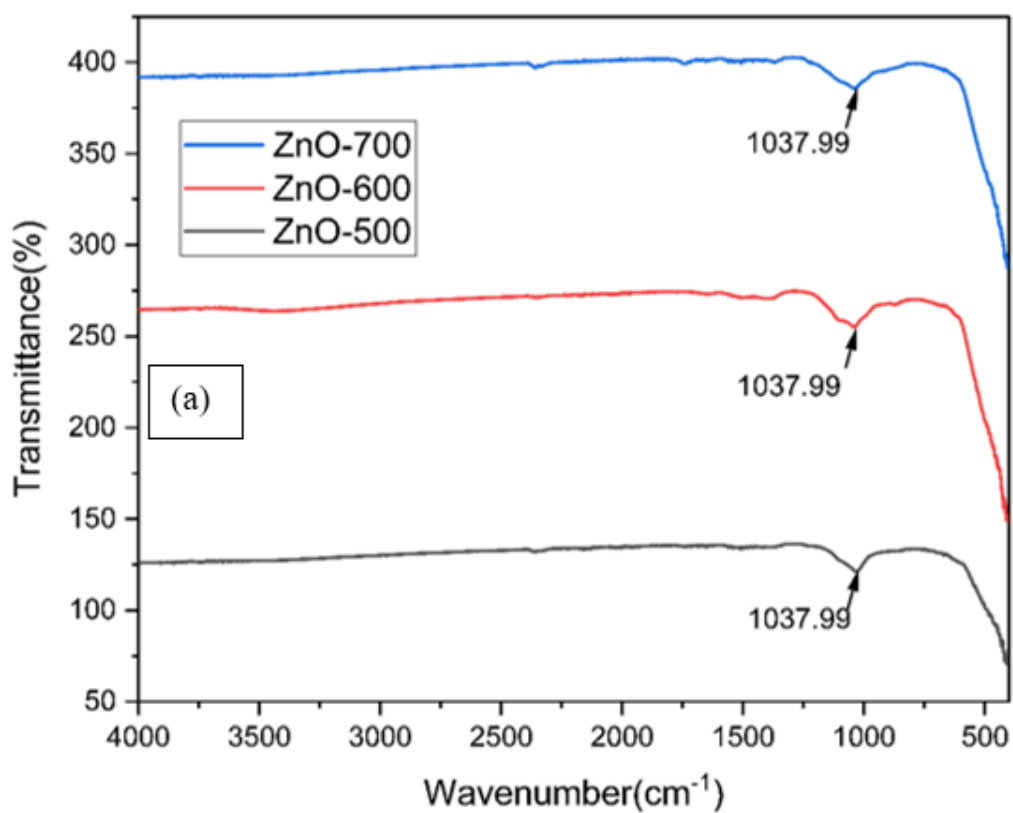


Figure 5

FTIR of ZnO nanoparticles: (a) full spectra for ZnO-500, ZnO-600, and ZnO-700; (b) enlarged low-wavenumber region for ZnO-500; (c) enlarged region for ZnO-600; (d) enlarged region for ZnO-700.

