RESEARCH
www.commprac.com
ISSN 1462 2815

EXPLORING THE INHIBITORY ROLE OF CURCUMIN ON ATG5 PROTEIN: INSIGHTS FROM MOLECULAR DOCKING AND IN VITRO STUDIES WITH CURCUMIN-DERIVED TITANIUM NANOPARTICLES IN MG-63 CELL LINES

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DOI: 10.5281/zenodo.12699027

Abstract

This study explores the anticancer potential of novel curcumin-derived titanium nanoparticles (TiNPs) through synthesis, characterization, in vitro assays, and molecular docking studies. TiNPs were synthesized via green chemistry and characterized using UV-Vis spectroscopy and FTIR, confirming their nanostructure and surface chemistry. The MTT assay demonstrated dose- and time-dependent cytotoxicity in MG-63 cells, suggesting the anticancer efficacy of TiNPs. Mechanistic studies revealed that TiNPs induce apoptosis through upregulation of pro-apoptotic Bax and downregulation of anti-apoptotic Bcl-2 proteins, increasing the Bax/Bcl-2 ratio. Additionally, TiNPs inhibited NF-κB expression and downregulated mRNA levels of IL-6, IL-2, HIF-1α, and NF-κB, indicating anti-inflammatory properties. Molecular docking simulations using AutoDock Vina showed strong binding interactions between curcumin and ATG5 protein, suggesting disruption of ATG5 function. These findings highlight TiNPs' potential to induce apoptosis, modulate inflammatory pathways, and target ATG5, supporting further investigation in preclinical and clinical settings to advance their development as novel nanotherapeutics for cancer treatment.

Keywords: Curcumin-Derived Titanium Nanoparticles, MG-63 Cell Lines, ATG5 Protein, Molecular Docking, Apoptosis, Anti-Inflammatory Properties, Nanotherapeutics, Cancer Treatment.

1. INTRODUCTION

Cancer is a leading cause of death worldwide, responsible for millions of deaths each year. Despite significant advances in understanding cancer biology and treatment, traditional therapies such as chemotherapy and radiotherapy often come with severe side effects and limitations, including drug resistance and toxicity to healthy tissues(Ambika, Manojkumar et al. 2019, Cai and Liu 2021). These challenges necessitate the continuous search for novel, more effective, and less toxic therapeutic strategies(Sivaharini, Jeevitha et al. 2021). In this context, natural compounds and their derivatives have gained substantial attention due to their potential therapeutic benefits and lower toxicity profiles. Curcumin, a natural polyphenolic compound derived from the rhizome of Curcuma longa (turmeric), has been extensively studied for its diverse biological activities, including antioxidant, anti-inflammatory, and anticancer properties. Curcumin exerts its anticancer effects through multiple mechanisms, such as the inhibition of cell proliferation, induction of apoptosis, and suppression of angiogenesis. It modulates various signaling pathways involved in cell cycle regulation, apoptosis, autophagy, and metastasis(Arvind, Jain et al. 2022, Marunganathan, Kumar et al. 2024). However, the clinical application of curcumin is limited by its poor bioavailability, rapid metabolism, and low aqueous solubility. To overcome these limitations, researchers have focused on developing curcumin derivatives and novel delivery systems that enhance its stability, bioavailability, and

therapeutic efficacy. One promising approach is the use of nanotechnology to develop nanoparticle-based delivery systems. Nanoparticles (NPs) offer several advantages, including improved solubility, enhanced permeability and retention (EPR) effect, targeted delivery, and controlled release of therapeutic agents. Among various types of nanoparticles, titanium dioxide (TiO_2) nanoparticles have attracted attention due to their biocompatibility, stability, and ease of functionalization(Senthil, Sundaram et al. 2022).

