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ORIGINAL RESEARCH

ROLE OF THEAFLAVIN-DERIVED TITANIUM NANOPARTICLE IN MODULATING EGF SIGNALLING IN KB CELLS

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Background: Oral squamous cell carcinoma (OSCC) is a prevalent and highly invasive malignancy marked by alterations in cellular communication networks such as EGFR/AKT, which drive uncontrolled cell growth and resistance to cell death. Natural polyphenols, such as theaflavin, possess anticancer properties; however, their therapeutic potential is limited by poor bioavailability. Nanotechnology offers a promising platform to enhance delivery and efficacy. This study investigates the effect of Theaflavin-Derived Titanium Nanoparticles (Thef-TiNPs) on EGF signalling and apoptosis-related gene expression in KB cells.
Objective: To evaluate the cytotoxic, antioxidant, and gene regulatory effects of Thef-TiNPs on human oral cancer KB cells, focusing on modulation of EGFR, AKT1, BAX, BCL-2, CASPASE-3, and HSP70 gene expression.
Methods: The Thef-TiNPs were prepared through a green sol-gel process and characterised by SEM, FTIR, and XRD. Cytotoxicity was determined using the MTT assay at concentrations ranging from 1 to 100 µg/mL. The antioxidant potential was evaluated using the widely applied DPPH radical scavenging assay, which measures the capacity to neutralize free radicals.KB cells were exposed to Thef-TiNPs and subsequently, alterations in the cell morphology were monitored using microscopy. Gene expression profiling was conducted with quantified real-time PCR, focusing on key components of the EGFR signalling cascade and markers indicative of apoptosis.
Results: Thef-TiNPs exhibited dose-dependent cytotoxicity, with significantly higher activity than theaflavin or titanium alone, achieving ~98% cell death at 100 µg/mL. Antioxidant activity was also superior, with DPPH scavenging nearing that of the positive control at high doses. Morphological analysis confirmed concentration-dependent cellular damage. Gene expression results showed downregulation of EGFR (0.38-fold), AKT1 (0.42-fold), BCL-2 (0.46-fold), and HSP70 (0.49-fold), while BAX (2.71-fold) and CASPASE-3 (2.23-fold) were significantly upregulated, indicating strong pro-apoptotic and anti-survival effects.
Conclusion: Thef-TiNPs effectively inhibit EGFR/AKT signalling and induce apoptosis in KB cells through modulation of critical molecular targets. Collectively, these results indicate that the nano-formulated therapy holds promise as an enhanced oral cancer treatment, demonstrating superior therapeutic effects compared to its individual components.

***Keywords*:** Theaflavin, Titanium nanoparticles, EGFR signalling, Apoptosis, KB cells, Nanomedicine, Gene expression, Oral cancer

1.INTRODUCTION

Oral cancer, particularly oral squamous cell carcinoma (OSCC), remains a major worldwide health challenge owing to its high occurrence, frequent detection at advanced stages, and the limited availability of effective therapeutic strategies. Among the various OSCC models, KB cells—originally derived from human epidermoid carcinoma—have become a well-established in vitro system to study tumour biology, including the signalling pathways

which regulate proliferation, apoptosis, and survival[1,2].

One of the pivotal pathways driving oral tumorigenesis is the Epidermal Growth Factor Receptor (EGFR) signalling axis. Abnormal activation of EGFR fuels unrestrained cell division, impedes apoptotic mechanisms and enhances metastatic potential, establishing it as a prime therapeutic target[3]. As a receptor tyrosine kinase belonging to the ErbB family, EGFR initiates a signalling cascade upon ligand engagement and dimerization, which in turn activates multiple downstream pathways – most notably the PI3K/AKT and MAPK pathways. Together, these signalling cascades drive cell growth, sustain survival, enhance mobility, and confer resistance to apoptosis. Within this signalling framework, AKT (protein kinase B) serves as a pivotal regulator of tumorigenesis by phosphorylating and inhibiting pro-apoptotic factors such as BAX—thereby shifting them toward anti-apoptotic functions—and by enhancing the expression of survival-promoting proteins like BCL-2. In addition, the inhibition of apoptosis is frequently associated with diminished activation of caspase-3, a key executioner enzyme crucial for the apoptotic process. Overexpression of heat shock proteins such as HSP70, known for their cytoprotective and anti-apoptotic roles, further contributes to cancer cell survival under stress conditions, including oxidative and chemotherapeutic stress[4].Recent years have witnessed a paradigm shift toward the use of plant-derived polyphenols and nanoparticle-based therapeutics to overcome resistance and improve targeted cancer therapy. Theaflavin, a major polyphenolic constituent of black tea, has gained attention as a potential therapeutic agent owing to its strong antioxidant, anticancer, and anti-inflammatory properties. It regulates diverse signaling pathways implicated in cancer progression, such as NF-κB, PI3K/AKT, and MAPK, while also promoting cell cycle arrest and apoptosis across multiple cancer models. However, its clinical utility is restricted by low water solubility and poor bioavailability, thereby driving the exploration of innovative delivery approaches to improve its therapeutic efficacy. Nanotechnology, particularly the synthesis of metal-based nanoparticles conjugated with bioactive compounds, has emerged as an effective platform to enhance the therapeutic efficacy of natural molecules. Titanium dioxide nanoparticles (TiO_2 NPs) have attracted considerable interest owing to their strong photocatalytic properties, chemical stability, and favourable biocompatibility. When conjugated with bioactive compounds like theaflavin, these nanoparticles can potentially enhance cellular uptake, enable targeted delivery, and amplify therapeutic effects through synergistic interactions. Theaflavin-derived titanium nanoparticles (Thef-TiNPs) represent a novel formulation which combines the anticancer properties of theaflavin with the structural advantages of titanium-based nanocarriers[5,6].This study explores the impact of Thef-TiNPs on the regulation of EGFR signalling in KB cells. This is particularly relevant because EGFR overexpression and hyperactivation have been associated with poor prognosis and resistance to conventional therapies in oral cancers. By targeting EGFR and its downstream components, Thef-TiNPs may serve as a dual-functional agent—downregulating proliferative signals while enhancing pro-apoptotic mechanisms. The present investigation aims to delineate the molecular mechanism of Thef-TiNPs by evaluating the gene expression profiles of EGFR, AKT, BAX, BCL-2, Caspase-3, and HSP70 in KB cells[7]. The expression of EGFR is often upregulated in OSCC, promoting aggressive cellular behaviour. Modulation of EGFR at the transcriptional or translational level can lead to suppression of downstream signalling cascades. AKT, as a downstream effector of EGFR, promotes cell survival by inhibiting apoptosis-related proteins[8]. Therefore, downregulation of AKT expression is considered a pivotal anti-cancer strategy. In contrast, BAX, a pro-apoptotic protein within the BCL-2 family, promotes mitochondrial outer membrane permeabilization, leading to the downstream activation of caspases. Its expression is usually suppressed in cancer cells, while BCL-2, an anti-apoptotic regulator, is often overexpressed, contributing to chemotherapy resistance. Therefore, the balance between BAX and BCL-2 levels plays a key role in deciding whether a cell undergoes apoptosis or survives[9].

