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Anti-Angiogenic Activity of Theaflavin-Coated Copper Nanoparticles Through VEGF Inhibition in Lung Cancer Cells

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Abstract

Background: *Lung cancer continues to be the major cause for deaths by cancer, with urgent need for novel therapeutics. Theaflavin, a natural polyphenol, exibits anti-cancer properties, and copper nanoparticles (CuNPs) are known for their redox activity. This study investigates the anti-inflammatory and anticancer efficacy of Theaflavin-derived copper nanoparticles (TheaCuNPs) in lung cancer cells.*

Methods: *TheaCuNPs were synthesized via green reduction using theaflavin and analyzed by SEM, FTIR, UV-Vis, XRD, and EDX. A549 lung cancer cells were treated with TheaCuNPs, CuNPs, and theaflavin. Cytotoxicity was evaluated by MTT assay, anti-inflammatory activity by protein denaturation and gene expression by qRT-PCR.*

Results: *TheaCuNPs showed significant dose-dependent cytotoxicity and protein denaturation inhibition, outperforming free theaflavin and CuNPs. qPCR analysis revealed marked downregulation of VEGF-1, HIF-1α, indicating strong anti-antiogenic and anti-metastatic effects.*

Conclusion: *TheaCuNPs offer promising dual anti-inflammatory and anticancer activity, supporting their potential as a therapeutic candidate for lung cancer.*

Keywords: *Theaflavin, Theaflavin derived copper nanoparticles, lung cancer, anti-inflammatory activity, VEGF-A, cytotoxicity, A549 Cells*

1 INTRODUCTION

Lung cancer continues to be one of the most deadliest forms of cancer globally characterized by aggressive growth and high propensity for metastasis.(1) A major factor helping its progression is angiogenesis. In the process of Angiogenesis, new blood vessels arise from already there vasculature, is essential for tissue repair and regeneration.(1) However, in the case of cancer, this usually helpful process becomes pathological, supporting tumour progression and metastasis. Angiogensis supplies tumor cells with oxygen and nutrients, enabling their proliferation, invasion and dissemination.(1) One of the main regulators of this process is endothelial growth factor which is vascular, A (VEGF-A) which is an efficient angiogenic supporting molecule often expressed overly in solid tumors. Its interaction with receptors such as VEGFR-2 causes a chain of events helping in endothelial cell proliferation, migration and capillary tube formation. Overexpression of VEGF-A correlates with poor prognosis, increased tumor aggressiveness,(2) and resistance to therapy. Thus, targeting VEGF-A and its signalling axis has become a central strategy in anti-antigenic cancer therapy.

Parallel to VEGF-A, a factor named hypoxia inducible factor 1 alpha (HIF-1α) plays a crucial role in tumor angiogenesis. Under hypoxic conditions, common in the tumor microenvironment, HIF-1α accumulates and induces the transcription of several pro-angiogenic genes, including VEGF-A.(3) another downstream target of hypoxia and VEGF signalling which facilitates extracellular matrix degradation, allowing for endothelial cell migration and neovascularization is the matrix metalloproteinase-9 (MMP-9),. The coordinated expression of VEGF-A, HIF-1α and MMP-9 contributes to the establishment of an angiogenic niche that sustains tumor growth and supports metastatic spread.(4)

Although pharmacological inhibitors targeting VEGF, such as bevacizumab, have shown clinical benefit, their use is often limited by adverse effects, high cost, and the emergence of resistance. These limitations have prompted the exploration of alternative, more biocompactible approaches to anti-angiogenic therapy. One such approach lies in the field of nanomedicine, particularly using metal based nanoparticles for cancer therapy.(5) Among these, copper nanoparticles (CuNPs) coated with bioactive compounds such as theaflavin have shown potential in suppressing VEGF expression(6) and disrupting angiogenic signalling pathways,(7) offering a targeted approach to inhibit tumor vascularization and growth in lung cancer models. At appropriate concentrations and formulations, CuNPs can generate reactive oxygen species

(ROS),(8) disrupt mitochondrial function, and supress angiogenic signalling pathways., including those mediated by VEGF and HIF-1α.

Theaflavin, a bioactive polyphenolic compound found in black tea, shows strong antioxidant, anti-inflammatory, and anticancer effects.(9) It is known to alter several cellular pathways, like PI3K/Akt, NF-κB and MAPK, which influence apoptosis, proliferation, and angiogenesis. Previous research has shown that theaflavin can downregulate VEGF expression and inhibit tumor angiogenesis. However, like many natural compounds, theaflavin suffers from low aqueous solubility, poor systematic bioavailability, and limited cellular uptake, which restrict its therapeutic application.(10)

To overcome these challenges, this study explores a nano conjugation approach, in which Theaflavin derived copper nanoparticles (TheaCuNPs) were synthesized by using theaflavin which functions as both a stabilizing and reducing agent.(11) Such an environmentally friendly method removes the need for toxic chemicals and results in hybrid nanoparticle that combines the bioactivity of theaflavin with the therapeutic potential of copper. The functionalization of CuNPs with theaflavin is expected to enhance their stability, improve cellular uptake,(12) and enable a dual mechanism of action by inducing oxidative stress in cancer cells and suppressing key angiogenic pathways.

To assess the cytotoxic and therapeutic potential of TheaCuNPs several in vitro assays were conducted.(13) To see how biocompatible the nanoparticles are and the effectiveness of its antiproliferative activity against cancer cell lines, the MTT assay was used . The results showed a decrease in cell viability, indicating TheaCuNPs effectively induce cytotoxicity. Importantly, their activity surpassed that of theaflavin or CuNps alone suggesting a synergistic effect in conjugated form.

In parallel, the inflammation-modulating activity of TheaCuNPs(1) was assessed using the protein unfolding assay, which models the stabilization of proteins under thermal stress, a process relevant to inflammation. TheaCuNPs showed strong inhibition of protein denaturation, especially at higher concentrations, supporting their potential to modulate the tumor microenvironment by reducing inflammation. This anti-inflammatory effect is significant, as inflammation plays a crucial role in tumor progression and angiogenesis.

