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Original Article

Theaflavin Attenuates Arecoline-induced M1 Polarization and Promotes Homeostasis in Human Periodontal Ligament Fibroblasts

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ABSTRACT

Background: Periodontal Ligament cells (PDL) functions as a bridge between the tooth and bone and playing a vital role in oral health. Periodontitis is an inflammatory condition that leads to dysfunction of Periodontal Ligament (PDL) cells, resulting in pyroptosis. Arecoline is a well-known alkaloid that produce excessive pro-inflammatory cytokines, reactive oxygen species lead to Pyroptosis of PDL cell line. Hence, many bioactive compounds play a vital role on anti-inflammatory activities. **Objectives:** This study aimed to the ameliorative effect of Theaflavin on Arecoline induced inflammation of human PDL fibroblasts based on the periodontal regeneration. **Materials and Methods:** IC_{50} of Arecoline, Dexamethasone and Theaflavin, was determined by MTT assay. Inflammation was induced by 64.44 µM of arecoline and further treated with different concentration (1, 5, and 10 µM) of theaflavin and evaluated for its morphological changes. Dexamethasone was used as positive control. To explore the molecular effects, quantitative Real-Time PCR (qRT-PCR) was used to examine the mRNA expression of five key osteogenic and extracellular matrix genes: RUNX2, ALPL, COL1A1, SPP1, BGLAP, with GAPDH serving as the internal control. Statistical analysis was done using one-way ANOVA with Dunnett's *post-hoc* test, setting significance at $p<0.05$. **Results:** The Arecoline reduced the viability and osteogenesity of the PDL cells. Theaflavin treatment resulted in the restoration of PDL cells even under Arecoline exposure. Eventually it modified the expression of RUNX2, ALPL, COL1A1 indicating a reactivation of osteogenic differentiation pathways and redox balance. **Conclusion:** These findings highlighted TF's potential as a natural, biocompatible option for supporting periodontal health and countering the oral toxicity associated with arecanut.

Keywords: Arecoline, Cancer, Health risk, Periodontal ligament fibroblasts, Public health, Theaflavin, Tobacco use.

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INTRODUCTION

Periodontal disease is a chronic inflammatory condition that slowly destroys the supporting tissues of the teeth. This includes the gums, Periodontal Ligament (PDL), cementum, and alveolar bone. It is one of the most common oral diseases around the world. The disease involves complex interactions among microbial plaque, host immune responses, and environmental or lifestyle factors (Aga *et al.*, 2023). The delicate balance between inflammation and tissue remodelling in the periodontium determines whether the disease progresses or heals. Ongoing inflammation causes the breakdown of the extracellular matrix, resorption of alveolar bone, and ultimately, tooth loss. Thus, the ability of PDL fibroblasts to keep tissue stable and regenerate periodontal structures is key to maintaining oral health (Bilal *et al.*, 2025).

As far as external agents causing harmful effects to periodontal inflammation, the chewing of betel nut (*areca catechu*) is particularly destructive. Betel nut is chewed regularly by countless Asian populations and is implicated in periodontal destruction, oral Submucous Fibrosis (OSMF), and oral carcinogenesis. Arecoline is the major alkaloid in betel nut that evokes severe cytotoxic, genotoxic, and inflammatory responses in oral tissues. The effect of arecoline alters the metabolism of fibroblasts, decreases collagen biosynthesis, and increases the release of inflammatory cytokines. Repeated exposure leads to the fibrotic remodeling of the connective tissue and loss of periodontal architecture. The harmful effects of arecoline underscore the

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necessity of finding agents that we can recover from arecoline induced cytotoxic affects, and restore cellular activity in periodontal tissues (Cen *et al*., 2025; Dandagi *et al*., 2024).

Mechanistically, arecoline disturbs the redox equilibrium of cells by depleting intracellular Glutathione (GSH) and generating excessive Reactive Oxygen Species (ROS). The resulting oxidative stress activates a network of stress responsive signaling pathways, including Nuclear Factor -κB (NF-κB), Mitogen-Activated Protein Kinases (MAPKs), and transforming growth factor-β/Smad (TGF-β/Smad) cascades. Activation of these pathways amplifies the expression of Interleukin-1β (IL-1β), Interleukin-6 (IL-6), Tumor Necrosis Factor-α (TNF-α), inducible Nitric Oxide Synthase (iNOS), and Cyclooxygenase-2 (COX-2), driving fibroblasts toward a sustained M1-like pro-inflammatory phenotype (De Jong *et al*., 2017). This polarization state disrupts the natural reparative balance by favouring catabolic over anabolic processes. In parallel, arecoline enhances the expression the expression of α-Smooth Muscle Action (α-SMA), Collagen type 1 (CO1A1), TGF-β1, promoting fibrogenesis rather than regeneration. These molecular changes collectively compromise the vitality, proliferation, and migration capacity of PDL fibroblasts, which are essential for periodontal repair following injury (Dong *et al*., 2025).

Recent evidence indicates that oxidative stress and inflammation are intimately linked phenomena in periodontal pathology. Persistent ROS generation not only causes direct cellular damage but also sustains the inflammatory cycle by activating redox-sensitive transcription factors. Conversely, inadequate antioxidant defense contributes to prolonged cytokine release and impaired wound healing. Therefore, agents that can simultaneously modulate oxidative stress and inflammatory signalling are of high therapeutic relevance for maintaining PDL homeostasis. In this context, natural dietary polyphenols have attracted significant interest due to their broad pharmacological activity, minimal toxicity, and cost effectiveness (Heinämäki *et al*., 2025).

Polyphenols such as curcumin, resveratrol, and Epigallocatechin Gallate (EGCG) are well-known for their ability to scavenge free radicals, inhibit inflammatory cascades, and promote differentiation in oral cells. Among these, Theaflavin (TF), a characteristic pigment derived from the enzymatic oxidation of catechins during black tea fermentation, has emerged as a potent bioactive molecule. Structurally, TFs possess a benzotropolone ring that confers strong radical scavenging ability and facilitates metal ion chelation, contributing to their antioxidant efficacy. Several derivates exist, including theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate, and theaflavin-3,3′-digallate, all of which have shown distinct biological properties in various tissue systems (Kapasi *et al*., 2026).