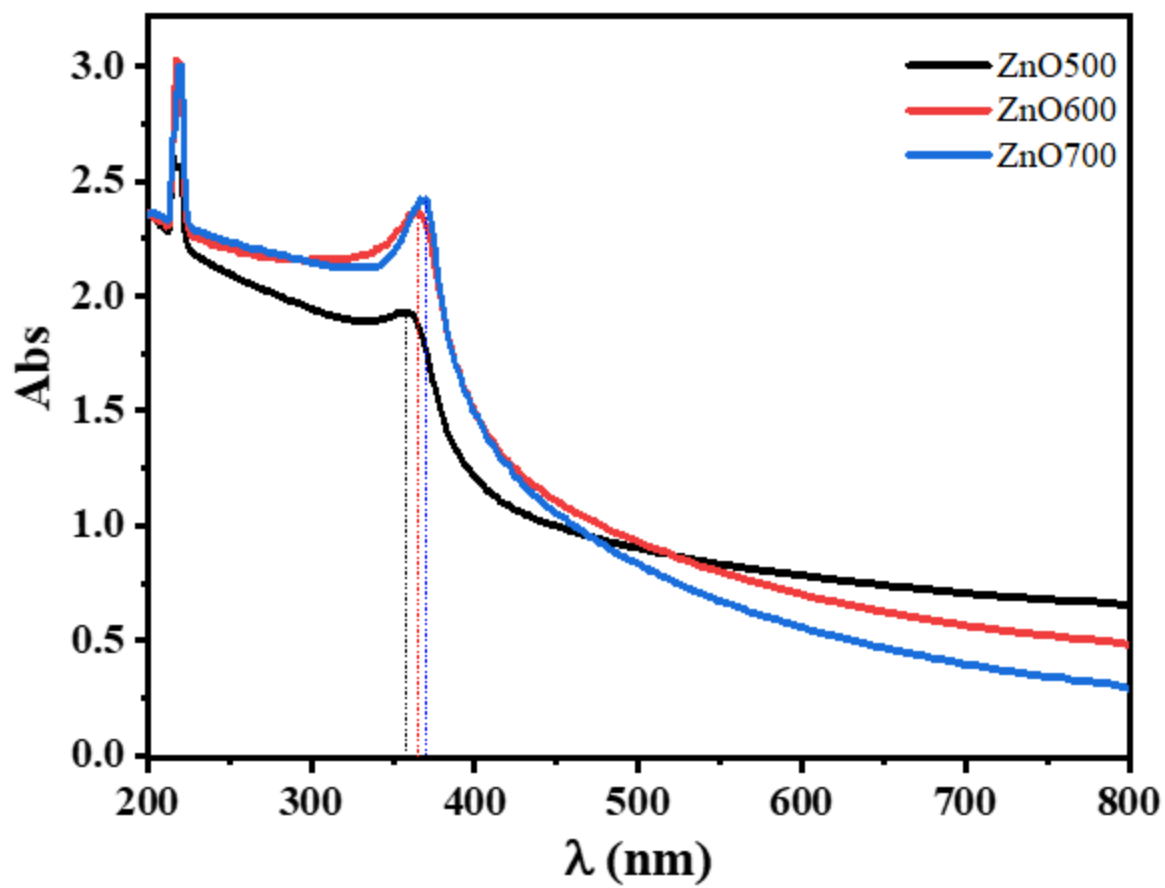


Figure 6

UV-vis absorbance of ZnO at various temperature calcination

