International Journal of Environmental Sciences
ISSN: 2229-7359
Vol. 11 No. 23s, 2025
https://theaspd.com/index.php

A Biocompatible Nanoparticle Approach To Wound Healing: Betanin-Znonps Modulate TGF-B/SMAD Signaling And Enhance Skin Regeneration

Amy Elizabeth Jacob[1], **Mrs.S. Sangeetha** [2]
[1]Saveetha Dental College and Hospital, Saveetha Institute of Medical and Technical Science, Saveetha University, Chennai - 600077, 152401003.sdc@saveetha.com
[2]Assistant Professor, Department of anatomy, Saveetha Dental College and Hospital, Saveetha Institute of Medical and Technical Science, Saveetha University, Chennai - 600077, Sangeethas.sdc@saveetha.com

ABSTRACT

Background: *A balance between tissue regeneration, apoptosis and inflammation is necessary for effective wound healing. Promising approaches to improving this process include systems based on nanotechnology and bioactive chemicals produced from plants. Using zebrafish and PDL cell cultures, the wound healing abilities of betanin-derived zinc oxide nanoparticles (Betan-ZnONPs), with an emphasis in angiogenesis and apoptotic pathways.*

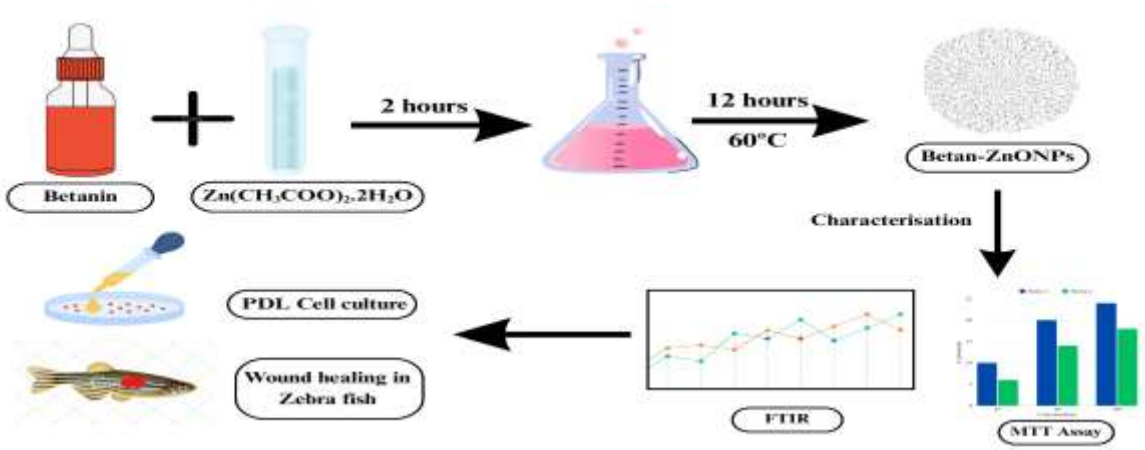
Methods: *Green synthesis was used to create Betan-ZnONPs, which were then characterized by FTIR, XRD, SEM, and EDAX. Using the MTT assay, cytotoxicity was evaluated in PDL cells at doses of 10, 50, and 100 µg/mL. In order to assess cell migration at a non-toxic concentration (28.32 µg/mL), a scratch wound healing assay was used. Using adult zebrafish with dorsal skin injury, in vivo wound healing was investigated by comparing the treatment and control groups in day 7 and day 14. To investigate tissue regeneration and expression of apoptotic and angiogenic markers, histopathological examination and qPCR based gene expression profiling were conducted.*

Results: *The successful synthesis of crystalline, spherical Betan-ZnONPs with the proper functional group stabilization was validated by characterization. With a biocompatible concentration of 28.32 µg/mL, MTT analysis revealed dose-dependent cytotoxicity. In the scratch assay, treated cells migrated more readily. By day 14, the group that received Betan-ZnONP treatment showed better wound healing and re-epithelialization in zebrafish. In treated samples, histological investigation showed less inflammation and well-organized tissue regeneration. Increased apoptosis and angiogenesis were supported by gene expression analysis that revealed downregulation of Bcl-2 and over-expression of VEGF, HIF-1a, Caspase-3, Caspase-8, Caspase-9.*

Conclusion: *Betan-ZnONPs significantly promote wound healing by modulating apoptotic and angiogenic pathways. These findings highlight their potential as a biocompatible, plant based nanotherapeutic for regenerative medicine.*

Keywords: *Betanin, wound healing, zebrafish, PDL cells, apoptosis, angiogenesis, Caspase, VEGF*

Graphical Abstract



1. INTRODUCTION

Wound healing is a complex and dynamic process involving different cell types, proceeding through various stages like hemostasis, inflammation, proliferation and remodeling. An efficient and successful healing requires the regulation of cellular migration, apoptosis, angiogenesis, and inflammatory resolution in a coordinated manner(1).Wound closure slows down if these events are not properly regulated, leading to chronic wounds, fibrosis or excessive scar formation. Bioactive chemicals and

nanotechnology-based systems that can target and modulate multiple important pathways involved in tissue repair have been the focus of recent advances in wound healing. The control of cell death and survival is one of the most important molecular processes in wound healing(2). During the inflammatory and remodeling stages, apoptosis, also known as programmed cell death, is essential for eliminating damaged cells. Capase-3, Caspace-8, Caspace-9, and Bcl-2 are important apoptosis regulators. While Caspase-3 is the primary execution caspase that breaks down cellular structures, Caspase-8 and Caspase-9 are initiator caspases that initiate intrinsic and extrinsic apoptotic pathways, respectively. However, Bcl-2 functions as an anti-apoptotic protein that protects the integrity of the mitochondrial membrane and stops downstream classes from being activated(3).To effectively resolve wounds without causing undue tissue loss, proapoptotic indicators must be balanced.

The development of new blood vessels, or angiogenesis, is crucial for supplying oxygen, nutrients and immune cells to the wound site, in addition to apoptosis. Endothelial cell proliferation and vessel sprouting are stimulated by vascular endothelial growth factor (VEGF), a crucial proangiogenic agent (4). Hypoxia Inducible Factor-1 alpha (HIF-1α) is a transcription factor that is frequently present in injured tissue and is tightly regulated in its expression. VEGF and HIF-1α work together to initiate vascular repair, which makes them key players in the development of wound healing treatments. Thus, by improving the transport of bioactive substances, modifying cellular signaling, and regulating biological interactions, nanotechnology presents a viable strategy to support wound healing(5) .Zinc-based nanoparticles (ZnNPs), among the different metallic nanoparticles studied, have become powerful wound healing agents because of zinc's critical functions in tissue regeneration, immunological regulation, and cell proliferation, Zinc also supports structural, catalytic, regulatory roles in a number of important proteins and enzymes that are involved in DNA synthesis and repair, inflammatory regulation, and antioxidant defense. Zinc oxide is found to exhibit antioxidant, antibacterial as well as anticancer properties(6). Green nanoparticle synthesis employing phytochemicals obtained from plants has gained a lot of interest in recent years(7). Several nanoparticles combined with natural substances like Rosmarinus officinalis leaf extract have been found to have apoptotic activities. (8).By using natural substances as stabilizing and reducing agents instead of dangerous chemicals, this technique increases biocompatibility. The betalain pigment betanin, which is mostly derived from Beta vulgaris, is one such substance that has both substantial biological activity and reducing potential. In a variety of scenarios, betanin has been shown to have anticancer, anti-inflammatory, and antioxidant properties as well as help in controlling apoptotic signaling and oxidative stress. For the manufacture of biogenic nanoparticles, required for regenerative therapy, betanin is a suitable choice due to these properties(9).