Theaflavins have been reported to suppress inflammatory responses through inhibition of NF-κB and MAPK signalling, thereby downregulating COX-2, iNOS, and pro-inflammatory cytokine. Concurrently, they activate the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, leading to increased expression of cytoprotective enzymes such as heme oxygenase-1 (HO-1), NAD(P)H Quinone Oxidoreductase-1 (NQO1), and Superoxide Dismutase (SOD). By orchestrating this drug action, suppressing pro-inflammatory mediators while enhancing antioxidant defenses, TFs may restore the redox and signaling balance disrupted by arecoline exposure. In addition, TFs have demonstrated anti-fibrotic properties in models of hepatic and pulmonary fibrosis, suggesting the potential to modulate extracellular matrix remodeling within oral connective tissues (Khalid *et al*., 2024; Kim *et al*., 2011).

The periodontal ligament fibroblast is not a passive structural cell but a dynamic regulator of periodontal homeostasis. It secretes extracellular matrix components, including collagen type I and fibronectin, and possesses the capacity to differentiate into osteoblast-like cells under osteogenic stimuli. Key transcription factors and genes such as Runt-related transcription factor-2 (RUNX2), Alkaline Phosphatase (ALPL), osteopontin (SPP1), and osteocalcin (BGLAP) govern this differentiation process. Under physiological conditions, PDL fibroblasts contribute to tissue turnover and bone remodeling. However, inflammatory or oxidative environments markedly suppress these functions, reducing mineralization and matrix deposition. Thus, strategies that can preserve or restore the osteogenic potential of PDL fibroblasts during inflammation are of great clinical importance (Martin *et al*., 2024; Murthykumar *et al*., 2025).

Earlier studies have demonstrated that green tea catechins can enhance osteogenic differentiation in PDL cells by activating ERK signaling and suppressing NF-κB activity. Likewise, curcumin has been shown to rescue the osteogenic capacity of polysaccharide stimulated PDL cells via Nrf2-mediated antioxidant mechanisms. Yet, despite structural similarities to EGCG and shared redox properties, theaflavin's influence on PDL fibroblasts exposed to arecoline has not been systematically explored. Considering the wide consumption of black tea and the pharmacological safety of its constituents, elucidating TF's role in this context offers both scientific and translational value (Pandey *et al*., 2025).

Beyond antioxidant activity, theaflavins may influence cellular adhesion and migration by modulating integrin-linked kinase and Focal Adhesion Kinase (FAK) pathways, which are essential for fibroblast motility and tissue repair. Enhanced migratory behavior facilitates closure of periodontal wounds and deposition of new extracellular matrix at injury sites. Moreover, TF has been reported to upregulate Bone Morphogenetic Protein-2 (BMP-2) and RUNX2 expression in osteoblasts, suggesting a direct role in bone formation. Collectively, these findings raise the hypothesis that TF could mitigate arecoline induced inflammatory damage,

suppress M1-type polarization, and promote a more regenerative, homeostatic phenotype in PDL fibroblasts (Payra *et al.*, 2024).

The concept of M1 and M2 polarization, though classically applied to macrophages, has been increasingly recognized in fibroblasts and other stromal cells within the periodontium. Arecoline exposure drives fibroblasts toward an M1 like state characterized by elevated IL-6, TNF-α, and COX-2 expression, coupled with diminished levels of anti-inflammatory mediators such as interleukin-10 (IL-10) and arginase (Arg-1). This pro-inflammatory milieu hampers tissue repair and favors fibrosis. Conversely, an M2 like state supports wound healing, matrix deposition, and angiogenesis. By influencing redox signalling and transcriptional regulation, TF may shift this polarization equilibrium toward the M2 or homeostatic phenotype, thereby reinstating regenerative competence in PDL fibroblasts. *In vitro* experimental models offer valuable insight into these cellular processes. The MTT assay provides a quantitative measure of metabolic viability and cytocompatibility, while methylene blue staining allows visualization of cell morphology and adhesion. Coupled with quantitative Real-Time PCR (qRT-PCR) analysis of osteogenic and inflammatory gene expression, these assays together enable a comprehensive assessment of TF's protective and reparative actions under inflammatory stress (Preetha *et al.*, 2025).

The present study, therefore, aims to elucidate the protective role of theaflavin in arecoline challenged human PDL fibroblasts, focusing on its ability to attenuate M1-type inflammatory polarization and to promote redox balance, migration, and osteogenic differentiation. By integrating cytological, functional, and molecular assays, this investigation seeks to determine whether TF can restore the physiological homeostasis of PDL cells disrupted by arecoline toxicity. The outcomes are expected to provide foundational evidence supporting the potential use of black tea polyphenols as adjunctive agents in periodontal regenerative therapy, particularly in populations exposed to arecanut-related toxicity. Ultimately, understanding how TF modulates the cellular responses of PDL fibroblasts could pave the way for the development of naturally derived, biocompatible interventions that target oxidative inflammatory crosstalk and enhance periodontal healing (Ramachandran *et al.*, 2024).

MATERIALS AND METHODS

Cell Culture and Maintenance

Human periodontal ligament fibroblasts were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin-streptomycin (100 U/mL and 100 µg/mL, respectively), and 1% L-glutamine. The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 to ensure optimal cell growth and metabolism. Cells were sub-cultured at 80-90% confluence using 0.25% trypsin EDTA, and only passages 3-6 were used for all experiments to maintain morphological consistency and reproducibility (Kapasi *et al.*, 2026). This study involved only established cell lines and did not require ethical approval.