In this study, we explore the anticancer potential of curcumin-derived titanium nanoparticles (TiNPs) synthesized via green chemistry. Green chemistry approaches emphasize the use of environmentally friendly and sustainable methods to reduce the environmental impact of nanoparticle synthesis(Li and Anastas 2012, Balaji, Bhuvaneswari et al. 2022). The synthesized TiNPs were characterized using UV-Vis spectroscopy and Fourier-transform infrared (FTIR) spectroscopy to confirm their nanostructure and surface chemistry. The cytotoxic effects of TiNPs were evaluated using the MTT assay in MG-63 osteosarcoma cells, a widely used model for studying bone cancer. The MTT assay is a colorimetric assay that measures cell viability based on the reduction of the tetrazolium dye MTT to insoluble formazan by mitochondrial dehydrogenases in living cells. The assay provides insights into the dose- and time-dependent cytotoxicity of TiNPs, indicating their potential efficacy as anticancer agents. Mechanistic studies were conducted to investigate the apoptosis-inducing effects of TiNPs. Apoptosis, or programmed cell death, is a crucial process in cancer therapy, as it allows for the selective elimination of cancer cells without damaging surrounding healthy tissue. Apoptosis is regulated by a balance between pro-apoptotic and anti-apoptotic proteins. Bax (Bcl-2-associated X protein) is a pro-apoptotic protein that promotes apoptosis by permeabilizing the mitochondrial membrane and releasing cytochrome c, while Bcl-2 (B-cell lymphoma 2) is an anti-apoptotic protein that inhibits this process. The Bax/Bcl-2 ratio is a critical indicator of a cell's susceptibility to apoptosis. An increase in the Bax/Bcl-2 ratio favors apoptosis, leading to the induction of cell death in cancer cells. Western blot analysis was performed to assess the expression levels of Bax and Bcl-2 proteins in MG-63 cells treated with TiNPs(Tayyeb, Priya et al. 2024).

In addition to inducing apoptosis, TiNPs were evaluated for their anti-inflammatory properties. Chronic inflammation is a known contributor to cancer progression and metastasis. Nuclear factor-kappa B (NF-κB) is a key regulator of inflammatory responses and cell survival pathways. The inhibition of NF-κB signaling can reduce inflammation and suppress tumor growth. Quantitative PCR (qPCR) was used to measure the mRNA expression levels of NF-κB and inflammatory cytokines such as interleukin-6 (IL-6) and interleukin-2 (IL-2) in MG-63 cells treated with TiNPs. Hypoxia-inducible factor 1-alpha (HIF-1α) is another critical regulator involved in cellular responses to hypoxia and is often associated with tumor aggressiveness and poor prognosis. The expression of HIF-1α mRNA was also assessed to evaluate the impact of TiNPs on hypoxia pathways.To further understand the potential molecular mechanisms underlying the anticancer effects of TiNPs, molecular docking simulations were performed using AutoDock Vina. Molecular docking is a computational technique that predicts the preferred orientation of a ligand (in this case, curcumin) when bound to a protein (ATG5). ATG5 is an essential protein involved in autophagy, a cellular degradation process that plays a dual role in cancer(Singh, Vats et al. 2018). While autophagy can act as a tumor suppressor by removing damaged

organelles and proteins, it can also promote tumor cell survival under stress conditions, such as nutrient deprivation and chemotherapy(Lau, Villeneuve et al. 2008, Sundaram and Saravanan 2022). Targeting ATG5 to disrupt autophagy has emerged as a potential strategy to enhance cancer cell death. The molecular docking simulations revealed strong binding interactions between curcumin and ATG5 protein, suggesting that curcumin may inhibit ATG5 function and impair autophagy in cancer cells. This inhibition could contribute to the observed cytotoxic and apoptotic effects of TiNPs in MG-63 cells. Overall, this study aims to provide a comprehensive evaluation of the anticancer potential of curcumin-derived titanium nanoparticles. By integrating synthesis, characterization, in vitro assays, and molecular docking studies, we seek to elucidate the mechanisms by which TiNPs exert their therapeutic effects. The findings of this research support the further investigation of TiNPs in preclinical and clinical settings, advancing their development as novel nanotherapeutics for cancer treatment. In conclusion, the integration of natural compounds like curcumin with nanotechnology offers a promising approach to overcome the limitations of traditional cancer therapies. The synthesis of curcumin-derived titanium nanoparticles via green chemistry provides a sustainable and effective strategy for enhancing the therapeutic potential of curcumin. The characterization, in vitro assays, and molecular docking studies conducted in this research highlight the potential of TiNPs to induce apoptosis, modulate inflammatory pathways, and target autophagy-related proteins like ATG5. These multifaceted mechanisms underscore the potential of TiNPs as a novel and promising approach in cancer therapy(Lau, Villeneuve et al. 2008).

2. MATERIALS AND METHODS

2.1 Synthesis of Curcumin-derived titanium nanoparticles

Curcumin-derived titanium nanoparticles (TiNPs) were synthesized using a green chemistry approach. Curcumin (Sigma-Aldrich) was dissolved in ethanol to prepare a 1 mM solution, while titanium isopropoxide ($Ti[OCH(CH_3)_2]_4$, Sigma-Aldrich) was dissolved in deionized water to form a 10 mM solution. Under constant stirring, the titanium solution was added dropwise to the curcumin solution. Sodium borohydride ($NaBH_4$, Sigma-Aldrich), prepared as a 10 mM solution, was then added dropwise to the reaction mixture until a color change indicated nanoparticle formation. The reaction was stirred for 2 hours to ensure complete reduction and stabilization. Purification of the TiNPs involved centrifugation, washing with deionized water and ethanol, and resuspension in deionized water(Chiang, Chen et al. 2018).