In this study, betanin-derived zinc nanoparticles (Betan-ZnONPs) are synthesized and evaluated for their potential in improved wound healing. The process of synthesis combines the regenerative qualities of Zinc with the antioxidant and medicinal qualities of betanin to create a unique hybrid nanomaterial with dual activity, that stimulates angiogenesis and regulates apoptosis. A multifaceted approach was used to evaluate the effectiveness of Betan-ZnONPs in wound healing. The impact of these nanoparticles in cell migration, a crucial event in wound closure, was assessed using fibroblast and keratinocyte cell lines using in-vitro scratch assay experiments(10). An indirect indicator of cellular motility and proliferation in response to the therapy was the rate of wound closure over time. Quantitative real-time PCR (qPCR) was used to validate the molecular pathways by comparing the expression levels of Bcl-2, VEGF, HIF-1α, Caspase-3, Caspase-8, and Caspase-9 in treated and untreated groups. This made it possible to determine whether Betan-ZnONPs enhance pro-survival and angiogenic signals to encourage tissue regeneration or induce a pro-apoptotic shift to eliminate damaged cells in order to facilitate wound healing. The zebrafish (Danio rerio) model, a well-established vertebrate system, was used for observing tissue regeneration and angiogenesis, to further confirm the translational potential of Betan-ZnONPs. Several studies have been conducted in zebrafish to observe the efficacy of nanoparticles like silver(11). Organism level validation of the in-vitro and in-silico data were obtained through gene expression analysis of apoptotic and angiogenic markers in zebrafish tissues after treatment(12).

The unique aspect of this research is the integrated design of a green synthesized nanoparticle that uses zinc and vitamins complementary biological roles to control angiogenesis and apoptosis, two essential wound healing processes, at the same time. Betan-ZnONPs offer a biocompatible and environment friendly alternative to traditional synthetic nanoparticles which is consistent with the development of

suitable nanomedicine(13). Additionally, the application of zebrafish as an in-vivo model provides a more comprehensive understanding of the healing process by bridging the gap between clinical relevance and cell based assays. Betan-ZnONPs can improve wound healing by modifying the Caspase cascade, anri-apoptotic processes, and angiogenic signaling through VEGF and HIF-1α, according to this study's significant experimental and mechanical data. By combining zebrafish validation, qPCR, and cell migration experiments, we showed that Betan-ZnONPs are a promising naturally occurring nanoparticle for regenerative medicine. A basis for future translational research is laid by this through assessment, which also creates new opportunities for wound care powered by nanotechnology(13),(14).

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

All of the compounds used in this investigation were analytical grade and were utilized exactly as supplied, requiring no further purification. The phytochemical used in the green synthesis of zinc nanoparticles, Betanin (≥95% purity) was acquired from Sigma-Aldrich (St. Louis, MO, USA). We purchased the zinc precursor, zinc acetate dihydrate ($Zn(CH_3COO)_2.2H2O$), and the pH- adjusting agent, sodium hydroxide (NaOH), from Himedia Laboratories (Mumbai, India). Penicillin-Streptomycin, Fetal Bovine Serum (FBS), Phosphate Buffered Saline (PBS), and Dulbecco's Modified Eagle medium (DMEM) were acquired from Gibco (Thermo Fisher Scientific, USA) for use in cell culture assays. The TRIzol reagent (Invitrogen, USA) was used to isolate total RNA, and a first strand cDNA synthesis kit (Applied Biosystems) was used for reverse transcription. For qPCR, the SYBR Green master mix was utilised, and Eurofins Genomics (India) custom synthesised gene-specific primers for Caspase-3, Caspase-8, Caspase-9, Bcl-2, VEGF, HIF-1α, and β-actin. Standard laboratory settings were used to keep the zebrafish used for the toxicity study at 28.5℃, and Sigma-Aldrich provided the tricaine methanesulfonate (MS-22) for the anesthesia.

2.2 Synthesis of Betan-ZnONPs

A green, one pot approach was used to carry out the green synthesis of Betan-ZnONPs. A reducing and stabilizing agent, betanin (≥ 95% purity), was bought from Sigma-Aldrich. A 10mM solution of zinc acetate dihydrate ($Zn(CH_3COO)_2.2H2O$) was prepared in deionized water and stirred at 60℃. To the zinc acetate solution, a freshly prepared Betanin solution (1mg/mL in deionized water) was added dropwise in a 1:2 volume ratio while being continuously stirred. To promote nanoparticle production, 1M NaOH was used to progressively raise the mixture's pH to 8.5. The successful synthesis of ZnONPs were identified by a noticeable colour shift from purplish red to light pink during the two hours that the reaction mixture was continually stirred. Following completion, the mixture was centrifuged for 20 minutes at 12,000 rpm after being allowed to cool. After three rounds of washing with ethanol and deionized water to get rid of any unreacted components, the final pellet was dried for 12 hours at 60℃ in a hot air oven. For additional characterization and biological tests, the Betan-ZnONPs powder was collected and stored at room temperature in airtight containers(14).

2.3 Characterization of Betan-ZnONPs

A wide range of analytical methods were used to characterize the synthesized Betan-ZnONPs. A JOEL JSM IT800 Scanning Electron Microscope (JOEL Ltd., Tokyo, Japan) was used to analyze the nanoparticles' surface morphology and structural characteristics. The results showed a quasi-spherical morphology and a particle size distribution with slight aggregation. Energy Dispersive X-ray analysis (EDX), which was connected to the SEM equipment , was used to establish the elemental composition. It found carbon signals that were attributed to the chitosan backbone and strong peaks for zinc and oxygen, which confirmed the creation of ZnO nanoparticles. To determine the functional groups in charge of stabilizing nanoparticles, Fourier Transform Infrared Spectroscopy (FTIR) was performed using an Agilent Cary 630 FTIR Spectrometer (Agilant Technologies, Gurugram, India) in the 500-4000 cm-1 range using KBr pellet method. The O-H, N-H, C-N, and Cu-O peaks validated the interaction between copper ions and β-chitosan. X-ray diffraction(XRD) analysis was used to determine the nanoparticles crystalline structure using Cu Kα radiation on a Rigaku Miniflex 600 X-ray diffractometer (Rigaku Corporation, Tokyo, Japan). The monoclinic phase of Zno was matched by the diffraction peaks at 2θ values, indicating that the Betan-ZnONPs were crystalline and phase pure(15) .

2.4 PDL Cell line and culture conditions

The National Centre for Cell Science (NCCS), Pune, India, provided the human Periodontal Ligament (PDL) cells, which were cultivated in a conventional aseptic environment. Dulbecco's Modified Eagle Medium (DMEM, Gibco, Thermo Fisher Scientific, USA) was used to keep the cells alive. 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin (100 U/mL penicillin and 100 µg/mL streptomycin) were added. The cultures were kept at 37℃ in a humidified environment with 5% CO2. When the cells were between 80 and 90% confluence, subculturing was carried out by using a 0.25% trypsin-EDTA solution (Gibco). To guarantee the best cellular response and reproducibility, cells from passages three and six were used in all tests(16).

2.5 MTT Cytotoxicity Assay

The MTT assay was used to evaluate the cytotoxicity of Betan-ZnONPs on PDL cells. After being seeded in 96 well plates (1×10^4), the cells were incubated for 24 hours and then treated with Betan-ZnONPs for an additional 24 hours at concentrations of 10, 50, and 100µg/mL. Following the treatment, 100µL of DMSO was added to dissolve the formazan crystals, then 20µL of MTT solution (5mg/mL) was added and incubated for 4 hours. A microplate reader (Bio-Rad iMark, USA) was used to measure absorbance at 570nm, and cell viability was represented as a percentage in relation to untreated controls. A concentration-dependent decrease in cell viability was seen in the assay, which was carried out in triplicate. The cytotoxicity was low at 10µg/mL, moderate at 50µg/mL and significant at 100µg/ml(17).

2.6 Cell migration assessment via Scratch Assay

The impact of Betan-ZnONPs on cell migration was assessed in PDL cells using the scratch wound healing assay. Six well plates were used to seed the cells, which were then cultivated to 90-100% confluence. A sterile 200µL pipette tip was used to make a homogeneous scratch over the cell monolayer. The cells were then washed with PBS to remove detached cells. Further, the cultures were treated with serum reduced media containing 10, 50, and 100µg/mL of Betan-ZnONPs, while untreated wells were used as controls. Using an inverted microscope, pictures of the wound region were taken at 0 and 24 hours. Using Image J software, the extent of wound closure was measured, and the migration rate was calculated as a percentage of wound closure. The results suggested the Betan-ZnONPs modulate PDL cell motility in a dose-dependent manner. At 10 and 50µg/mL, the cell migration was enhanced, while at 100 µg/mL, the migration rate was decreased(18).