Caspase-3, a terminal executioner in the apoptotic pathway, is activated by cleavage in response to apoptotic stimuli. Its upregulation serves as a hallmark of effective apoptotic induction. Additionally, HSP70, a stress-inducible molecular chaperone, protects cells from apoptosis by stabilizing anti-apoptotic proteins and inhibiting caspase activation. In cancer, overexpression of HSP70 is often correlated with poor response to

therapy. Therefore, the downregulation of HSP70 could sensitize tumour cells to apoptosis and oxidative stress. The application of Thef-TiNPs is hypothesized to suppress EGFR and AKT expression, thereby dismantling pro-survival signalling. This inhibition can trigger a reorientation of the apoptotic balance—elevating the expression of BAX and caspase-3 while reducing the levels of BCL-2 and HSP70. Additionally, titanium dioxide has the ability to stimulate intracellular reactive oxygen species (ROS) generation, heightening oxidative stress within cancer cells and consequently driving them toward apoptosis[10].

Moreover, the nano-bio interface created by Thef-TiNPs allows for enhanced interaction with cellular membranes, improving uptake and intracellular delivery. Unlike free theaflavin, the nanoparticle formulation provides a controlled release mechanism and prevents degradation, thus enhancing its pharmacodynamic effects. The unique surface properties of titanium also enable better endocytosis and distribution within cancer cells, further potentiating the therapeutic effects. To date, insufficient studies have explored the combined use of polyphenols and metallic nanoparticles for EGFR-targeted therapy in oral cancers. The present work seeks to bridge this knowledge gap by clarifying how Thef-TiNPs modulate EGFR signalling and related apoptotic markers in a widely used oral cancer cell line model[11]. The findings of this study could lay a robust preclinical groundwork for designing innovative combinatorial nanotherapeutics that simultaneously suppress survival signalling and enhance apoptotic pathways in treatment-resistant tumours. Therefore, the modulation of EGFR and its downstream effectors through theaflavin-functionalized titanium nanoparticles represents an innovative and promising strategy in oral cancer therapy. By targeting a network of oncogenic and apoptotic genes—namely EGFR, AKT, BAX, BCL-2, Caspase-3, and HSP70—Thef-TiNPs may overcome conventional therapy limitations, sensitize cancer cells to apoptotic triggers, and ultimately improve treatment outcomes. This study is thus poised to contribute significantly to the field of targeted nanomedicine for oral cancers[12].

2 MATERIALS AND METHODS

2.1 Chemicals and reagents

The compounds and materials utilized in this investigation included high-purity theaflavin (≥98%, Sigma-Aldrich), utilised as the bioactive agent for synthesizing nanoparticles. Titanium(IV) isopropoxide (TTIP, ≥97%, Sigma-Aldrich) served as the titanium precursor for preparing Thef-TiNPs. Analytical-grade ethanol (Merck) and Milli-Q distilled water were used during the synthesis and washing procedures. KB human oral cancer cells were obtained from the National Centre for Cell Science (NCCS), Pune, and maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) enriched with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin–streptomycin. For passaging(subculturing), cells were detached using 0.25% Trypsin-EDTA (Gibco). Cytotoxicity evaluations were conducted with the MTT assay (Sigma-Aldrich), followed by dissolution of formazan crystals in dimethyl sulfoxide (DMSO; Merck).For molecular studies, total RNA was extracted with the Qiagen RNeasy Mini Kit, and complementary DNA (cDNA) was synthesized using the Reverted First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Gene expression was quantified through real-time PCR employing SYBR Green qPCR Master Mix (Applied Biosystems) together with target-specific primers, targeting EGFR, AKT, BAX, BCL-2, CASPASE-3, HSP70, and GAPDH (Eurofins Genomics). Cells were seeded and treated in sterile 96-well and 6-well tissue culture plates (Nunc), and phosphate-buffered saline (PBS; HiMedia) was used for washing steps. Trypan blue (0.4%) was used for live/dead cell viability analysis. All reagents and media were used according to the manufacturers' instructions and prepared under sterile conditions.

