RESEARCH PAPER

Anti-inflammatory Role of Theobromine derived zinc in NFκB Pathway Inhibition in Wound healing Models

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Abstract

Background: Chronic inflammation inhibits wound healing by altering the balance of signaling. The NF-κB pathway drives inflammation. Phytochemicals including theobromine obtained from green-synthesized zinc oxide nanoparticles play important roles in controlling inflammation and, consequently facilitate healing.

Objective: To explore the downregulation of NF-κB pathway by Theobromine-loaded zinc oxide nanoparticles (Theobro-ZnONPs) and its potential anti-inflammatory and wound healing effects in vitro and in vivo.

Materials and Methods: Theobro-ZnONPs were synthesized in a green path, cyto-compatibility test was performed on PDL cells (5, 10 and 25 µg/mL) through MTT assay. Scratch assay was performed at optimal dose for the assessment of wound healing. NF-κB, IκBα, COX-2, TNF-α IL-6 and IL-10 expression was determined by qPCR. The healing evaluation was performed through histopathology in these zebrafish wound models on Days 7 and 14.

Results: Theobro-ZnONPs demonstrated concentration-dependent cytocompatibility (10 µg/mL). Treated cells showed enhanced migration. NF-κB, COX-2, TNF-α and IL-6 were reduced when IκBα and IL-10 are increased. At Day 14, zebrafish wounds healed faster compared with the mammalian wound model, with less inflammation and organized tissue regeneration.

Conclusion: Theobro-ZnONPs are highly effective in reducing inflammation and expediting wound healing associated with modulating NF-κB/IκBα pathway which is responsible for restoring integrity of tissue as biocompatible nanotherapeutics against inflammation-driven wounds.

Keywords: Theobromine, Zinc oxide nanoparticles, NF-κB pathway, inflammation, wound healing, PDL cells, Zebrafish models, medicine

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1 Introduction

Wound healing is an intrinsic and dynamic biological process that restores tissue integrity after injury. It involves a tightly coordinated series of events, including haemostasis, inflammation, proliferation and remodelling.(Kalogeromitros et al., 2026) Among these, the trajectory of healing is primarily determined by the inflammatory phase. Dysregulated or prolonged inflammation can result in delayed wound healing, chronic wounds, or excessive scarring. (Majie et al., 2026) The inflammatory response is largely regulated by transcriptional mediators, one of the most central regulators of pro-inflammatory gene expression being the nuclear factor kappa light chain enhancer of activated B cells (NF-κB) pathway. NF-κB activation leads to several cytokines, chemokines, and inflammatory enzymes being transcripted, including TNF-α, IL-6, COX-2 and IL-1β, through which the inflammatory response is initiated and sustained. Therefore, targeted modulation of the NF-κB pathway has emerged as a promising strategy to promote

inflammation resolution and accelerate wound healing. One key regulatory element of this pathway is IκBα, an inhibitory protein, which binds to NF-κB in the cytoplasm and prevents its nuclear translocation. (Jafarbeglou et al., 2026)IκBα undergoes phosphorylation and degradation upon stimulation by inflammatory signals, allowing NF-κB to translocate to the nucleus and activate target genes. Interventions that stabilize IκBα to inhibit NF-κB activation can effectively suppress the inflammatory cascade, leading to enhanced tissue repair. In this context, the development of bioactive agents that can modulate these pathways without inducing toxicity is of immense therapeutic interest. (Dong et al., 2026)

Recent advances in nanomedicine have offered new tools for inflammation modulation and regenerative therapy.(Fahrurroji et al., 2026) Among these, zinc oxide nanoparticles (ZnONPs) have attracted popularity due to their multifunctional properties, including antibacterial activity, wound healing promotion and immune modulation.(Marzok et al., 2026) Zinc is an element found in trace amounts and is essential for numerous enzymatic processes. It is also known to play a critical role in cellular proliferation, immune regulation, and tissue regeneration.(Madan et al., 2026) In nanoparticulate form, zinc exhibits enhanced bioavailability and surface reactivity, making it more effective in modulating biological responses compared to its bulk counterpart. To improve the biocompatibility and therapeutic efficacy of ZnONPs, green synthesis methods using plant-based bioactive compounds have been extensively explored. (Nazish et al., 2026)These eco-friendly approaches avoid the use of harsh chemical reducing agents and stabilize the nanoparticles using phytochemicals that themselves possess therapeutic benefits. (Poorani et al., 2026)Theobromine, a naturally occurring methylxanthine alkaloid found in cacao beans (*Theobroma cacao*), is one such compound with established pharmacological activities. Traditionally known for its cardiovascular and neuromodulatory properties, theobromine has recently drawn interest for its anti-inflammatory and antioxidant activities. Studies have shown that theobromine can inhibit inflammatory cytokine production and modulate redox sensitive signalling pathways, including NF-κB, making it a promising candidate for bio reductive nanoparticle synthesis.

In this study, Theobromine derived Zinc Oxide nanoparticles (Theobro-ZnONPs) were synthesized via a green chemistry approach and evaluated for their anti-inflammatory and wound wound healing potential. (Govea-Alonso et al., 2026)The objective was to explore the capability of Theobro-ZnONPs to inhibit NF-κB signalling and promote tissue regeneration with the help of assays in both in vitro and in vivo settings. The biological effects were assessed in human periodontal ligament (PDL cells), a well-established model for wound healing studies due to their regenerative potential and inflammatory responsiveness. Initial cytocompatibility and dose selection were carried out using the MTT assay, which measures mitochondrial metabolic activity as a surrogate for cell viability. Theobro-ZnONPs were tested at concentrations of 5, 10, and 25 µg/mL, and the optimal non-toxic dose was selected for further analysis. To evaluate the effect on cell migration, a scratch wound healing assay was performed using PDL cells treated with the selected concentration. Enhanced cell migration into the scratched region would indicate a pro-regenerative effect of the nanoparticles.