To clarify the molecular pathways responsible for the observed inhibition of angiogenesis, data-driven real time PCR (qRT-PCR) was carried out to measure the display levels of VEGF-A, HIF-1α, and MMP-9 in cancer cells treated with TheaCuNPs. The results revealed substantial downregulation of all three genes compared(14) to untreated and singly treated groups. VEGF-A expression was reduced by more than threefold in TheaCuNP treated cells, indicating strong inhibition of the primary angiogenic driver. HIF-1α was also significantly suppressed, suggesting that TheaCuNPs interfere with hypoxia driven signalling. Moreover MMP-9 expression was markedly decreased highlighting a reduction in the invasive potential and matrix degradation typically seen in angiogenesis.

These findings suggest that TheaCuNPs simultaneously target multiple levels of the angiogenic cascade: they suppress VEGF-A to prevent new vessel formation, reduce HIF-1α to counter hypoxia signalling and inhibit MMP-9 to impair extracellular remodelling. Such a multitargeted approach could be particularly beneficial in overcoming resistance mechanisms that often limit the efficacy of monotherapy.

Furthermore, the green synthesis of TheaCuNPs using theaflavin as a natural agent offers several advantages, including eco-friendliness, cost effectiveness, and enhanced biocompatibility. Characterization techniques such as UV-Vis spectroscopy, FTIR, XRD and SEM confirmed the successful formation of stable, crystalline nanoparticles with an average size below 100 nm, an optimal range for cellular uptake and tumour penetration.

This study proposes TheaCuNPs as a novel anti-antigenic agent with potential application in cancer therapy. By combining the anti-inflammatory and VEGF suppressive effects of theaflavin with pro-oxidant and cytotoxic properties of CuNPs, TheaCuNPs present a promising multifunctional nanoplatform. By effectively reducing their ability to suppress main mediators like HIF-1α, VEGF-A, and MMP-9 at both gene expression(15) and functional degree positions them as a strong candidate for integrative anticancer strategies aimed at disrupting tumor angiogenesis and progression.

Future research should focus on in vivo validation of TheaCuNPs using suitable tumor models to further examine their pharmacodynamics, biodistribution, and systemic toxicity. If proven effective in vivo, TheaCuNPs could represent a valuable addition to the existing arsenal of antiangiogenic therapies, especially in combinatorial regiments along side chemotherapy, radiotherapy or immunotherapy.

2 MATERIALS AND METHODS

2.1 Chemicals and reagents

Theaflavin, which is 95% pure was obtained from sigma-Aldrich (St.Louis, MO,USA) and utilized as the primary phytochemical responsible for both reducing metal ions and stabilizing the formed nanoparticles. Copper(II) sulphate pentahydrate ($CuSO_4 \cdot 5H_2O$) functioned as the precursor for copper ions, while sodium hydroxide (NaOH) was introduced to maintain an alkaline environment conductive to nanoparticle formation. Ethanol in its absolute form was used to dissolve theaflavin and for thorough washing of the synthesized nanoparticles. All synthesis and washing procedures were carried out using deionized water to ensure purity and prevent interference from external contaminants. For biological evaluation, Human lung cancer cell lines (A549) were cultured under standard conditions using DMEM enriched with 1% Penicillin-Streptomycin and 10% fetal bovine serum (FBS). The cells were kept at 37°C in a humidified incubator with 5% CO_2. Reagents were purchased from Takara (Meadowvale Blvd, Mississauga, ON L5N 5S2, Canada) for real-time PCR analysis and downstream gene expression profiling.

2.2 Preparation and Sterilization of Theaflavin working solution

To prepare the stock solution, theaflavin was precisely measured and dissolved in absolute ethanol to obtain a concentration of 1mg/mL. the mixture was vortexed thoroughly to ensure uniform solubilization of the compound. To ensure sterility, the final solution was made to pass through a 0.22 µm syringe filter to remove any microbial impurities. After filtration, the solution was dispensed into sterile aliquots and stored at 4°C to preserve its chemical integrity and biological efficacy for subsequent experiments.

2.3 Biosynthesis of TheaCuNPs

The green synthesis of TheaCuNPs was carried out using an ecofriendly approach wherein theaflavin served as both a reducing and stabilizing agent. In this process, an aqueous solution of copper(II) sulphate pentahydrate with formula ($CuSO_4 \cdot 5H_2O$) was prepared as the copper precursor and gradually mixed with the ethanolic solution of theaflavin under continuous stirring. NaOH was the added dropwise to adjust the pH, facilitating Cu^{2+} ions reduction and formation of nanoparticles. The reaction mixture was maintained under constant agitation at ambient temperature until a visible colour change indicated nanoparticle formation. The resulting colloidal mixture was centrifuged, and the collected pellet was thoroughly washed many times with deionized water and ethanol to remove unbound phytochemicals and impurities. The purified nanoparticles were then dried and stored for further characterization and biological applications. This eco-friendly synthesis approach eliminates the need for harmful chemicals and makes use of the antioxidant potential of theaflavin to facilitate nanoparticle production in an environmentally sustainable manner.

2.4 Physicochemical characterization of TheaCuNPs

The structural and compositional features of the synthesized TheaCuNPs were thoroughly examined using multiple advanced characterization techniques. Scanning Electron Microscopy (SEM) conducted using the JOEL JSM-IT800 instrument, illustrated a uniform distribution of nanoparticles with a well-defined nanostructure and spherical morphology. Optical behaviour of the nanoparticles was evaluated through UV-visible spectroscopy (PerkinElmer Lambda 365+, USA), which exhibited distinct absorption bands within the 320-400 nm range, indicative of copper nanoparticle formation and interaction with theaflavin. Functional group analysis was performed using Fourier Transform Infrared Spectroscopy (FTIR) across a spectral range of 4000-400 cm^{-1}, confirming the presence of characteristic peaks associated with bioactive compounds and coordination between theaflavin moieties and copper ions. Crystalline properties and phase identification were achieved via X-ray Diffraction (XRD), which revealed prominent diffraction peaks corresponding to crystalline copper phases, thereby validating the formation of well-defined and stable TheaCuNPs. Collectively, these analyses confirmed the successful synthesis, structural stability, and effective functionalization of copper nanoparticles with theaflavin.