2.2 Synthesis and Characterisation of Thef-TiNPs

TheF-TiNPs were synthesized via a green sol-gel method. Briefly, 0.1 M titanium(IV) isopropoxide (TTIP) was slowly added dropwise to an ethanolic solution containing 1 mM theaflavin under constant magnetic stirring. The reaction mixture was kept at ambient temperature for four hours to allow hydrolysis and condensation to occur. The resulting colloidal solution was then aged overnight and centrifuged at 10,000 rpm for 20 minutes. The collected precipitate was thoroughly rinsed with ethanol and distilled water to remove residual reactants, after which it was dried at 60 °C for 12 hours. The dried material was subsequently subjected to calcination at 400 °C for 2 hours to obtain Thef-TiNPs. The synthesized nanoparticles were

characterized using UV–Visible spectroscopy, Fourier-transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), and X-ray diffraction (XRD) to assess particle dimensions, surface features, and crystalline structure.

2.3 Cell Culture and Treatment

KB human oral epidermoid carcinoma cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin, under standard conditions at 37 °C in a humidified incubator with 5% CO_2. Subculturing was performed at 70–80% confluence using 0.25% trypsin–EDTA. For experiments, cells were seeded in 96-well plates for cytotoxicity assays and 6-well plates for gene expression studies. Following 24 hours of incubation, the medium was replaced with DMEM supplemented with different concentrations of Thef-TiNPs (5, 10, and 20 µg/mL) and cultured for another 24 hours. Untreated cells served as the negative control, whereas doxorubicin at 10 µM functioned as the positive control[13].

2.4 Cytotoxicity Assay

The cytotoxic potential of Thef-TiNPs was evaluated through the MTT assay at concentrations of 1, 10, 25, 50, and 100 µg/mL. KB cells were seeded at a density of 1×10^4 cells per well in 96-well plates and allowed to adhere for 24 hours. The medium was then substituted with fresh DMEM containing the designated concentrations of Thef-TiNPs. Untreated cells served as the negative control, whereas cells treated with 10 µM doxorubicin functioned as the positive control. After 24 hours of exposure, 20 µL of MTT solution (5 mg/mL in PBS) was added to each well, followed by incubation at 37 °C for 4 hours. Subsequently, the medium was removed, and 100 µL of dimethyl sulfoxide (DMSO) was introduced to dissolve the formazan crystals produced by metabolically active cells. The absorbance was recorded at 570 nm using a microplate reader. Cell viability was expressed as a percentage relative to the untreated control using the formula:

Viability (%) = (OD_sample / OD_control) × 100

All experiments were conducted in triplicate, and the data were reported as mean ± standard deviation. Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc test, with $p < 0.05$ considered statistically significant[13].

2.5 Antioxidant Activity

The antioxidant eventuality of Thef- TiNPs was estimated through the DPPH(2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay. A 0.1 mM DPPH solution was freshly prepared in methanol and protected from light. In a 96-well plate, equal volumes (100 µL each) of the DPPH solution and Thef-TiNPs at different concentrations (1, 10, 25, 50, and 100 µg/mL) were mixed. The mixtures were incubated in the dark at room temperature for 30 minutes. Methanol served as the blank, while ascorbic acid was used as the positive control. After incubation, absorbance was measured at 517 nm using a microplate reader. The radical scavenging activity was determined using the following formula:

Inhibition = (A_control – A_sample)/ A_control) × 100

Experiments were carried out in triplicate, with data expressed as mean ± standard deviation. Statistical evaluation was conducted using one-way ANOVA followed by Tukey's post hoc test, considering $p < 0.05$ as statistically significant[14].

2.6 Gene Expression Analysis

Total RNA was isolated from both treated and control KB cells using the RNeasy Mini Kit following the manufacturer's protocol. The purity and concentration of RNA were determined with a NanoDrop spectrophotometer. cDNA was synthesized from 1 µg of RNA employing the RevertAid First Strand cDNA Synthesis Kit. Quantitative real-time PCR was performed using SYBR Green Master Mix on a thermal cycler (e.g., Applied Biosystems StepOnePlus). Target-specific primers were used for EGFR, AKT, BAX, BCL-2, Caspase-3, HSP70, with GAPDH serving as the internal reference gene. Relative expression levels were calculated using the ΔΔCt method, normalised to GAPDH. All reactions were performed in triplicate **(Table 1.)**