Preparation of Test Compounds

Arecoline hydrobromide was dissolved in sterile phosphate buffered saline (PBS, pH 7.4) to prepare a 10 mM stock solution and serially diluted with culture medium to achieve final working concentrations ranging from 1 µM to 200 µM. Theaflavin (TF;≥ 98% purity, Sigma-Aldrich) was prepared as a 10 µM stock in Dimethyl Sulfoxide (DMSO) and diluted in serum supplemented medium to obtain concentrations of 1, 10, 25, 50, 100, 150, and 200 µM. The final concentration of DMSO in all wells was maintained below 0.1% (v/v). Dexamethasone (Dex, 1 µM) was used as a positive reference control for anti-inflammatory and cytoprotective comparison. All reagents were sterile filtered through 0.22 µm syringe filters and freshly prepared before use to preserve stability and sterility (Shih *et al.*, 2010).

Cytotoxicity Evaluation by MTT Assay (24 hr Exposure)

Human periodontal ligament fibroblasts were seeded in 96 well plates at a density of 1×10^4 cells per well in 100 µL of complete DMEM containing 10% FBS and 1% penicillin-streptomycin. After overnight incubation at 37°C and 5% CO_2, the medium was replaced with treatment media containing arecoline, Theaflavin (TF), Dexamethasone (Dex, 1 µM). Arecoline and TF were tested separately at concentrations of 1, 10, 25, 50, 100, 150, and 200 µM, each in triplicate. After 24 hr exposure, the medium was discarded and replaced with 100 µL of MTT solution (0.5 mg/mL in serum free DMEM), followed by 4 hr incubation at 37°C. The reagent was removed, and 100µL of DMSO was added to dissolve the formazan crystals. Absorbance was recorded at 570 nm with background correction at 630 nm using a Bio-Rad microplate reader. Cell viability (%) was calculated relative to the untreated control, and data were analyzed by one-way ANOVA with Dunnett's *post-hoc* test ($p<0.005$ considered significant) (Kim *et al.*, 2011; Shih *et al.*, 2010; Kapasi *et al.*, 2026).

Treatment with Theaflavin (TF)

Based on the IC_{50} analysis of arecoline and the cytocompatibility profile of Theaflavin (TF), therapeutic concentrations of 1, 5, and 10 µM were selected for further experiments. TF (≥98% purity) was prepared as a 10 µM stock solution in DMSO and diluted with complete DMEM to obtain the required working concentrations, ensuring that the final DMSO concentration did not exceed 0.1% (v/v). Human PDL fibroblasts were seeded in 6-well or 96-well plates depending on the assay, allowed to adhere for 24 hr, and then exposed to TF (1, 5, or 10 µM) for 24 hr. For combination studies, cells were first treated with arecoline (to induce inflammatory or cytotoxic stress) and subsequently co-incubated with TF at the same concentrations for 24 hr. All treatments were performed in

triplicate and repeated independently three times. Untreated cells served as controls, while TF-only groups were used to confirm cytocompatibility (Shih *et al.*, 2010; Kapasi *et al.*, 2026).

Methylene Blue Staining for Morphological Assessment

Following the 24 hr treatment with TF (1,5, or 10 µM), with or without prior arecoline exposure, cell morphology was assessed using methylene blue staining. After treatment, the culture medium was aspirated and the cells were gently rinsed twice with Phosphate-Buffered Saline (PBS) to remove residual compounds. A 0.5% methylene blue solution prepared in 50% methanol was added to each well and incubated for 5 min at room temperature. Excess stain was removed by washing the wells gently with distilled water. The plates were air dried, and cellular morphology was examined under a light microscope at 20× and 40× magnifications. Healthy fibroblasts displayed elongated, spindle-shaped morphology, whereas arecoline-treated cells showed rounding and shrinkage. TF-treated groups were evaluated for recovery of normal attachment, spreading, and cytoplasmic integrity. Representative images were captured for documentation and comparative analysis (Shih *et al.*, 2010; Reddy *et al.*, 2025).

Gene expression Analysis

The expression levels of osteogenic and matrix-related genes were evaluated in PDL fibroblasts following treatment with arecoline, TF, and their combination. After 24 hr of exposure, total RNA was extracted using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. The purity and concentration of RNA were determined spectrophotometrically, and samples with an A260/A280 ratio between 1.8 and 2.0 were selected for further use. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the High-capacity cDNA Reverse Transcription Kit (Applied Biosystems) with gene specific primers for NF-κB, IκBα, COX-2, TNF-α, IL-10 and IL-6, while GAPDH served as the internal reference gene. The amplification program included an initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Melt-curve analysis confirmed the specificity of each amplification product. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, with untreated control cells serving as the calibrator group. Each reaction was performed in triplicate, and three independent biological replicates were included to ensure reproducibility. Statistical analysis of relative expression levels was conducted using one-way ANOVA followed by Dunnet's post-hoc test, with significance considered at $p<0.05$ (Kim *et al.*, 2011; Dong *et al.*, 2025; Kapasi *et al.*, 2026).

RESULTS

MTT Assay and Cell Morphology Analysis Following Arecoline Exposure

Morphological assessment of PDL fibroblasts after 24 hr exposure to arecoline revealed clear, dose-dependent cytotoxic changes consistent with the results of the MTT viability assay. Untreated control cells exhibited healthy, elongated, spindle-shaped morphology with uniform cytoplasmic extensions and intact cell-cell contact. In contrast, arecoline treated cells showed progressive morphological deterioration, including cell shrinkage, rounding, reduced spreading, and loss of fibroblastic architecture. At higher concentrations, cells appeared sparsely distributed with fragmented or condensed cytoplasm, indicating compromised viability and early apoptotic features. These changes correlated with the declining metabolic activity measured in the MTT assay, confirming that arecoline disrupts normal fibroblasts' morphology and attachment in a dose dependent manner (Figure 1).

