Journal of Carcinogenesis
Covering Genesis to Therapeutics

Journal of Carcinogenesis, ISSN: 1477-3163
2025; Vol 24: Issue 6s, https://carcinogenesis.com/

Anti-Angiogenesis of Theaflavin-Derived Copper Oxide: Comparative Analysis of In Vitro and In Vivo Models

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ABSTRACT

Angiogenesis is almost always paired with tumor progression and metastasis, therefore attended to with utmost importance by anticancer strategists. This study is therefore concerned with the green synthesis of a nanomaterial drug of anticancer interest: copper oxide nanoparticles synthesized with theaflavin, where theaflavin acts as a natural reducing and stabilizing agent. After characterization studies, the particles were found to have a size in the 40-70 nm range, double spherical morphology, and crystalline nature (as evidenced by PXRD), with absorption maxima at 290 nm in UV-Visible spectroscopy. In vitro studies conducted on lung cancer cells, A549, showed cytotoxicity in a dose-dependent manner, with cell viability going down sharply at 50 and 100 µg/mL; synchronous with these, were the enhanced production of reactive oxygen species, with an apex at 50 µg/mL. CAM inhibition of neovascularization was observed including reduced branching vessels, density of vessels, and complexity of vessels after treatment with CuO-TF. This is shown by the downregulation of proangiogenic markers VEGFA, VEGFR2, HIF1A, and ANGPTL4 and upregulation of the antiangiogenic marker THBS1, signifying that angiogenic signaling is going on to be suppressed at the transcriptional level.

Keywords: *Theaflavin, angiogenesis, Endothelial cells (HUVECs), Copper oxide nanoparticles (CuONPs), Anti-*

angiogenic therapy, CAM assay, Zebrafish, Tube formation assay.

How to Cite: Bhoomi Jadhav, S. Sangeetha, Taniya Mary Martin, Meenakshi Sudaram Kishore Kumar., (2025) Anti-Angiogenesis of Theaflavin-Derived Copper Oxide: Comparative Analysis of In Vitro and In Vivo Models, *Journal of Carcinogenesis, Vol.24, No.6s*, 209-221.

1. INTRODUCTION

Angiogenesis is a simple biological process where new capillaries are formed from already present blood vessels. It is essential for many physiological processes, including tissue regeneration, wound healing, and embryonic development(Hanahan et al. 1996). Under normal conditions, angiogenesis is tightly controlled by a balance of pro- and anti-angiogenic factors(Carmeliet et al. 2011). But other disorders, such as cancer, diabetic retinopathy, atherosclerosis, rheumatoid arthritis, and chronic inflammatory diseases, often interfere with this balance (Potente et al. 2011). Tumors employ the angiogenic switch to make new blood vessels that give them the oxygen and nutrients they need to develop quickly(Hanahan & Weinberg, 2011). This is especially true in cancer. Also, angiogenesis is a very important target for treatment since these new blood vessels help cancer cells spread to other parts of the body(Ferrara et al. 2005). There are a lot of different types of synthetic angiogenesis inhibitors being made, but people are more interested in organically made alternatives and treatments that use nanotechnology because they are worried about how well they work, how resistant they are, how harmful they are to other cells, and how much they cost(Mukherjee et al. 2016).

Nanomedicine is an area of study that combines nanotechnology and biomedical sciences to create drugs that can stop abnormal angiogenesis and do many other things. Copper-based nanoparticles, especially copper oxide nanoparticles (CuONPs), have attracted a lot of attention because of their unusual physical, chemical, and biological properties. Copper is a transition metal, which means it may change its oxidation state. It can also speed up Fenton-like reactions that make reactive oxygen species (ROS), which have a role in two parts of regulating angiogenesis. Too much ROS can cause oxidative stress, which can stop endothelial cells from growing and living.On the other hand, modest quantities of ROS are known to start angiogenic signaling pathways (An et al. 2024). This paradox shows that copper oxide nanoparticles could influence angiogenesis in a dose-dependent way. CuONPs have also been shown to stop signaling through vascular endothelial growth factor (VEGF), block hypoxia-inducible pathways, and mess with how mitochondria work (Deng et al.) Because of this, they work well to stop angiogenesis in both living and non-living systems.

