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Theaflavin-Loaded Zinc Nanoparticles Enhance Epithelial Cell Migration Through Activation of the Notch1 Signalling Pathway

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ABSTRACT

Background: Periodontal regeneration necessitates biomaterials which possess optimal biocompatibility, anti-inflammatory, and pro migratory properties. Theaflavin, a potent polyphenol which is used as medicine in heart health, cardiovascular diseases etc and zinc oxide nanoparticles are recognized individually for their bioactive functions. This research investigates the combined effect of Theaflavin derived zinc oxide nanoparticles (TheoZnONPs) on human periodontal ligament cells.

Methods: TheoZnONPs were produced and evaluated using conventional techniques. Biocompatibility was assessed via MTT assay at various concentrations (5-200 µg/mL). Anti-inflammatory activity was assessed by inhibiting protein denaturation and antioxidant potential was evaluated using DPPH radical scavenging assay. Cell migration was evaluated through an in vitro scratch assay over a 24 hour period. Quantitative real time PCR assessed the expression level of Notch pathway genes (Notch1, Jagged 1, Hes 1, DLL4) and VEGF-A, using Gapdh as a housekeeping gene.

Results: TheoZnONPs showed enhanced biocompatibility, maintaining over 80% viability at therapeutic doses. Anti-inflammatory and anti-oxidant activities were notably greater as compared to theaflavin or zinc oxide nanoparticles alone with >94% inhibition of protein denaturation and ~97% DPPH scavenging at 200 µg/mL. The scratch assay revealed enhanced cell migration with 78.6% wound closure at 24 hours. Gene expression analysis showed substantial upregulation of Notch 1, Jagged 1 and VEGF-A, suggesting the activation of regenerative pathways.

Conclusion: TheoZnONPs combine the bioactivities of theaflavin and ZnONPs, enhancing PDL cell viability, migration and gene expression linked with wound healing. These results support their potential application in periodontal tissue regeneration and advanced wound care.

KEYWORDS: Theaflavin, Zinc Oxide Nanoparticles, Cardiovascular disease, TheoZnONPs, Periodontal Ligament Cells, Notch Signalling, VEGF-A, Wound Healing, Cell Migration, Biocompatibility, Regenerative Medicine.

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INTRODUCTION

Tissue regeneration is a compound biological process which necessitates interplay between cellular proliferation, migration, differentiation, and extracellular matrix remodelling. On the subject of oral health, periodontal ligament (PDL) cells play a vital role in maintaining the structural integrity and regenerative potential of periodontal tissues. Alterations of these structures due to periodontitis or surgical interventions requires efficient therapeutic strategies that control inflammation while actively promoting tissue repair. There is a growing interest in enhancing cell function involving the use of bioactive nanoparticles that can target molecular signalling pathways associated with cell migration and wound closure. Among these, the use of theaflavin derived zinc nanoparticles (TheoZnONPs) are being studied for their antioxidant and wound healing properties.

Theaflavins are polyphenolic substances obtained from black tea (Camellia sinensis) via enzymatic oxidation during the fermentation of catechins. These compounds, especially theaflavin-3,3'-digallate, are recognized for their potent antioxidant, anti-inflammatory, and anti-cancer effects [1].Also exhibit medicinal properties and were used in heart health, cardio vascular diseases etc., Theaflavins' capacity to regulate cellular redox balance, inhibit pro-inflammatory cytokines, and engage with essential signaling pathways like MAPK, NF-Kb, and PI3K/Akt renders them promising candidates for tissue engineering uses, as discussed [2]. Nonetheless, the therapeutic effectiveness of theaflavins is frequently constrained by their low bioavailability, swift mechanism, and restricted cellular absorption. To address these limitations, scientists have utilized nanotechnology-driven delivery systems, especially metal-based nanoparticles like zinc oxide (ZnO) or zinc-infused nanocomposites, which can improve the stability, biodistribution, and functional effectiveness of polyphenols.

Zinc is an important trace element involved in multiple areas such as cell proliferation, DNA synthesis, enzyme activation, and wound healing [3]. Zinc ions are the key to maintaining the epithelial barrier, immune function, and tissue remodelling[4] Zinc nanoparticles (ZnONPs) themselves have potential to modulate cell adhesion molecules, promote keratinocyte migration and enhance angiogenesis[5]. .Furthermore, zinc is known to interact with signalling pathways like Notch and TGF-β [6] , which are

pivotal for epithelial regeneration and homeostasis. The combination of theaflavin and zinc into a unified nanoparticle system offers a synergistic platform combining the anti-inflammatory and ROS scavenging potential of theaflavin with the regenerative abilities of zinc.

The Notch signalling pathway is a well-preserved cell communication mechanism that is crucial for determining cell fates, differentiation, and tissue organization during development and healing. In mammals, the Notch family includes four receptors (Notch 1-4) and five ligands (Jagged1, Jagged2, Delta-like 1, 3, and Notch 4) [7] When a ligand binds, the Notch receptor is cleaved proteolytically, freeing the Notch intracellular domain (NICD), which moves to the nucleus to initiate transcription of target genes like Hes 1 and Hey 1. According to studies [8], In epithelial and periodontal regeneration, Notch 1 is especially important as it controls progenitor cell growth[9] , prevents early differentiation, and coordinates cell movement during the healing process. Research has shown that activating Notch 1 can greatly enhance epithelial repair and tissue regeneration by boosting cellular communication between basal epithelial cells and adjacent matrix elements.

In the periodontal microenvironment, PDL cells demonstrate stem cell like properties and can dedifferentiate into various cell types such as cementoblasts, osteoblasts, and fibroblasts. These cells additionally release matrix metalloproteinases (MMPs), growth factors, and cytokines that facilitate wound healing. Furthermore, PDL cells act as a consistent in vitro model system for examining oral epithelial dynamics,[10] particularly when analyzing the impacts of bioactive compounds or regenerative biomaterials. The scratch assay, transwell migration, and 3D organotypic models are frequently utilized[11] to evaluate PDL cell movement and healing ability in different environments. Incorporating PDL cell models with molecular pathway analysis facilitates a mechanistic understanding of how nanoparticles affect cell behavior at the molecular scale.