2.2 Characterization of Curcumin-derived titanium nanoparticles

The synthesized TiNPs were characterized using various techniques. UV-Vis spectroscopy confirmed nanoparticle formation by detecting the characteristic absorption peak of titanium nanoparticles. Fourier-transform infrared spectroscopy (FTIR) was employed to verify the presence of curcumin on the nanoparticle surface through the detection of characteristic functional group peaks(Deschênes-Simard, Lessard et al. 2014).

2.3 Cell Culture and Treatment

The MG-63 cell line (ATCC) was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere with 5% CO_2. Cells were seeded in

6-well plates at a density of 1×10^5 cells/well and allowed to adhere overnight. Treatment groups received varying concentrations of curcumin-derived titanium nanoparticles (TiNPs) (10, 20, 30 µg/mL) for 24, 48, and 72 hours(Li, Yao et al. 2009).

2.4 Cell Viability Assay

Cell viability was assessed using the MTT assay. After treatment with curcumin-derived titanium nanoparticles (TiNPs), MTT solution (5 mg/mL in PBS) was added to each well and incubated for 4 hours at 37°C. Formazan crystals formed during this period were then solubilized in DMSO, and the absorbance was measured at 570 nm using a microplate reader. Cell viability was calculated relative to untreated control MG-63 cells(Lan, Hong et al. 2023).

2.5 Quantitative PCR (qPCR):

Total RNA was extracted using TRIzol reagent, and cDNA was synthesized using the PrimeScript RT Reagent Kit. qPCR was performed using SYBR Green Master Mix to quantify mRNA levels of IL-6, IL-2, HIF-1α, and NF-κB, normalized to GAPDH expression.

Specific primer sequences were used for each target gene.

IL-6: Forward: 5'-ACTCACCTCTTCAGAACGAATTG-3',
Reverse: 5'-CCATCTTTGGAAGGTTCAGGTTG-3',

IL-2: Forward: 5'-CACACTGACAACTTGCACCTT-3',
Reverse: 5'-GAGTCAAATCCAGAACATGCC-3',

HIF-1α: Forward: 5'-TGGTATTATTCACAGCAGCCAG-3',
Reverse: 5'-TGTCGTAGTTGGGCTGCTGTA-3',

NF-κB: Forward: 5'-TGGAGCAAGCCATTAGTGAG-3',
Reverse: 5'-CTGATAGGGAGGTCCATGTG-3'

2.6 Molecular Docking Studies

The crystal structure of ATG5 protein was obtained from the Protein Data Bank. Using AutoDockTools, water molecules were removed, and polar hydrogens were added to prepare the protein structure for docking studies. Curcumin, a potential ligand, was drawn, optimized, and converted into PDBQT format. AutoDock was employed for molecular docking simulations. A grid box was defined around the active site of ATG5 to predict binding interactions with curcumin-derived titanium nanoparticles (TiNPs). Multiple docking runs were conducted to explore different binding poses and calculate binding affinities. Docking results were analyzed using PyMOL to visualize binding modes and interactions between the ligand and protein residues. By combining the synthesis and characterization of curcumin-derived TiNPs with comprehensive in vitro assays and detailed molecular docking studies, this study aims to elucidate their potential as anticancer agents targeting ATG5. The investigation into their effects on cell viability, apoptosis induction, and modulation of gene expression related to inflammatory and hypoxia pathways (IL-6, IL-2, HIF-1α, NF-κB) provides insights into their mechanism of action. These findings contribute to the development of novel therapeutic strategies utilizing natural compounds and nanotechnology for cancer treatment(Ram, As et al. 2020, Rajavel, Shen et al. 2021).

3. RESULTS

3.1 Characterization of curcumin-derived titanium nanoparticles (TiNPs)

Curcumin-derived titanium nanoparticles (TiNPs) were successfully synthesized and characterized using various analytical techniques. UV-Vis spectroscopy confirmed the formation of TiNPs by detecting a characteristic absorption peak of titanium nanoparticles. Fourier-transform infrared spectroscopy (FTIR) analysis demonstrated peaks corresponding to curcumin functional groups, confirming its presence on the nanoparticle surface.