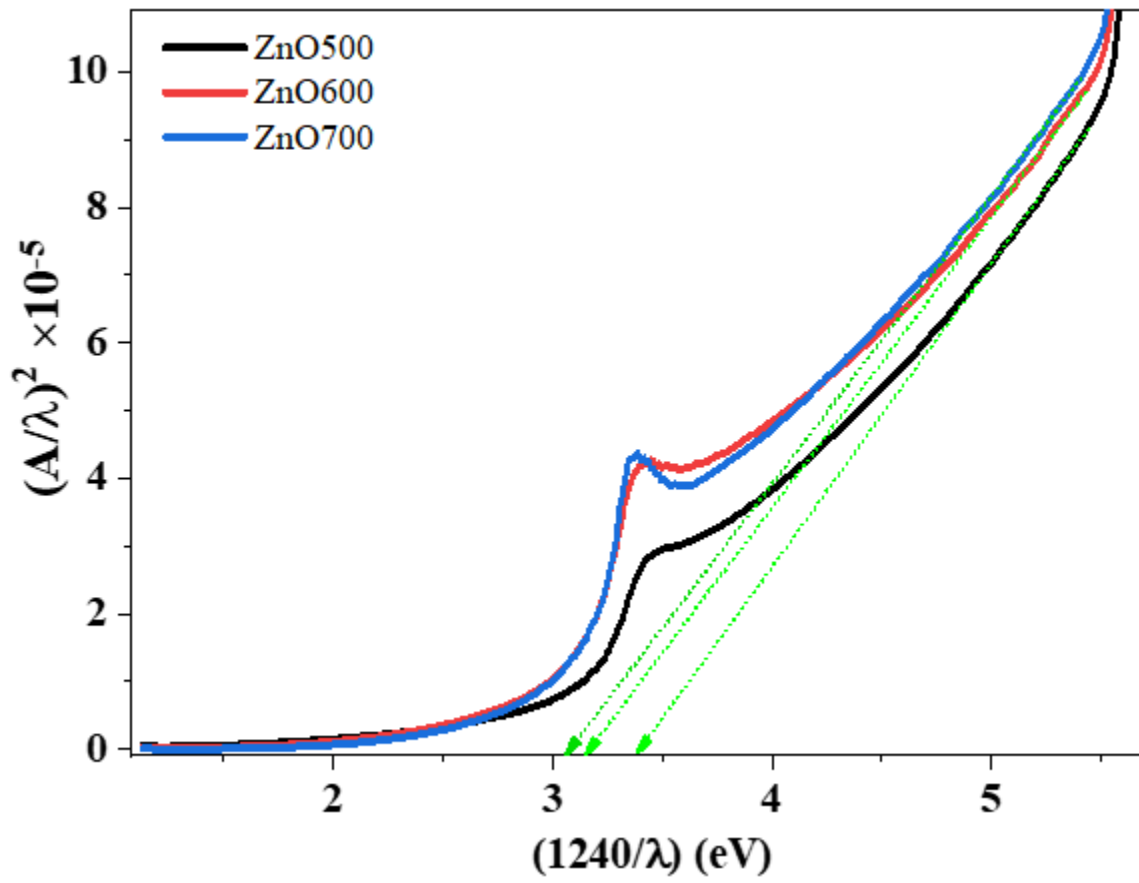


Figure 7

optical bandgap energy calculations based on ASF method of ZnO at various temperature.