To assess the molecular mechanism of action, quantitative real time PCR (qPCR) was done and gene expression was analysed. Levels of expression of key genes associated with inflammation were measured, including NF-κB, IκBα, COX-2, TNF-α, IL-6, and IL-10. Upregulation of anti-inflammatory markers such as IL-10, along with downregulation of pro inflammatory genes like TNF-α, IL-6 and COX-2, would confirm the anti-inflammatory role of Theobro-ZnONPs, particularly through modulation of NF-κB pathway. Furthermore, the wound healing efficacy of Theobro-ZnONPs was evaluated in an in vivo zebrafish model, a well-accepted vertebrate model for studying tissue repair and inflammation. (Santhosh et al., 2025)Zebrafish offer advantages including rapid healing capacity, transparent tissues for observation, and conserved immune and genetic pathways. Dorsal skin wounds were induced in adult zebrafish, and the healing response was assessed at two time points, Day 7 and Day 14, to compare the temporal progression of wound closure between the control and treated groups.(Park et al., 2026)

Histopathological evaluation of the wounded zebrafish tissues provided detailed insights into tissue remodelling, inflammation and epithelial regeneration. Haematoxylin and eosin (H&E) stained sections were analysed for the presence of inflammatory cell infiltration, re-epithelialization, dermal organization, and neovascularization. Enhanced tissue architecture, reduced inflammation, and restored epidermal structure in the treated group would further support the therapeutic efficacy of Theobro-ZnONPs. The integrated findings from cell viability assays, migration studies, gene expression

profiling, zebrafish wound healing assessment, and histopathological evaluation aimed to provide comprehensive evidence of properties of Theobro-ZnONPs such as those of anti-inflammatory and wound healing. By targeting the NF-κB/IκBα pathway and modulating the inflammatory mediators' expression, Theobro-ZnONPs present a promising therapeutic option for the treatment of wounds characterized by excessive or chronic inflammation.(Begon-Pescia et al., 2025)

Importantly, this study also underscores the relevance of using natural bioactives in nanoparticle synthesis to not only reduce environmental impact but also enhance therapeutic functionality. Theobromine serves as a dual functional agent acting both as a reducing/stabilizing agent in nanoparticle synthesis and as a bioactive component with direct biological effects. The resulting hybrid nanomaterial, Theobro-ZnONPs harnesses the wound healing and anti-inflammatory potential of both zinc and theobromine, offering a biocompatible and multifunctional solution for regenerative medicine. Thus, this study provides novel insights into the wound healing application of Theobro-ZnONPs, with a mechanistic focus on blocking of NF-κB pathway and promotion of expression of anti-inflammatory genes. Through the integration of in vitro assays, molecular analysis and in vivo validation, it establishes a strong foundation for the future translational development of plant based nanotherapeutics for managing inflammation driven wound pathologies.

2 Materials and Methods

2.1 Chemicals and Reagents

Analytical grade reagents were used as received throughout the course of this study, Theobromine (≥99% purity), used as the green reducing and stabilizing agent, was purchased from Sigma Aldrich (St.Louis, MO, USA). Zinc acetate dihydrate ($Zn(CH_3COO)_2.2H_2O$) was used as the zinc precursor and obtained from Himedia Laboratories (Mumbai,India). Sodium hydroxide (NaOH) was used for pH adjustment during nanoparticle synthesis. For cell culture studies, Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, and phosphate buffered saline (PBS) were procured from Gibco, Thermo Fisher Scientific (USA). The MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and dimethyl sulfoxide (DMSO) for the cytotoxicity assay was obtained from Sigma-Aldrich. For RNA extraction, TRIzol reagent was used (Invitrogen, USA) and the high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) was used to perform reverse transcription. SYBR Green PCR Master Mix (Applied Biosystems, USA) with gene specific primers was used to carry out qPCR reactions synthesized by Eurofins Genomics (India). Tricaine methanesulfonate for zebrafish anaesthesia was purchased from Sigma Aldrich, and all other solutions for histopathology were procured from standard histology suppliers.

2.2 Synthesis of Theobro-ZnONPs

Theobro-ZnONPs were synthesized using a green chemistry approach. Deionised water was used to dissolve zinc acetate dihydrate ($Zn(CH_3COO)_2.2H_2O$) to prepare a 10 mM zinc precursor solution. In Parallel, theobromine was dissolved in warm deionized water to obtain a 1 mg/ML solution, which serves as the reducing and stabilizing agent. Under constant stirring at 60°C, Theobromine was added to the zinc acetate solution in a dropwise manner, maintaining a 1:2 volume ratio (Theobromine: zinc acetate). The reaction mixture pH was adjusted to pH 9 using 1M NaOH, which facilitated the nucleation and growth of ZnO nanoparticles. A pale yellowish white precipitate was formed after the reaction was allowed to proceed under continuous stirring for 2 hours, indicationg the successful synthesis of ZnO nanoparticles . Following this, centrifucation of the resulting suspension was done at 12000 rpm for 20 minutes, and the pellet was washed three times with ethanol and distilled water to remove unreacted precursors and loosely bound biomolecules. The purified nanoparticles were dried in a hot air oven at 60°C for 12 hours and stored in an airtight container for further characterization and biological evaluation.

2.3 Multimodal Characterization of Theobro-ZnONPs

The physicochemical properties of the synthesized Theobro-ZnONPs were evaluated using Scanning Electron Microscopy (SEM), X-ray Diffraction (XRD) and Fourier Transform Infrared Spectroscopy (FTIR). SEM analysis, performed using a JOEL JSM-IT800 (JOEL Ltd., Tokyo, Japan), revealed that the nanoparticles exhibited a predominantly spherical to oval morphology with slight agglomeration, and the particle size was within the nanometer range. FTIR analysis, conducted using a PerkinElmer FTIR spectrometer (USA), identified characteristic functional groups involved in the reduction and stabilization process, including O-H, N-H, C=O, and C-N bonds, confirming the capping action of theobromine. A distinct Zn-O vibration band further verified the formation of zinc oxide. XRD analysis, carried out using a Bruker D8 Advance diffractometer (Bruker, Germany), confirmed the crystalline nature

of the nanoparticles. The diffraction patterns corresponded to the typical crystalline structure of ZnO, with no indications of amorphous content or secondary phases, indicating successful synthesis of highly pure and crystalline Theobro-ZnONPs.