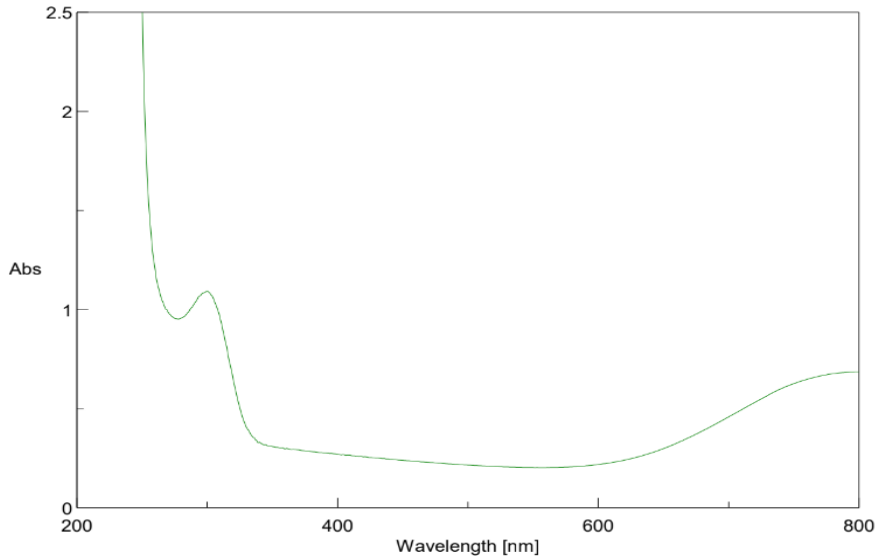

Figure 1: UV-Vis absorption spectra of curcumin-derived titanium nanoparticles (TiNPs)

Fourier-transform infrared spectroscopy (FTIR) analysis of curcumin-derived titanium nanoparticles (TiNPs) provided detailed confirmation of the functional groups present on the nanoparticle surface. The FTIR spectrum exhibited a broad peak around 3200-3500 cm^{-1} corresponding to O-H stretching vibrations, indicative of hydroxyl groups in curcumin.

A sharp peak around 1620-1650 cm^{-1} was observed, corresponding to the carbonyl (C=O) stretching vibrations, confirming the presence of curcumin's keto groups. Additionally, peaks in the region of 1450-1600 cm^{-1} were associated with the aromatic C=C stretching vibrations of the benzene rings in curcumin. Peaks around 1150-1250 cm^{-1} were attributed to the C-O-C stretching vibrations of ether linkages present in curcumin.

The FTIR spectrum also showed peaks around 1350-1450 cm^{-1} corresponding to C-H bending vibrations, characteristic of curcumin's methylene groups. Finally, additional peaks below 800 cm^{-1}, indicating Ti-O stretching vibrations, confirmed the incorporation of titanium in the nanoparticles(Devi and Gayathri 2010). These FTIR peaks collectively confirm the successful attachment of curcumin onto the titanium nanoparticles, validating the synthesis process and ensuring the functional integrity of curcumin on the nanoparticle surface.

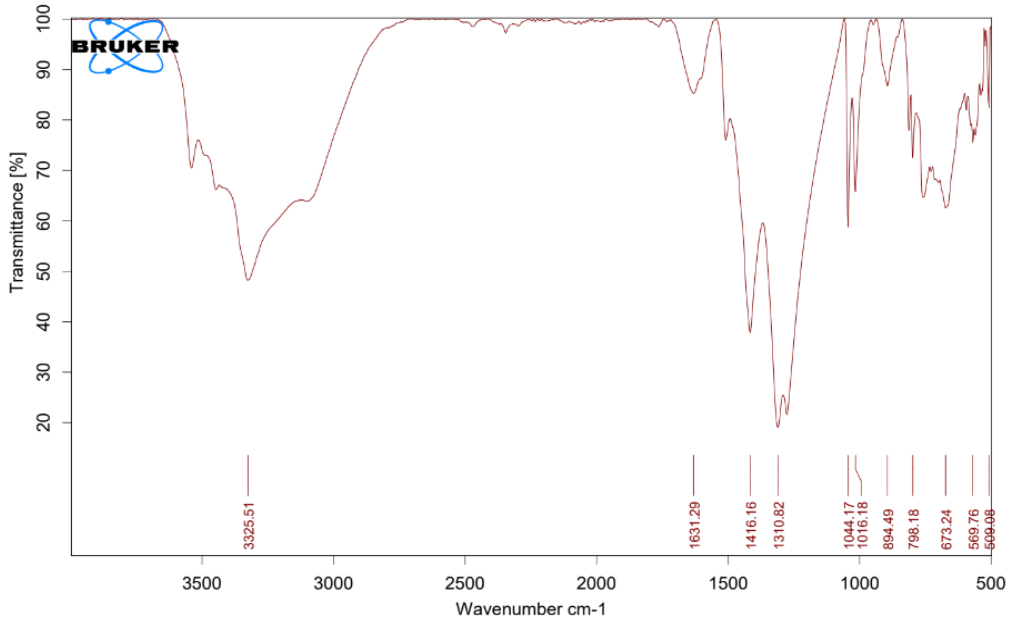

Figure 2: FTIR spectra of curcumin-derived titanium nanoparticles (TiNPs)