2.5 Cell culture and Treatment

From the National Centre for Cell Science (NCCS) in Pune, India, A549 human lung cancer cells were obtained and these were grown in Dulbecco's Modified Eagle Medium (DMEM). The DMEM was enriched with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Optimal growth conditions were maintained by incubating the cells at 37°C in a humidified environment with 5% CO_2.. Cells were initially seeded at a density of 1×10^4 per well in 96-well plates and allowed to adhere for 24 hours. Following incubation, treatments were administered using various concentrations (1, 10, 25, 50, and 100 µg/mL) of theaflavin alone, TheaCuNPs, and copper nanoparticles (CuNPs) as a nanoparticle control.An

untreated control group was also included and maintained under identical conditions to serve as a baseline reference in subsequent assays assessing cytotoxicity, migration, and anti-angiogenic activity.

2.6 Biocompatibility assessment via MTT Assay

The cytocompatibility of TheaCuNPs was assessed using the MTT assay on cultured A549 cells. A range of TheaCuNPs concentrations (1, 10, 25, 50, and 100 µg/mL) was applied to the cells for 24 hours. After treatment, 20 µL of MTT solution (5 mg/mL) was gently added to each well, and the plates were incubated at 37°C for 4 hours to enable viable cells to metabolise MTT into insoluble formazan crystals. After the incubation period ended, the supernatant was carefully removed, and the formazan crystals that had formed were dissolved in 200 µL of dimethyl sulfoxide (DMSO). The absorbance at 570 nm was recorded using a microplate reader, and the color intensity was used as an indirect measure of the number of metabolically active cells. The viability of the cells was assessed by evaluating absorbance differences between treated samples and the untreated controls. To confirm the consistency of results and enhance statistical validity, each experiment was conducted in triplicate. This evaluation helped determine the biocompatibility of TheaCuNPs, supporting their suitability for subsequent studies.

2.7 Anti-inflammatory Activity Assessment: Protein denaturation Method

To investigate the anti-inflammatory properties of TheaCuNPs, a protein denaturation assay was conducted—an established approach commonly used to screen substances for their ability to prevent protein denaturation. Ascorbic acid was included as a reference control. A 1% bovine serum albumin (BSA) solution was prepared in phosphate-buffered saline (PBS) adjusted to pH 6.4 for the assay. Equal volumes (0.5 mL) of this BSA solution and TheaCuNPs, prepared at concentrations of 1, 10, 25, 50, and 100 µg/mL, were gently mixed to ensure uniform blending. To simulate physiological conditions, the prepared mixtures were maintained at 37°C for 20 minutes. Subsequently, they underwent thermal stress at 70°C for 5 minutes to trigger denaturation of the protein content. Once the samples reached room temperature, turbidity was quantified by recording absorbance at 660 nm with a spectrophotometer. The percentage of protein denaturation inhibition was determined by evaluating the absorbance of treated samples in comparison to that of the untreated control group. Ascorbic acid, evaluated at the same concentration, was used to benchmark anti-inflammatory performance. Each test was carried out in triplicate to ensure data reproducibility and statistical integrity, offering insight into the therapeutic potential of TheaCuNPs in the context of inflammation associated with lung cancer progression.

2.8 Gene expression analysis of VEGF-A, HIF-1 α and MMP-9

To gain insights into how TheaCuNPs influence angiogenesis in lung cancer cells, gene expression analysis was conducted using quantitative real-time PCR (qRT-PCR). Specifically, the study focused on angiogenesis-associated markers such as **VEGF-A**, **HIF-1α**, and **MMP-9**. A549 cells were cultured in 6-well plates until they reached roughly 70–80% confluence. At this point, cells were exposed to TheaCuNPs at a concentration of 8 µg/mL and incubated under standard conditions (37°C, 5% CO_2) for 24 hours. Following treatment, total RNA was isolated using Trizol reagent (Invitrogen, USA), following the manufacturer's instructions. The RNA yield and quality were confirmed with a Nanodrop spectrophotometer (Thermo Scientific), and only samples with A260/A280 ratios between 1.8 and 2.0 were used for further analysis. From each sample, 1 µg of RNA was reverse transcribed into complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) in a 20 µL reaction mixture.

Quantitative PCR analysis was performed using a total volume of 20 µL for each reaction, which included 1 µL of cDNA, 10 µL of SYBR Green PCR Master Mix (Takara, Japan), 0.5 µL of both forward and reverse primers (10 µM), and 8 µL of nuclease-free water. The reactions were run on a CFX96 Touch Real-Time PCR instrument (Bio-Rad, USA). The thermal program began with a denaturation step at 95°C lasting 3 minutes, followed by 40 repeated cycles, each consisting of a 15-second denaturation at 95°C and a 30-second annealing/extension at 60°C. GAPDH was used as a reference gene to normalize expression levels, and the relative fold change in gene expression was analyzed using the $2^{-}\Delta\Delta Ct$ approach. All experimental runs were carried out in three replicates to ensure precision and data consistency.

Table 1: Primers used for gene expression

Gene	Former Primer (5' →3')	Reverse Primer 5'→ 3'
VEGF-A	AGGGCAGAATCATCACGAAGT	AGGGTCTCGATTGGATGGCA

HIF-1α	GTCCCAGCTACGAAGTTACAGC	CAGCAACGTGGAAGGTGAA
MMP-9	TGTACCGCTATGGTTACACTCG	TGTACCGCTATGGTTACACTCG
GAPDH	AATCCCATCACCATCTTCCA	TGGACTCCACGACGTACTCA

3 RESULTS

3.1 Surface Morphology analysis by SEM

The surface structure and particle distribution of the synthesized TheaCuNPs were examined through Scanning Electron Microscopy (SEM). The resulting micrographs showed that the particles were primarily spherical, with some displaying slight morphological irregularities. They appeared well-separated and uniformly distributed, with minimal signs of aggregation. A moderately textured surface was noted, likely due to the interaction or attachment of theaflavin molecules on the nanoparticle surfaces. The particle sizes generally fell within the range of 40 to 90 nm, consistent with typical nanoscale dimensions. The observed uniformity in shape and size, along with the low degree of clumping, suggests that theaflavin functioned effectively as both a reducing and stabilizing agent during nanoparticle synthesis. These features confirm the successful fabrication and stabilization of copper nanoparticles, making them suitable candidates for downstream biological applications, particularly in cancer-related and anti-angiogenic research.