2.4 McCoy cell line and Culture conditions

The McCoy cell line (mouse fibroblast-like cells) was used as an in vitro model to evaluate the cytocompatibility and anti-inflammatory potential of Theobro-ZnONPs. Cells were obtained from the National Centre for Cell Science (NCCS) and maintained in DMEM Medium (Gibco, thermos Fisher Scientific, USA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cultures were incubated at 37°C in a humidified atmosphere and under standard conditions with 5% CO_2. Cells were subcultured upon reaching 89-90 % confluence using 0.25% trypsin-EDTA solution, and all assays were performed using cells between passage 3 and 8 to ensure reproducibility and biological consistency. Prior to experimental treatments, cells were seeded at the required density and allowed to adhere for 24 hours before exposure to Theobro-ZnONPs at selected conditions.

2.5 Cell Viability Evaluation Following Theo-ZnONP Exposure

The cytotoxicity of Theobro-ZnONPs was evaluated in McCoy cells using the MTT assay. Cells were seeded in a 96 well plate at a density of 1×10^4 /well and incubated for 24 hours at 37°C in a 5% CO_2 atmosphere to allow cell attachment. After incubation, cells were treated with Theobro-ZnONPs at concentrations of 5,10 and 25µg/mL for 24 hours. Following treatment, each well was incubated for 4 hours after the addition of 20µL of MTT reagent (5 mg/mL in PBS). 100 µL of DMSO was used to dissolve the resulting formazan crystals, and absorbance was measured at 570 nm using a microplate reader. Cell viability was expressed as a percentage relative to untreated control wells.

2.6 Cell Migration assay by Scratch method

The scratch wound healing assay was performed to assess the effect of Theobro-ZnONPs on the migratory ability of McCoy cells. Cells were seeded into 6 well plates and cultured until they reached 90-100% confluence. A uniform scratch was made across the center of each well using a sterile 200 µL pipette tip. The wells were then gently washed with PBS to remove non-adherent and detached cells. Based on prior MTT assay findings indicating minimal cytotoxicity, a concentration of 1µg/mL Theobro-ZnONPs was selected for the migration study. Cells were treated with the nanoparticles in serum reduced medium, while the control group received medium without nanoparticles. Plates were incubated at 37°C with 5% CO_2 for 24 hours, after which images of the wound area were captured using an inverted phase contrast microscope at 0 and 24 hours.

2.7 Zebrafish Maintenance under standard laboratory conditions

All animal handling procedures were carried out in compliance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Aqnimals (CPCSEA), Government of India. Ethical approval for the study was obtained from the Institutional Animal Ethical Committee (IAEC) of Saveetha Dental College and Hospitals, SIMATS, Chennai, India under protocol number BRUAC/SDCH/SIMATS/IAEC/03-2024/12. Adult zebrafish (*Danio rerio),* AB strain, aged 6-8 months, were procured from a certified local vendor and acclimatized in the laboratory for one week prior to experimentation. Fish were maintained in glass aquaria under controlled environmental conditions, including a temperature of 28±1°C, a 14 hour light/10 hour dark photoperiod, and continuous aeration and filtration. Water quality parameters such as pH, ammonia, nitrite levels, and dissolved oxygen were monitored and maintained within optimal limits. Fish were fed a commercially available balanced diet twice daily, and partial water changes were performed regularly to ensure a clean aquatic environment. Environmental enrichment, including hiding structures, was provided to minimize stress and support natural behaviour. All zebrafish were handled humanely, and procedures were performed to minimize discomfort throughout the experimental period.

2.8 Zebrafish wound Infliction and Treatment Protocol

Wound infliction was performed on adult zebrafish following standard microsurgical procedures. Fish were anesthetized using tricaine methanesulfonate (MS-222,0.016%), and a controlled dorsal skin abrasion was created using a sterile scalpel under a dissection microscope. Post injury, zebrafish were transferred to individual treatment tanks under continuous aeration. The animals were divided into two groups, a control group maintained in system water, and a treatment group exposed to Theobro-ZnONPs. Separately, a fish acute toxicity test was

conducted in accordance with OECD Test Guideline No 236 and the IC_{50} value was calculated to be 300 µg/mL (data not added). Based on this, a sub toxic treatment concentration of 25.3 µg/mL was selected for the in vivo wound healing experiment. The treatment tanks were maintained with the designated Theobro-ZnONP concentration, and partial water changes were performed daily to sustain nanoparticle stability and water quality. Fish behaviour and general health were monitored throughout the study period. Observations and sampling were carried out a Day 7 and Day 14 post wounding.

2.9 Histopathological Analysis

On day 14, zebrafish from both control and treated groups were anesthetized using MS-222 (0.016%) and euthanized. The wounded dorsal skin tissues were carefully dissected and immediately fixed in 10% neural buffered formalin for 24 hours. Fixed samples were processed by dehydration in graded ethanol series, cleared with xylene, and embedded in paraffin wax. A rotary microtome was used to obtain sections that were 4-5µm and they were mounted on glass slides. The sections were then stained with Hematoxylin and Eosin (H&E) to assess tissue morphology, inflammatory infiltration, re-epithelialization, and dermal organization. Light microscope was used to examine stained slides, and representative images were captured for qualitative evaluation of healing response across groups.

2.10 Gene expression analysis by qPCR

To evaluate the molecular response involved in inflammation and wound healing, total RNA was isolated from wounded zebrafish tissues on Day 14 using TRIzol reagent following the manufacturer's instructions. A Nanodrop spectrophotometer was used to assess the concentration and purity of RNA, and 1µg of RNA was reverse transcribed into cDNA. qPCR was performed using SYBR Green Master Mix in a QuantStudio 5 Real-Time PCR system. Gene specific primers were used for NF-κB, IκBα, COX-2, TNF-α, IL-6 and IL-10, with β-actin serving as the endogenous control. Each reaction was carried out in triplicate, and thermal cycling conditions were optimized as per standard protocols. The relative gene expression levels were calculated using the 2^−ΔΔCt method, and results were expressed as fold changes compared to the control group.