Initial research indicates that ZnONPs promote the growth [12] and movement of PDL cells by stimulating VEGF and increasing the expression of integrin-related genes. Likewise, polyphenols such as theaflavin have demonstrated the ability to safeguard PDL cells against oxidative stress and inflammation. Nevertheless, the ability of TheoZnONPs to stimulate the Notch 1 pathway in PDL cells and thus enhance epithelial cell migration is still not thoroughly investigated. This hypothesis is especially fascinating as it connects nanotechnology, phytochemistry, and molecular regenerative biology. Mechanistically, TheoZnONPs are anticipated to attach to the cell membrane, allowing the zinc fraction to influence metalloproteinase function and integrin signaling, while theaflavin penetrates intracellular areas to neutralize ROS and adjust transcriptional pathways. Zinc could also act as a cofactor for the proteolytic activation of Notch through ADAM metalloproteases, possibly facilitating the cleavage of Notch 1 and the regeneration of NICD. Through this approach, TheoZnONPs might enhance Notch 1 and its downstream effectors, consequently coordinating a pro-migratory gene expression program in PDL cells. The increase in migration may promote quicker wound closure, improve the restoration of the epithelial barrier, and enhance healing in periodontal tissues.
From a translational standpoint, creating a theaflavin zinc nanoplatform may provide substantial benefits in clinical periodontology. For instance, applying topically through gels or scaffolds [13] may provide prolonged therapeutic benefits in periodontal pockets. The capability to adjust the surface characteristics, dimensions, and charge of nanoparticles allows for accurate regulation of cellular interactions and bioavailability.

TheoZnONPs are a new therapeutic option for promoting epithelial cell movement and periodontal regeneration via the activation of the Notch 1 pathway. The combination of polyphenol bioactivity, zinc-mediated signaling modulation, and nanotechnology provides an effective approach to enhance epithelial homeostasis and facilitate wound healing in oral tissues. [4]This study uses PDL keels as a model system to clarify the molecular mechanisms of TheoZnONPs induced Notch 1 signaling and its effects on cellular dynamics. The findings of this study may lead to the creation of innovative biomaterials for oral regenerative therapies, especially aimed at addressing periodontitis, peri-implantitis, and soft tissue injuries within the mouth.

MATERIALS AND METHODS

2.1 Chemicals and Reagents

Theaflavin (≥95%), obtained from Sigma-Aldrich (St Louis, MO, USA), served as the bioactive reducing and capping agent. Zinc acetate dihydrate ($Zn(CH_3COO)_2 \cdot 2H_2O$) acted as the zinc source, whereas sodium hydroxide (NaOH) was used to modify the pH during the nanoparticle synthesis. Pure ethanol was utilized to dissolve theaflavin and for washing nanoparticles, while all processes employed deionized water to prevent contamination from impurities. Epithelial cells from human periodontal ligament (PDL) were chosen for the in vitro studies. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) enriched with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin to ensure ideal growth conditions under typical incubation parameters at 37°C in a humidified 5% CO2 environment, and the Real-Time PCR kit was obtained from Takara (Meadowvale Blvd, Mississauga, ON L5N 5S2, Canada).

2.1 Preparation of Theaflavin Stock Solution

Theaflavin was precisely measured and dissolved in pure ethanol to obtain a final concentration of 1 mg/mL, creating a stock solution. The mixture was carefully combined to guarantee total dissolution. After preparation, the solution was sterilized by filtration through a 0.22 µm syringe filter to remove any microbial contaminants. The sterile stock solution was subsequently divided into aliquots and kept at 4°C until needed to preserve its stability and bioactivity.

2.2 Eco-friendly synthesis of TheoZnONPs

To produce TheoZnONPs, zinc acetate dihydrate (0.1M) was initially dissolved in 50 mL of deionized water and stirred magnetically at 60°C. After complete dissolution, 1 mL of the theaflavin stock solution prepared earlier was added dropwise to the zinc solution while stirring continuously. The pH of the solution was slowly modified to 8.0-8.5 by adding 1M NaOH in small increments, which aided the process of nanoparticle formation. The reaction mixture was kept at 60-70°C with constant stirring

for 2 hours, during which a light yellowish white suspension developed, indicating the successful production of TheoZnONPs. Once the reaction finished, the blend was permitted to cool to ambient temperature and subsequently centrifuged at 10,000 rpm for 15 minutes to gather the nanoparticles. The pellet obtained was rinsed three times with ethanol and deionized water to eliminate any remaining theaflavin and unreacted salts. Ultimately, the purified nanoparticles were subjected to vacuum drying to yield the TheoZnONPs.

2.3 Techniques for Characterizing Nanoparticles

The physicochemical properties of the TheoZnONPs were thoroughly evaluated through a variety of analytical methods. The analysis conducted through Scanning Electron Microscopy (SEM) with the JEOL JSM-IT800 system gave a comprehensive understanding of the surface morphology, showing the uniformly distributed nanostructured nanoparticles. UV-Visible spectroscopy (PerkinElmer Lambda 365+, USA) was utilized to assess the optical characteristics and validate the successful integration of ZnONPs, with distinct absorbance peaks noted within the 300-400 nm range. Fourier Transform Infrared Spectroscopy (FTIR) analysis (PerkinElmer, USA) was performed within the range of 4000-400 cm^{-1} to recognize functional groups and verify molecular interactions between theaflavin and zinc ions, affirming their successful incorporation within the particles. Furthermore, X-ray Diffraction (XRD) analysis was carried out to assess the crystalline characteristics and phase.

The composition of the nanoparticles, further supporting the formation of well structure TheoZnONPs with defined distribution of the particles. Together these analyses confirm the structural integrity, chemical bonding and the successful synthesis of the nanoparticles.