2.7 Animal Ethics and Zebra fish maintenance

The Institutional Animal Ethical Committee of Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences (SIMATS) granted ethical clearance prior to the start of in vivo experiments with Betan-ZnONPs, with approval number
BRULAC/SDCH/SIMATS/IAEC/03-2024/12. A certified local source provided the adult zebrafish, AB strain which were between 6 and 8 months old and acclimated in a controlled laboratory setting. The zebrafish were fed a nutritionally balanced meal twice a day and kept on a 14:10 hour light-dark cycle to support their natural circadian rhythms. To guarantee the best possible water quality, the aquatic system underwent constant filtration and aeration. Important variables including temperature, dissolved oxygen, and pH were consistently tracked and maintained within optimal physiological limits. Periodically, partial water changes were carried out in order to maintain a healthy aquatic habitat and remove organic garbage. Tanks containing hiding places and visual cues that evoked a natural habitat were added to reduce stress and promote natural behaviours, which helped the zebrafish survive and thrive during the study (19).

2.8 Zebrafish wound infliction and treatment protocol

In order to assess the ability of Betan-ZnONPs to heal wounds, two groups of adult zebrafish were created. Both Group Ⅰ (Negative Control) and Group Ⅱ (Treated Group) were exposed to 28.32 µg/mL of Betan-ZnONPs, a quantity determined to non-cytotoxic based on previous MTT assay results. Group Ⅰ received treatment for a month after the injury. Under a dissection microscope, zebrafish were given a normal wound infliction under anesthesia with 0.016% tricaine methanesulfonate (MS-222). The treated group was kept in tanks with freshly prepared Betan-ZnONPs solution after wounding, whereas the control groups kept in system water free of nanoparticles. To maintain aquatic health and nanoparticle-concentration, water parameters were partially adjusted and monitored everyday. To evaluate the regenerate outcomes between the treated and untreated groups, wound healing progress was monitored

and documented using a dissection microscope at two critical time points, which is at Day 7 and Day 14 (20).

2.9 Histopathological evaluation of wounded region

Zebrafish from the control group and the Betan-ZnONPs group (28.32 µg/mL) were put to death on Day 14 after wounding by administering an excessive dosage of tricaine methanesulfonate. After careful removal using sterile microsurgical scissor, the injured areas were promptly preserved in 10% neutral buffered formalin for a full day, Following this, the fixed tissues were treated using common histological methods such as paraffin embedding, xylene clearing, and graded ethanol dehydration. Using a rotatory microtome, serial sections with a thickness of 4-5 µm were created and placed on glass slides. For the purpose of assessing overall tissue architecture, cellular infiltration, and re-epithelialization, the sections were stained with Hematoxylin and Eosin (H&E) and regenerative images were captured for comparative analysis. The histological results were evaluated to compare the inflammatory response, tissue regeneration and wound resolution between the control and treated groups (21).

2.10 Gene expression analysis

Following the treatment with Betan-ZnONPs, qPCR was used to measure the expression levels of genes linked to angiogenesis and apoptosis in zebrafish. TRIzol reagent was used to extract total RNA from pooled zebrafish tissues on the 14th day after injury. Nanodrop spectrophotometry was used to verify the concentration and purity of the collected RNA. Then, using a first strand cDNA Synthesis Kit, 1 mg of RNA was reverse transcribed into cDNA. Using real-time PCR equipment and SYBR Green Master Mix, gene expression was measured. Caspase-3, Caspase-8, Caspase-9, Bcl-2, VEGF, and HIF-1α were amplified by qPCR with be β-actin serving as the internal reference. Using the $2^{-\Delta\Delta Ct}$ technique, the relative gene expression levels were determined. When compared to the control group, the results showed a considerable overexpression of Caspase-3, Caspase-8, and Caspase-9 in the treatment group, indicating that both intrinsic and extrinsic apoptotic pathways were activated. Bcl-2 was shown to be downregulated concurrently, suggesting a change in signaling towards pro-apoptosis. The groups treated with Betan-ZnONPs showed a significant elevation of VEGF and HIF-1α in terms of angiogenesis indicators, indicating improved neovascularization potential that is essential for wound healing. Three duplicates of each experiment were carried out, and statistical analysis validated the significance of these changes in gene expression (22).

Table 1: Primers used for gene expression

Gene	Former Primer (5'→3')	Reverse Primer (5'→3')
Caspase-3	AGTGGAACTGACGATGATGGC	CGCATCTGTACCAGACCGTT
Caspase-8	TGACAGTGTTGATCGTGCTG	AGGACTTGCAGTTTGTGCCT
Caspase-9	GACTGGATGAGCAGAGATGG	TTCTCGATCAGGAGGTCTTG
Bcl-2	CTGGCATCTTCTCCTTCCAG	AGATGCACCCAGGGTGATG
VEGF	GTGTGCGGACAGTGGTAGAG	TCCAGGAGTGGGAGTTGAGG
HIF-1α	ACTCAGGACACAGATTTAGACTG	CTGCTCATAACCCACAGGTT
β-actin	TGGCATCACACCTTCTACAA	AGGAAGGAAGGCTGGAAGAG

3. RESULTS

3.1 Physiochemical properties of biogenically synthesized zinc nanoparticles

The Betan-ZnONPs were primarily spherical to quasi-spherical in shape, with smooth surfaces and a small aggregation according to SEM analysis. This was probably caused by betanin's natural capping. At lower magnification, the nanoparticles seemed to be evenly scattered and distinct, but at higher magnification,

they showed occasional clustering. The successful nanoscale creation was confirmed by the estimated average particle size, which ranged between 20 and 50 nm. The elemental makeup of the produced nanoparticles was validated by the EDAX spectrum. The zinc oxide was confirmed by string signals corresponding to oxygen and zinc. The phytochemical betanin, which was employed as a stabilizing and reducing agent, is also responsible for the carbon peaks that were seen. The high purity of the synthesized Betan-ZnONPs were indicated by the lack of any unexpected elemental peaks. FTIR analysis revealed important functional groups that were involved in the stabilization and production of nanoparticles. There was a large absorption peak at about 3453cm^{-1}, which corresponded to O-H and N-H stretching vibrations and showed the presence of hydroxyl and amine groups of betanin. Peaks at about 1369cm^{-1} were thought to be C-N or -COO bending vibrations, whereas peaks at about 1635 cm^{-1} were thought to be C=O or C=C stretching. Notably, the synthesis of zinc oxide nanoparticles was indicated by a clear band close to 875 cm^{-1} , which verified the establishment of Zn-O bonds. XRD investigation revealed distinctive diffraction peaks with values of 2θ. The peaks matched with the typical diffraction pattern of ZnO's hexagonal wurtzite structure. The clarity and intensity of the peaks demonstrated that the nanoparticles were crystalline. Phase purity of the synthesized Betan-ZnONPs were indicated by the absence of additional peaks (Figure 1).