Table 1: Primers used for gene expression

Gene	Former Primer (5'→3')	Reverse Primer 5'→3'
NF-κB	GCTTCTGCCCACAGATCAAA	TCGGAGGAGCTGTTTTCTGT
IκBα	ACCCTGTCAGGTTCTCCAGA	GAGGAGGTTGGTGTTGGTGT
COX-2	TGTGAGCAGGTGTGTTTGGA	GCTTCCAGTATTGAGGAGAAGG
TNF-α	CTGAGGCTTCCAGAAAACGA	CTTCAGCGTCTCGTGTGTTT
IL-6	CTGCTCCTGGTGATGCTACA	GGTGGTGGTATCCTCTGTGA
IL-10	GAGGAGGTGATGCCCCAAG	CTGCTCCACTGCCTTGCT
β-actin	TGGCATCACACCTTCTACAA	AGGAAGGAAGGCTGGAAGAG

3 Results

3.1 Physicochemical properties of Theobroma-ZnONPs

The synthesized Theobro-ZnONPs were successfully characterized using SEM, FTIR and XRD techniques to evaluate their morphological, structural, and chemical properties. SEM analysis revealed that the nanoparticles exhibited a predominantly spherical to oval morphology with moderate uniformity and minimal agglomeration (Figure 1). The particle sizes were in the nanometer range, confirming the nanoscale dimension suitable for biomedical applications. identification of the functional groups involved in the reduction and stabilization of the nanoparticles was done using FTIR spectroscopy. The spectra displayed prominent absorption bands corresponding to O-H/N-H stretching (3400 cm^{-1}), C=O/C=C stretching (1600-1650 cm^{-1}), and C-N bending vibrations (1400 cm^{-1}), which are characteristic of theobromine. A distinct peak in the 500 cm^{-1} regions indicated the presence of Zn-O stretching, confirming the formation of zinc oxide. Crystalline nature of the nanoparticles was confirmed by analysis via XRD. The diffraction pattern corresponded to the typical hexagonal wurtzite structure of ZnO, as reported in standard JCPDS data, with no detectable amorphous background or impurity peaks, indicating high purity and well-defined crystallinity of the synthesized Theobro-ZnONPs (Figure 2). These characterization results collectively confirm that the green synthesis method using theobromine produced stable, crystalline and well structured ZnO nanoparticles with functional surface chemistry suitable for biological applications.

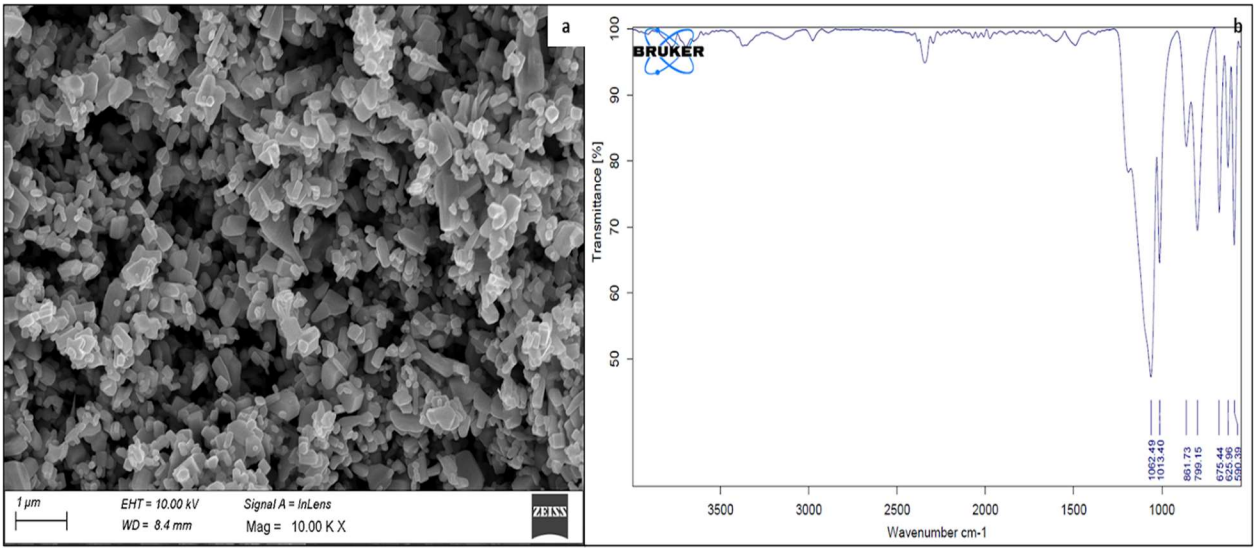

Figure 1: Characterization of Theobro-ZnONPs

a) Scanning Electron Microscopy image showing Theobro-ZnONPs with spherical to oval morphology.

b) Fourier Transform Infrared spectrum of Theobro-ZnONPs displaying functional groups confirming the successful formation and stabilization of ZnO nanoparticles using theobromine.

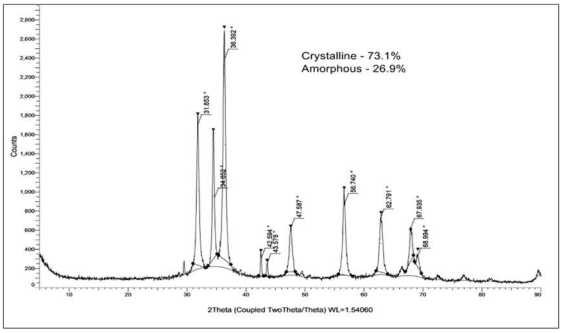

Figure 2: X-ray diffraction pattern of Theobro-ZnONPs .

The diffraction peaks correspond to the characteristic planes of hexagonal wurtzite crystal structure of ZnO indicating the crystalline nature of the nanoparticles.

3.2 Morphological assessment of McCoy cells Post treatment

Microscopic images were captured to assess the morphological characteristics of McCoy cells after 24 hour exposure to Theobro-ZnONPs at concentrations of 5,10, and 25 µg/mL, alongside the untreated control (Figure 3). The control group showed healthy, elongated fibroblast like morphology with uniform cell distribution and high confluency. Cells treated with 5 µg/mL Theobro-ZnONPs retained a morphology similar to the control, indicating good cytocompatibility. At 10 µg/mL, confluency was slightly reduced and in certain areas cell shrinkage and other minor morphological changes were observed. At 25 µg/mL, a notable decrease in cell density and alterations in cell shape were evident, consistent with dose dependent cytotoxicity observed in the MTT assay. These visual findings support the biocompatibility of lower nanoparticle concentrations and highlight the threshold beyond which toxicity becomes significant.