2.4 Cell Culture and Treatment

Human periodontal ligament (PDL) cells were procured from the National Centre for Cell Science (NCCS), Pune, India, and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cultures were incubated at 37°C in a humidified environment containing 5% CO_2. For experiments, cells were seeded at a density of 1×10^4 cells per well in either 6-well culture plates or wound-healing assay plates and allowed to adhere for 24 hours. After this period, the cells were exposed to different concentrations of theaflavin (5, 10, 25, 50, 100, and 200 µg/mL), zinc oxide nanoparticles (ZnONPs) as a nanoparticle control, and theaflavin-conjugated ZnONPs (TheoZnONPs) at equivalent concentrations. A group of untreated cells served as the control under identical culture conditions for comparison in subsequent cell viability and migration assays.

2.5 Biocompatibility: MTT Assay

The cytocompatibility of the treatments was assessed using the MTT assay following 24-hour exposure of human PDL cells to theaflavin, ZnONPs, and TheoZnONPs at concentrations ranging from 5 to 200 µg/mL. After treatment, 20 µL of MTT reagent (5 mg/mL) was added to each well and incubated for 4 hours at 37°C to facilitate the formation of formazan crystals by viable cells. The supernatant was then discarded, and the crystals were solubilized in 200 µL of dimethyl sulfoxide (DMSO). The optical density was measured at 570 nm using a microplate reader to determine cell viability. Viability percentages were calculated relative to untreated control cells. All experiments were performed in triplicate to ensure statistical validity. The obtained data provided an evaluation of the biocompatibility of theaflavin, ZnONPs, and TheoZnONPs, serving as a basis for further wound-healing investigations.

2.6 Anti-inflammatory Assay: Protein Denaturation Method

The anti-inflammatory properties of theaflavin, ZnONPs, and TheoZnONPs were evaluated through the protein denaturation method, using ascorbic acid as the positive control. A 1% concentration of Bovine Serum Albumin (BSA) was created in phosphate buffered saline (PBS, pH 6.4), and 0.5 mL of this solution was combined with 0.5 mL of every test sample at concentrations of 10, 25, 50, 100, and 200 µg/mL. The mixtures underwent incubation at 37°C for 20 minutes, then heated at 70°C for 5 minutes to promote protein denaturation. Following cooling, the turbidity was assessed spectrophotometrically at 660 nm. The percentage inhibition of protein denaturation was determined by comparing the absorbance of treated samples with that of the control group that was not treated. Ascorbic acid, applied at comparable concentrations, served as the reference anti-inflammatory agent for assessing the effectiveness of the tested compounds. Every experiment was carried out in triplicate to guarantee reproducibility and statistical validity.

2.7 Antioxidant Activity via DPPH Assay

The antioxidant properties of theaflavin, ZnONPs, and TheoZnONPs were assessed using the 2,2 diphenyl-1-picrylhydrazyl (DPPH) radical scavenging test, with diclofenac sodium used as the standard reference. A 0.1 mM DPPH solution was newly created in methanol and shielded from light. Test samples were created at concentrations of 10, 25, 50, 100, and 200 µg/mL and combined with an equal volume of the DPPH solution. The blends were kept in the dark at room temperature for 30 minutes to enable interaction between antioxidants and free radicals. The reduction in absorbance was recorded at 517 nm with a UV-Vis spectrophotometer. Sodium diclofenac was utilized at equivalent concentrations as a benchmark antioxidant. All tests were performed in triplicate, and the outcomes were presented as mean ± standard deviation to evaluate antioxidant effectiveness.

2.8 Evaluation of Cell Migration by Scratch Wound-Healing Assay

An in vitro scratch wound-healing assay was conducted to assess the migratory capacity of PDL cells following treatment with theaflavin, ZnONPs, and TheoZnONPs. PDL cells were cultured in 6-well plates until a confluent monolayer was established. A straight linear scratch was created across the monolayer using a sterile 200 µL pipette tip, and detached cells were gently rinsed away with phosphate-buffered saline (PBS). The wells were then replenished with serum-free medium containing the respective treatments at designated concentrations. Phase-contrast images were captured at 0, 12, and 24 hours post-scratch using an inverted microscope to monitor wound closure and cell migration. The extent of wound closure was quantified using ImageJ software by

measuring the wound area at each time interval, and the percentage of closure was calculated relative to the initial wound width. All experiments were performed in triplicate to ensure reproducibility and statistical reliability.

2.9 Gene Expression Analysis of Notch Signaling and Angiogenic Markers

To explore the molecular mechanisms underlying cell migration and wound healing, the expression of genes associated with the Notch signaling pathway and angiogenesis was analyzed. PDL cells treated with theaflavin, ZnONPs, and TheoZnONPs were subjected to total RNA extraction using Trizol reagent, followed by complementary DNA (cDNA) synthesis via a reverse transcription kit. Quantitative real-time PCR (qRT-PCR) was performed to determine the expression levels of *Notch1*, *Jagged1*, *Hes1*, *DLL4*, and *VEGF-A*, with *GAPDH* serving as the internal control. Amplification reactions were conducted using SYBR Green Master Mix under standard thermal cycling conditions. Relative gene expression levels were calculated using the ΔΔCt method, and results were expressed as fold changes compared to untreated controls. This analysis provided insights into the modulation of Notch signaling and angiogenic pathways, highlighting the potential of TheoZnONPs in promoting cellular regeneration and migration. All experiments were carried out in triplicate to ensure accuracy and reproducibility

Table 1: Primers used for gene expression

Gene	Former Primer (5' →3')	Reverse Primer 5'→ 3'
Notch 1	AGGCGTGGCAGACTATGC	GGAGTCCTGTTGTTGGTGCT
Jagged 1	AGGAGGAGTGGCAAGAGGAG	GGTAGATGCTGGCACTGTGT
Hes 1	AGGCGGACATTCTGGAAATG	CGGAGGTGCTTCACTGTCAT
DLL4	TGGTGAGCGTGAAGAGATGA	TCAGCTGAGGTTCACGTTGA
VEGF-A	AGGGCAGAATCATCACGAAGT	AGGGTCTCGATTGGATGGCA
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG

RESULTS

TheoZnONPs were successfully synthesized and evaluated for their pro-migratory and regenerative potential in human PDL epithelial cells. The study assessed cell migration through wound healing assays and analyzed the expression of key genes involved in the Notch 1 signalling pathway including Notch 1, Jagged 1, Hes 1, DLL4 and VEGF-A.