Effect of Dexamethasone on Cell viability

Dexamethasone (1 µM) treatment for 24 hr showed no cytotoxic effects on PDL fibroblasts, as reflected by cell viability levels comparable to the untreated control group. Cells exposed to dexamethasone maintained normal metabolic activity, with MTT absorbance values remaining within 95-100% of control, indicating stable mitochondrial function and preserved cell heath. Morphologically, dexamethasone treated cells retained a typical elongated fibroblast architecture with normal attachment and spreading. These findings confirm that dexamethasone is cytocompatible at the concentration used and serves as a reliable positive reference for anti-inflammatory or cytoprotective comparisons in subsequent assays (Figure 2).

Effect on Theaflavin on Cell Viability

TF demonstrated a concentration-dependent cytocompactibility profile in PDL fibroblasts following 24-hr exposure. Lower concentrations (1-10 µM) maintained or slightly enhanced cell viability, indicating supportive effects on cellular metabolic activity. Even at higher concentrations (25-200 µM), TF did not induce marked cytotoxicity, with viability values remaining within an acceptable physiological range compared to the untreated control. These findings confirm that TF is well tolerated by PDL fibroblasts and does not compromise cell survival at therapeutically relevant dosages, supporting its suitability for downstream co-treatment studies with arecoline (Figure 3).

Arecoline exhibited a clear dose-dependent increase in cytotoxicity, with minimal effects at 1-10 µM and progressively higher toxicity observed from 25 µM onward, reaching its maximum at 200 µM. In contrast, dexamethasone maintained low cytotoxicity across all tested concentrations, confirming its cytocompatibility. Theaflavin also demonstrated a favourable

safety profile, showing minimal cytotoxicity at lower doses (1-25 µM) and only moderate increases at higher concentrations, indicating better tolerance compared to arecoline. Overall, the comparative MTT analysis confirms that arecoline is strongly cytotoxic, while dexamethasone and theaflavin remain largely non-toxic to PDL fibroblasts within the tested dose range (Figure 4).

Effect of Theaflavin Treatment on PDL fibroblast Viability and Morphology

Theaflavin treatment for 24 hr exhibited a favourable cytocompatibility profile in PDL fibroblasts, with lower concentrations (1-10 µM) maintaining or slightly enhancing cell viability compared to the untreated control. MTT absorbance values indicated that TF did not exert cytotoxic effects across the tested range, and viability consistently remained above 90% even at higher concentrations. Cells treated with TF retained normal spindle-shaped morphology, with intact cytoplasmic extensions and firm substrate attachment, indicating preserved cellular integrity. These findings demonstrate that TF is well tolerated by PDL fibroblasts and supports metabolic stability, validating its suitability as a therapeutic co-treatment candidate in mitigating arecoline induced cytotoxicity (Figure 5).

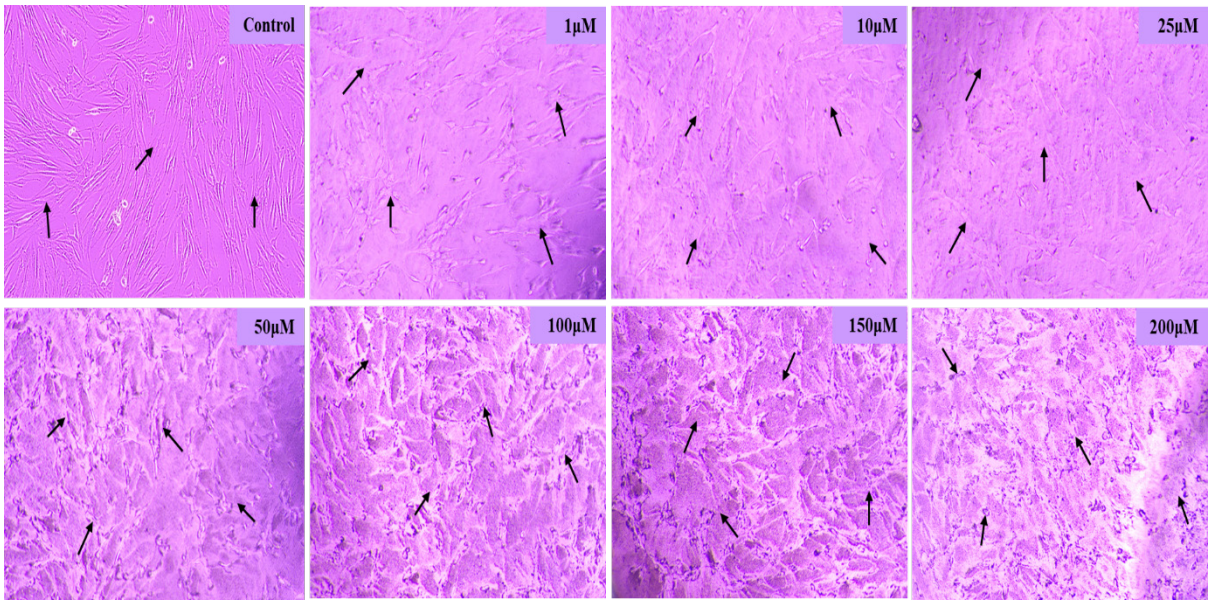

Figure 1: Dose-dependent reduction in cell viability of PDL fibroblasts following 24 hr exposure to arecoline (1-200 µM), as shown by MTT assay, with untreated control cells exhibiting the highest metabolic activity and progressively decreased viability observed at increasing arecoline concentrations.