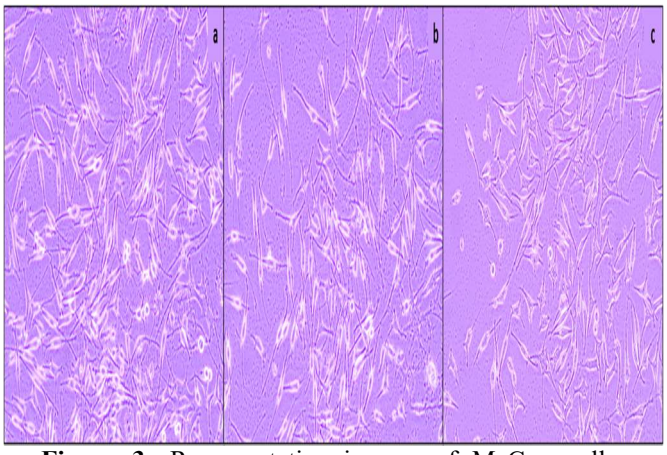

Figure 3: Representative images of McCoy cells following treatment with varying concentrations of Theobro-ZnONPs

The image shows a) Control b) the highest concentration of 25 µg/mL c) the lowest concentration of 5 µg/mL

3.3 MTT Cytotoxicity assay in McCoy cells

The cytocompatibility of Theobro-ZnONPs was assessed using MTT assay in McCoy cells. Cells were exposed to three different concentrations, 5,10, and 25µg/mL of Theobro-ZnONPs for 24 hours, and cell viability was determined based on mitochondrial metabolic activity. The results demonstrated a concentration dependent response. At 5µg/mL, Theobro-ZnONPs exhibited minimal cytotoxicity, with cell viability remaining above 90%, indicating good biocompatibility. At 10 µg/mL, moderate cytotoxic effects were observed, with viability decreasing slightly but remaining within an acceptable range for further biological assays. At the highest concentration of 25 µg/mL,cell viability was significantly reduced, suggesting dose-dependent cytotoxicity at elevated levels. Overall, The MTT assay confirmed that 1-10 µg/mL Theo-ZnONPs are biocompatible with McCoy cells, while 25 µg/mL may approach the cytotoxic threshold. Based on these results, I µg/mL was selected as the optimal working concentration for subsequent cell migration and gene expression assays to ensure cellular safety during treatment (Figure 4).

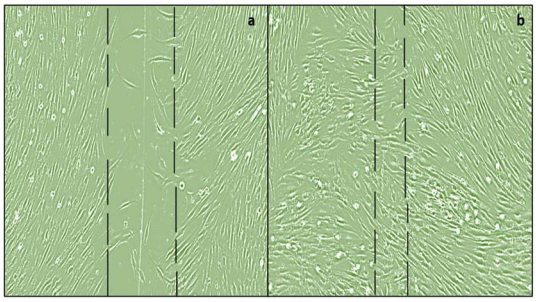

Figure 4 : MTT assay showing the effect of Theobro-ZnONPs on McCoy cell viability.

treatment of cells with Theobro-ZnONPs at concentrations of 5, 10 and 25µg/mL for 24 hours, and MTT assay was used to assess cell viability. Absorbance was measured at 570 nm, and viability was expressed as a percentage relative to the untreated control group. A dose-dependent decrease in viability was observed with minimal cytotoxicity at 5 µg/mL and notable reduction at 25 µg/mL, indicating concentration dependent cytotoxic effects of Theobro-ZnONPs.

3.4 Cell Migration Assessment by Scratch Assay

The scratch wound healing assay was performed to assess the effect of Theobro-ZnONPs on migration ability of McCoy cells. A sterile scratch was made on a confluent monolayer, and the cells were treated with 1µg/mL Theobro-ZnONPs while the control group remained untreated (Figure 5). After 24 hours of incubation, through visual comparison under an inverted microscope, a greater degree of wound closure was observed in the treated group when compared to the control. The edges of the scratch in the nanoparticle treated wells appeared more closed, indicating increased cell movement into the wound area. In contrast, the control group showed relatively less closure of the scratched region. These observations suggested that Theobro-ZnONPs at a non-toxic concentration enhanced cellular migration and supported the potential role of the nanoparticles in promoting wound healing (Figure 6).

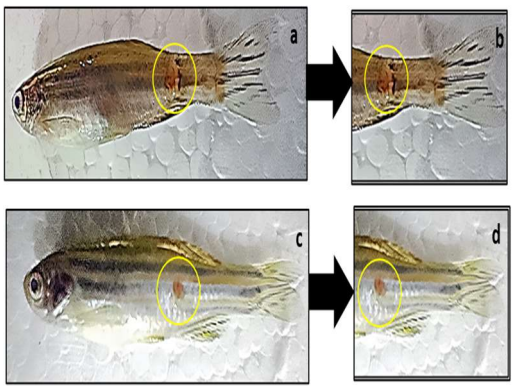

Figure 5: Scratch wound healing assay showing the effect of Theobro-ZnONPs on McCoy cell migration .

Images were captured at 24 hours following scratch creation. The treated group received 1µg/mL Theobro-ZnONPs while the control group remained untreated.

Figure 6: Bar graph showing the percentage of wound closure after 24 hours.

McCoy cells treated with 1µg/mL Theobro-ZnONPs exhibited a higher degree of wound closure compared to the untreated control group, indicating enhanced cell migration.

3.5 Wound closure Observation In Zebrafish Post-Treatment

Wound healing was evaluated in adult Zebrafish subjected to dorsal skin injury and treated with Theobro-ZnONPs at a concentration of 25.3 µg/mL. Observations were made on Day 7 and Day 14 post injury and compared with an untreated control group. On Day 7, zebrafish in the control group showed partial closure of the wound with signs of persistent inflammation and incomplete tissue remodelling. In contrast, the Theobro-ZnONPs treated group exhibited more advanced wound closure with smoother wound edges and early signs of tissue regeneration. By Day 14, the treated group displayed nearly complete re-epithelialization of the wounded area, with restored skin texture and coloration. The control group, however, continued to show incomplete healing and visible scar tissue. These visual observations suggest that Theobro-ZnONPs contribute to accelerated wound healing and enhanced tissue regeneration in the zebrafish model (Figure7).