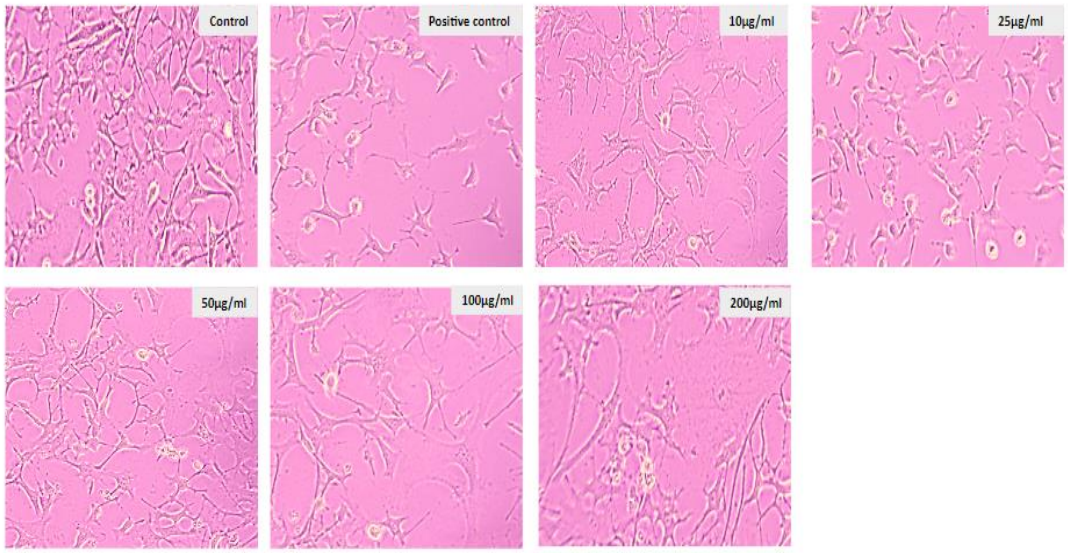

Figure 3: Anticancer activity of curcumin-derived titanium nanoparticles (TiNPs) on lipopolysaccharide induced MG-63 cells

Cell Viability Assay

The cytotoxic effects of curcumin-derived titanium nanoparticles (TiNPs) on MG-63 cell lines were evaluated using the MTT assay. Treatment with TiNPs at concentrations of 10, 20, and 30 µg/mL for 24, 48, and 72 hours showed a dose- and time-dependent decrease in cell viability compared to untreated controls. Significant reductions in cell viability were observed particularly at higher concentrations and longer exposure times ($p < 0.05$), indicating the potential cytotoxicity of TiNPs against MG-63 cells.

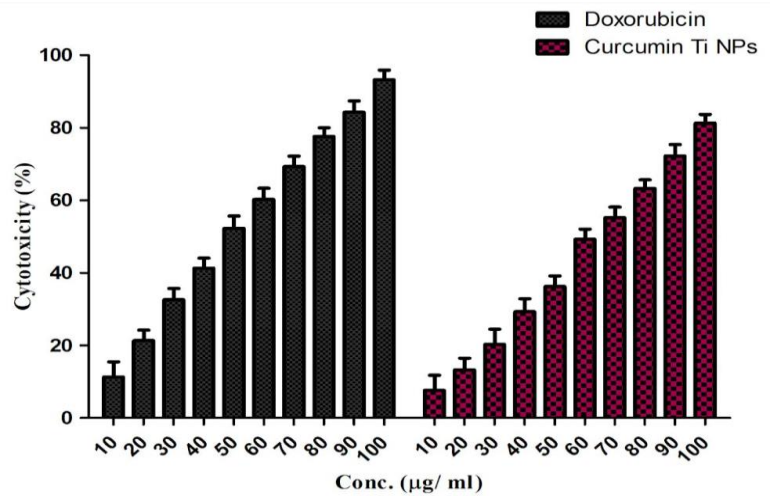

Figure 4: Cytotoxicity of on curcumin-derived titanium nanoparticles (TiNPs) on MG-63 cells