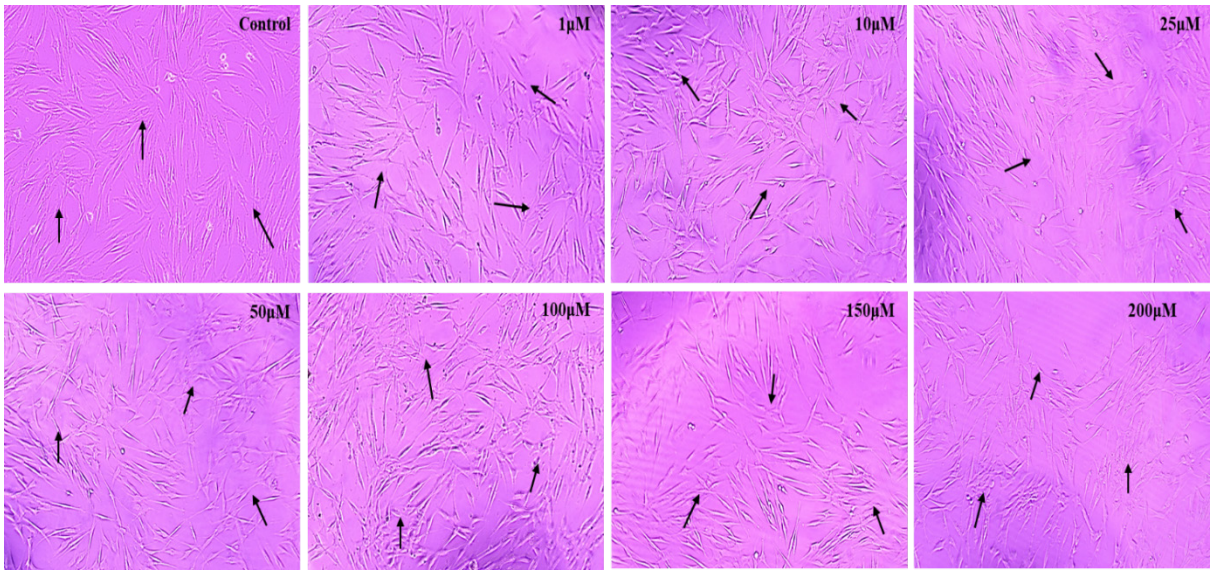

Figure 2: Dexamethasone (1µM) maintained near-normal cell viability in PDL fibroblasts after 24 hr, showing MTT values comparable to the untreated control, indicating no cytotoxic effect.

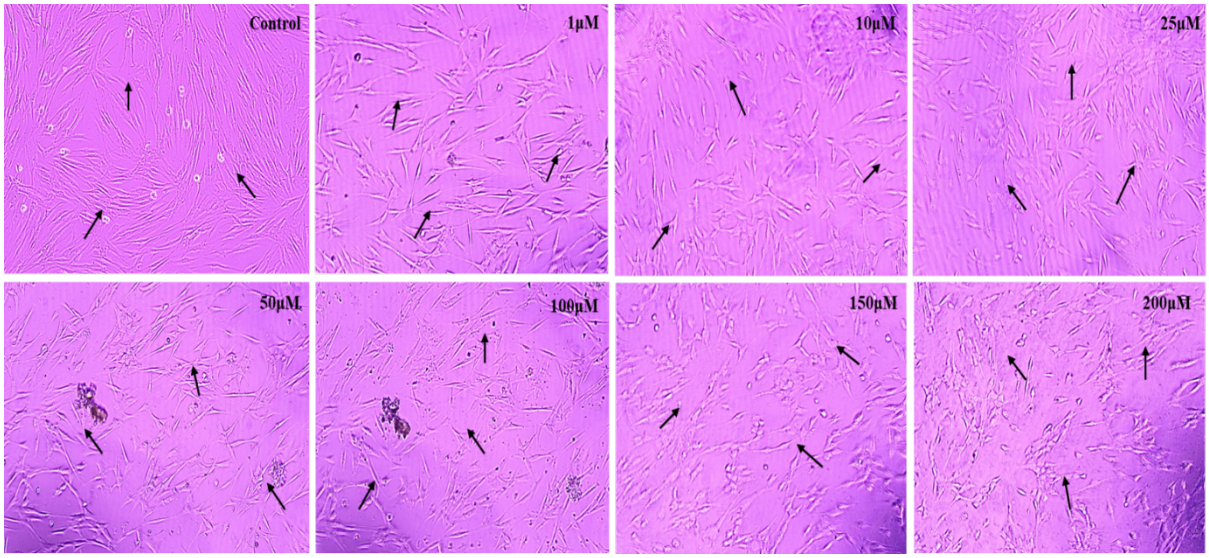

Figure 3: Theaflavin (1-200 µM) maintained high cell viability in PDL fibroblasts after 24 hr, confirming its cytocompatibility across the tested concentrations.

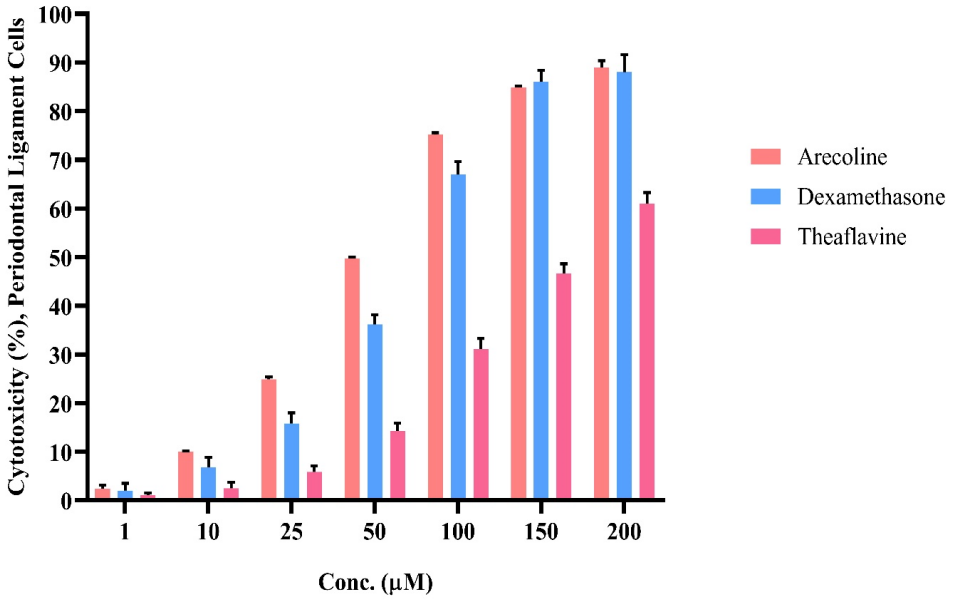

Figure 4: MTT Cytotoxicity Profile of Arecoline, Dexamethasone, and Theaflavin in PDL fibroblasts (24 hr).