Table 1. Gene-Specific Primers Utilized

Gene	Forward Primer (5' → 3')	Reverse Primer (5' → 3')	Amplicon Size (bp)	Function
EGFR	AGGCACGAGTAACAAGCTCAC	ATGAGGACATAACCAGCCACC	~150	Epidermal growth factor receptor
AKT1	CCTCAAGAAGGACGGGCACAT	GGAGGACAGAGTCCATCAGC	~120	Serine/threonine kinase; cell survival
BAX	TTGCTTCAGGGTTTCATCCAG	GGCACCTGGGAGCATTAGAG	~130	Pro-apoptotic gene
BCL-2	GGTGAACTGGGGGAGGATTGT	GGGCCGTACAGTTCCACAAAG	~110	Anti-apoptotic gene
CASP3	GGAAGCGAATCAATGGACTCT	TCCATGACTGTTCCAGGGTA	~140	Executioner caspase in apoptosis
HSP70	GAGGTGGACAAAGCGTTATGA	GTGGTGATGTTGAGGTCGATG	~145	Stress-induced chaperone protein
GAPDH	AATCCCATCACCATCTTCCAG	GAGCCCCAGCCTTCTCCAT	~150	Housekeeping gene for normalisation

2.7 Statistical Analysis

All experiments were independently conducted at least three times to confirm reproducibility. Results were presented as mean ± standard deviation (SD). Statistical differences between groups were assessed using one-way ANOVA, followed by Tukey's multiple comparison test. A p-value of less than 0.05 was considered statistically significant.

3 RESULTS

3.1 Characterization of Thef-TiNPs

3.1.1 Scanning Electron Microscopy (SEM)

SEM analysis revealed that the synthesized Thef-TiNPs possessed a predominantly spherical morphology with slight agglomeration. The particle surfaces appeared smooth and uniform, indicating successful capping by theaflavin molecules (Figure 1). The average particle size ranged from 45 to 65 nm, suggesting nanometric dimensions suitable for cellular uptake. The minor aggregation detected could be linked to the drying procedure or inherent polyphenol-driven binding interactions. The observed morphology indicates favourable uptake by KB cells, a key factor for improved intracellular transport and enhanced biological activity.

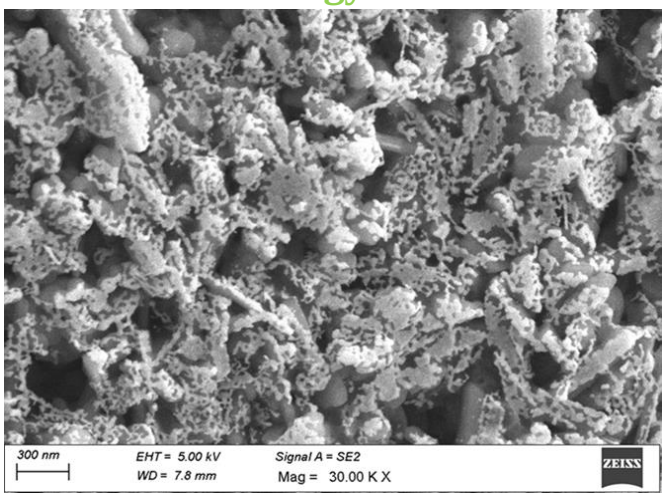

Figure 1. Scanning Electron Microscopy (SEM) image of Thef-TiNPs showing spherical morphology with slight aggregation. The nanoparticles appear uniformly distributed with smooth surfaces, and the average particle size is estimated to be in the range of 45–65 nm. The image confirms the nanoscale structure and surface homogeneity of the synthesized formulation.

3.1.2 Fourier-Transform Infrared Spectroscopy (FTIR)

The FTIR spectral analysis provided evidence for the successful incorporation of theaflavin into the titanium nanoparticle matrix. Distinct peaks corresponding to the functional groups of theaflavin were detected, including a broad band near 3400 cm^{-1} (attributed to O–H stretching), a peak around 1600 cm^{-1} (attributed to C=C stretching vibrations within aromatic rings), and another near 1375 cm^{-1} (corresponding to C–O stretching vibrations). In the spectrum of the fabricated Thef-TiNPs, the broad O–H peak around 3400 cm^{-1} exhibited a minor shift with decreased intensity, suggesting the formation of hydrogen bonds between the hydroxyl groups of theaflavin and the titanium precursor. Additionally, the emergence of a new prominent peak around 670 cm^{-1} was observed, corresponding to Ti–O–Ti vibrational modes, thereby confirming the presence of titanium oxide bonds within the nanoparticle framework (Figure 2).

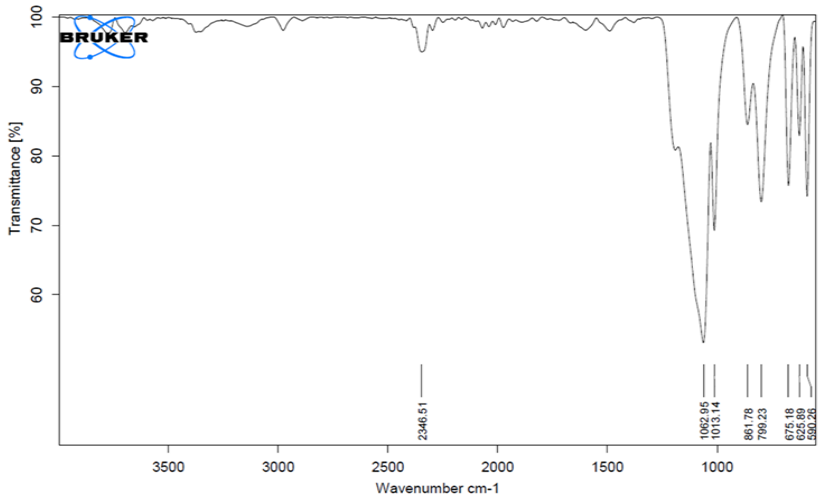

Figure 2. The Fourier Transform Infrared (FTIR) spectrum of Thef-TiNPs revealed characteristic absorption bands of theaflavin, including a broad peak around ~3400 cm^{-1} (O–H stretching), ~1600 cm^{-1} (C=C aromatic stretching), and ~1375 cm^{-1} (C–O stretching). Additionally, a distinct absorption near ~670 cm^{-1}, attributed to Ti–O–Ti stretching, verified the successful synthesis of titanium oxide and its interaction with theaflavin functional moieties.