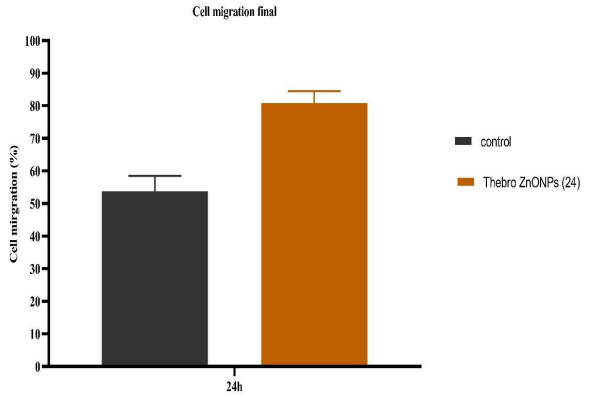

Figure 7: Histopathological evaluation of dorsal skin tissue in zebrafish on Day 14 post wounding.

a) Control group showing incomplete tissue organization and persistent inflammation.
b) Magnified view of the wound site in the control group with visible inflammatory infiltration and delayed epithelial restoration.
c) Treated group (25.3µg/mL Theobro-ZnONPs) showing well organised epidermal and dermal layers.
d) Magnified view of the wound site in the treated group, indicating reduced inflammation, re-epithelialization, and enhanced tissue regeneration.

3.6 Histopathological Evaluation of Wounded tissue

Histopathological analysis of zebrafish dorsal skin tissue was performed on Day 14 post wounding to assess the structural differences between the control and treated groups. In the control group, H&E-stained sections revealed disrupted tissue architecture, with incomplete re-epithelization, disorganised dermal layers, and persistent inflammatory cell infiltration at the wound site. The epidermis appeared thin and irregular, and signs of delayed regeneration were evident. In contrast tissue sections from the group treated with 25.3 µg/mL theorbo-ZnONPs displayed notable improvements in wound healing. The epidermal layer was continuous and well organized, and the dermis showed restored structure with increased cellular density and reduced inflammatory infiltration. The wound site exhibited evidence of re-epithelization, tissue remodelling, and early neovascularization, indicating advanced healing and reduced inflammation. These histological findings supported the therapeutic role of Theobro-ZnONPs in enhancing tissue repair and accelerating wound closure in the zebrafish model.

3.7 Gene Expression Analysis

To assess the levels of expression of key genes playing a role in inflammation and immune regulation in zebrafish skin tissue on Day 14 post wounding a qPCR was conducted. The group treated with Theobro-ZnONPs (25.3µg/mL) was compared to the untreated control group. The pro-inflammatory transcription factor NF-κB was significantly downregulated in the treated group, indicating suppression of central inflammatory signalling. Correspondingly, the expression of IκBα, the inhibitory regular of NF-κB, was upregulated, suggesting effective prevention of NF-κB nuclear translocation. Furthermore, the mediators of inflammation - COX-2, TNF-α, and IL-6 were markedly downregulated following nanoparticle treatment, reflecting a reduction in inflammation at the gene expression level. Conversely, the anti-inflammatory cytokine IL-10 was significantly upregulated in a treated sample, indicating a shift toward inflammation resolution and tissue recovery. These gene expression patterns support the anti-inflammatory mechanism of Theobro-ZnONPs primarily through modulation of the NF-κB/IκBα axis and downregulation of downstream cytokine expression involved in wound associated inflammation (Figure 8).

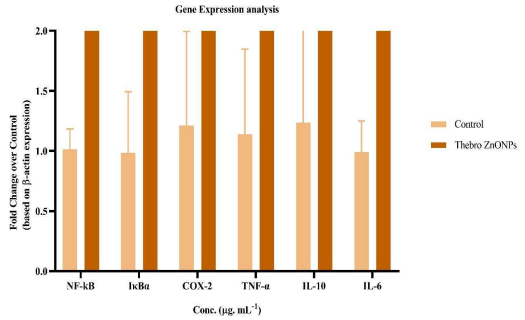

Figure 8: Relative gene expression of inflammation related markers in zebrafish skin tissue on Day 14 post treatment.

4 Discussion

The process of wound healing involves a complex interplay of cellular and molecular events, including inflammation, migration, proliferation, and remodeling. Inflammation is a critical early phase that orchestrates downstream regenerative responses, but persistent or excessive inflammation can impair healing and lead to chronic wounds. (Eissa et al., 2023) The regulation of inflammatory responses by the induction of genes that promote inflammation is one of the key roles played by the NF-κB signaling pathway. In this contest, we explored the potential of Theobro-ZnONPs to modulate inflammation and enhance wound healing through NF-κB pathway inhibition using both in vitro and in vivo models.(Afzal et al., 2025)

Theobromine, a naturally occurring methylxanthine, possesses antioxidant and anti-inflammatory properties. When used as a green reducing and capping agent for ZnO nanoparticle synthesis, it imparts biocompatibility and functional activity to the resulting nano formulation. (Li et al., 2026)In our study, the physicochemical characterization of Theobro-ZnONPs using SEM, FTIR and XRD confirmed successful synthesis. SEM showed that the nanoparticles were predominantly spherical with a uniform nanoscale size distribution. FTIR analysis confirmed the presence of characteristic theobromine functional groups along with Zn-O bonding, indicating successful capping. (Sharma et al., 2026)XRD revealed the crystalline wurtzite structure of ZnO with no amorphous content, validating the high purity and crystalline integrity of the nanoparticles. To evaluate cytocompatibility, McCoy cells were treated with 5,10 and 25 µg/mL of Theobro-ZnONPs, and an MTT assay was used to assess cell viability. The nanoparticles exhibited concentration dependent cytotoxicity, with 5 µg/mL maintaining over 90% viability, while 25 µg/mL significantly reduced cell survival. Based on this data, a safe and non-toxic working concentration of 1 µg/mL was chosen for subsequent cell based functional assays. The scratch wound healing assay in McCoy cells demonstrated that 1 µg/mL Theobro-ZnONPs significantly enhanced cellular migration into the wounded area compared to the untreated control. Migration is a fundamental aspect of the proliferative phase in wound healing., facilitating re-epithelialization and matrix deposition. The observed increase in migration suggests that even at low, biocompatible concentrations, Theobro-ZnONPs can positively influence cell motility, likely through modulation of intracellular signalling pathways involved in cytoskeletal rearrangement and chemotaxis.