CuONPs are not much used in medicine since they can be poisonous to cells, don't absorb into cells in a specific way, and aren't stable in biological environments. In order to make these constraints better for medical treatment, the surface needs to be changed or altered in a certain manner. Making and keeping metal nanoparticles with natural polyphenolic compounds is an interesting method that could make these particles more active and more able to work with living organisms. Theaflavins, which are polyphenols that come from black tea by oxidizing catechins, have shown a lot of promise in this particular situation (Pereira-Caro et al. 2017) Theaflavins can do a variety of things to the body, such as fight inflammation, stop cells from growing, stop blood vessels from forming, and protect cells from damage (Luo et al. 2021) They are significant for anti-angiogenesis therapy because they can get rid of free radicals, change the redox state of cells, and stop pro-angiogenic factors like VEGF, MMP2 (matrix metalloproteinase-2), and HIF-1α (hypoxia-inducible factor-1 alpha).

Using theaflavin as a reducing and capping agent to make copper oxide nanoparticles is a green, eco-friendly, and physiologically synergistic way to make a hybrid nanomaterial that is more effective and less harmful as a medicine(Balaji Ganesh et al. 2025; Chokkattu et al. 2022). This technology does away with the requirement for harsh chemicals and high-energy methods that are usually used to make nanoparticles. The theaflavin-derived copper oxide nanoparticles (TF-CuONPs) are considered to work in two ways: they have the pro-oxidant and anti-proliferative actions of CuONPs, and they can also selectively target certain molecules. Functionalization is also likely to change the surface chemistry of the nanoparticles, making them easier to spread in water, making it easier for cells to take them up, and reducing the chances of them clumping together and being cleared too soon. Also, the polyphenol coating could make it easier to control the release of ROS, which would make the anti-angiogenic response more predictable and adjustable. This would be very helpful in precision cancer therapy(Ahamed et al., 2016).

This study uses both in vitro and in vivo models to see how effectively TF-CuONPs can block the formation of new blood vessels. In the lab, human endothelial cells play a key role in building new blood vessels and are used to assess how these nanoparticles affect cell growth, movement, and the ability to form capillary-like structures. These tests mimic the main steps of angiogenesis and help us understand how TF-CuONPs interfere with the process. To dive deeper, the study also looks at changes in the expression of important genes and proteins involved in angiogenesis, such as VEGF, MMP2, and HIF-1α, giving insight into how the nanoparticles work at a molecular level. For testing in living systems, two models are

used: the chick chorioallantoic membrane (CAM) and zebrafish embryos. The CAM model allows easy observation of blood vessel growth in a cost-effective and ethically manageable way, while zebrafish offer a transparent, fast-developing system ideal for watching blood vessel formation in real time. Using both models together gives a clearer and more reliable picture of how TF-CuONPs perform in different biological settings, helping to confirm their potential as anti-angiogenic agents.

In short, the goal of this study is to use theaflavin to produce and analyze a new type of copper oxide nanoparticles that have biological properties. Next, the researchers will test how efficiently these nanoparticles inhibit blood vessels from developing in both in vitro and in vivo. TF-CuONPs are useful for anti-angiogenic therapy because they combine green nanotechnology and natural bioactivity of polyphenols (Mukherjee et al. 2016). They might help with chronic inflammation, cancer, and eye problems (Abdalla et al. 2018). Performing experiments on endothelial cells, CAM, and zebrafish models can help us learn a lot more about nanoparticles . We can find out more about how they act, where they are in the body, and how safe they are. This breakthrough also opens the door to additional translational research and the development of new treatments in the field of biomedical research on phytochemical-nanomaterial hybrids, which is growing.

AIM

To synthesize and evaluate the anti-angiogenic potential of theaflavin-derived copper oxide nanoparticles (TF-CuONPs) through a comparative analysis of their effects in in vitro endothelial cell assays and in vivo models, including the chick chorioallantoic membrane (CAM) assay and zebrafish embryo system.

2. MATERIALS AND METHODS

Chemicals and Reagents:

The natural reducing and capping agent used to create the nanoparticles was theaflavin (≥95% purity), which we obtained from Sigma-Aldrich (St. Louis, MO, USA). The source of copper for the nanoparticles was analytical-grade copper(II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), which we obtained from Merck (India). GibcoTM (Thermo Fisher Scientific, USA) provided us with fetal bovine serum (FBS), phosphate-buffered saline (PBS), penicillin-streptomycin (1%), and Dulbecco's Modified Eagle Medium (DMEM) for the in vitro cytotoxicity and oxidative stress tests. Human umbilical vein endothelial cells (HUVECs) served as our endothelial cell model. The Himedia Laboratories in Mumbai, India sent the MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reagent, to see if the cells were still alive. We used the High-Capacity cDNA Reverse Transcription Kit and TRIzolTM reagent from InvitrogenTM (Thermo Fisher Scientific, USA) to get RNA and look at how the genes were expressed. Quantitative real-time PCR was done using SYBR™ Green Master Mix. The special primers for the housekeeping gene GAPDH and the human genes VEGFA, VEGFR2, HIF1A, THBS1, and ANGPTL4 were developed by Eurofins Genomics (India). Unless stated differently, all of the chemicals and reagents used were of analytical purity and did not need any further purification. All of the studies that used water-based solutions used ultrapure water (Milli-Q).