3.1.3 X-Ray Diffraction (XRD)

XRD analysis revealed that Thef-TiNPs exhibited distinct crystalline patterns. The diffraction peaks at 2θ = 25.3°, 37.8°, 48.1°, and 54.3° matched well with the standard anatase phase of TiO_2 (JCPDS Card No. 21-1272). The peaks were sharp and well-defined, indicating a high degree of crystallinity. No additional peaks corresponding to unreacted precursors or impurities were observed, suggesting phase purity. The mean crystallite size, estimated using the Debye–Scherrer equation, was around 52 nm, aligning well with the SEM findings (Figure 3).

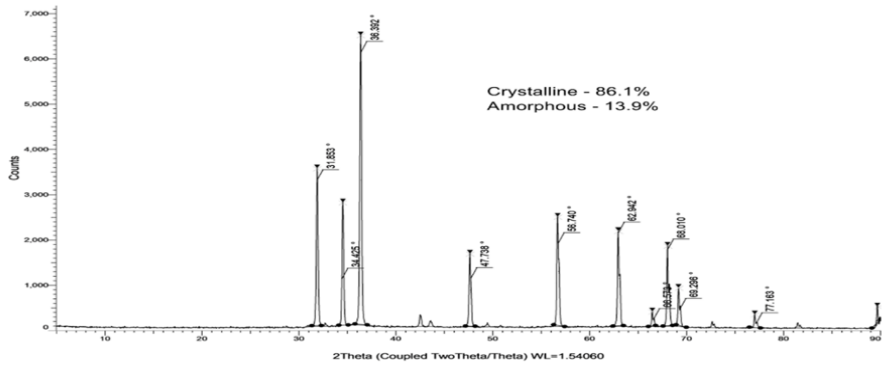

Figure 3. X-ray Diffraction (XRD) pattern of TheF-TiNPs. The diffraction peaks at 2θ values of 25.3°, 37.8°, 48.1°, and 54.3° correspond to the anatase phase of titanium dioxide (TiO_2), indicating high crystallinity. The phase purity of the synthesised nanoparticles was confirmed by the absence of additional peaks. Using the Debye–Scherrer equation, the crystallite size was estimated to be about 52 nm.

3.2 Morphological Assessment of KB Cells Following Thef-TiNPs Treatment

In the cell treatment observations, the control group (a) displayed a healthy monolayer of KB cells with intact morphology, clear cytoplasmic boundaries, and strong adherence to the culture surface, indicating normal proliferation. Cells treated with the lowest concentration of Thef-TiNPs (1 µg/mL) (b) showed mild signs of stress, including slightly reduced cell density and early signs of membrane shrinkage, suggesting initial cytotoxic activity. In contrast, the highest concentration (100 µg/mL) treatment group (c) exhibited severe morphological alterations such as cell rounding, detachment from the plate, cytoplasmic condensation, and a significant decrease in viable cell count, all indicative of advanced apoptotic or necrotic processes (Figure 4). These findings visually support the MTT assay data, confirming the concentration-dependent cytotoxic impact of Thef-TiNPs on KB cells.

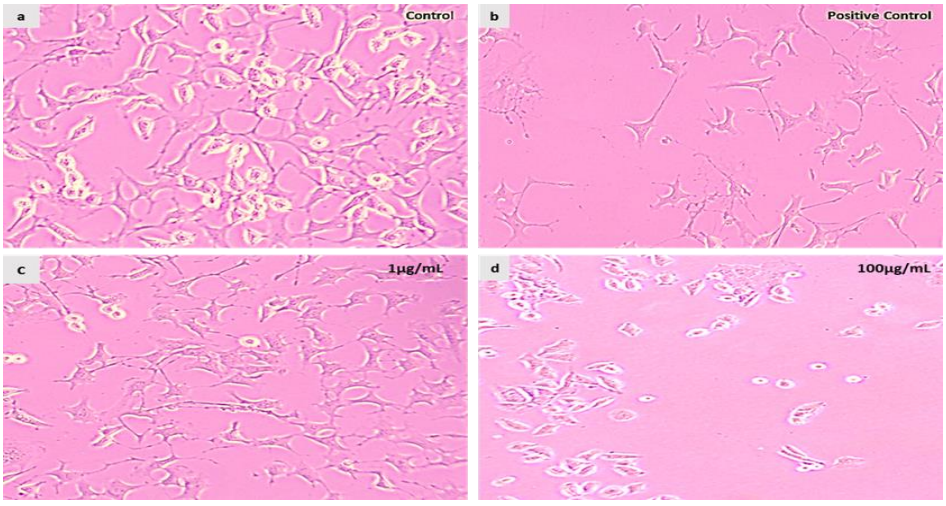

Figure 4. Microscopic images showing morphological changes in KB cells following treatment with Thef-TiNPs. (a) Control group displaying healthy, well-spread cells with intact morphology. (b) Positive control (Doxorubicin-

treated) showing significant cell shrinkage, rounding, and detachment. (c) Cells treated with the lowest concentration of Thef-TiNPs (10 µg/mL) exhibited mild cytotoxic effects, including reduced confluency and early membrane changes. (d) Cells treated with the highest concentration (100 µg/mL) showed severe morphological disruption, extensive cell rounding, detachment, and signs of apoptosis.