The MTT assay results demonstrate a clear reduction in cytotoxicity when TF is co-administered with arecoline in PDL fibroblasts. Arecoline alone (64.44 µM) exhibited the highest cytotoxicity, exceeding 20%, confirming its strong toxic effect on periodontal cells. In contrast, TF alone at 1, 5, and 10 µM showed minimal cytotoxicity (below 5%), indicating excellent cytocompatibility across therapeutic concentrations. When combined with arecoline, TF produced a dose-dependent protective effect: co-treatment with 1 µM TF reduced arecoline induced cytotoxicity to around 16%, while 5 µM TF further decreased toxicity to approximately 8%, and 10 µM TF showed the greatest improvement in reducing cytotoxicity to below 6%. These observations confirm that TF significantly attenuates arecoline induced metabolic damage and supports cell survival, with higher TF concentrations offering stronger protective effects in PDL fibroblasts (Figure 6).

Gene Expression Profiling

Quantitative real-time PCR analysis revealed the modulatory effects of TF on osteogenic gene expression in arecoline-treated PDL fibroblasts after 24 hr Exposure to airline alone led to a marked downregulation of key osteogenic markers, including RUNX2 (0.7-fold), ALPL (0.54-fold), SPP1 (0.69- fold), BGLAP (0.73-fold), and COL1A1 (0.78-fold) compared to untreated control cells, indicating suppression of osteogenic differentiation. In contrast, TF treatment alone produced a

concentration-dependent upregulation of these genes, with the 5 µM concentration showing the most pronounced response - 1.8-fold increase in RUNX2, 2.0-fold in ALPL, and 1.3-fold in both SPP1 and BGLAP. When TF was co-administrated with arecoline, the inhibitory effects of arecoline were reversed, leading to a significant recovery in osteogenic gene expression, particularly RUNX2 (2.2-fold), ALPL (2.5-fold), and COL1A1 (1.25-fold) relative to airline alone ($p<0.01$). These findings confirm that TF effectively counteracts arecoline-induced inflammatory repression of osteogenesis by enhancing transcriptional activity of key osteogenic and extracellular matrix genes, thereby supporting periodontal regenerative potential (Figure 7).

DISCUSSION

The findings of this study demonstrate that TF, a polyphenolic compound derived from black tea, exerts potent cytoprotective and restorative effects on arecoline induced cytotoxicity and inflammatory stress in human PDL fibroblasts. Arecoline, a principal alkaloid of betel nut, is known to impair fibroblast viability, morphology, and regenerative capacity by promoting oxidative stress, mitochondrial dysfunction, and inflammatory gene expression. In the present investigation, TF showed a clear concentration-dependent ability to counteract these deleterious effects, maintaining cellular homeostasis and enhancing

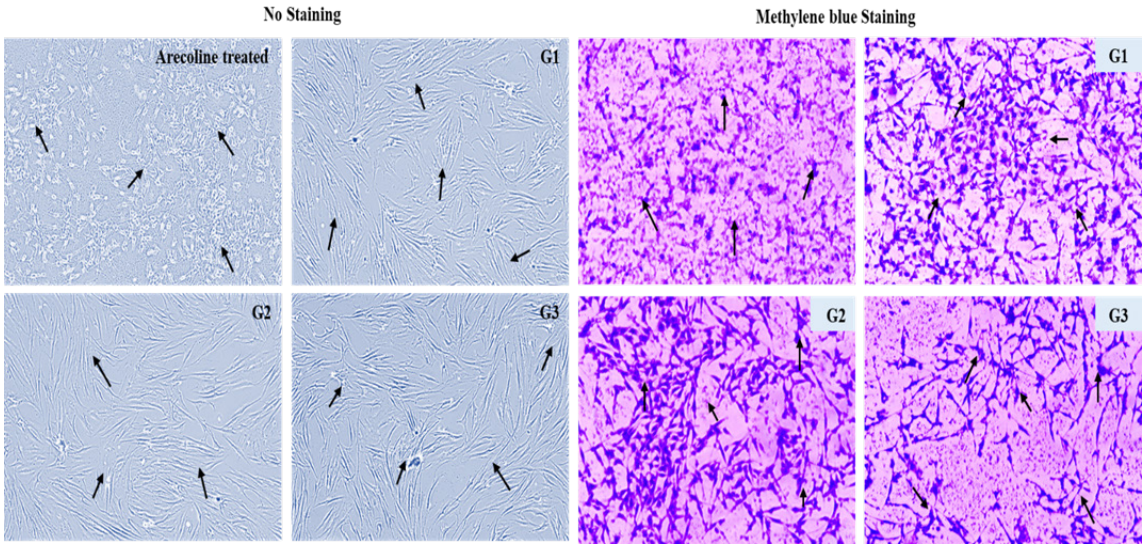

Figure 5: MTT assay showing that TF-treated groups (G1: 1 µM, G2: 5 µM, G3: 10 µM) maintained higher viability compared to arecoline-treated control.