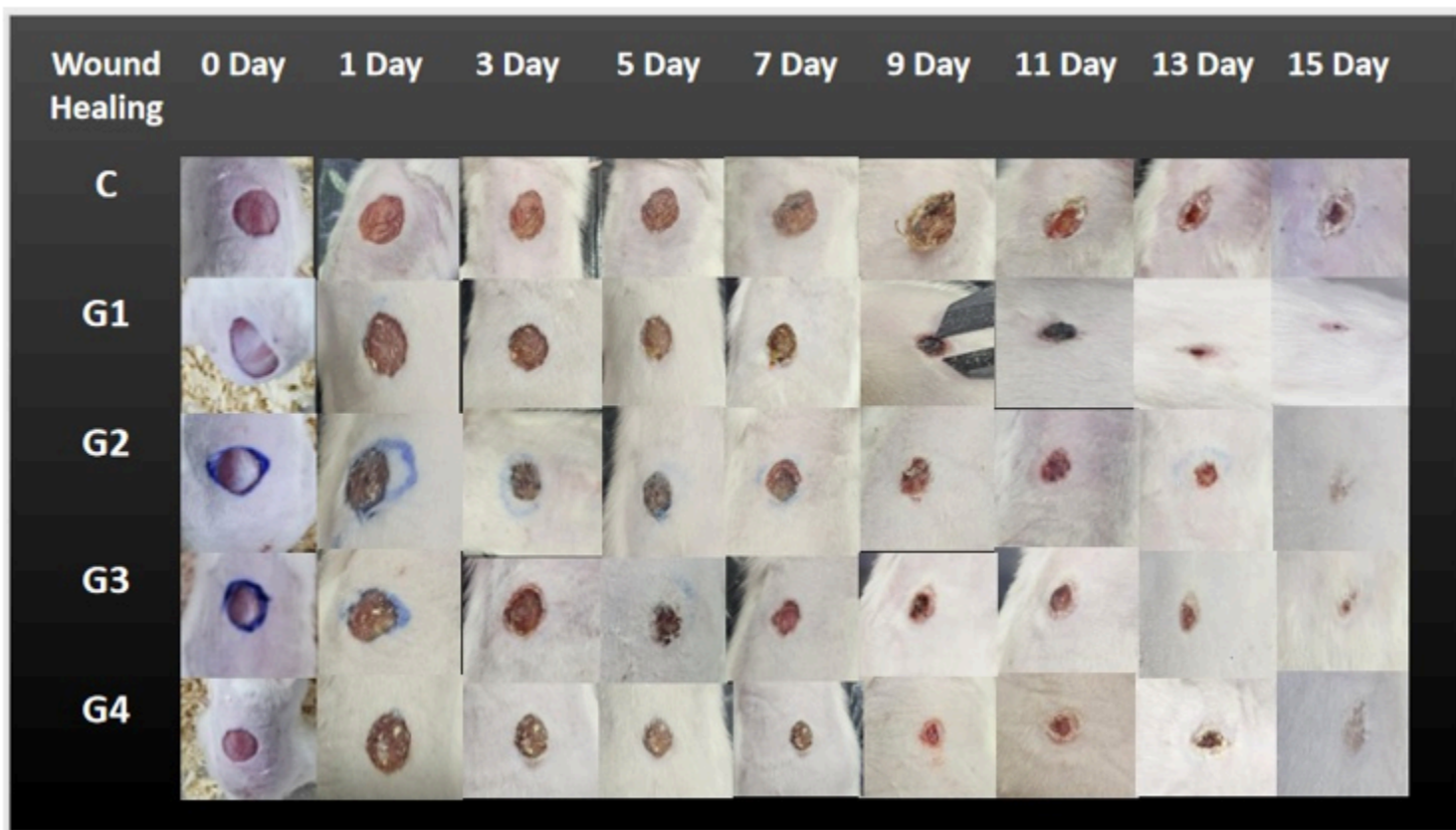


Figure 8

Macroscopic evaluation of excisional wound healing in albino rats over 15 days; representative images show wound closure in different experimental groups: Control (C), Chitosan (G1), and green synthesized ZnO nanoparticles from *Anagyris foetida* leaf extract calcined at 500 °C (G2), 600 °C (G3), and 700 °C (G4). Images were taken on Days 0, 1, 3, 5, 7, 9, 11, 13, and 15. Progressive wound contraction and scab formation are evident across all groups, with G3 and G4 demonstrating markedly accelerated epithelialization and almost complete closure by Day 15 compared with delayed healing in the control group.