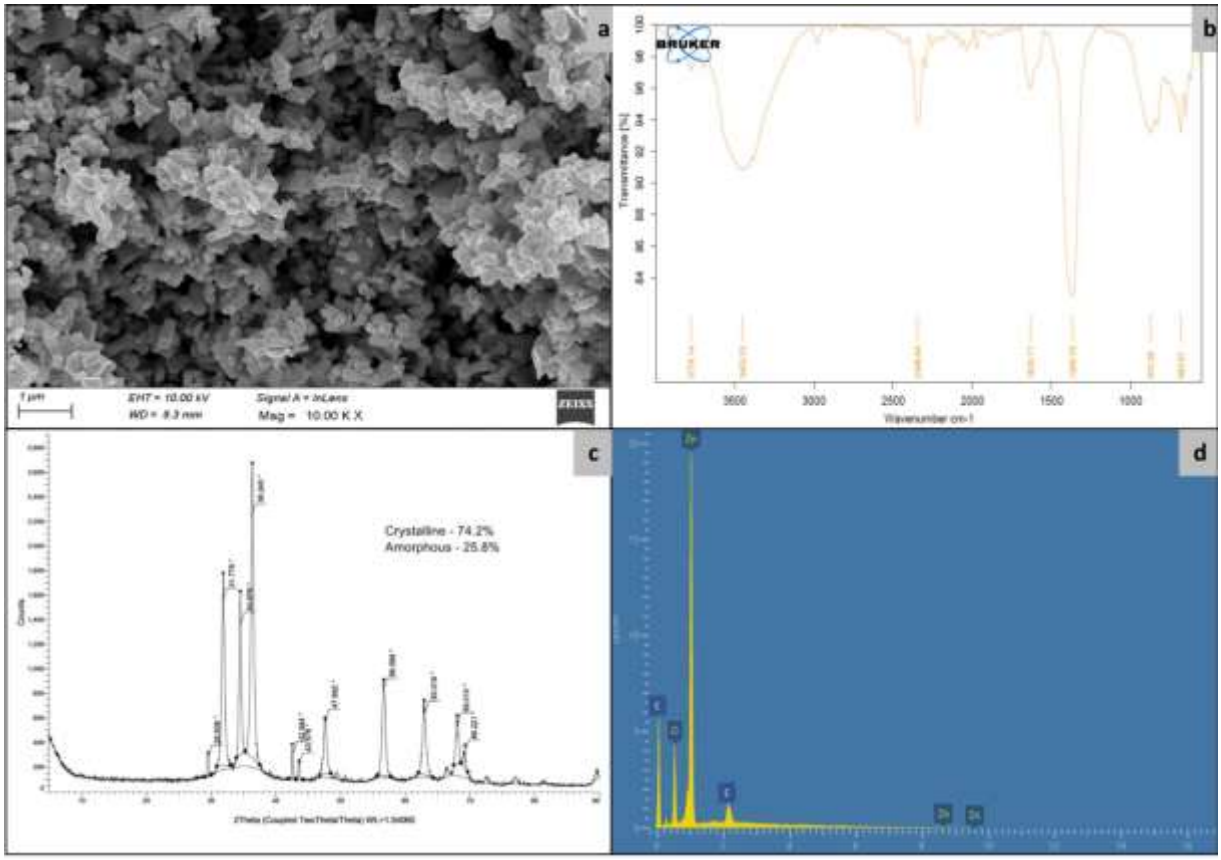


Figure 1: Comprehensive physicochemical characterization of Betan-ZnONPs where,
a) Scanning electron microscopic image of synthesized nanoparticles b) FTIR spectrum displaying characteristic functional groups. c) X-ray diffraction pattern which shows distinct diffraction peaks d) Energy dispersive X-ray spectrum confirming elemental composition of nanoparticles

3.2 Cell viability assessment using MTT Assay

MTT assay was conducted to assess the cytotoxicity of Betan-ZnONPs on PDL cells at concentrations 10, 50, and 100 µg/mL. The results demonstrated a dose-dependent reduction in cell viability, with minimal cytotoxicity observed at 10 µg/mL, moderate cytotoxicity at 50 µg/mL, and significant cell death at 100

µg/mL. These findings confirm that lower concentrations are biocompatible, while higher concentrations exert a stronger antiproliferative effect on PDL cells (Figure 2).

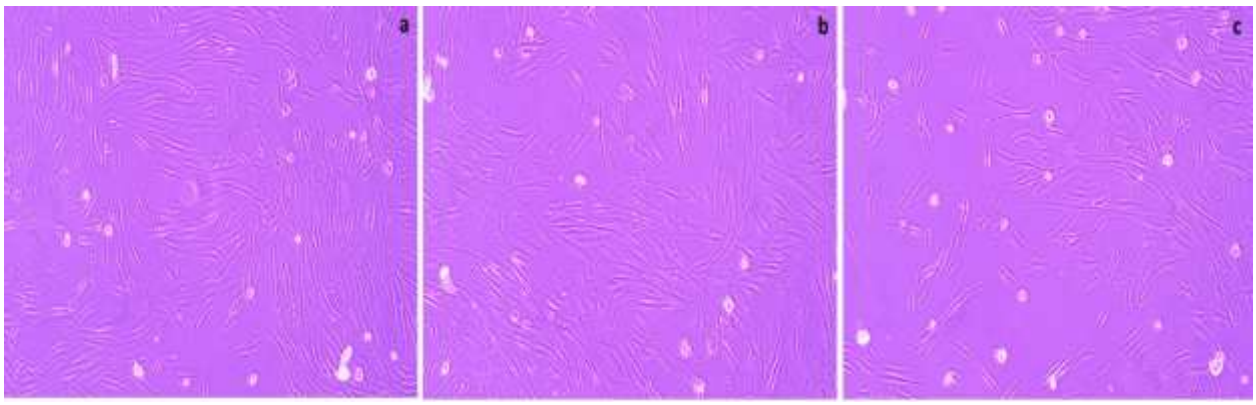


Figure 2: Cytotoxic effect of Betan-ZnONPs on PDL cells evaluated by MTT assay. Cells were treated with a) 10 µg/mL , b) 50 µg/mL, c)100 µg/mL of Betan-ZnONPs for 24 hours.

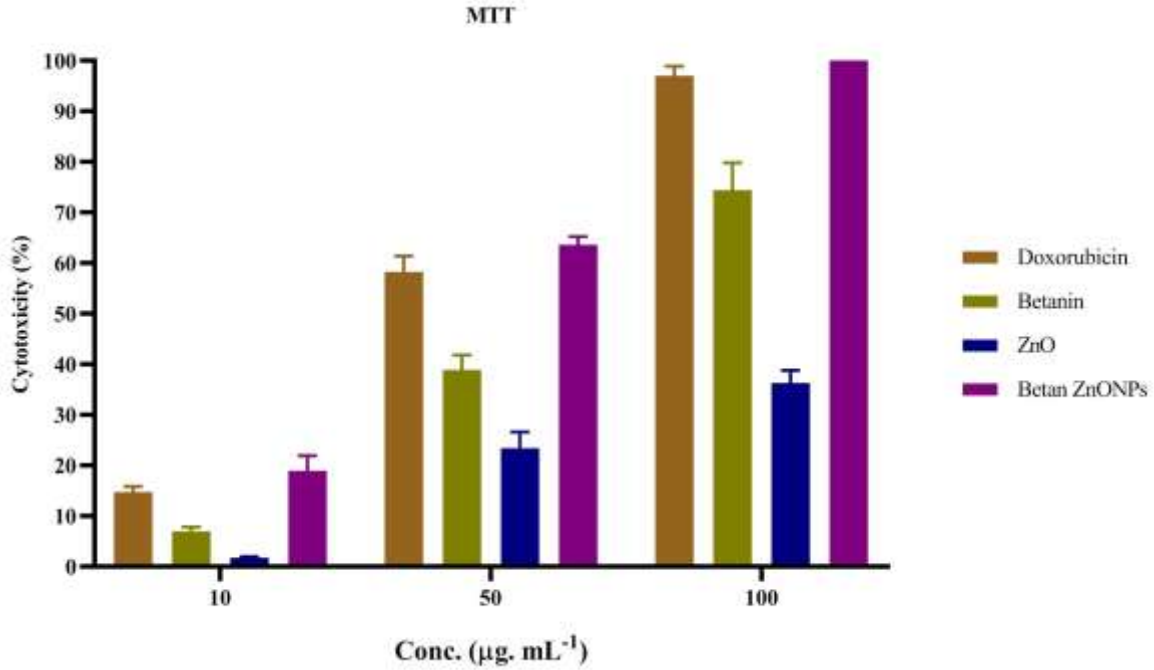


Figure 3: Bar graph representing the effect of Betan-ZnONPs on PDL cell viability was determined by MTT assay. PDL cells were treated with 10, 50, and 100 µg/mL of Betan-ZnONPs