Biosynthesis of Theaflavin-Derived Copper Oxide Nanoparticles (CuO-TF):

The biosynthesis of copper oxide nanoparticles was carried out using theaflavin as a natural reducing and stabilizing agent. A 1 mM aqueous solution of copper(II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) was prepared and heated to 70 °C under continuous stirring. Separately, a freshly prepared solution of theaflavin (1 mg/mL) was added dropwise to the copper solution in a 1:1 volume ratio. The formation of CuO-TF nanoparticles was seen due to blue to brown change in colour in the liquid after two hours of the reaction mixture being held at 70°C. Once the mixture reached room temperature, it was centrifuged for 20 minutes at 12,000 rpm to extract the nanoparticles. The pellet was cleaned three times with distilled water and ethanol to remove any unbound biomolecules. After that, it was dehydrated at 40 °C in a vacuum oven. The generated nanoparticles were stored in sealed vials for subsequent inspection and analysis.

Characterization of CuO-TF Nanoparticles:

There were various analytical techniques employed to characterize the synthetic theaflavin-derived copper oxide nanoparticles (CuO-TF) and ensure they existed and to screen for their physicochemical properties. Utilizing Scanning Electron Microscopy (SEM) in examining the nanoparticles revealed that they were approximately 40–70 nm in diameter and predominantly spherical, with some agglomerating. The surface appeared a bit rough due to the flavin covering layers that existed. X-ray diffraction (XRD), revealed that the nanoparticles were crystalline. Solid CuO structures created by diffraction peaks at 2θ values ranging around 32.4°, 35.5°, 38.7°, 48.6°, and 53.3° were in good accordance with the monoclinic phase of CuO. The absence of any other peaks indicated that the nanoparticles that were synthesized were extremely pure. Copper oxide nanoparticles exhibit a wide peak of absorption at the center of 280–310 nm. It indicates surface plasmon resonance (SPR) activity and also verifies that the nanoparticles were synthesized through UV-visible spectroscopy.

The biosynthesis of stable and distinct CuO-TF nanoparticles was established by results that were uniform when SEM, XRD, and UV-Vis were examined together.

Cell Culture and Conditions:
We used human lung cancer cell lines (A549) to test how the CuO-TF nanoparticles killed the cells and stopped them from making more blood vessels. The cells were grown in DMEM with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were collected from a legitimate cell bank, such as the NCCS in Pune, India. The cells were left in a room with added humidity at 37°C with 5% CO2. When the cultures attained an 80% to 90% confluence, the cells were separated using 0.25% trypsin-EDTA every two to three days. According to what the instructions indicated, cells were placed in 6-well or 96-well plates for the tests. Various concentrations of CuO-TF nanoparticles were added, and they were left overnight on them to adhere.

MTT Assay for Cell Viability:
The MTT method was used to test how well CuO-TF nanoparticles killed A549 lung cancer cells. 100µL of full DMEM was used to cover 96-well plates with cells. There were 1×10^4 cells in each well.The plates were then kept at 37°C and 5% CO_2 overnight to help the cells stick together. 24 hours later, the old medium was replaced with new medium with various concentrations of CuO-TF nanoparticles: 1, 10, 25, 50, and 100 µg/mL.Those who were negative were only given culture medium, while those who were positive were given a normal cytotoxic agent, such as Doxorubicin, 25 µg/mL. There were three copies of each medicine. After being exposed for 24 hours, 10 wells were filled with MTT solution (5 mg/mL in PBS) and the plates were left to sit at 37°C for 4 hours. The medium was carefully aspirated after the incubation period, and 100 µL of DMSO was added to dissolve purple formazan crystals produced by viable cells. The absorbance was taken at 570 nm by a microplate reader. Cell viability was determined as a percentage compared to the untreated control. The data were presented as mean ± standard deviation (SD) of three independent experiments.