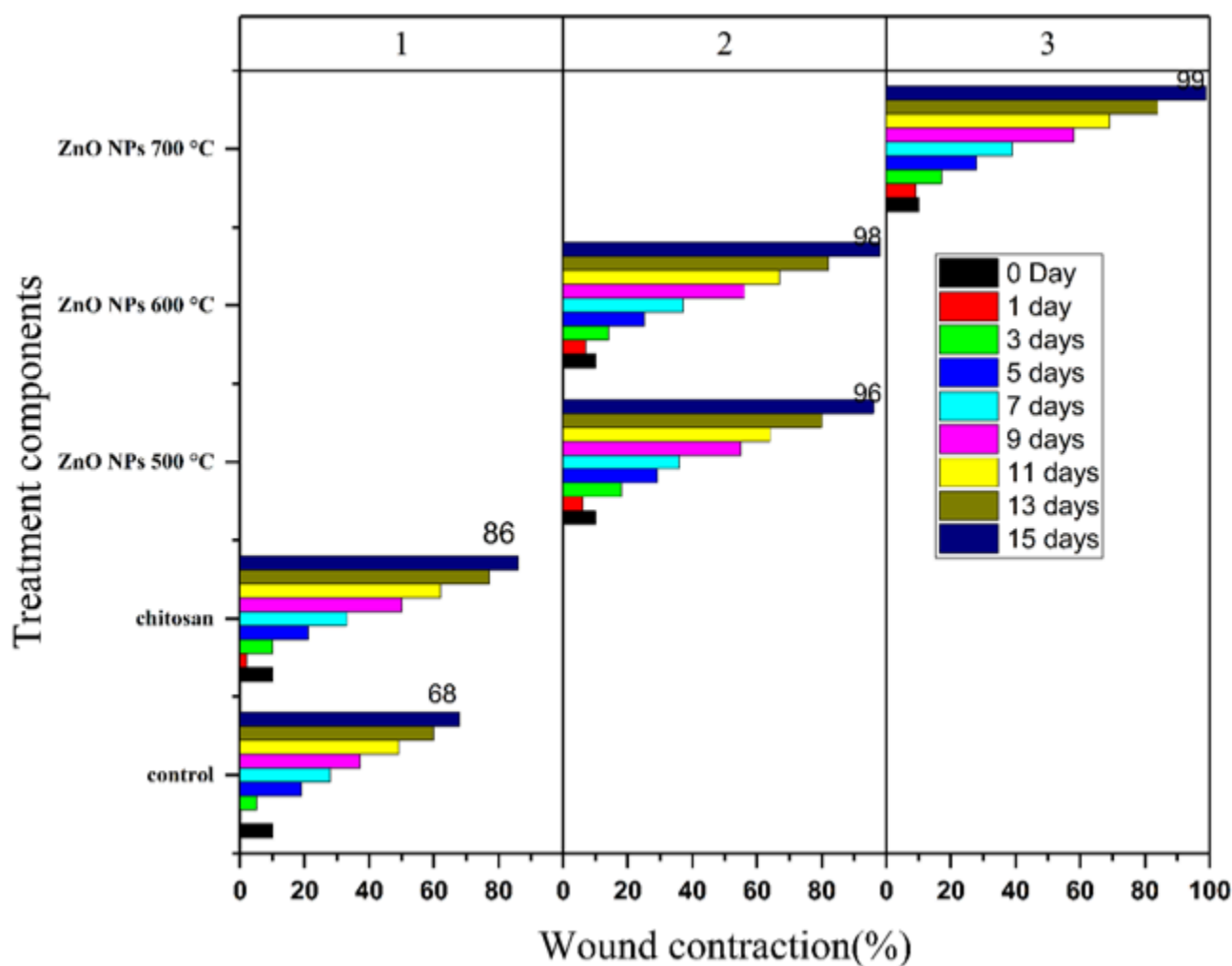


Figure 9

Macroscopic wound contraction (%) over 15 days in albino rats treated with different formulations; Control (C) showed the slowest healing with 68% contraction by Day 15. Chitosan (G1) accelerated healing to 86%. Green synthesized ZnO nanoparticles (ZnO NPs) from *Anagyris foetida* leaf extract, calcined at 500 °C (G2), 600 °C (G3), and 700 °C (G4), markedly enhanced wound healing in a temperature-dependent manner. By Day 15, contraction reached 96% in G2, 98% in G3, and 99% in G4, indicating that ZnO NPs at higher calcination temperatures significantly promoted wound closure compared with control and chitosan groups.