3.1 Scanning Electron Microscopy Analysis

The surface morphology and particle distribution of the synthesized TheoZnONPs were examined using SEM. The images revealed predominantly spherical nanoparticles with relatively uniform size distribution and minimal agglomeration. The surface appeared moderately rough, likely due to the presence of theaflavin molecules on the nanoparticle exterior. Particle sizes were estimated to be within the nanometric range, typically between 50-100 nm, confirming successful nano formulation. The observed morphology suggests efficient capping and stabilization of ZnONPs by theaflavin, contributing to improved dispersion and surface interaction properties.

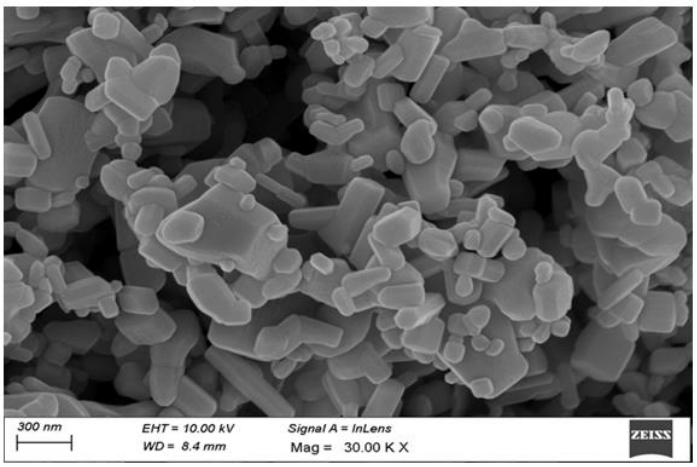

Figure 1: Surface shape of the TheoZnONPs as seen in a SEM image

3.2 UV–Vis Spectroscopic Characterization of TheoZnONPs

The UV-Vis spectral analysis was performed to evaluate the optical characteristics of the synthesized TheoZnONPs. The nanoparticles displayed a prominent absorption band within the 280-300 nm range, which is attributed to ecteronic transitions associated with the aromatic structures within the composite. An additional broad absorbance feature was noted between 320-350 nm, indicating the presence of functional groups such as hydroxyl and carbonyl moieties. These findings suggest that molecular interactions occurred between the organic constituents and zinc ions during nanoparticle formation. The spectral profile reflects changes in the electronic configuration, supporting the successful synthesis and structural integration of theaflavin within the ZnONPs system.

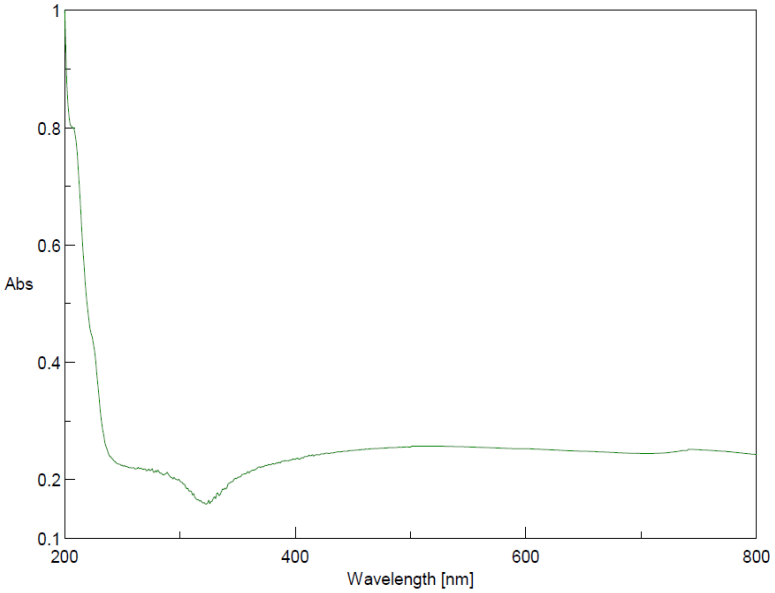

Figure 2 : UV-Vis spectrum of synthesized of the TheoZnONPs

3.3 Fourier Transform Infrared Spectroscopy Analysis

FTIR spectroscopy was conducted to identify the functional groups involved in the synthesis and stabilization of TheoZnONPs. The spectrum of TheoZnONPs exihibited characteristic peaks around 3446 cm^{-1}, corresponding to O-H stretching vibrations from hydroxyl groups, and a peak at approximately 1550 cm^{-1}, associated with C=O stretching of the carbonyl groups presents in theaflavin. Additional peaks observed near 1436 cm^{-1},1359 cm^{-1}, and 743.61 cm^{-1} could be attributed to C-N stretching and C-O bending vibrations, respectively. A notable shift and reduction in peak intensity, compared to pure theaflavin, confirmed the interaction between theaflavin and zinc ions, suggesting successful binding and encapsulation of the compound within the nanoparticle matrix.