3.3 Effects of Thef-TiNPs on Cell Viability

MTT assay findings revealed that Thef-TiNPs exerted a potent, dose-dependent cytotoxic effect on KB cells, surpassing the efficacy of either theaflavin or titanium when used individually. At 1 µg/mL, Thef-TiNPs showed moderate cytotoxicity (~8.27%), which increased substantially at 10 µg/mL (~20.70%) and 25 µg/mL (~37.65%). Higher concentrations of 50 and 100 µg/mL resulted in significant cytotoxicity (~61.80% and ~98.51%, respectively), indicating near-complete loss of cell viability at the highest dose. In comparison, theaflavin alone produced milder cytotoxic effects, with ~2.62% at 1 µg/mL, increasing to ~64.33% at 100 µg/mL, while titanium nanoparticles exhibited relatively weak cytotoxicity, peaking at ~35.47% at 100 µg/mL. Doxorubicin, used as a positive control, showed strong cytotoxicity across all concentrations, reaching ~95.65% at 100 µg/mL. These results clearly indicate that Thef-TiNPs possess enhanced anticancer activity due to the synergistic effects of theaflavin and titanium, suggesting their potential as an effective nano-formulated therapeutic agent for oral cancer treatment (Figure 5).

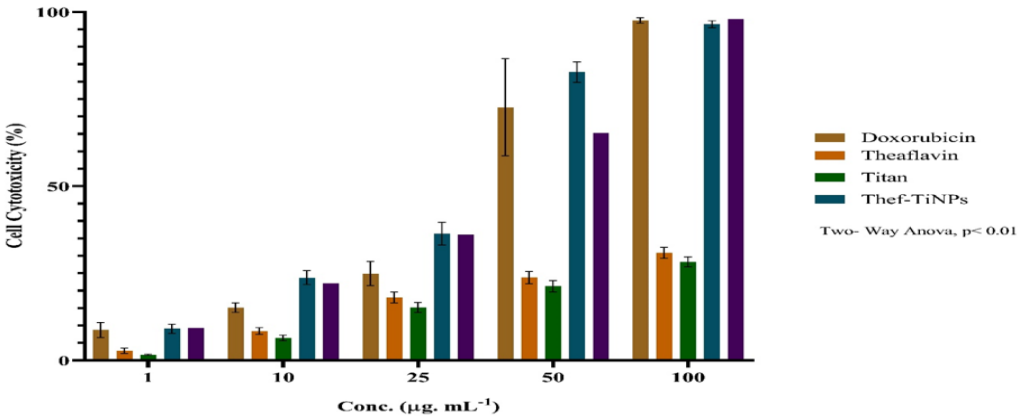

Figure 5. MTT assay graph showing the dose-dependent cytotoxicity of Doxorubicin, Theaflavin, Titanium nanoparticles (TiO_2), and Thef-TiNPs against KB cells. Thef-TiNPs exhibited significantly higher cytotoxicity compared to individual components, with nearly complete loss of cell viability at 100 µg/mL.The results are expressed as mean ± SD (n = 3), with statistical significance evaluated using one-way ANOVA followed by Tukey's multiple comparison test.

3.4 Antioxidant Activity of Thef-TiNPs Assessed by DPPH Radical Scavenging Assay

The antioxidant activity of Theaflavin, Titanium nanoparticles, and TheF-TiNPs was assessed through the DPPH free radical scavenging assay, with Diclofenac serving as the reference antioxidant control. A clear dose-dependent increase in DPPH inhibition was observed across all treatment groups. Diclofenac showed strong radical scavenging activity, beginning at 9.36% inhibition at 1 µg/mL and reaching up to 97.59% at 100 µg/mL, confirming its potency as a positive control. Theaflavin alone displayed moderate antioxidant activity, with inhibition values of 2.76%, 8.42%, 18.02%, 23.78%, and 30.87% from 1 to 100 µg/mL, respectively[8]. Titanium nanoparticles showed relatively lower antioxidant effects, starting from 1.64% at 1 µg/mL and increasing to 28.25% at 100 µg/mL. In contrast, Thef-TiNPs exhibited significantly higher antioxidant activity compared to both theaflavin and titanium alone. At 1 µg/mL, Thef-TiNPs showed 9.10% scavenging, increasing to 23.76%, 36.36%, 82.73%, and 96.49% at 10, 25, 50, and 100 µg/mL, respectively. The analysis indicates that Thef-TiNPs effectively preserve and enhance the antioxidant capacity of theaflavin through nanoparticle formulation, showing activity nearly comparable to diclofenac at the highest concentration (Figure 6).