The MTT assay was used to assess the dose-dependent cytotoxic potential of doxorubicin, ZnO, betanin, and Betan-ZnONPs. All the test chemicals show a steady decline in cell viability as concentration increases. Betan-ZnONPs had more cytotoxicity (18.95%) at 5 µg/mL than betanin (6.93%) and ZnO (1.77%). However, doxorubicin exhibited 14.75% cytotoxicity. Betan-ZnONPs remained more cytotoxic at 10 µg/mL than both betanin (38.87%) and ZnO (23.45%), and marginally more than doxorubicin (58.25%). Comparably high cytotoxicity values were demonstrated by doxorubicin and Betan-ZnONPs at 25 µg/mL (96.99% and 96.46% respectively), whereas betanin and ZnO attained 74.40% and 36.27% respectively. At higher dosages, the pattern persisted. ZnONPs produced cytotoxicity values between 61.96% and 65.21% at 50 µg/mL, which was substantially higher than those of ZnO (20.71%-26.95%) and Betanin (35.95%-41.84%). Betan-ZnONPs showed strong cytotoxicity at 100 µg/mL (95.74%-98.95%), which was close to the effects of doxorubicin (94.80%-98.26%) and much greater than that of betanin (68.38%-78.92%) and ZnO (33.96%-38.94%). These results show how Betan-ZnONPs have improved cytotoxic efficacy, outperforming the effects of either betanin or ZnO alone and coming close to the potency of the common chemotherapeutic drug doxorubicin, especially at higher concentrations

(Figure 3). The IC_{50} value was determined as 2.49 µg/mL, hence the in vitro scratch assay was done with 1µg/mL.

3.3 Scratch Assay based evaluation of cell migration

The impact of Betan-ZnONPs on the ability of migration of PDL cells were evaluated using the scratch wound healing assay (Figure 4). Cell migration was considerably increased by treatment with 1 µg/mL of Betan-ZnONPs in comparison to the untreated control. The treated group exhibited more wound closure after 24 hours, which suggests that there was accelerated cell movement into the scratched area. According to quantitative image analysis, the group treated with Betan-ZnONPs had a wound closer rate of about 80-85% compared to 40-50% in the control group. These findings support the possible use of Betan-ZnONPs to improve wound healing by showing that they encourage cell migration (Figure 5).

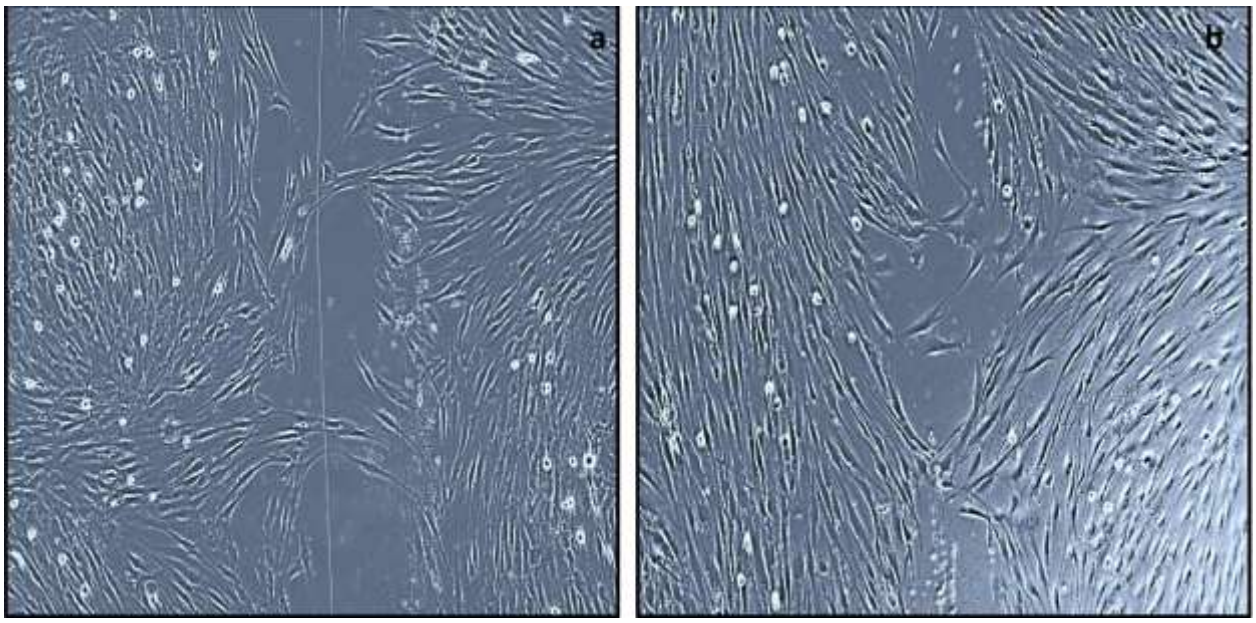


Figure 4: Scratch wound healing assay demonstrating the effect of Betan-ZnONPs on PDL cell migration at 24 hours, where a) represents untreated control group and b) 1µg/mL treated Betan-ZnONPs.

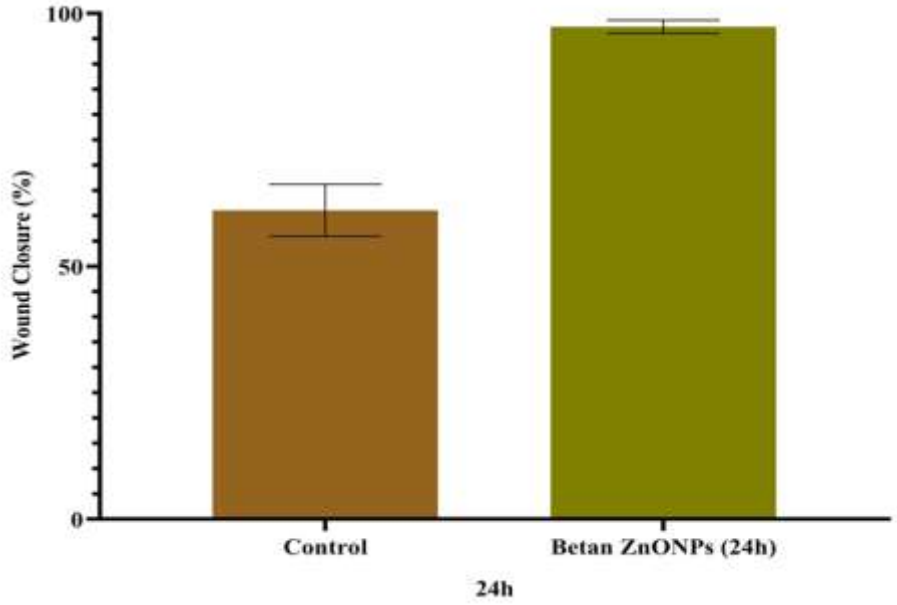


Figure 5: Wound closure percentage of PDL cells at 24 hours post-scratch. Cells treated with 1 µg/mL Betan-ZnONPs showed significantly enhanced migration compared to the untreated control group.