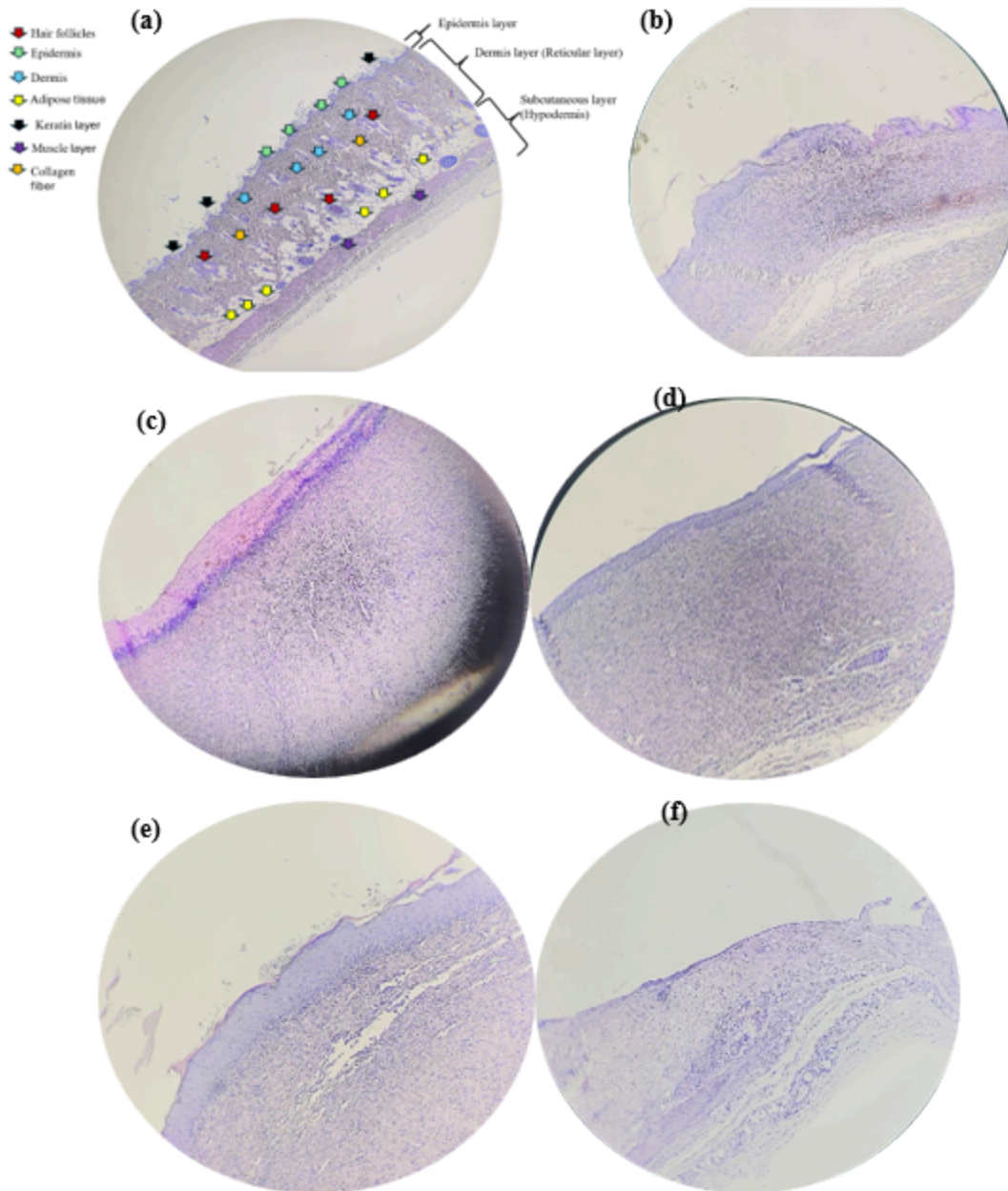


Figure 10

Histopathology of excisional wounds on Day 15 (H&E, 10×; same magnification across panels). (a) Normal rat skin (baseline reference). (b) Control (no treatment): incomplete re-epithelialization with exuberant granulation and persistent inflammation. (c) Chitosan (G1): nearly continuous neo epidermis and early stratum corneum with immature, loosely organized collagen. (d) ZnO-500 (G2): complete re-epithelialization, denser collagen bundles, reduced leukocytic infiltrate, (e) ZnO-600 (G3), near-normal epidermal architecture with better-aligned collagen and sparse inflammation, and (f) ZnO-700 (G4), uniformly stratified keratinized epidermis and mature, well-organized dermis approaching normal skin architecture.