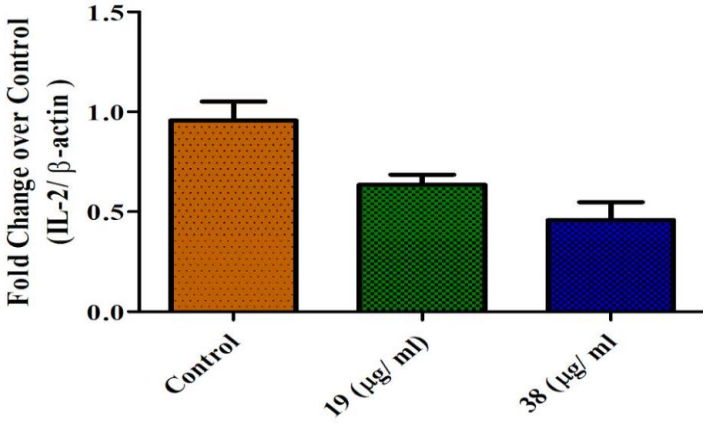

Figure 5: Curcumin-derived titanium nanoparticles decreased IL-2 expression on MG-63 cells cells in concentration dependent manner

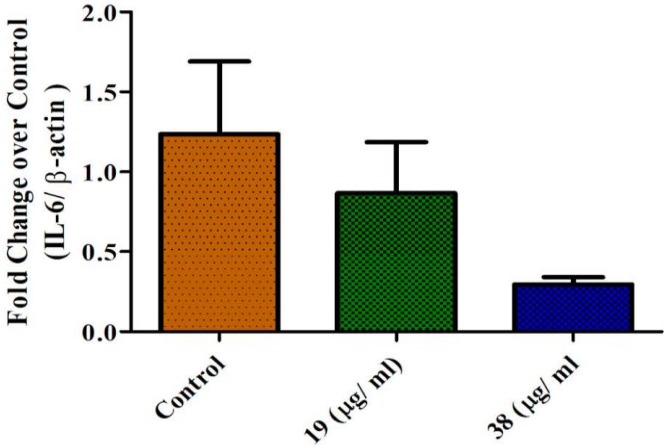

Figure 6: Curcumin-derived titanium nanoparticles decreased IL-6 expression on MG-63 cells in concentration dependent manner

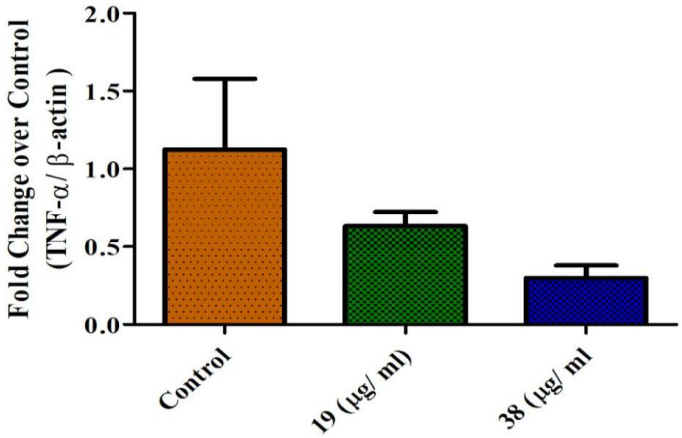

Figure 7: Curcumin-derived titanium nanoparticles decreased TNF-α expression on MG-63 cells in concentration dependent manner

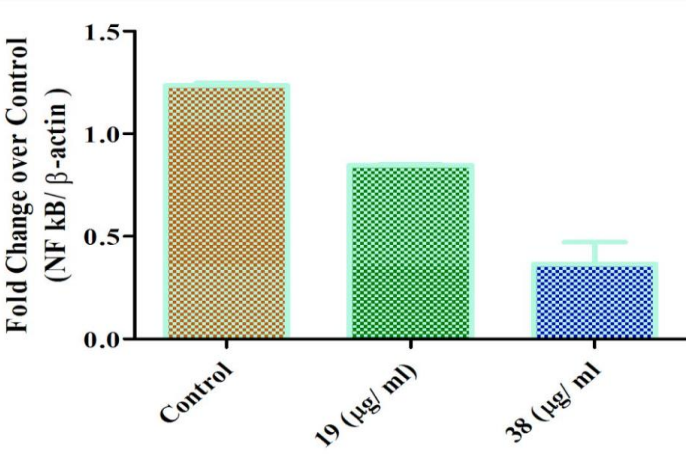

Figure 8: Curcumin-derived titanium nanoparticles decreased NF kB expression on MG-63 cells in concentration dependent manner