3.4 Wound healing progression in control and treated zebrafish

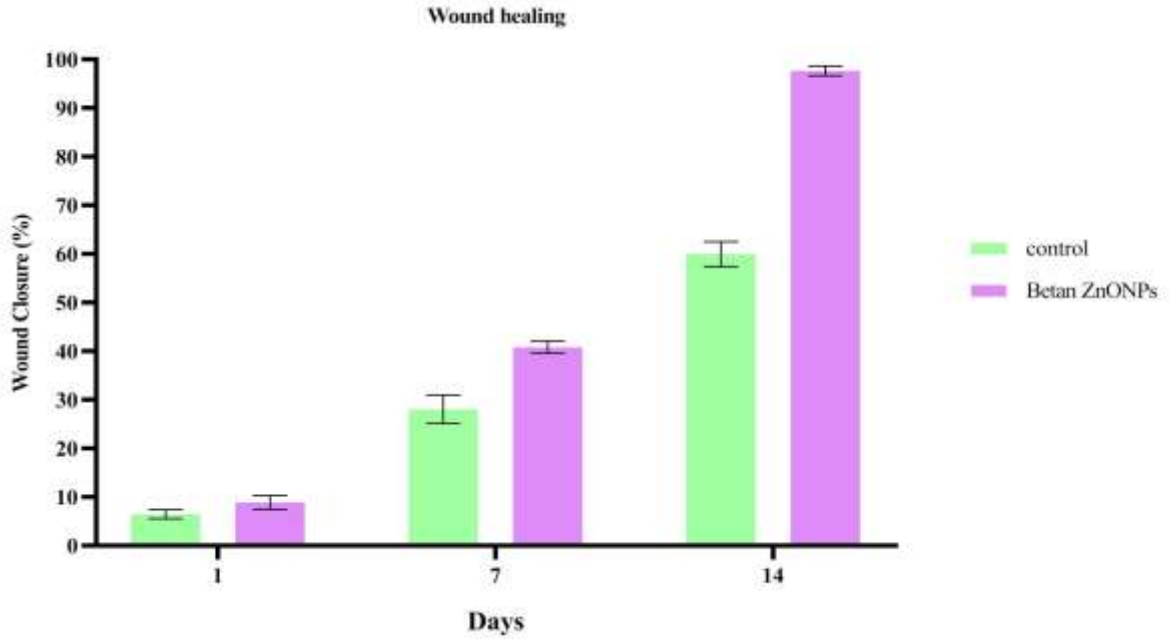


Figure 6: Graph showing percentage reduction in wound size in Day 7 and Day 14 post injury. The Betan-ZnONPs treated group (28.32 µg/mL) exhibited significantly greater wound closure compared to the untreated group.

On Day 7 and day 14 after injury, the dorsal skin region of zebrafish was used to track the healing process. There was minimal skin regeneration in the control group by day 7, and by day 14, there was still evidence of persistent inflammation and inadequate wound closure (Figure 6). An independent test for fish toxicity (OECD Test No 236) was conducted and the IC_{50} was determined to be 300 µg/mL (Data not given). The treatment concentration was established at 28.37 µg/mL based on the IC_{50} value. In contrast, the group that received Betan-ZnONPs treatment (28.37 µg/mL) showed much better healing, as seen by decreased erythema, smoother wound edges, and visible re-epithelialization as early as day 7. By day 14, the injured dorsal skin area of the treated zebrafish had almost fully healed, suggesting increased capacity for regeneration. Given their pro-regenerative, anti-inflammatory, and antioxidant qualities, our findings imply that Betan-ZnONPs help zebrafish heal their skin more quickly and efficiently (Figure 7).

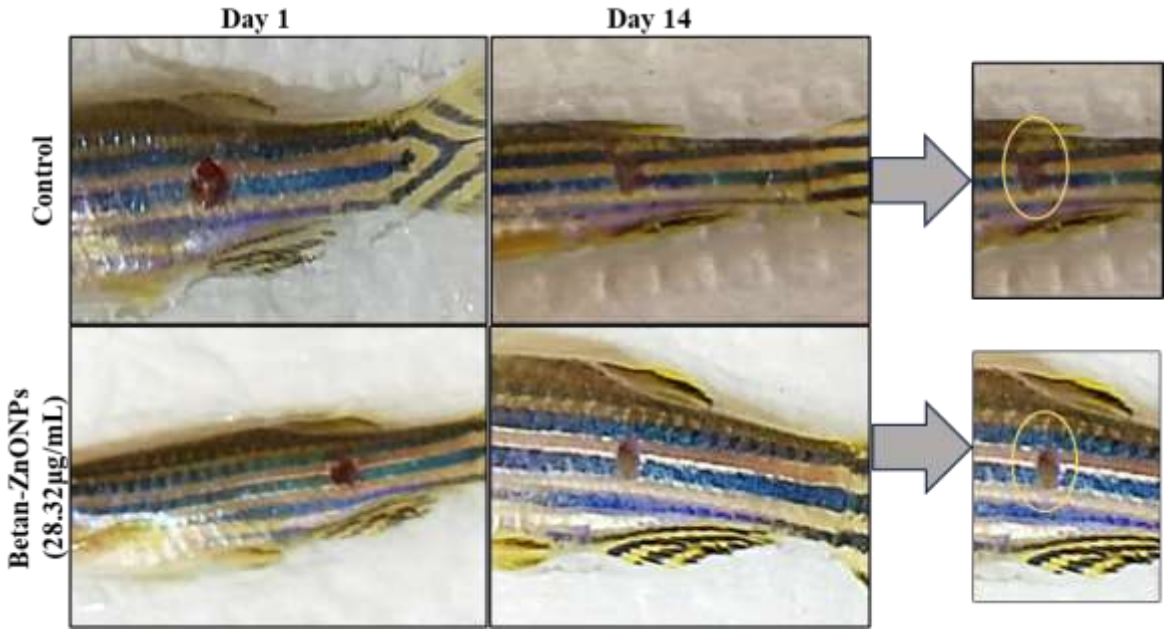


Figure 7: Representative images of wound closure in zebrafish on Day 7 and Day 14 post-treatment.

3.5 Histopathological analysis of wounded skin tissue

The control and Betan-ZnONPs related groups differed significantly, according to histological analysis of dorsal skin tissue in Day 14 after wounding. H&E stained sections of the control group revealed delayed or impaired healing loosely organized dermal tissue, chronic inflammatory cell infiltration, and partial epithelial repair. On the other hand, the treated group showed dense, organized dermal tissue, re-established epithelial layers, and well-regenerated skin architecture. The samples treated with Betan-ZnONPs showed clear signs of re-epithelialization and improved cellular organization, confirming the function of the nanoparticles in promoting efficient wound closure and fast tissue regeneration (Figure 8).

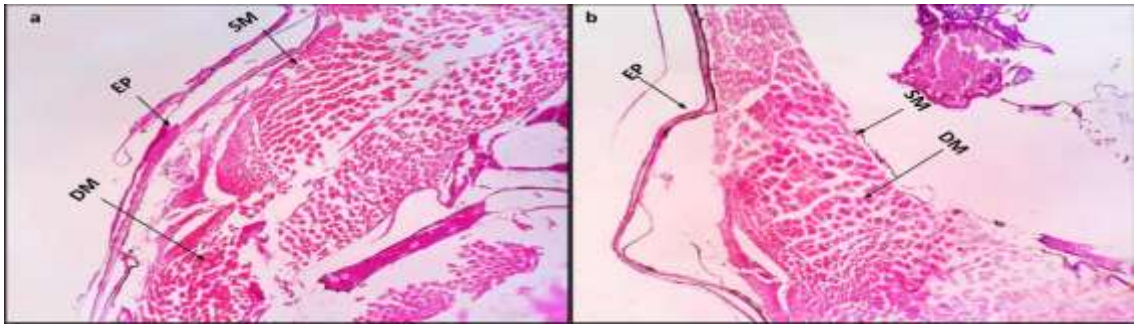


Figure 8: Histopathological comparison of wounded dorsal skin in zebrafish on day 14. Representing a) control group shows incomplete epithelial restoration, persistent inflammatory infiltration, and disorganized dermal structure and b) Betan-ZnONPs (28.32 µg/mL) displays complete re-epithelialization and reduced inflammation.
(SM-Skeletal Muscle, EP-Epithelium, DM-Densely packed muscle cells)

3.6 Gene expression analysis

The expression of genes linked to angiogenesis and apoptosis was assessed in zebrafish skin tissue on day 14 after wounding using qRT-PCR analysis. A considerable increase of pro-apoptotic markers Caspase-3, Caspase-8 and Caspase-9 was observed in the Betan-ZnONPs treated group (28.32 µg/mL) in comparison to the control group. This suggests that the programmed cell death pathways necessary for tissue remodeling have been activated. On the other hand, the anti-apoptotic gene Bcl-2's expression was significantly downregulated, which further supports the treated group's proapoptotic shift. VEGF and HIF-1α, two genes linked to angiogenesis, were also markedly increased in the treated samples, indicating improved vascularization and a hypoxic response as part of the healing process. These findings indicate that Betan-ZnONPs facilitate wound repair through a dual mechanism involving apoptosis mediated clearance of damaged cells and promotion of new blood vessel formation of new blood vessel formation (Figure 9).