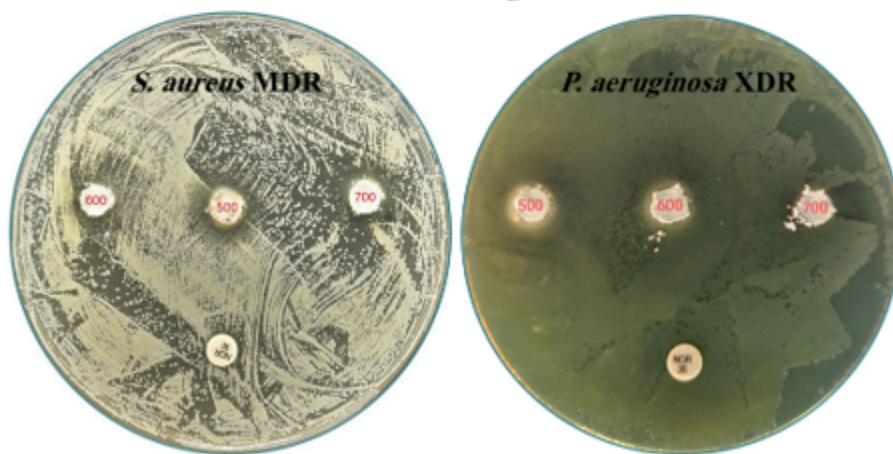
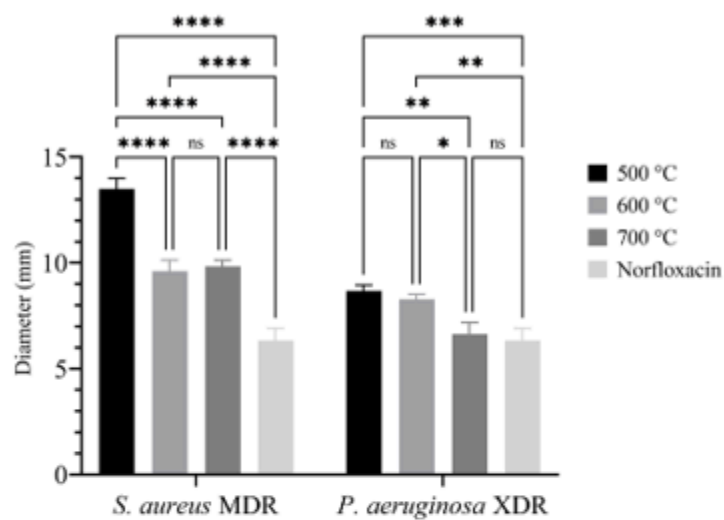


Figure 11

Antibacterial activity of green-synthesized ZnO nanoparticles against MDR *Staphylococcus aureus* and XDR *Pseudomonas aeruginosa* at different calcination temperatures (500 °C, 600 °C, and 700 °C), as evaluated by the disc diffusion assay. Data represent mean inhibition zone diameters (mm $\pm$ SD, $n = 3$).