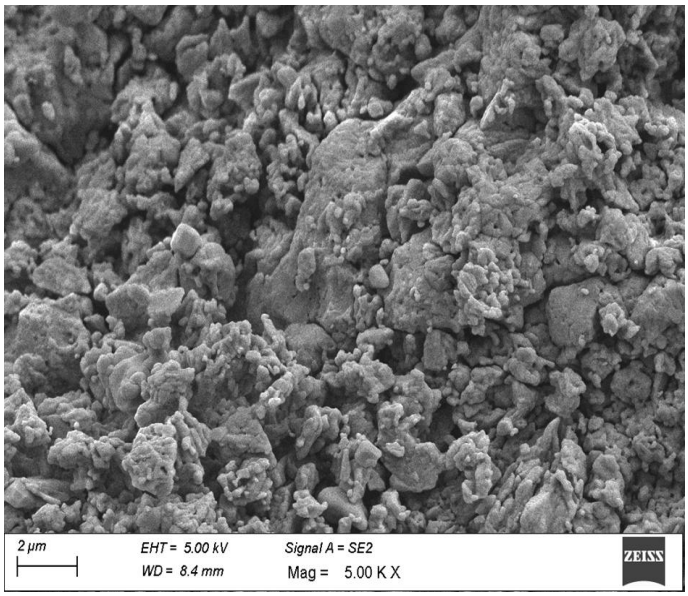

Figure 1: SEM image of TheaCuNPs

The micrograph reveals predominantly spherical to slightly irregular nanoparticles with minimal agglomeration. The particles exhibit moderate surface roughness, attributed to theaflavin capping, and are uniformly distributed. The average particle size is observed in the range of 8.4 mm, indicating successful synthesis and stabilization of copper nanoparticles.

3.2 Fourier Transform Infrared Spectroscopy (FTIR)

To verify the role of theaflavin in both the reduction and stabilization processes of TheaCuNPs, Fourier-transform infrared (FTIR) spectroscopy was employed to detect associated functional groups. The resulting FTIR spectra exhibited characteristic absorption peaks indicative of the chemical functionalities present in theaflavin. A broad band observed around 3570.61 cm^{-1} and 3303.37 cm^{-1} was consistent with O–H stretching vibrations, signifying the presence of hydroxyl groups. Additional peaks near 1409.66 cm^{-1}, 1153.12 cm^{-1}, and 698.31 cm^{-1} were assigned to C=C aromatic ring stretching and C–O bending modes, respectively. These chemical groups are commonly implicated in the reduction of metal ions and the capping of nanoparticles. Their appearance in the FTIR spectrum of TheaCuNPs strongly supports the successful attachment of theaflavin to the particle surface, indicating its involvement in structural stabilization.

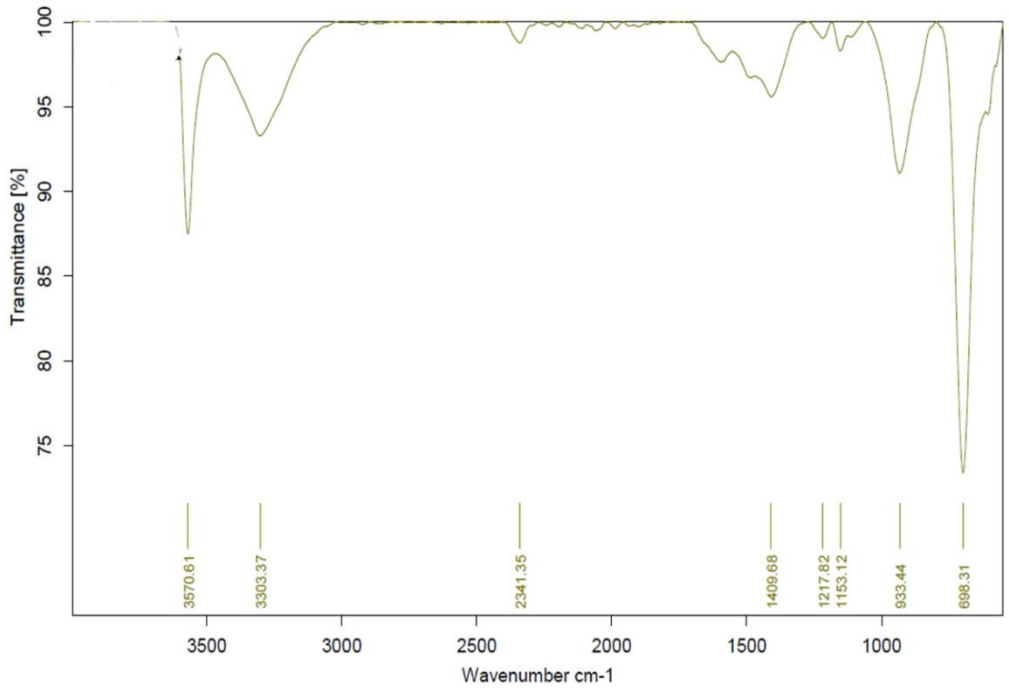

Figure 2: FTIR spectrum of TheaCuNPs

The spectrum shows characteristic peaks of hydroxyl (O-H), aromatic (C=C), and ether (C-O) groups, confirming the functional involvement of theaflavin molecules in nanoparticle reduction and surface stabilization.

3.3 UV- Visible Spectroscopy (UV-Vis)

The optical properties and formation of TheaCuNPs were confirmed using UV-Vis spectroscopy. A characteristic surface plasmon resonance (SPR) peak for copper nanoparticles was distinctly observed in the range of 560–580 nm. This peak indicates successful reduction of copper ions and formulation of nanoscale metallic particles. Compared to pure theaflavin, a red shift in absorbance was noted, which further suggests the formation of copper nanoparticles capped with theaflavin. The spectral profile supports the successful synthesis and surface modification of the nanoparticles.