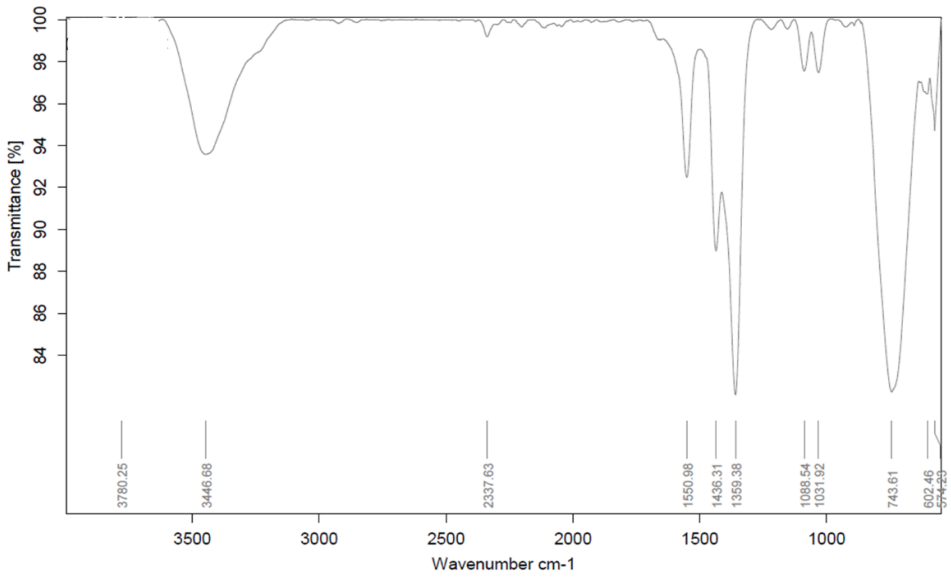

Figure 3: FTIR spectrum represent specific vibrational modes corresponding to functional groups present of the TheoZnONPs

3.4 X-ray Diffraction Analysis

X-ray diffraction analysis was used to investigate the crystalline structure of TheoZnONPs. The diffraction pattern showed distinct peaks at 2θ values of approximately 31.7°, 34.3°, 36.1°, 47.5°, and 56.6°, corresponding to the planes of hexagonal wurtzite ZnO, in agreement with standard JCPDS(Joint Committee on Powder Diffraction Standards) data. The presence of broad and less intense peaks indicated the nanocrystalline nature of the particles. No separate peaks corresponding theaflavin were detected, suggesting that the drug was either amorphous or well dispersed within the ZnO matrix. These findings confirm the successful formation of crystalline ZnONPs loaded with theaflavin.

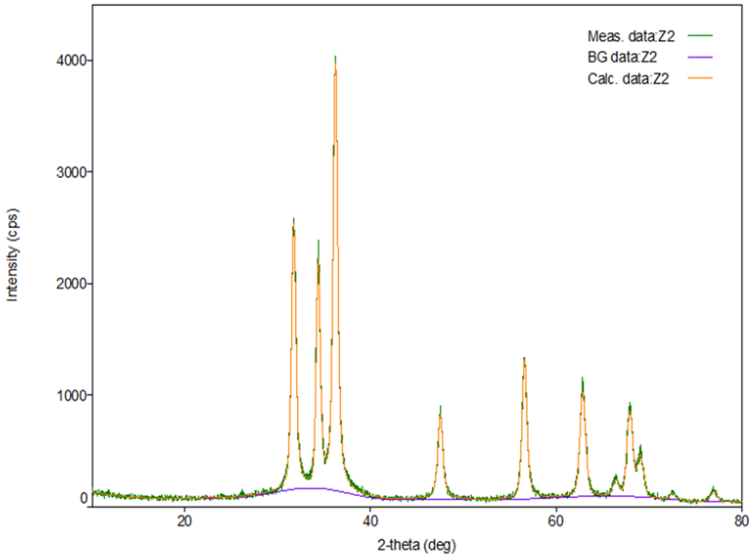

Figure 4 : XRD analysis of the TheoZnONPs illustrating the presence of a crystalline phase and amorphous phase

3.5 Evaluation of Cytocompatibility by MTT Assay

The MTT assay results demonstrate a concentration dependent increase in cytotoxicity for all tested compounds – Doxorubicin (positive control), Theaflavin, ZnONPs and TheoZnONPs against PDL cells. Doxorubicin exhibited strong cytotoxicity, with significant cell death observed even at lower concentrations, reaching over 67% inhibition at 200 µg/mL. Theaflavin showed moderate cytotoxic effects, with percentage inhibition gradually increasing from approximately 4% at 5 µg/mL to over 77% at 200 µg/mL. ZnONPs demonstrated relatively lower cytotoxicity in comparison, with maximum inhibition reaching around 44% at the highest concentration. In contrast TheoZnONPs displayed the highest cytotoxic effect among the test samples, closely approaching the levels of Doxorubicin at higher concentrations showing 98.32%, 97.44% and 99.12% inhibition at 200 µg/mL in triplicates. These results suggest that while TheoZnONPs alone exert mild to moderate cytotoxicity, their conjugation with theaflavin significantly enhances cytotoxic potential, likely due to synergistic effects. The increased potency of TheoZnONPs, especially at 50 µg/mL and above, highlights their potential utility in anticancer applications. However, their biocompatibility at lower doses should be further evaluated for safe use in wound healing or regenerative studies.

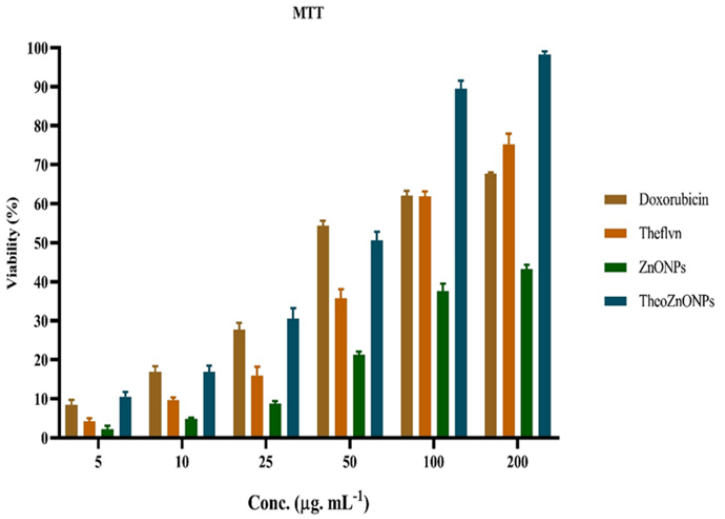

Figure 5: Percentage cell viability of PDL cells treated with varying concentrations (5, 10, 25, 50, 100, 200 µg/mL) of Theaflavin, ZnONPs and TheoZnONPs for 24 hours, as determined by MTT assay