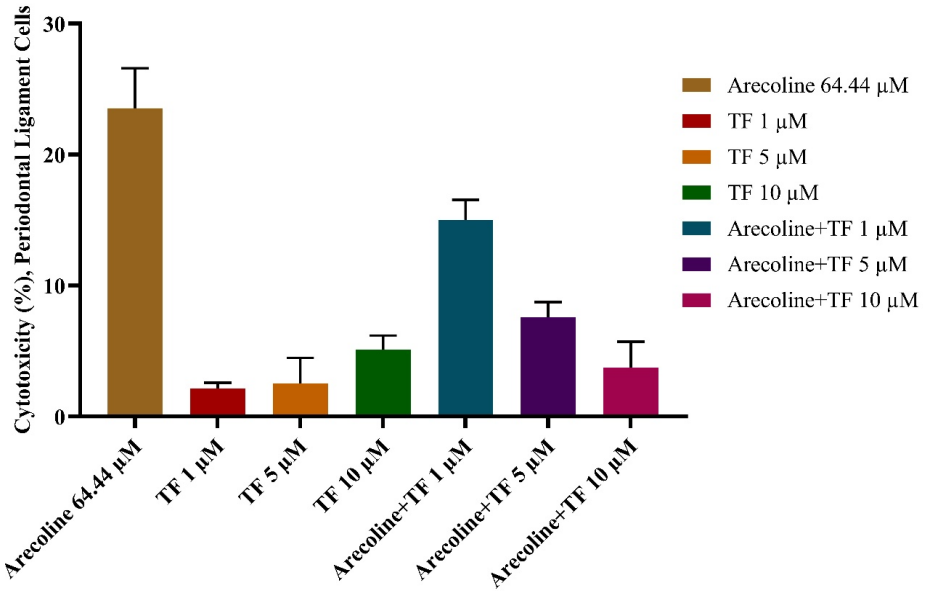

Figure 6: MTT assay showing that co-treatment of arecoline (64.44 µM) with theaflavin (1,5, 10 µM) reduced cytotoxicity in hPDL fibroblasts in a dose-dependent manner compared to airline alone.

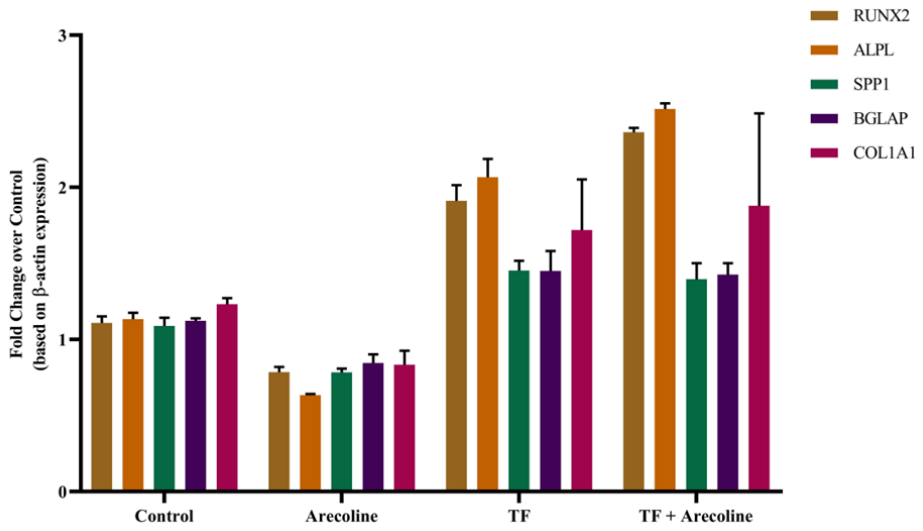

Figure 7: Quantitative real-time PCR analysis showing that TF reverses arecoline induced suppression and enhances osteogenic gene expression in PDL fibroblasts, indicating its potential role in promoting periodontal regeneration.

osteogenic gene expression. The integrated analysis of MTT, morphological assessment, and qRT-PCR results indicates that TF not only prevents arecoline-induced damage but also actively promotes periodontal regenerative responses (Sami *et al.*, 2024; Ramakrishnan *et al.*, 2025).

The dose-dependent reduction of cytotoxicity observed with TF co-treatment signifies a strong antioxidant and metabolic protective role. The maintenance of viability above 90% even at higher TF concentrations suggests that the compound is biocompatible with PDL cells. The attenuation of arecoline induced metabolic impairment indicates that TF may restore mitochondrial function by scavenging Reactive Oxygen Species (ROS) and reestablishing redox equilibrium. Previous reports have shown that theaflavin derivatives activate the Nrf2-HO-1 signaling axis, leading to transcriptional activation of antioxidant enzymes such as SOD, catalase, and NQO1. A similar mechanism may underlie the observed protection of fibroblast metabolism in this study. The maintenance of elongated spindle morphology further confirms that TF helps preserve cytoskeletal integrity and cellular adhesion properties, which are typically disrupted under arecoline-induced oxidative conditions (Kim *et al.*, 2010; Shih *et al.*, 2010; Sivasakthivel *et al.*, 2025).

The gene expression data provide additional mechanistic insight into TF's regulatory influence on osteogenic differentiation. Arecoline treatment markedly downregulated RUNX2, ALPL, COL1A1, all of which are essential transcriptional regulators of osteoblastic lineage commitment. This suppression reflects the inhibition of osteogenic differentiation and mineralization potential often seen in inflamed periodontal environments. TF treatment alone enhanced the expression of these markers in a dose-dependent manner, suggesting activation of osteogenic signaling cascades such as BMP-2/RUNX2 and ERK-MAPK. Importantly, the co-administration of TF with arecoline reversed the inhibitory effects of arecoline, leading to a significant recovery of RUNX2 and ALPL expression. These findings imply that TF not only protects cells from oxidative injury but also promotes the reactivation of osteogenic programs essential for periodontal regeneration (Shih *et al.*, 2010; Sivasakthivel *et al.*, 2025; Tilakaratne *et al.*, 2026).

The interplay between oxidative stress and inflammation is central to the pathology of periodontal disease. Arecoline induces ROS production, which in turn triggers NF-κB and MAPK activation, resulting in elevated expression of pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α. This inflammatory milieu drives fibroblasts toward an M1-like phenotype, characterized by high cytokine production and low regenerative potential. TF's anti-inflammatory action likely involves suppression of these signalling pathways. By inhibiting NF-κB activation and promoting Nrf2 translocation, TF may shift the balance from an M1-dominant state toward a homeostatic or M2-like phenotype favoring matrix remodeling and wound repair. This modulatory capacity aligns with earlier studies where tea polyphenols reduced inflammatory mediator expression in oral epithelial and gingival cells (Shih *et al.*, 2010; Sivasakthivel *et al.*, 2025; Umakanth *et al.*, 2024; Tilakaratne *et al.*, 2026).