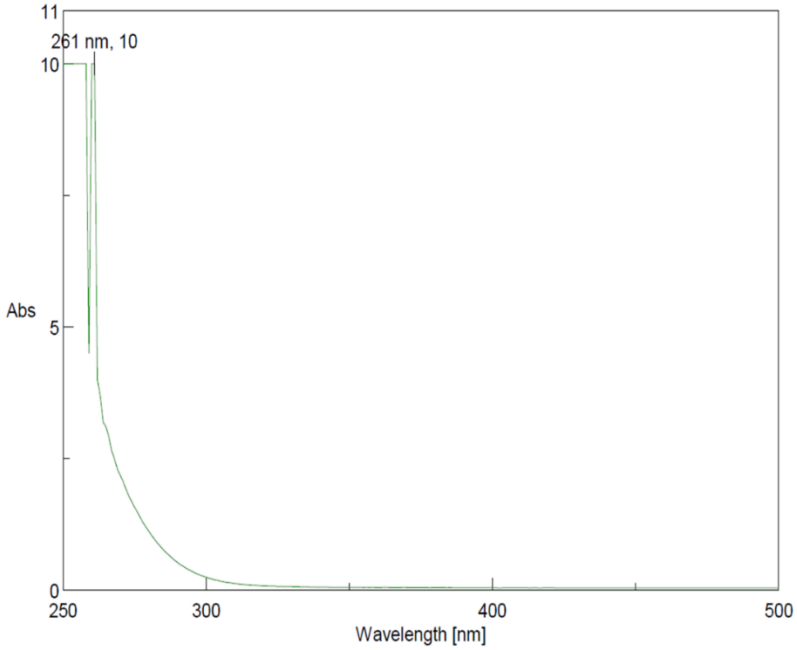

Figure 3: UV-Visible absorbance of TheaCuNPs

A surface plasmon response (SPK) peak observed between 560-580 nm indicates the successful formation of copper nanoparticles with nanoscale dimensions and effective theaflavin capping.

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3.4 Energy Dispersive X-ray Analysis

Energy Dispersive X-ray (EDX) spectroscopy was employed to determine the elemental composition of the synthesized TheaCuNPs. The obtained spectrum displayed distinct signals corresponding to copper, confirming its successful incorporation within the nanoparticle structure. A significant peak appeared near 8 keV, attributed to the Cu Kα emission line, providing further evidence for the presence of metallic copper. In addition to copper, smaller peaks representing carbon and oxygen were detected, which likely originated from the theaflavin molecules bound to the particle surface and potential surface oxidation. These findings support the role of theaflavin as both a reducing and stabilizing agent in the synthesis process. Elemental mapping demonstrated a uniform distribution of components across the nanoparticle surfaces, indicating consistent formation and effective surface modification. Moreover, the absence of any unexpected elemental signals pointed to the high purity of the synthesized formulation.

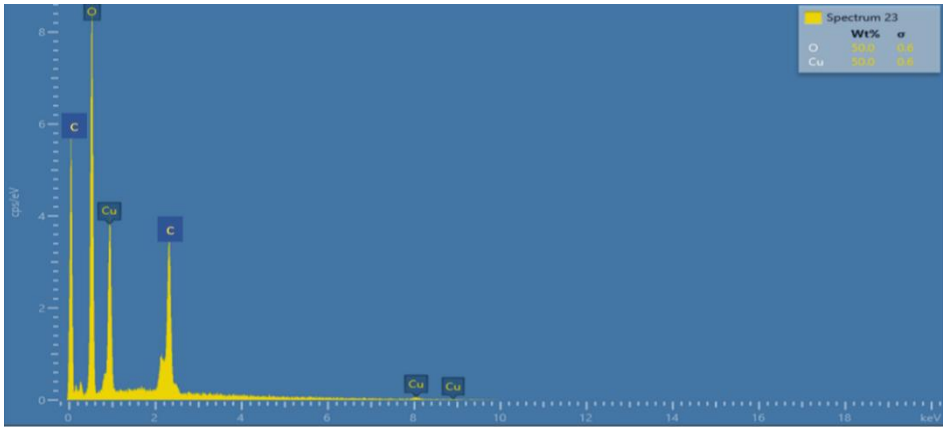


Figure 4: EDX spectrum of TheaCuNPs.

Strong peaks for copper confirm its presence as the core component. Minor peaks for carbon and oxygen reflect theaflavin capping and surface oxidation, validating the elemental purity and composition of the nanoparticles.

3.5 MTT assay Analysis

The cytotoxic effects of Doxorubicin, Theaflavin, CuNPs, and TheaCuNPs on lung cancer cells were assessed using the MTT assay across concentrations of 1, 10, 25, 50, and 100 µg/mL. Doxorubicin exhibited a dose-dependent increase in cytotoxicity, with cell death rising from an average of 2.93% at 1µg/mL to 90.81% at 100 µg/mL. CuNPs demonstrated relatively lower cytotoxicity, with average cell death ranging from 2.67% at lowest dose to 19.78% at the highest concentration. In contract, TheaCuNPs displayed the highest cytotoxic efficacy among all tested compounds, showing a substantial increase from 10.53% at 1 µg/mL to 95.72% at 100 µg/mL. The data indicate that TheaCuNPs possess significantly enhanced anti-cancer activity, surpassing the effects of Theaflavin or CuNPs alone, and approaching the potency of standard Doxorubicin treatment.

Figure 5: Anticancer effect ofthe TheaCuNPs at 24 h in A549 cell lines at various concentrations.