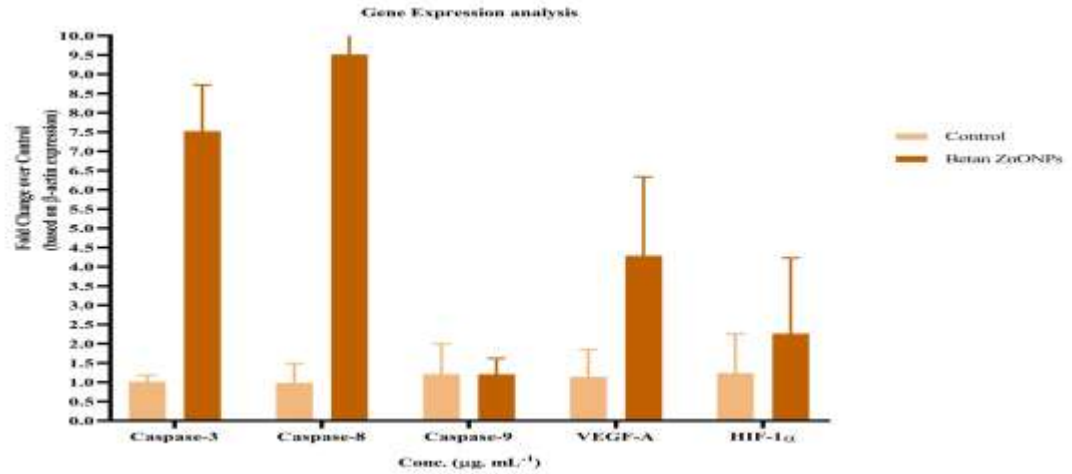


Figure 9: Relative gene expression of apoptosis and angiogenesis markers if day 14 post treatment. Bar graph representing the fold change in expression of Caspase-3, Caspase-8, Caspase-9, BCl-2, VEGF and HIF-1α in Betan-ZnONPs treated zebrafish (28.32 µg/mL) compared to the untreated control group.

4. DISCUSSION

Angiogenesis, migration, inflammation, cell proliferation, and tissue remodeling are all involved in the biologically intricate and strictly controlled process of wound healing. In recent years, the incorporation of nanotechnology based on phytochemicals has become a viable strategy to improve wound healing (23).Nanoparticles like zinc in particular have exhibited effective activity against microbes, as antioxidants and anti-inflammatory agents(24). Naturally occurring substances like cinnamon, mint and karpooravalli have also been probed to exhibit antimicrobial properties against wound pathogens(25). This work assessed the effectiveness of Betan-ZnONPs in wound healing utilizing both in vitro and in vivo models, paying special attention to cytocompatibility, cell migration, histopathological alterations, and gene expression linked to angiogenesis and apoptosis. Betanin, a naturally occurring pigment found in beetroot, was used in the green synthesis of zinc oxide nanoparticles producing stable, spherical, and crystalline nanoparticles that were verified by SEM, FTIR, EDAX, and XRD investigations. With particle sizes in the nanometer range and a consistent morphology, the SEM pictures supported their potential for interaction with biological membrane and cellular uptake. Important functional groups were detected by FTIR analysis, such as the hydroxyl and amine groups from betanin, which most likely helped to stabilize and reduce zinc ions during synthesis. The crystalline nature of the nanoparticles was confirmed by XRD patterns, and elemental purity was confirmed by EDAX, which displayed trace carbon from the capping agent and prominent peaks for oxygen and zinc (26).

According to the MTT assay, cytotoxicity showed a dose-dependent decrease in PDL cell viability, with 10 mg/mL exhibiting significant cytotoxic effects. For additional testing, a concentration of 28.32 µg/mL was chosen as it offered a balance between biocompatibility and bioactivity. Since it generated notable cellular reactions, this concentration was found suitable for wound healing applications without causing stress or cell death. The results of the scratch assay showed that Betan-ZnONPs promoted migration in PDL cells. In comparison to untreated controls, cells treated with the nanoparticle demonstrated improved wound closure after 24 hours. The capacity of Betan-ZnONPs to stimulate cell migration is crucial since dermal regeneration and wound re-epithelialization depend on fibroblast and epithelial migration. These results are consistent with other studies that highlighted the bioactivity of betanin and the regeneration potential of zinc oxide nanoparticles (27).

In vivo wound healing experiments on zebrafish provided additional confirmation of the regenerative capacity of Betan-ZnONPs . Zebrafish act as a perfect vertebrate model because of their transparent body, quick regeneration, and human-like genetic makeup. Wounds on the dorsal skin of zebrafish healed noticeably more quickly in the treated group than in the control group. The control group showed delayed epithelialization and unresolved tissue remodeling by day 14, whereas the group treated with Betan-ZnONPs showed nearly total wound closure. Histological evidence provides further support for these macroscopic observations.

Further understanding of the biological effects of the nanoparticles was made possible by histopathological analysis of the injured skin tissues. There was minimal inflammatory infiltration and no evidence of neovascularization in the tissues of the Betan-ZnONPs treated group. The epithelial and dermal layers were well organized. Opposed to this, the control group continued to exhibit loosely distributed dermal structures, inflammation and insufficient epithelial regeneration. These results confirm the anti-inflammatory and regenerative properties of Betan-ZnONPs in vivo. Using gene expression analysis, the molecular mechanisms behind the improved wound healing were examined. Pro-apoptotic markers like Caspase-3, Caspase-8, Caspase-9 were significantly upregulated in the treated group. By eliminating damaged or senescent cells, apoptosis aids in tissue remodeling and regeneration, which is essential for wound healing. The transition of the healing tissue towards regulated apoptosis was further supported by the downregulation of the anti-apoptotic protein Bcl-2. Furthermore, VEGF and HIF-1alpha, two angiogenic indicators, were shown to be significantly upregulated in the treated group. It is well known that VEGF and HIF-1α are important regulators of angiogenesis, which is necessary for providing oxygen and nutrients to the tissues that are regenerating. The antioxidant qualities of betanin may also help stabilize HIF-1α in hypoxic environments, while zinc ions are known to activate the signaling cascade. This dual modulation probably accounts for the faster vascularization and repair seen in the Betan-ZnONP group (28).

According to the collective findings, Betan-ZnONPs improve wound healing via a variety of mechanisms, including encouraging PDL cell migration and proliferation, modifying apoptotic signaling to promote tissue turnover and upregulation of angiogenic pathways to support neovascularization and nutrient supply. Given their combined anti-inflammatory, antioxidant and tissue repair capabilities, zinc oxide and betanin's synergistic effect is likely what propels its combined biological effects. Although the focus of the

study is on zebrafish and in vitro models, it is important to note that the results provide a solid foundation for translational research into mammalian models. To further the molecular knowledge, more research utilizing protein level validation, oxidative stress markers and time-course expression profiling would be beneficial.

Furthermore, the green synthesis method used in this study emphasizes the significance of environmentally friendly, non-toxic nanoparticle production techniques. Betan-ZnONPs are attractive prospects for wound healing as well as for more broader applications in tissue engineering, regenerative medicine and dermatology due to their efficacy and biocompatibility (29).

5. CONCLUSION

According to this study, Betan-ZnONPs considerably improve wound healing in both in vivo and in vitro models. At specific concentrations, the nanoparticles exhibited excellent physicochemical characteristics and biocompatibility. Histopathology indicated that Betan-ZnONPs enhanced tissue regeneration, accelerated the closure of dorsal skin wounds in zebrafish, and encouraged cell migration in PDL cells. Important apoptotic and angiogenic markers were shown to be upregulated by gene expression analysis, while Bcl-2 was downregulated. The potential of Betan-ZnONPs as a biocompatible and effective nanotherapeutic agent for wound healing applications is supported by these findings.