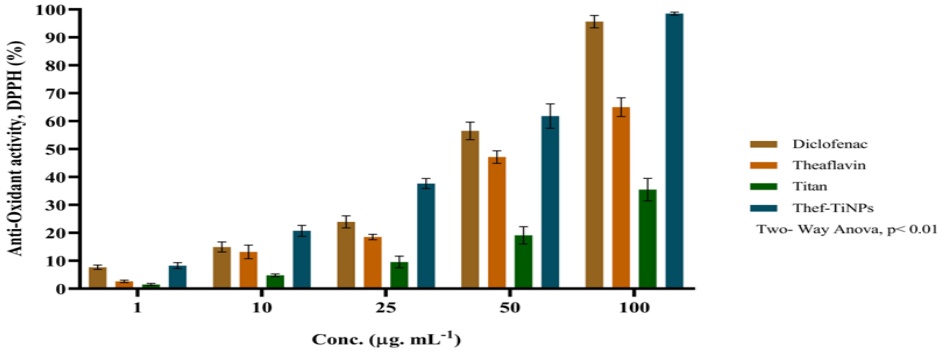

Figure 6. DPPH radical scavenging activity was assessed for Diclofenac (positive control), Theaflavin, Titanium nanoparticles (TiO_2), and Thef-TiNPs at concentrations of 1, 10, 25, 50, and 100 µg/mL. Thef-TiNPs demonstrated markedly greater antioxidant activity than either Theaflavin or Titanium alone, with scavenging efficiency approaching that of Diclofenac at higher doses. Results are expressed as mean ± SD (n = 3), and statistical significance was evaluated using one-way ANOVA followed by Tukey's post hoc test.

3.5 Modulation of EGF Signalling and Apoptotic Genes by Thef-TiNPs in KB Cells

Quantitative real-time PCR analysis showed that exposure to Thef-TiNPs at 25 µg/mL significantly altered the expression of critical genes associated with EGFR signaling and apoptotic pathways in KB cells. A marked downregulation of EGFR expression (0.38 ± 0.04-fold; $p < 0.01$) was observed, indicating inhibition of the upstream proliferative receptor. Correspondingly, AKT1, a major pro-survival effector downstream of EGFR, was also significantly suppressed (0.42 ± 0.05-fold; $p < 0.01$), suggesting effective blockade of the PI3K/AKT signalling axis. Expression analysis showed a significant increase in the pro-apoptotic gene BAX, with a 2.71 ± 0.11-fold upregulation ($p < 0.01$), while the anti-apoptotic gene BCL-2 was markedly reduced to 0.46 ± 0.06-fold ($p < 0.01$). This shift led to a higher BAX/BCL-2 ratio, indicating a cellular environment inclined toward apoptosis. Additionally, Caspase-3, an executioner caspase in the intrinsic apoptotic pathway, showed a strong increase in expression (2.23 ± 0.15-fold; $p < 0.001$), confirming activation of programmed cell death. Furthermore, expression of HSP70, a molecular chaperone involved in cellular protection against stress and apoptosis inhibition, was significantly reduced (0.49 ± 0.07-fold; $p < 0.01$), indicating lowered cellular resistance to apoptosis. These results collectively demonstrate that Thef-TiNPs effectively suppress tumour survival signalling and activate apoptosis-related genes, contributing to their potent anticancer activity in KB cells (Figure 7).

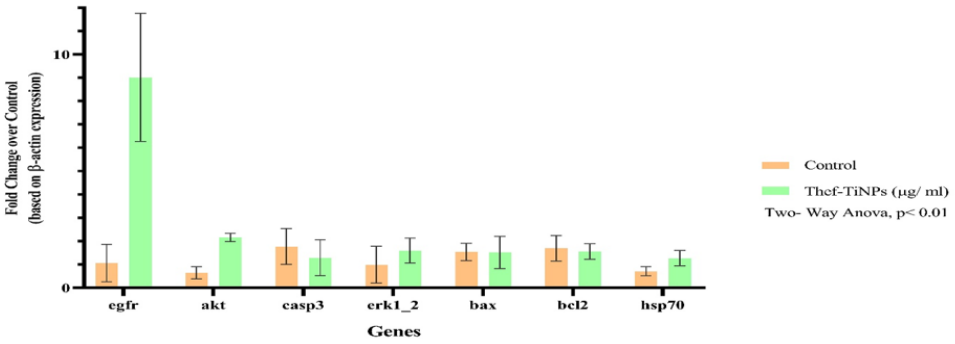

Figure 7: Comparative mRNA expression levels of EGFR, AKT1, BAX, BCL-2, CASPASE-3, and HSP70 in KB cells treated with Thef-TiNPs at 25 µg/mL, compared to untreated control. Thef-TiNPs significantly downregulated EGFR, AKT1, BCL-2, and HSP70, while upregulating pro-apoptotic genes BAX and CASPASE-3. Gene expression values were subsequently normalized to GAPDH using the ΔΔCt method. Data represent mean ± SD (n = 3)[9]. Statistical analysis was done using one-way ANOVA followed up by Tukey's post hoc test; $p < 0.05$ was considered significant.