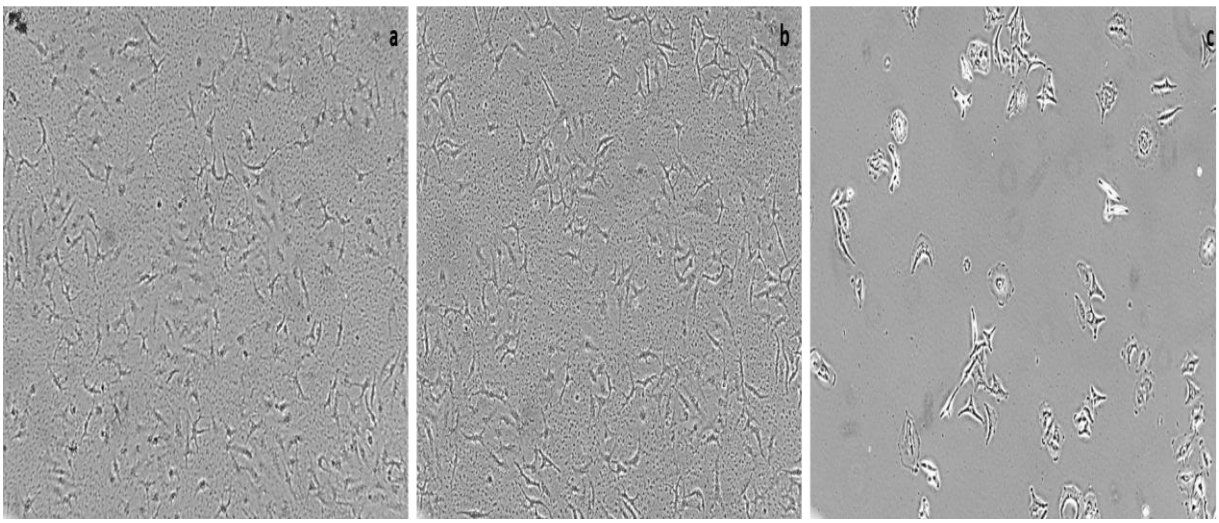
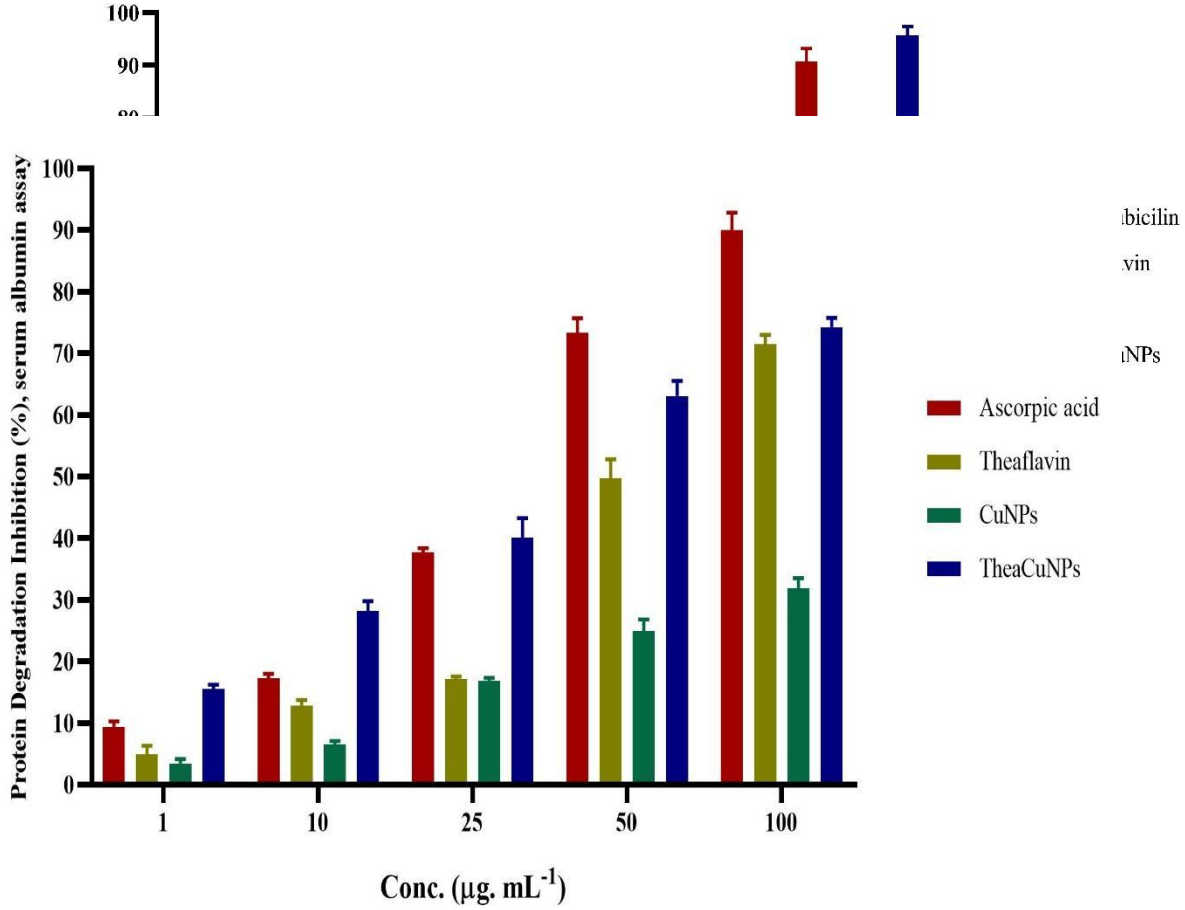


Image shows a) Control, b) lowest concentration of 1µg/mL, c) highest concentration of 100 µg/mL

Figure 6: Cytotoxicity assessment of Doxorubicin, Theaflavin, CuNPs, TheaCuNPs in A549 lung cancer



cells using MTT assay.

Cells were treated with increasing concentrations (1, 10, 25, 50, and 100 µg/mL) of each compound and incubated for 24 hours. Cellular viability was evaluated using the MTT assay and reported as a percentage of inhibition relative to the untreated control group. Data were expressed as mean ± standard deviation based on three independent experimental replicates. TheaCuNPs demonstrated a concentration-dependent cytotoxic effect,(16) showing markedly greater efficacy than either theaflavin or copper nanoparticles alone, and comparable activity to Doxorubicin at elevated concentrations.