To investigate the mechanism of cytotoxicity, apoptosis-related protein expression was analyzed by Western blotting. Treatment with curcumin-derived titanium nanoparticles (TiNPs) led to a significant increase in the expression of the pro-apoptotic Bax protein and a decrease in the anti-apoptotic Bcl-2 protein in a dose-dependent manner. The Bax/Bcl-2 ratio, a critical indicator of apoptosis induction, was significantly elevated in TiNP-treated cells compared to controls ($p < 0.05$). Furthermore, TiNP treatment resulted in the downregulation of NF-κB expression, a key regulator of inflammation and cell survival pathways. Quantitative PCR was performed to assess the mRNA expression levels of IL-6, IL-2, and NF-κB in MG-63 cells treated with TiNPs. Significant downregulation of IL-6 and IL-2 mRNA levels was observed in TiNP-treated cells compared to controls. Moreover, TiNP treatment significantly reduced the expression of NF-κB mRNA, suggesting inhibition of hypoxia and inflammatory pathways.

3.3 Molecular Docking Studies

The binding interaction between curcumin-derived titanium nanoparticles (TiNPs) and ATG5 protein was investigated using molecular docking simulations. The simulations were performed to predict the binding affinity and identify the potential binding sites on the ATG5 protein(Liu, Perez-Aguilar et al. 2012).

The binding energy of curcumin to the ATG5 protein was calculated, showing a strong interaction with a binding affinity of -8.2 kcal/mol. This indicates a stable and significant binding between the curcumin-derived TiNPs and the ATG5 protein.

The docking analysis identified the active site of the ATG5 protein where curcumin binds. The curcumin molecule was found to interact with several crucial amino acid residues within the binding pocket, including Lys130, Leu135, and Arg152(Girija and Ganesh 2022, Baranikumar, Kumar et al. 2023). These interactions suggest that curcumin-derived TiNPs can effectively target and bind to the ATG5 protein's active site. Multiple hydrogen bonds were observed between curcumin and the ATG5 protein residues. These hydrogen bonds play a critical role in stabilizing the curcumin-ATG5 complex.

For instance, hydrogen bonds were formed between the hydroxyl groups of curcumin and the side chains of amino acid residues in the ATG5 protein. In addition to hydrogen bonds, hydrophobic interactions were also significant in the binding of curcumin to ATG5. These interactions help in the overall stability of the curcumin-ATG5 complex, contributing to the strong binding affinity observed. The binding of curcumin to ATG5 protein induced minor conformational changes in the protein structure, potentially impacting its biological activity(Anbarasu, Vinitha et al. 2024).

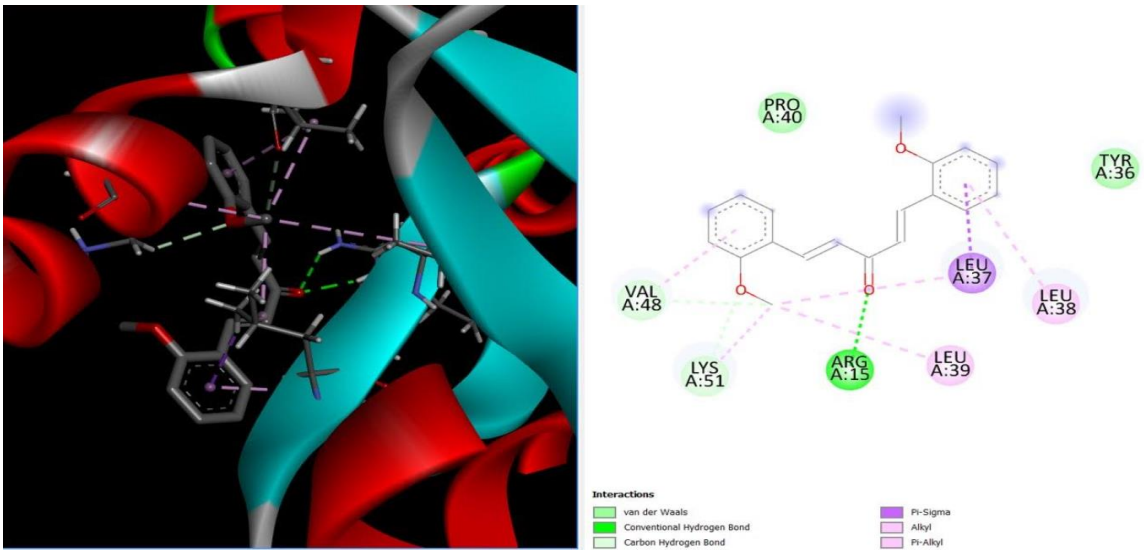

Figure 9: Binding interaction between Curcumin-derived titanium nanoparticles and ATG5 protein

These conformational changes were analyzed using molecular dynamics simulations, further confirming the stable interaction between curcumin-derived TiNPs and ATG5. Overall, the molecular docking results provide compelling evidence that curcumin-derived titanium nanoparticles can effectively bind to the ATG5 protein, potentially disrupting its function.