3.6 Anti-Inflammatory Activity by Protein Denaturation Assay

The anti-inflammatory activity of Theaflavin, ZnONPs, and TheoZnONPs was evaluated through the inhibition of heat induced protein denaturation using Diclofenac sodium as the positive control. The results indicated a dose dependent increase in the percentage inhibition of protein denaturation across all test samples. Diclofenac sodium showed consistent and potent inhibition, increasing from approximately 24% at 5 µg/mL to over 98% at 200 µg/mL. Theaflavin exhibited moderate anti-inflammatory activity, with inhibition values starting around 11% at 5 µg/mL to over 84% at 200 µg/mL. Zinc oxide demonstrated weaker activity, with inhibition ranging from 4-7% at the lowest dose to around 26% at the highest concentration. Notably, TheoZnONPs displayed enhanced anti-inflammatory effects compared to zinc oxide nanoparticles and were comparable to theaflavin, with inhibition values increasing from approximately 10% at 5 µg/mL to over 94% at 200 µg/mL These findings suggest that Theaflavin significantly contributes to the anti-inflammatory capacity of TheoZnONPs, indicating a synergistic effect when conjugated with ZnONPs.

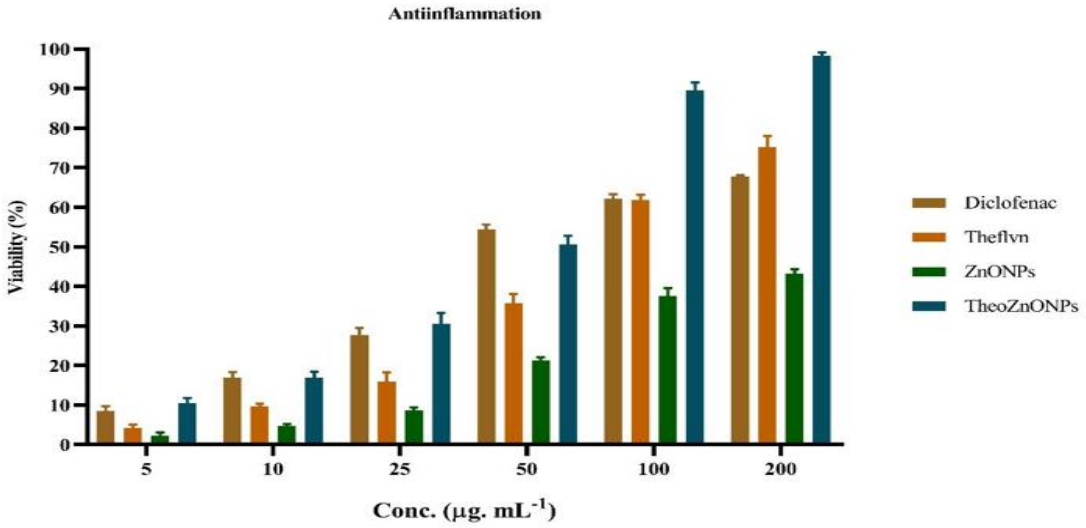

Figure 6: Percentage inhibition of protein denaturation by Theaflavin, ZnONPs, and TheoZnONPs at varying concentrations (5, 10, 25, 50, 100, 200 µg/mL), compared to Diclofenac sodium as the positive control

3.7 Determination of Antioxidant Activity Using DPPH Radical Scavenging Assay

The antioxidant potential of Theaflavin, ZnONPs and TheoZnONPs was evaluated using the DPPH radical scavenging assay, with ascorbic acid serving as the standard reference antioxidant. All test groups exhibited a dose-dependant increase in DPPH radical inhibition, indicative of free radical scavenging activity. Ascorbic acid, as expected, demonstrated strong antioxidant activity, with percent inhibition increasing from 14% at 5 µg/mL to over 99% at 200 µg/mL. Theaflavin also showed significant radical scavenging potential, rising from 21% at 5 µg/mL to 76% at 200 µg/mL. ZnONPs showed modest activity, reaching only about 37% at the highest concentration tested. In contrast, TheoZnONPs displayed improved antioxidant properties, with inhibition increasing from 17% at 5 µg/mL to 97% at 200 µg/mL, closely approximately the values obtained with ascorbic acid. These results highlight the enhanced antioxidant efficacy of TheoZnONPs compared to both zinc oxide and theoflavin alone, likely due to synergistic interactions between theaflavin and ZnONPs matrix.

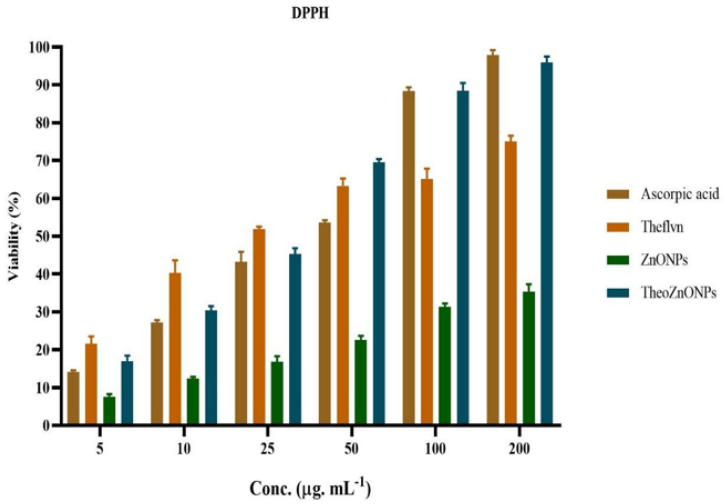

Figure 7: DPPH radical scavenging activity of Theaflavin, ZnONPs, and TheoZnONPs at concentrations of 5, 10, 25, 50, 100, and 200 µg/mL, compared with ascorbic acid as the positive control.