REFERENCES

1. Richardson R, Slanchev K, Kraus C, Knyphausen P, Eming S, Hammerschmidt M. Adult zebrafish as a model system for cutaneous wound-healing research. J Invest Dermatol. 2013 Jun;133(6):1655–65.
2. Sharif F, Steenbergen PJ, Metz JR, Champagne DL. Long-lasting effects of dexamethasone on immune cells and wound healing in the zebrafish. Wound Repair Regen. 2015 Nov-Dec;23(6):855–65.
3. Viswanath V, Wu Y, Boonplueang R, Chen S, Stevenson FF, Yantiri F, et al. Caspase-9 activation results in downstream caspase-8 activation and bid cleavage in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinson's disease. J Neurosci. 2001 Dec 15;21(24):9519–28.
4. Balaji Ganesh S, Aravindan M, Kaarthikeyan G, Martin TM, Kumar MSK, Chitra S. Embryonic toxicology evaluation of novel , bioceramics and tendon extracellular matrix incorporated scaffolds for periodontal bone regeneration using zebrafish model. J Oral Biol Craniofac Res. 2025 Mar 25;15(3):563–9.
5. Interactions of nanomaterials with cell signalling systems – Focus on purines-mediated pathways. Colloids and Surfaces B: Biointerfaces. 2022 Dec 1;220:112919.
6. Lakshmi, Dean -International Affairs, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences. Biomedical potential of zinc oxide nanoparticles synthesized using plant extracts. Int J Dent Oral Sci. 2021 Aug 21;4160–3.
7. Ravikumar OV, Marunganathan V, Kumar MSK, Mohan M, Shaik MR, Shaik B, et al. Zinc oxide nanoparticles functionalized with cinnamic acid for targeting dental pathogens receptor and modulating apoptotic genes in human oral epidermal carcinoma KB cells. Mol Biol Rep. 2024 Feb 24;51(1):352.
8. Vimal TG, Selvaraj J. GREEN SYNTHESIS OF SILVER NANOPARTICLES USING Rosmarinusofficinalis LEAF EXTRACT AND STUDY OF ANTICANCER EFFECT AND APOPTOSIS INDUCTION ON PROSTATE CANCER CELL LINE (PC-3). Journal of Pharmaceutical Negative Results. 2022 Oct 7;13.
9. Anbarasu M, Martin TM, Priya P, Sivamurugan V, Kumar MSK, Shaik MR, et al. Assessing the impact of Ag-ZnO nanoparticle on the induction of oxidative stress, hematological, and molecular changes in zebrafish (Danio rerio) and McCoy fibroblast cell lines. Aquac Int. 2024 Aug;32(4):5373–92.
10. Ragavendran C, Imath M, Kamaraj C, Nakouti I, Manoharadas S. Eco-friendly synthesis of betanin-conjugated zinc oxide nanoparticles: antimicrobial efficacy and apoptotic pathway activation in oral cancer cells. Mol Biol Rep. 2024 Nov 7;51(1):1128.
11. Garapati B, Malaiappan S, Rajeshkumar S, Murthykumar K. Cytotoxicity of lycopene-mediated silver nanoparticles in the embryonic development of zebrafish-An animal study. J Biochem Mol Toxicol. 2022 Oct;36(10):e23173.
12. Patton EE, Zon LI, Langenau DM. Zebrafish disease models in drug discovery: from preclinical modelling to clinical trials. Nat Rev Drug Discov. 2021 Aug;20(8):611–28.
13. Esteves LC, Pinheiro AC, Pioli RM, Penna TC, Baader WJ, Correra TC, et al. Revisiting the Mechanism of Hydrolysis of Betanin. Photochem Photobiol. 2018 Sep;94(5):853–64.
14. Kosa SA, Zaheer Z. Betanin assisted synthesis of betanin@silver nanoparticles and their enhanced adsorption and biological activities. Food Chem. 2019 Nov 15;298:125014.
15. Vinitha V, Anbarasu M, Priya P, Preeyanghaa M, Neppolian B, Prathap L, et al. Depolymerization of PET wastes catalysed by Sn and Ag doped ZnO nanoparticles and evaluation of Embryonic Toxicity using Zebrafish. 2023 Mar 1 [cited 2025 Aug 27]; Available from: https://www.researchsquare.com/article/rs-2626685/latest.pdf
16. Takayama S, Murakami S, Miki Y, Ikezawa K, Tasaka S, Terashima A, et al. Effects of basic fibroblast growth factor on human periodontal ligament cells. J Periodontal Res. 1997 Nov;32(8):667–75.
17. Bahuguna A, Khan I, Bajpai VK, Kang SC. MTT assay to evaluate the cytotoxic potential of a drug. Bangladesh J Pharmacol. 2017 Apr 8;12(2):8.
18. Bobadilla AVP, Arévalo J, Sarró E, Byrne HM, Maini PK, Carraro T, et al. In vitro cell migration quantification method for scratch assays. J R Soc Interface. 2019 Feb 28;16(151):20180709.
19. Lawrence C, Mason T. Zebrafish housing systems: a review of basic operating principles and considerations for design and functionality. ILAR J. 2012;53(2):179–91.

20. Ramachandran T, Mohanraj KG, Mary Martin T, K MS. Enhanced Wound Healing With β-Chitosan-Zinc Oxide Nanoparticles: Insights From Zebrafish Models. Cureus. 2024 Sep;16(9):e69861.
21. Edirisinghe SL, Nikapitiya C, Dananjaya SHS, Park J, Kim D, Choi D, et al. Effect of Polydeoxyribonucleotide (PDRN) Treatment on Corneal Wound Healing in Zebrafish (). Int J Mol Sci [Internet]. 2022 Nov 4;23(21). Available from: http://dx.doi.org/10.3390/ijms232113525
22. Okuno S, Shimizu S, Ito T, Nomura M, Hamada E, Tsujimoto Y, et al. Bcl-2 prevents caspase-independent cell death. J Biol Chem. 1998 Dec 18;273(51):34272–7.
23. Bhuyan A, Dey P, Gogoi H, Mandal S. Exploring Naturally-Derived Targeted Nano Delivery Therapy for Burn Wound Healing with Special Emphasis on Preclinical Outcomes. Curr Drug Deliv [Internet]. 2024 Dec 3; Available from: http://dx.doi.org/10.2174/0115672018343042241120072749
24. Barma MD. Synthesis of triphala incorporated zinc oxide nanoparticles and assessment of its antimicrobial activity against oral pathogens : An in-vitro study. Biosci Biotechnol Res Commun. 2020 Aug 25;13(7):74–8.
25. Monica K, Rajeshkumar S, Ramasubramanian A, Ramani P, Sukumaran G. Anti-inflammatory and antimicrobial effects of herbal formulation using karpooravalli, mint, and cinnamon on wound pathogens. J Adv Pharm Technol Res. 2022 Dec;13(Suppl 2):S369–73.
26. Probing the dominance of interstitial oxygen defects in ZnO nanoparticles through structural and optical characterizations. Ceramics International. 2014 Nov 1;40(9):14569–78.
27. Rattanasuwan K, Rassameemasmaung S, Kiattavorncharoen S, Sirikulsathean A, Thorsuwan J, Wongsankakorn W. Platelet-rich plasma stimulated proliferation, migration, and attachment of cultured periodontal ligament cells. Eur J Dent. 2018 Oct-Dec;12(4):469–74.
28. Brentnall M, Rodriguez-Menocal L, De Guevara RL, Cepero E, Boise LH. Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis. BMC Cell Biol. 2013 Jul 9;14:32.
29. Amjadi S, Nazari M, Alizadeh SA, Hamishehkar H. Multifunctional betanin nanoliposomes-incorporated gelatin/chitosan nanofiber/ZnO nanoparticles nanocomposite film for fresh beef preservation. Meat Sci. 2020 Sep;167:108161.

AUTHOR CONTRIBUTIONS

A) Amy Elizabeth Jacob - contributed in designing the study, execution of the project, statistical analysis, manuscript drafting.
B) Dr.K.Meenakshi Sundaram - contributed in designing the study, execution of the project, statistical analysis, manuscript drafting.
C) Mrs.S.Sangeetha - contributed in study design, guiding the research work, manuscript correction and publication.

ACKNOWLEDGMENT

We extend our sincere gratitude to Saveetha Dental College and hospitals for their constant support and successful completion of this work.

CONFLICT OF INTEREST

The authors hereby declare that there is no conflict of interest in this study.