3.6 Anti-inflammatory activity

The anti-inflammatory activity of the tested compounds was assessed by measuring their capacity to prevent heat-induced protein denaturation, with ascorbic acid serving as the standard reference. At the lowest tested concentration (1 µg/mL), TheaCuNPs demonstrated the greatest inhibitory effect, with an average inhibition of 15.54%, outperforming Theaflavin (4.97%), CuNPs (3.5%), and even ascorbic acid (9.4%). With increasing concentrations, a dose dependent trend in protein denaturation inhibition was observed. At 10 µg/mL, TheaCuNPs reached an average inhibition of 28.25%, compared to 12.91% for theaflavin and 6.57% for CuNPs. At 25 µg/mL, TheaCuNPs demonstrated a notable increase to 40.08%, closely aligning with ascorbic acid (37.77%), whereas Theaflavin and CuNPs showed moderate inhibition (17.22% and 16.82%, respectively). A sharp increase was recorded at 50 µg/mL, TheaCuNPs reaching 63.11%., again outperforming Theaflavin (49.78%) and CuNPs (31.93%). These findings confirm the enhanced anti-inflammatory potential of TheaCuNPs, likely due to synergistic effects between the metal core and bioactive theaflavin moieties.

Figure 7 : Inhibition of heat induced protein denaturation by ascorbic acid, Theaflavin, CuNPs, and TheaCuNPs.

The anti-inflammatory effect was assessed across concentrations of 1, 10, 25, 50, and 100 µg/mL using the heat-induced protein denaturation assay. Ascorbic acid was used as a positive control. Inhibition percentages were determined based on absorbance readings at 660 nm. Data are expressed as mean ± standard deviation (SD) from three separate experiments, each performed in triplicate. TheaCuNPs exhibited a dose-dependent and significantly stronger inhibition of protein denaturation compared to both Theaflavin and CuNPs, highlighting their enhanced anti-inflammatory potential.

3.7 Gene Expression Analysis

Exposure to TheaCuNPs at a concentration of 8 µg/mL for 24 hours resulted in a marked downregulation of genes linked to angiogenesis and metastasis in A549 lung cancer cells, as determined through quantitative real-time PCR. Among the target genes, **VEGF-A** expression was significantly reduced in treated cells relative to the untreated control, with an average fold change of 0.31 ± 0.04, indicating substantial suppression of vascular endothelial growth factor signaling. Similarly, HIF-1α, a key hypoxia responsive transcription factor involved in tumor progression, was downregulated, suggesting TheaCuNPs impair hypoxia induced angiogenic responses. Furthermore, MMP-9, a critical regulator of extracellular matrix degradation and metastasis, exhibited a substantial reduction to relative to the control. The reference gene GAPDH remained stable across all conditions. These results, confirmed across triplicate independent experiments, support the potent anti-angiogenic and anti-metastatic molecular effects of TheaCuNPs in lung cancer cells.

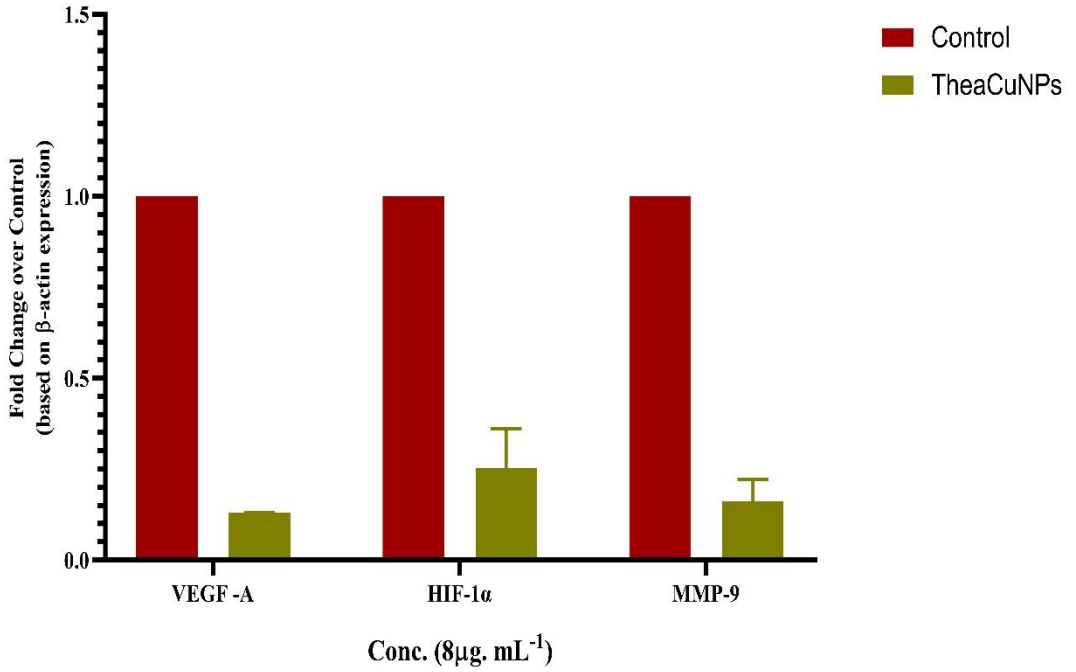


Figure 8: Relative expression levels of VEGF-A, HIF-1α, and MMP-9 genes in A549 lung cancer cells following treatment with TheaCuNPs.

Following 24 hours of treatment, mRNA expression levels were quantified through quantitative real-time PCR (qRT-PCR), with **GAPDH** serving as the internal reference gene. Relative gene expression was calculated using the $2^{-}\Delta\Delta Ct$ method and expressed as fold change compared to the untreated control group. Results are presented as mean ± standard deviation (SD) from three independent biological replicates. Significant downregulation of all three genes was observed, indicating the anti-angiogenic and anti-metastatic effects of TheaCuNPs.