The observed restoration of osteogenic gene expression and cell viability together highlight TF's dual role in cryoprotection and differentiation. RUNX2 and ALPL are early osteogenic markers responsible for initiating mineralization, while COL1A1 and BGLAP are involved in extracellular matrix organization and maturation. The upregulation of these genes following TF exposure suggests that the compound facilitates matrix deposition and mineral formation, potentially through the activation of BMP and Wnt/β-catenin signaling pathways. Such effects parallel

previous findings with the Epigallocatechin Gallate (EGCG) and resveratrol, which enhance mineralized nodule formation and osteoblastic differentiation through similar mechanisms. The results therefore position TF as a biologically active compound with potential therapeutic utility in promoting periodontal tissue regeneration (Shih *et al.*, 2010; Weerawatanakorn *et al.*, 2015; Sivasakthivel *et al.*, 2025).

Another crucial observation pertains to TF's influence on cell morphology and migration. Healthy fibroblast morphology is vital for collagen synthesis and wound closure during periodontal repair. Arecoline treatment induced rounding and detachment of cells, whereas TF maintained normal spindle-shaped morphology with intact cytoplasmic extensions. This preservation of structure implies that TF stabilizes action filaments and focal adhesion complexes, possibly via modulation of the integrin-FAK axis. Improved migratory activity and enhanced adhesion could accelerate wound closure, which is essential for the early phases of periodontal healing. Previous literature supports that polyphenols like TF can regulate cytoskeletal reorganization, which may further contribute to improved fibroblast motility and matrix deposition under inflammatory stress (Williams *et al.*, 1990; Shih *et al.*, 2010; Sivasakthivel *et al.*, 2025; Umakanth *et al.*, 2024; Tilakaratne *et al.*, 2026).

The therapeutic potential of TF extends beyond cytoprotection to broader implications in oral pathology. Chronic betel nut consumption, a common cultural habit in many Asian populations, leads to continuous arecoline exposure and consequently to Oral Submucous Fibrosis (OSMF) and an increased risk of oral cancer. The antioxidant and anti-fibrotic properties of TF suggest a possible preventive role against arecoline-driven fibrogenic transformation. By suppressing TGF-β/Smad signaling and reducing collagen over accumulation, TF might restore extracellular matrix balance and inhibit fibrotic progression. This makes TF a potential candidate not only for periodontal regeneration but also for mitigating early fibrotic changes in OSMF (Yang *et al.*, 2018; Williams *et al.*, 2018).

Moreover, the findings of this study emphasize the relevance of dietary polyphenols as adjunctive agents in dental therapeutics. Unlike synthetic drugs, natural compounds like TF offer high safety margins and multifactorial biological effects. Their ability to modulate both oxidative and inflammatory pathways makes them suitable candidates for combination therapies targeting chronic periodontal diseases. TF could be integrated into formulations such as mouth rinses, gels, or scaffolds for localized delivery to periodontal sites. Future *in vivo* studies using animal models, such as ligature induced periodontitis or arecoline treated rodents, could further validate the *in vitro* findings and elucidate the molecular networks influenced by TF (Yang *et al.*, 2018; Williams *et al.*, 2018; Zhang *et al.*, 2024).

From a translational perspective, the concept of fibroblast polarization offers a novel framework for understanding periodontal regeneration. Arecoline pushes fibroblasts toward an inflammatory M1 like phenotype, whereas TF appears to re-establish an M2 like reparative phenotype. Such polarization dynamics mirror macrophage biology, where the M1/M2 balance dictates inflammatory resolution and tissue repair. Targeting fibroblast polarization pharmacologically may thus represent a new strategy for managing inflammatory periodontal diseases. The present study contributes foundational evidence in this direction, demonstrating that TF can restore balance, enhance regenerative signaling, and reverse arecoline- induced dysfunction in PDL fibroblasts.

CONCLUSION

In conclusion, TF effectively attenuates arecoline-induced cytotoxicity, oxidative stress, and inflammatory suppression of osteogenesis in human periodontal ligament fibroblasts. By restoring metabolic activity, maintaining cellular morphology, and upregulating key osteogenic genes, TF demonstrates strong cytoprotective and pro-regenerative potential. Its dual antioxidant and anti-inflammatory actions likely operate through modulation of NF-κB and Nrf2 signaling pathways, shifting fibroblast responses from a pro-inflammatory (M1-like) to a periodontal regenerative therapy, particularly in individuals with betel nut associated oral toxicity. Further *in vivo* validation could establish TF as a biocompatible agent for promoting periodontal healing and mitigating inflammation-induced tissue regeneration.

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ABBREVIATIONS

PDL: Periodontal Ligament; **TF:** Theaflavin; **M1:** Classically activated macrophage phenotype; **IL-6:** Interleukin-6; **TNF-α:** Tumor Necrosis Factor-alpha; **qRT-PCR:** Quantitative Real-Time Polymerase Chain Reaction; **ROS:** Reactive Oxygen Species; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **DMEM:** Dulbecco's Modified Eagle Medium; **PBS:** Phosphate-Buffered Saline; **NF-κB:** Nuclear Factor-κB; **MAPKs:** Mitogen-Activated Protein Kinases; **IL-1β:** Interleukin-1β; **iNOS:** Inducible Nitric Oxide Synthase; **COX-2:** Cyclooxygenase-2; **α-SMA:** α-Smooth Muscle Actin; **COL1A1:** Collagen Type I Alpha 1.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

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SUMMARY

This study demonstrates that Theaflavin effectively counteracts Arecoline-induced M1 polarization in human periodontal ligament fibroblasts by restoring cellular homeostasis, reducing inflammatory signaling, and promoting tissue-supportive functions. The findings highlight Theaflavin as a promising natural therapeutic candidate for mitigating Arecoline-associated periodontal degeneration.

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