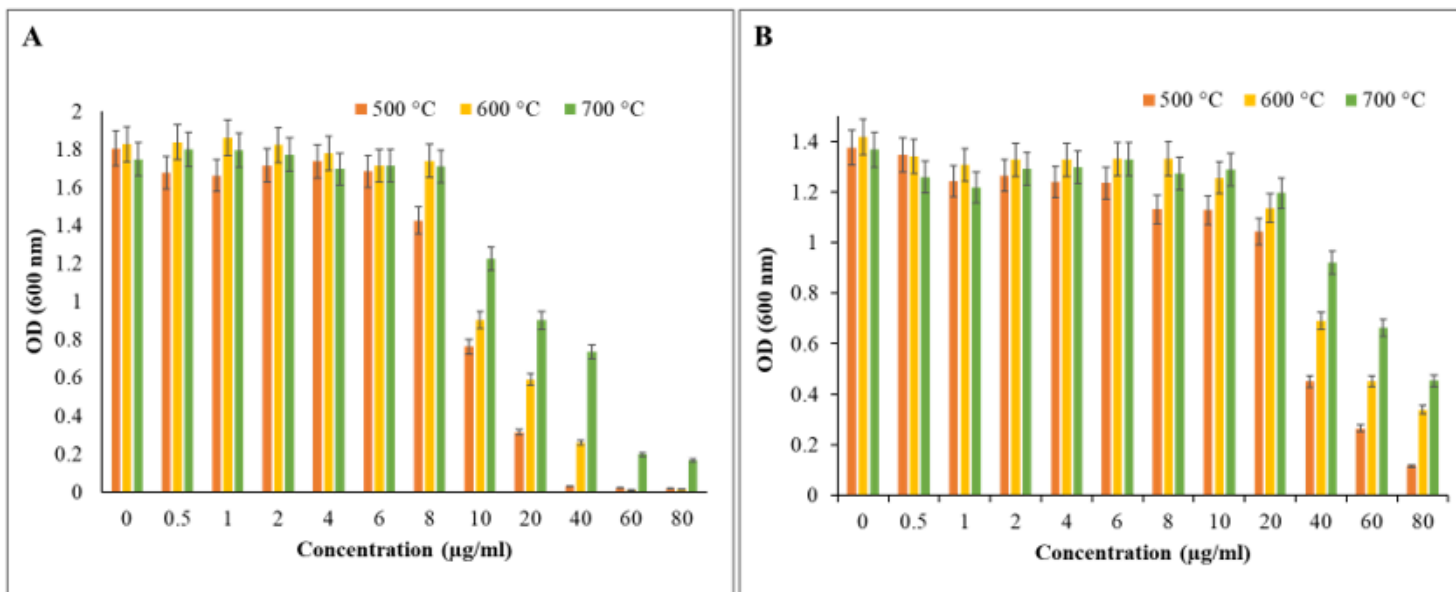


Figure 12

MIC and MBC of ZnO nanoparticles against (A) *Staphylococcus aureus* and (B) *Pseudomonas aeruginosa* at different calcination temperatures.

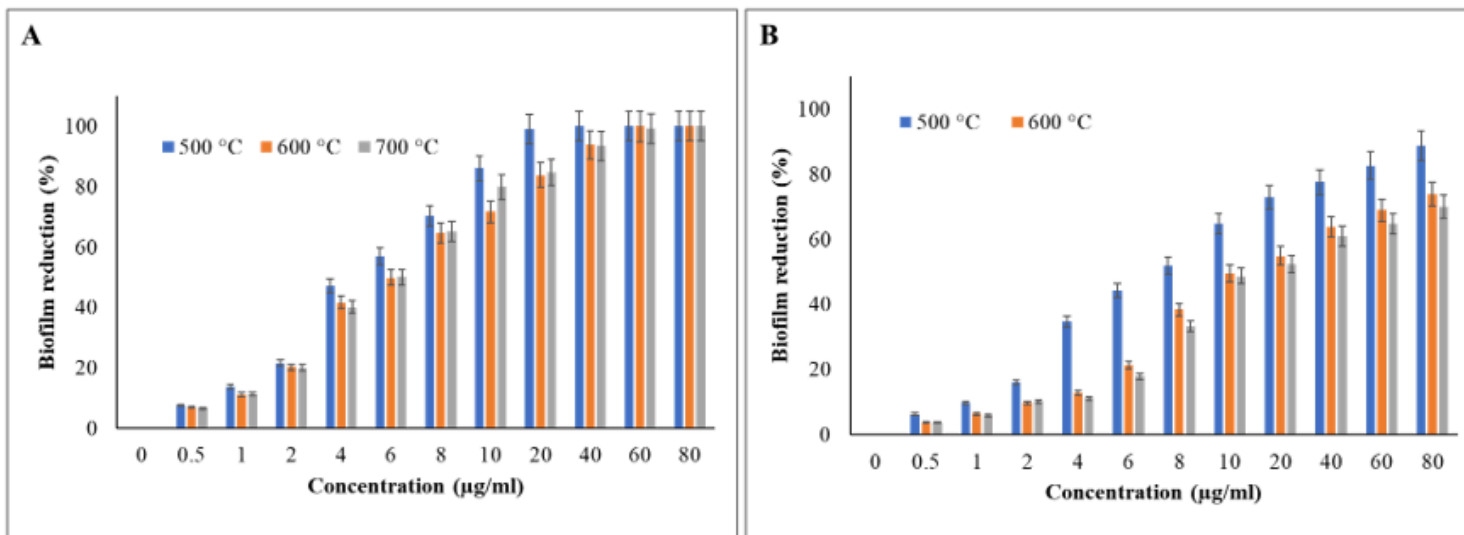


Figure 13

Percentage biofilm inhibition by green synthesized ZnO nanoparticles against (A) MDR *S. aureus* and (B) XDR *P. aeruginosa*.