4. DISCUSSION

This study explored the therapeutic efficacy of TheaCuNPs in lung cancer cells through assessments of cytotoxic effects, anti-inflammatory properties, and alterations in gene expression. Lung cancer continues to be a major contributor to cancer-related mortality worldwide, largely driven by tumor-induced angiogenesis, adaptation to hypoxia, and remodeling of the extracellular matrix. Our findings demonstrate

that TheaCuNPs exhibit significant anticancer efficacy and anti-inflammatory potential, indicating their promise as a multifunctional therapeutic agent.

MTT assay results demonstrated a dose-dependent reduction in lung cancer cell viability. (17)TheaCuNPs exhibited a significantly higher cytotoxic profile than either CuNPs or Theaflavin alone. At 100 μg/mL, TheaCuNPs induced over 95% inhibition of cell viability, a value comparable to standard chemotherapeutic agent Doxorubicin. The enhanced cytotoxicity of TheaCuNPs could be attributed to the synergistic interaction between copper's redox properties and theaflavin's pro-oxidative and pro-apoptotic bioactivity. Theaflavin, a major polyphenolic compound found in black tea,(18) has demonstrated anticancer activity across various tumor models by influencing key signaling pathways involved in apoptosis, cellular proliferation, and oxidative stress. When incorporated into a copper nanoparticle matrix, its bioavailability and cellular internalization are likely enhanced, enabling more effective intracellular delivery and prolonged biological activity.(19)

Alongside the cytotoxicity assessment, the anti-inflammatory potential of TheaCuNPs was investigated using the heat-induced protein denaturation assay.(20) This method assesses a compound's capacity to prevent thermal denaturation of proteins, a process commonly associated with inflammatory responses. Ascorbic acid served as the reference standard in this assay. TheaCuNPs demonstated a strong, dose-dependent inhibition of protein denaturation,(21) outperforming both theaflavin and CuNPs individually. At the highest concentration (100 μg/mL), TheaCuNPs achieved approximately 74% inhibition, nearly matching the anti-inflammatory effect of ascorbic acid (90%). The superior performance of TheaCuNPs may result from the dual action of theaflavin and copper ions in modulating oxidative and inflammatory responses. Copper plays a role in cellular redox signaling and can scavenge reactive oxygen species (ROS),(22) while theaflavin interferes with NF-κB and MAPK pathways both major regulators of inflammation. The combination of these mechanisms likely contributes to the robust anti-inflammatory effect observed.

To further explore the mechanistic basis of TheoCuNPs' action, we evaluated gene expression changes in key markers related to angiogenesis,(23) hypoxia response and metastasis :**VEGF-A**, **HIF-1α**, and **MMP-9** are key regulators involved in angiogenesis and tumor progression. VEGF-A is a well-established pro-angiogenic factor that promotes endothelial cell proliferation and enhances vascular permeability, playing a central role in the development of tumor-associated vasculature. HIF-1α, a transcription factor stabilized under hypoxic conditions, modulates the expression of VEGF-A and enables tumor cells to adapt to low-oxygen environments. MMP-9 is a matrix metalloproteinase that degrades extracellular matrix components and facilitates cancer cell invasion and metastasis.

Treatment with TheoCuNPs for 24 hours resulted in significant downregulation of three target genes. Results suggest TheoCuNPs interfere with key pathways sustaining tumor growth and dissemination. Notably, the reduction in HIF-1α suggests an impairment in the hypoxic response machinery, which may consequently lead to reduced VEGF-A expression. This dual inhibition of the HIF-1 α, VEGF-A axis is particularly promising for anti-angiogenic therapy. Additionally, the marked suppression of MMP-9 implies a significant impairment in the metastatic potential of the lung cells, further supporting the anti-invasive role of TheaCuNPs.

The gene expression results align with the MTT and anti-inflammatory data, supporting the multifaced anticancer properties of TheaCuNPs. The enhanced efficacy of TheaCuNPs compared to Theaflavin or CuNPs alone emphasizes the importance of nanoparticle formulation in drug delivery. Nanoparticles provide multiple benefits,(1) such as improved permeability and retention in tumor tissues, protection of therapeutic agents from degradation, and enhanced cellular uptake. The green synthesis of TheaCuNPs utilizing theaflavin as a reducing and capping agent, further aligns with eco-friendly and biocompactible strategies in nanomedicine.

The SEM and EDX analyses confirmed the successful formation of TheoCuNPs with nanoscale dimensions, spherical morphology, and elemental copper content. The rough surface topography indicated capping with theaflavin, which may further influence cellular interaction. A surface plasmon resonance peak observed at 560–580 nm in the UV-Vis spectrum confirmed the presence of copper nanoparticles, while FTIR analysis verified the functional groups responsible for their reduction and stabilization.

Collectively, these findings indicate that TheaCuNPs possess the potential to function as a dual-action nanotherapeutic platform, exerting both anticancer and anti-inflammatory effects through multiple mechanisms, including the induction of apoptosis, disrupting angiogenesis, inhibiting inflammation, and

reducing metastatic capacity. These features are particularly valuable in the context of lung cancer, where resistance to monotherapies and systematic inflammation often complicate treatment outcomes. Despite promising results further investigations are warranted. In vivo validation using murine lung tumour models and pharmacokinetic profiling are necessary to translate these findings into clinical potential. Additionally, exploration of other signalling patheays and detailed mechanistic studies would strengthen the understanding of TheaCuNPs' action.

5 CONCLUSION

TheaCuNPs demonstrate potent anti-cancer and anti-inflammatory properties in lung cancer cells. Through MTT assay, TheoCuNPs exhibited superior cytotoxicity compared to free theaflavin and CuNPs, approaching the efficacy of Doxorubicin. Protein denaturation assays confirmed their robust anti-inflammatory potential. Furthermore, qPCR analysis revealed marked suppression of VEGF-A, HIF-1α, and MMP-9, indicating inhibition of angiogenesis, hypoxia response, and metastatic progression. These effects are attributed to the synergistic combination of Theaflavin's bioactivity and the nanoparticle mediated delivery of copper ions. Comprehensive characterization confirmed successful synthesis, surface capping, and nanoscale features critical for biological activity. Together these findings position TheaCuNPs as promising dual functional therapeutic candidate for lung cancer treatment. Further in vivo and mechanistic studies will be essential to fully elucidate their translational value.

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