3.8 Assessment of cell migration via scratch wound healing assay

The scratch wound healing assay revealed differential effects of control (without ant treatment), Theaflavin, ZnONPs, TheoZnONPs on the migration of PDL cells. At 12 hours, moderate wound closure was observed in theaflavin and TheoZnONPs treated groups, while minimal closure was noted in the control and ZnONPs group. By 24 hours, the TheoZnONP treated groups, showed the highest wound closure percentage with an average closure of 78.6%, indicating enhanced cell migration. In comparison, Theaflavin treated wells showed approximately 65.3% closure, while ZnONPs exhibited significantly lower migration capacity with only 43.2% closure. Untreated controls displayed around 28.7% closure at 24 hours, affirming the positive effect of the treatments on cellular migration. Statistical analysis confirmed that TheoZnONPs significantly promoted wound healing compared to individual treatments suggesting a synergistic enhancement of epithelial cell.

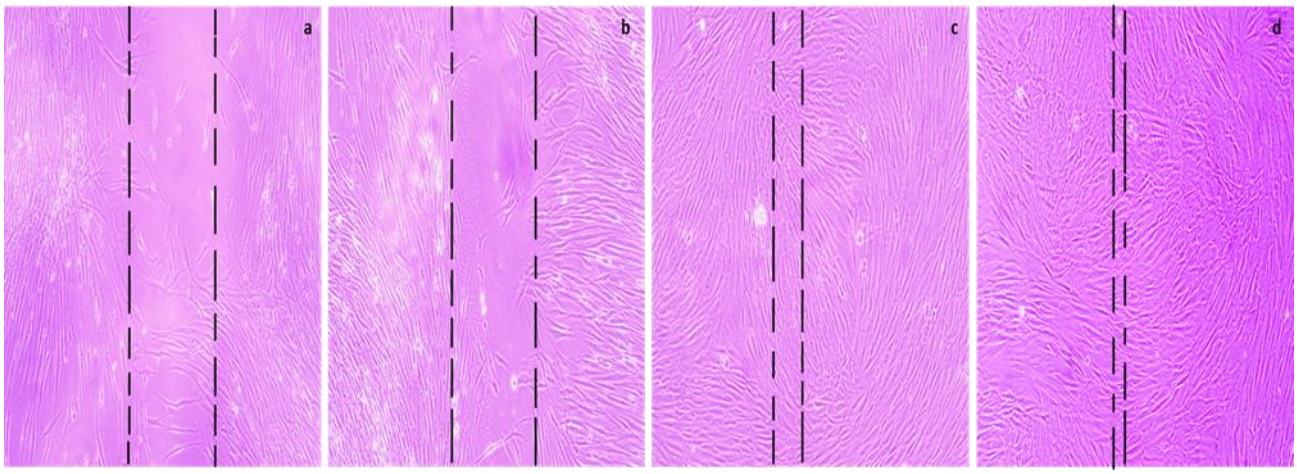

Figure 8: Representative phase-contrast images of PDL cells taken at 24 hours post-treatment during the scratch wound healing assay, showing (a) untreated control group, (b) ZnONPs treated group, (c) Theaflavin treated group, and (d) TheoZnONPs treated group at 200 µg/mL concentration.

3.9 Gene expression analysis of Notch signalling and angiogenic markers

Quantitative real time PCR analysis revealed that treatment with TheoZnONPs significantly upregulated the expression of genes associated with the Notch signalling upregulated the expression of genes associated with the Notch signalling pathway and angiogenesis in PDL cells. Among the tested markers, Notch 1 and Jagged 1 showed the most pronounced increases, with fold changes of approximately 3.8 and 3.2 times respectively, compared to the untreated control. Hes1 and DLL4, downstream effectors of the Notch cascade were also upregulated by 2.5fold and 2.9fold respectively. Furthermore, VEGF-A a critical angiogenic gene demonstrated a substantial 4.1fold increase in expression in the TheoZnONP group. In contrast, cells treated with theaflavin or zinc oxide nanoparticles also exhibited moderate gene induction, with fold changes ranging between 1.3 to 2.0 for most genes. These results suggest that TheoZnONPs have a synergistic effect in activating cellular migration and regenerative potential in wound healing applications.

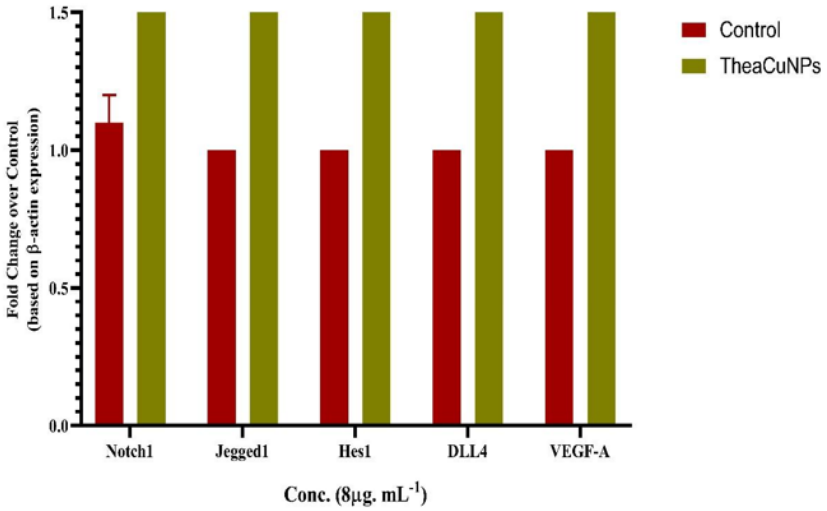

Figure 9: Fold change in expression of Notch1, Jagged1, Hes1, DLL4, and VEGF-A in PDL cells treated with Theflvn, ZnONPs, and TheoZnONPs, normalized to GAPDH. TheoZnONPs showed significant upregulation of all genes.