To validate the molecular level anti-inflammatory potential of Theobro-ZnONPs, we performed gene expression analysis via qPCR targeting six key markers. NF-κB, IκBα, COX-2, TNF-α, IL-6, and IL-10. The NF-κB transcription factor, which is central to inflammatory signalling, was significantly downregulated in nanoparticle treated zebrafish tissue, suggesting suppression of inflammatory transcriptional activity. IκBα, which inhibits NF-κB pathway by preventing its translocation to the nucleus, was upregulated, confirming stabilization of its inhibitory function. These findings clearly indicate that Theobro-ZnONPs downregulate inflammatory signaling at the transcriptional level via modulation of the NF-κB/IκBα axis.

Further downstream, expression of COX-2, an enzyme involved in prostaglandin synthesis and inflammation, was also markedly reduced. Similarly, cytokines TNF-α and IL-6, pro-inflammatory in nature, directly targeting NF-κB as well as the key mediators of acute inflammation, were significantly suppressed. These reductions suggest that Theobro-ZnONPs can effectively attenuate the cytokine cascade typically observed during the inflammatory phase of wound healing. Interestingly, the anti-inflammatory cytokine IL-10 was significantly upregulated in treated samples. IL-10 plays a crucial role interminating inflammation, limiting tissue damage, and promoting repair. The simultaneous downregulation of proinflammatory genes and upregulation of IL-10 further strengthens the case for Theobro-ZnONPs as a potent anti-inflammatory agent.To examine therapeutic potential in a living system, we implemented an in in vivo zebrafish wound healing model, a widely accepted vertebrate system due to its genetic homology, transparent tissues, and rapid healing capacity. A dorsal skin injury model was established,and zebrafish were treated with 25.3 µg/mL of Theobro-ZnONPs based on prior IC_{50} estimations (OECD Test No. 236). Wound observations on Day 7 and Day 14 in comparison with

the control, showed faster and more complete closure of the wound in the treated group. The visual improvement in wound healing correlated well with molecular and in vitro findings.(Pavel et al., 2025)

These macroscopic healing trends were further supported by histopathological analysis. In the control group, tissue sections revealed incomplete epithelial regeneration, high levels of inflammatory cell infiltration, and disorganized dermal layers even on Day 14. (Silva Alves et al., 2026)In contrast, Theobro-ZnONPs treated tissue showed a well-organized epidermis, reduced inflammation, and signs of neovascularization, indicative of tissue remodelling and repair. These histological improvements align with our gene expression findings and reinforce the therapeutic value of Theobro-ZnONPs in wound healing. (Nofal et al., 2026)Taken together, our results demonstrate that Theobro-ZnONPs act through multiple complementary mechanisms, they exhibit biocompatibility, promote cellular migration, suppress pro-inflammatory gene expression, upregulate anti-inflammatory mediators, and enhance tissue regeneration in vivo. Zinc as a trace element, plays a vital role in enzymatic activity, wound repair and immune modulation, and its nanoparticulate form ensures enhanced bioavailability and surface interaction. Theobromine, a bioactive compound derived from cacao, is known for its anti-inflammatory and antioxidant effects. Their combination through green synthesis yields a hybrid nanoparticle that is not only safe and eco-friendly but also therapeutically potent.

The use of green synthesis methods avoids toxic solvents and harsh chemicals, improving the overall safety profile of the nanoparticles and aligning with sustainability goals in nanomedicine. While our results are promising, further validation through protein level expression, oxidative stress markers, and long-term in vivo studies in mammalian models would enhance translational relevance.

5 Conclusion

This study demonstrates that Theobro-ZnONPs promote wound healing by modulating inflammation through suppression of the NF-κB signalling pathway. In vitro assays confirmed that Theobro-ZnONPs are biocompatible at low concentrations and enhance cell migration. In vivo zebrafish wound healing and histological assessments showed faster and more organised tissue regeneration. qPCR analysis revealed downregulation of NF-κB, COX-2, TNF-α, IL-6 and upregulation of IκBα and IL-10, supporting their potent anti-inflammatory action. These findings collectively establish Theobro-ZnONPs as a promising green synthesized nanotherapeutic for managing inflammation driven wound healing.

6. Conflict of Interest

There is no conflict of interest

References

Afzal, H.-S., Uttra, A.-M., Qasim, S., Malik, A., & Mobashar, A. (2025). Theobromine as a multi-target therapeutic agent: Analgesic, anti-inflammatory, and anti-arthritic potential with network pharmacology insights. *Annales Pharmaceutiques Francaises*, *83*(6), 1114–1129.

Begon-Pescia, C., Manchon, L., Özbilgiç, R., Boireau, S., Louis, A., Georges, S., & Nguyen-Chi, M. (2025). Temporal dynamics of macrophage transcriptional profiles during zebrafish wound healing. *Frontiers in Immunology*, *16*, 1721612.

Dong, J., Lang, Y., Cui, J., Cheng, B., Zhang, X., He, J., Wang, R., Liu, X., & Zhou, M. (2026). Oral microalgal nanoparticle hydrogel for multifaceted management of chronic oral ulcers in diabetes. *Journal of Nanobiotechnology*. https://doi.org/10.1186/s12951-026-04324-2

Eissa, I. H., Yousef, R. G., Elkady, H., Elkaeed, E. B., Alsfouk, A. A., Husein, D. Z., Ibrahim, I. M., Elhendawy, M. A., Godfrey, M., & Metwaly, A. M. (2023). Identification of new theobromine-based derivatives as potent VEGFR-2 inhibitors: design, semi-synthesis, biological evaluation, and studies. *RSC Advances*, *13*(33), 23285–23307.