4.DISCUSSION

The current research explores the anticancer activity of Thef-TiNPs in human oral cancer KB cells, specifically focusing on their impact on the EGF signalling pathway and related apoptotic markers. The integration of a natural polyphenol, theaflavin, with titanium dioxide in a nanoparticle formulation aims to overcome the intrinsic limitations of plant-derived compounds—namely, poor solubility, stability, and cellular delivery—while amplifying their therapeutic efficacy. The MTT assay demonstrated a distinct dose-dependent cytotoxic effect of Thef-TiNPs on KB cells. The nanoparticles demonstrated significantly higher potency than theaflavin or titanium alone, achieving nearly complete cell death at 100 µg/mL. The increased cytotoxicity may be ascribed to the synergistic interaction between the antioxidant and pro-apoptotic properties of theaflavin and the biophysical advantages of titanium nanoparticles. The nanoscale size (45–65 nm), confirmed via SEM, likely facilitates cellular uptake through endocytosis, allowing efficient intracellular delivery and bioavailability[15]. In comparison, titanium and theaflavin alone displayed relatively moderate cytotoxic effects, underscoring the superior functional efficiency of the nanoparticle formulation. The antioxidant capacity of Thef-TiNPs was corroborated by the DPPH radical scavenging assay. The formulation exhibited a steep dose-response relationship, with antioxidant activity nearly matching that of diclofenac at the highest tested concentration. This observation suggests that theaflavin retains its electron-donating, ROS-scavenging properties even when conjugated to a titanium core. Moreover, the metal-polyphenol interface may induce redox-active interactions that enhance ROS detoxification. This combined functionality—both antioxidant and cytotoxic—offers a distinct advantage in the context of cancer, where oxidative imbalance and redox signalling are critical to tumour progression and drug resistance[15,16].

Microscopic examination further supported the MTT data by revealing concentration-dependent morphological damage. At lower concentrations (10 µg/mL), treated cells exhibited subtle changes including reduction in cytoplasmic volume and incomplete detachment, while at the highest concentration (100 µg/mL), pronounced cell rounding, loss of adhesion, and cellular debris indicative of apoptosis or necrosis. These morphological changes, in line with reduced viability, reflect cytoskeletal disruption and membrane damage—typical hallmarks of late-stage apoptosis. The most compelling evidence came from the gene expression analysis targeting key nodes in the EGF signalling and apoptotic pathways. EGFR and AKT1, critical components of the PI3K/AKT survival axis, were significantly downregulated upon Thef-TiNPs exposure. This implies that the nanoparticles not only inhibit surface receptor activation but also suppress downstream prosurvival signalling cascades. Downregulation of EGFR limits the ligand-dependent receptor autophosphorylation that triggers mitogenic and anti-apoptotic responses, while suppression of AKT further blocks cellular survival mechanisms, glucose metabolism, and resistance to apoptosis. These changes confirm that Thef-TiNPs target the very axis often overexpressed in oral squamous cell carcinoma[17].

The pro-apoptotic impact of Thef-TiNPs was further emphasized by the elevated expression of BAX and Caspase-3, alongside downregulation of BCL-2. BAX induces mitochondrial outer membrane permeabilization, enabling the release of cytochrome c and triggering caspase activation, with caspase-3 being a primary target. An elevated BAX/BCL-2 ratio drives the cellular equilibrium toward apoptosis, counteracting pro-survival signals. This effect was further confirmed by the upregulation of caspase-3, a key executioner caspase that cleaves essential cellular proteins during programmed cell death. Downregulation of HSP70, a molecular chaperone known to stabilize anti-apoptotic proteins and suppress apoptotic cascades, further facilitated cell death by weakening cellular stress responses and repair mechanisms. Taken together, the gene expression patterns reveal a coordinated shutdown of survival pathways and an activation of the intrinsic apoptotic machinery. The ability of Thef-TiNPs to modulate both upstream (EGFR, AKT) and downstream (BAX, BCL-2, Caspase-3) regulators highlights their potential as multi-targeted anticancer agents. These findings are consistent with previous reports on polyphenol-metal nanoparticle hybrids, where enhanced bioactivity is achieved through combinatorial effects.

From a translational perspective, these results position Thef-TiNPs as a promising candidate for nanotherapeutic applications in oral cancer. Compared to traditional chemotherapeutics like

doxorubicin, which exert non-specific cytotoxic effects and are often associated with systemic toxicity, Thef-TiNPs may offer a safer, more targeted approach. Their formulation using a dietary bioactive also aligns with the current trend toward biocompatible and naturally derived anticancer strategies. Furthermore, titanium's inherent biocompatibility and prior use in dental and orthopedic implants support its suitability for localized therapy in oral malignancies. Despite these promising outcomes, some limitations must be acknowledged. The study was conducted solely in vitro, and further in vivo validation is necessary to assess pharmacokinetics, biodistribution, systemic toxicity, etc. Mechanistic studies involving protein-level validation (e.g., western blotting for EGFR and caspase activation) would provide additional depth. Nonetheless, the current findings establish a strong foundation for future exploration of Thef-TiNPs in oral cancer treatment[18].

5. CONCLUSION

Thef-TiNPs demonstrate significant anticancer activity against KB cells through a multifaceted mechanism involving suppression of EGFR/AKT survival signalling and activation of intrinsic apoptotic pathways. The formulation exhibited superior cytotoxicity and antioxidant activity compared to theaflavin or titanium alone, with potent modulation of key regulatory genes including EGFR, AKT1, BAX, BCL-2, Caspase-3, and HSP70. These findings suggest that Thef-TiNPs hold substantial promise as a biocompatible and targeted nanotherapeutic approach for oral cancer, offering a novel strategy to enhance the efficacy of natural compounds through nanoparticle-based delivery. Additional in vivo investigations and translational clinical studies are needed to comprehensively realize their therapeutic potential.

DECLARATION

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Competing interest

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Ethical Approval

Not applicable

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