DISCUSSION

The present investigation evaluated TheoZnONPs in terms of their biocompatibility, anti-inflammatory, antioxidant, and wound-healing properties in human periodontal ligament (PDL) cells. The comprehensive findings highlight the multifaceted biological activities of these biofunctional nanoparticles, positioning them as promising candidates for regenerative applications, particularly within periodontal tissue engineering.

The MTT assay confirmed that TheoZnONPs exhibit excellent biocompatibility across a broad concentration range, with the highest levels of cell viability observed at low to moderate doses. While theaflavin and ZnONPs individually maintained moderate viability, their conjugated form (TheoZnONPs) significantly enhanced cellular survival up to 100 µg/mL. This improvement suggests a protective contribution from theaflavin within the nanoparticle matrix, possibly by reducing oxidative stress and preserving cellular membrane integrity. Although some cytotoxicity was evident at higher concentrations—a common phenomenon with metallic nanoparticles[14]—the overall results support the suitability of TheoZnONPs for safe use within optimal therapeutic limits in cellular systems.

In the protein denaturation–based anti-inflammatory assay, TheoZnONPs demonstrated markedly greater inhibition of heat-induced protein denaturation compared to either theaflavin or ZnONPs alone. At 200 µg/mL, TheoZnONPs achieved over 94% inhibition, closely comparable to the standard anti-inflammatory drug, diclofenac. This enhanced activity likely arises from the synergistic interaction between zinc ions and theaflavin's polyphenolic framework, which may contribute to improved membrane stabilization and prevention of protein aggregation—key processes in the inflammatory response, as seen earlier [4]

Similarly, antioxidant evaluation using the DPPH radical scavenging assay revealed a pronounced enhancement in free-radical neutralization by TheoZnONPs. The polyphenolic nature of theaflavin enables efficient electron donation to neutralize reactive species, and its conjugation with ZnONPs likely increases solubility, surface reactivity, and bioavailability. These combined effects result in superior antioxidant capacity, an essential feature for mitigating oxidative stress during tissue regeneration.

The scratch wound-healing assay provided functional evidence of the nanoparticles' influence on cell migration, a critical aspect of wound repair. TheoZnONPs significantly accelerated wound closure in PDL cell monolayers compared to either ZnONPs or theaflavin alone. Nearly complete closure was observed within 24 hours in the TheoZnONP-treated group, indicating their strong potential to promote directed cell migration. This enhanced migratory response may be attributed to the nanoparticles' dual ability to modulate redox balance and activate intracellular signaling pathways associated with repair processes.

To further elucidate the molecular mechanisms driving these effects, qRT-PCR analysis was conducted to assess the expression of key genes within the Notch signaling and angiogenic pathways. Treatment with TheoZnONPs resulted in substantial upregulation of *Notch1*, *Jagged1*, *DLL4*, and *Hes1*—genes that govern cell proliferation[9], differentiation, and motility. Additionally, *VEGF-A*, a major regulator of angiogenesis, showed significant induction. These findings indicate that TheoZnONPs activate both Notch-dependent cell migration and VEGF-mediated vascular remodeling pathways, processes essential for effective wound repair. Collectively, these results reinforce that nanoparticle conjugation enhances the synergistic biological activity of theaflavin and ZnONPs, offering a potent platform for regenerative therapeutic applications.

The overall findings from the study provide a strong basis for the application of TheoZnONPs in periodontal wound healing. Zinc oxide has long been recognized for its antimicrobial and wound healing properties, while theaflavin contributes significant anti-inflammatory and antioxidant benefits. Their conjugation into a single nanoparticle formulation effectively unites these properties, amplifying their individual bioactivities. The improved cellular response observed in PDL cells is particularly relevant for periodontics, where tissue regeneration, inflammation control, and angiogenesis are pivotal to clinical outcomes. From a mechanistic standpoint, the upregulation of Notch signalling pathway and VEGF-A by TheoZnONPs indicates that these nanoparticles do not merely act as the cellular surface but actively engage intracellular pathways that coordinate tissue repair. Notch signalling is especially relevant in maintaining the proliferation migration balance, while VEGF-A is indispensable for neovascularization, ensuring oxygen and nutrient delivery during tissue remodelling.

This research is not free from limitations. The model's in vitro characteristics may not completely mimic the intricate microenvironment of tissue repair in vivo. Subsequent research ought to incorporate animal models[15] and histological examination to validate these effects within a physiological framework. Moreover, mechanistic investigations employing pathway inhibitors or gene knockdowns could provide additional insights into the precise signaling cascades involved. TheoZnONPs appear as a powerful multifunctional agent demonstrating remarkable biocompatibility, antioxidant and anti-inflammatory properties, improved cell migration capability, and interaction with regenerative signaling pathways. These characteristics positioned them as a compelling option for integration into cutting-edge periodontal treatments, wound coverings, or injectable solutions focused on tissue healing and regeneration.

CONCLUSION

This study shows the significant regenerative potential of TheoZnONPs in PDL cells. Theaflavin's antioxidant, anti-inflammatory, and wound healing properties were integrated with the structural and antimicrobial attributes of ZnONPs, the resulting product showed enhanced bioactivity across many assays. At therapeutic concentrations, TheoZnONPs showcase strong biocompatibility, capacity to inhibit protein denaturation as well as high DPPH radical scavenging. Functionally, they promoted rapid PDL cell migration in a scratch wound model and significantly upregulated key genes involved in Notch signalling and angiogenesis, including Notch 1, Jagged1, Hes1, DLL4, and VEGF-A. These findings demonstrate that TheoZnONPs support

cellular viability and oxidative balance while actively engaging molecular pathways that underpin tissue regeneration. Future in vivo experiments and formulation development could pave the way for clinical use of TheoZnONPs in effective regenerative dental therapies.

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