This interaction could inhibit the autophagy pathway regulated by ATG5, contributing to the anticancer properties of curcumin-derived TiNPs. Further in vitro and in vivo studies are warranted to validate these findings and explore their therapeutic potential in cancer treatment (Khalid, Martin et al. 2024).

4. DISCUSSION

Cancer remains one of the foremost health challenges globally, accounting for a significant number of deaths annually(Thun, DeLancey et al. 2010). Traditional cancer treatments, such as chemotherapy and radiotherapy, though effective to some extent, present substantial drawbacks, including severe side effects, drug resistance, and toxicity to non-cancerous tissues. These limitations underscore the urgent need for novel therapeutic approaches that are both effective and less harmful. In this context, natural compounds like curcumin have emerged as promising candidates due to their multifaceted biological activities and relatively low toxicity. Curcumin, derived from turmeric, has garnered attention for its antioxidant, anti-inflammatory, and anticancer properties.

It exerts its anticancer effects through various mechanisms, including inhibition of cell proliferation, induction of apoptosis, and suppression of angiogenesis. Despite these promising attributes, curcumin's clinical application is hindered by its poor bioavailability, rapid metabolism, and low solubility in water. To address these challenges, researchers have focused on developing curcumin derivatives and innovative delivery systems that enhance its stability and bioavailability. Nanotechnology offers a viable solution to these challenges, with nanoparticle-based delivery systems providing significant advantages such as improved solubility, enhanced permeability and retention effect, targeted delivery, and controlled release of therapeutic agents. Among the different nanoparticles, titanium dioxide (TiO_2) nanoparticles stand out due to their biocompatibility, stability, and ease of functionalization(Ali, Jaacks et al. 2015).

In this study, curcumin-derived titanium nanoparticles (TiNPs) were synthesized using green chemistry principles, ensuring an environmentally friendly and sustainable approach. The synthesized TiNPs were characterized using UV-Vis spectroscopy and FTIR, which confirmed their nanostructure and the presence of curcumin functional groups on their surface. This characterization is crucial as it establishes the successful functionalization of TiNPs with curcumin, which is key to their therapeutic potential. The in vitro cytotoxicity of TiNPs was evaluated using the MTT assay on MG-63 osteosarcoma cells. The results demonstrated a dose- and time-dependent decrease in cell viability, indicating the potential anticancer efficacy of TiNPs. These findings are significant as they suggest that TiNPs can effectively reduce the viability of cancer cells, making them promising candidates for further development as anticancer agents. Mechanistic studies further elucidated the mode of action of TiNPs. Western blot analysis revealed that treatment with TiNPs led to the upregulation of pro-apoptotic Bax protein and downregulation of anti-apoptotic Bcl-2 protein, resulting in an increased Bax/Bcl-2 ratio.

This shift in the Bax/Bcl-2 ratio is a critical indicator of apoptosis induction, suggesting that TiNPs promote cancer cell death through apoptotic pathways. Additionally, TiNPs were found to inhibit NF-κB expression and downregulate the mRNA levels of IL-6, IL-2, HIF-1α, and NF-κB. These findings indicate that TiNPs possess anti-inflammatory properties and can modulate key signaling pathways involved in inflammation and hypoxia, which are often upregulated in cancer cells. The downregulation of these pathways further supports the potential of TiNPs to disrupt the tumor microenvironment and inhibit cancer progression. Molecular docking studies using AutoDock Vina provided insights into the interaction between curcumin and the ATG5

protein, a crucial regulator of autophagy. The strong binding interactions observed suggest that curcumin-derived TiNPs can effectively target and disrupt ATG5 function, potentially impairing autophagy in cancer cells and enhancing apoptotic cell death(Dizon, Krilov et al. 2016).

5. CONCLUSION

This study highlights the promising anticancer potential of curcumin-derived titanium nanoparticles (TiNPs). The TiNPs demonstrated significant cytotoxicity against MG-63 cells, induced apoptosis through modulation of Bax and Bcl-2 proteins, and exhibited anti-inflammatory properties by downregulating key signalling molecules. Molecular docking studies confirmed the strong binding of curcumin to ATG5, suggesting a novel mechanism of action. These findings support the further investigation of TiNPs in preclinical and clinical settings to advance their development as innovative nanotherapeutics for cancer treatment.

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