Fahrurroji, A., Suhandi, C., Chaerunisaa, A. Y., Pratiwi, L., & Sriwidodo, S. (2026). Natural Bioactive-Based Advanced Wound Dressings for Diabetic Wound Healing: A Systematic Review of Emerging Biomaterial Platforms. *International Journal of Nanomedicine*, *21*, 588990.

Govea-Alonso, D. O., Garay-Barragán, M. I., Cazares-Rodríguez, E., & Villabona-Leal, E. G. (2026). Green Synthesis of ZnO Nanoparticles: A Sustainable Approach for Wound Care. *Nanotechnology, Science and Applications*, *19*, 550024.

Jafarbeglou, A., Abbasi, F., Davoodi, F., Mohammadi, R., Dezfoulian, O., Raisi, A., & Yahoo, A. (2026). Salvia miltiorrhiza promotes cutaneous wound healing by modulating inflammation, oxidative stress, and angiogenesis in mice. *Tissue & Cell*, *102*, 103560.

Kalogeromitros, M., Giannas, E., Bellen Nordahl, E. J., Pugliese, F., Manning, M., Jones, L. S., Chennupati, V., Gergoudis, F. R., & Ballou, J. H. (2026). Skin Problems in Search of Skin Solutions: A Wound Care Overview. *Annals of Plastic Surgery*.

https://doi.org/10.1097/SAP.0000000000004769

Li, M., Zhou, Y., Huang, J., Liu, X., Wang, W., Ge, Y., Zhao, Z., Gu, L., Gong, C., Zhu, X., He, S., & Liu, P. (2026). From clinic to ecosystem: sustainable zinc oxide nanoparticles as dual-purpose agents for Big Health. *Nanoscale*, *18*(14), 7342–7359.

Madan, S., Dhanraj, G., & Ramasubburayan, R. (2026). Antibacterial and Antibiofilm Activities of Streptomyces rochei-Mediated Zinc Oxide Nanoparticles against Dental Caries-Causing Streptococcus mutans - An In Vitro Study. *Indian Journal of Dental Research : Official Publication of Indian Society for Dental Research*. https://doi.org/10.4103/ijdr.ijdr_467_24

Majie, A., Roy, K., Jha, B., Lim, W. M., & Gorain, B. (2026). Establishing synergistic role of acacia honey and vitamin C: A promising strategy for faster wound tissue regeneration. *Injury*, *57*(7), 113339.

Marzok, M., Hassan, M., Elkawi, M. A., Al Mohamed, Z., Hereba, A., Hegazy, Y., & El-Sherif, M. (2026). Comparative efficacy of zinc oxide nanoparticles, MSC-derived microvesicles, and platelet-rich fibrin in rabbit tendon regeneration. *Scientific Reports*. https://doi.org/10.1038/s41598-026-46509-7

Nazish, M., Rahimova, S., Zubair, M., Khan, M. F., Khan, M. A., Bibi, F., Khan, M. I., Ulla, S., Bakhrom, J., Alwaznah, R., Abid, I., & Naseem, M. T. (2026). Phytofabricated zinc oxide nanoparticles (ZnO-NPs) from a medicinal salt-tolerant Oxystelma esculentum (L.f.) Sm. extract and their multifunctional biological activities. *Scientific Reports*, *16*(1). https://doi.org/10.1038/s41598-026-47491-w

Nofal, A. E., El-Sayyad, G. S., Saber, A. A., & Zedan, O. I. (2026). Ameliorative Effect of Green Bimetallic Zinc Oxide-Magnesium Oxide Nanoparticles Against Gastric Ulcer in Rats via Modulation of Oxidative Stress, Inflammatory Cascades, Nrf2/NF-κB Signaling, Angiogenic VEGF Expression. *Biological Trace Element Research*. https://doi.org/10.1007/s12011-026-05127-0

Park, S. A., Lupi, G., Ozbilgic, R., Kollár, M., Ožvat, A. A., Nguyen-Chi, M., & Mikula, K. (2026). The morpho-kinetic landscape of macrophage modes during wound healing in zebrafish. *Scientific Reports*, *16*(1), 6506.

Pavel, C. D., Chiţescu, C. L., Dragostin, O.-M., Dediu, L., Aprodu, I., Iacob, A. D., Vatcu, R., Zamfir, A.-S., & Zamfir, C. L. (2025). Evaluation of Oxidative Stress and Antioxidant Effects of Methylxanthines in Adult Zebrafish Exposed to Zinc Oxide Nanoparticles (ZnO-NPs). *Medicina (Kaunas, Lithuania)*, *62*(1). https://doi.org/10.3390/medicina62010021

Poorani, K., Rajkumar, M., Prajapati, B. G., Velmani, S., Sudha, P. N., Shanmugarathinam, A., & Paliwal, H. (2026). Biofabrication of -Mediated Chitosan-Zinc Oxide Nanocomposites for In Vitro Antioxidant, Antibacterial, Anti-Inflammatory and Wound-Healing Properties. *Pharmaceutics*, *18*(3). https://doi.org/10.3390/pharmaceutics18030390

Santhosh, K., Gunasekaran, R., Kannan, K., & Sivaperumal, P. (2025). Bioactive potential of : phytochemical profiling, antibacterial activity, and toxicity evaluation on zebrafish () larvae. *Natural Product Research*, 1–9.

Sharma, R., Shaheen, G., Rautela, I., Tyagi, S., Bachheti, R. K., Bachheti, A., & Worku, L. A. (2026). Synthesis and characterization of zinc oxide nanoparticles by the aerial part of extract and assessment of their biological activities. *Zeitschrift Fur Naturforschung. C, Journal of Biosciences*. https://doi.org/10.1515/znc-2025-0159

Silva Alves, L. K., Pairis-Garcia, M. D., Bonin Ferreira, J., Rocha Merenda, V., Mitalli Tomacheuski, R., Esteves Trindade, P. H., Siepker, C., & Lopez-Soriano, M. (2026). Topical wound-care products and their effects on healing, inflammatory biomarkers, and growth in piglets undergoing castration. *Porcine Health Management*, *12*(1). https://doi.org/10.1186/s40813-026-00492-7