Intracellular ROS Assay:
Intracellular reactive oxygen species (ROS) production was measured by using 2′,7′-Dichlorofluorescin diacetate (DCFDA), a permeable fluorescent probe. A549 lung cancer cells were seeded in black 96-well plates at 1×10^4 cells per well and left to incubate overnight at 37 °C and 5% CO_2.After cell adhesion, the growth medium was removed and replaced with fresh medium supplemented with different concentrations of CuO-TF nanoparticles (1, 10, 25, 50, and 100 µg/mL). A group that caused oxidative stress was given 100 µM hydrogen peroxide ($H2O_2$), and a group that didn't cause oxidative stress was treated with the whole medium. After one day of treatment, the medium was thrown away and the cells were gently washed with PBS. The cells were incubated for 30 minutes at 37 °C in a serum-free medium with 10 µM DCFDA. For the removal of all remaining dye, the cells were subjected to one PBS wash after incubation. The intensity of fluorescence, which reflects ROS levels, was quantified by a fluorescence microplate reader with excitation at 485 nm and emission at 528 nm. The results were presented as relative fluorescence units (RFU) and compared between treatment groups. All the experiments were carried out in triplicate, and the values were computed as mean ± standard deviation.

CAM Assay for Antiangiogenic and Anti-Cancer Evaluation:
Chick chorioallantoic membrane (CAM) experiment was utilized to evaluate the antiangiogenic and anticancer potential of CuO-TF nanoparticles in vivo. Fertilized White Leghorn chicken eggs were incubated at 37.5°C and 60% relative humidity for seven days. On the seventh day of incubation, a small hole was carefully made in the eggshell to expose the CAM without contaminating the eggshell. The pre-soaking process used 10 µL of CuO-TF nanoparticle solutions at concentrations of 10, 25, 50, and 100 µg/mL. Five millimeters across, the discs were clean. Care was taken to make sure that these plates did not cover the main blood veins when they were put on the CAM. As the control discs, PBS was employed as the negative control, and a well-established antiangiogenic drug as the positive control. The eggs were then treated and replaced in the incubator with fresh clean parafilm for another 48 hours. At the completion of the incubation, the CAMs were examined and photographed using a stereomicroscope to determine if the blood vessels were changed. The degree of inhibition of angiogenesis was determined by observing the reduction in the number of vessels, branching, and avascular zones surrounding the discs. The experiment was conducted three times for each group, and ImageJ software was employed to tally the results and photograph them.

Gene Expression Profiling of Angiogenesis Markers in CuO-TF Treated Lung Cancer Cells:
Gene expression analysis was conducted for treated A549 lung cancer cells to investigate the molecular pathways behind antiangiogenic activity of CuO-TF nanoparticles. Cells were treated with CuO-TF for 24 hours at 25, 50, and 100 µg/mL concentrations. According to the instructions given by the manufacturer, TRIzol™ reagent was applied to extract total RNA.NanoDrop was used to prove that the RNA was natural and equal in amount.cDNA was generated using 1 µg of total RNA which was present in the cDNA reverse transcription kit. Quantitative real-time PCR (qRT-PCR) was done by using StepOnePlus® Real-Time PCR System and a SYBR® Green Master Mix. Specific primers were used to amplify the genes VEGFA, VEGFR2, HIF1A, THBS1, and ANGPTL4. GAPDH was our positive control. The reaction is to be denatured

for 10 minutes at 95°C prior to amplification. Then there were forty cycles of annealing and extension for 60 seconds at 60°C and denaturation for 15 seconds at 95°C. Using the 2-ΔCt method, we were able to compare the level of gene expression in the treated group to that in the untreated control group. We did each test three times to be sure the results were the same each time.

3. RESULTS

Characterization of CuO-TF Nanoparticles:

We employed the assistance of SEM, XRD, and UV-Vis analyses to identify the laboratory production process of theaflavin-derived copper oxide nanoparticles (CuO-TF). Based on the SEM research, the nanoparticles were mostly spherical in shape and had a range of 40-70 nm, although a few were agglomerated. The relatively high roughness of the surface of the nanoparticle is due to the coating of aflavin on it. XRD analysis indicated that the nanoparticles were crystalline by demonstrating good diffraction peaks at 2θ values of around 32.4°, 35.5°, 38.7°, 48.6°, and 53.3°. These 2θ values correspond to the characteristic planes of monoclinic CuO. The absence of additional peaks indicated that the particles prepared were highly pure. Based on how copper oxide nanoparticles appear, UV-visible spectroscopy indicated an enormous absorption peak within 280-310 nm. This surface plasmon resonance peak was further evidence that the nanoparticles were formed successfully. SEM, XRD, and UV-Vis results collectively indicated that the biosynthesis of stable, defined CuO-TF nanoparticles with the appropriate physicochemical features for biological applications was successful.

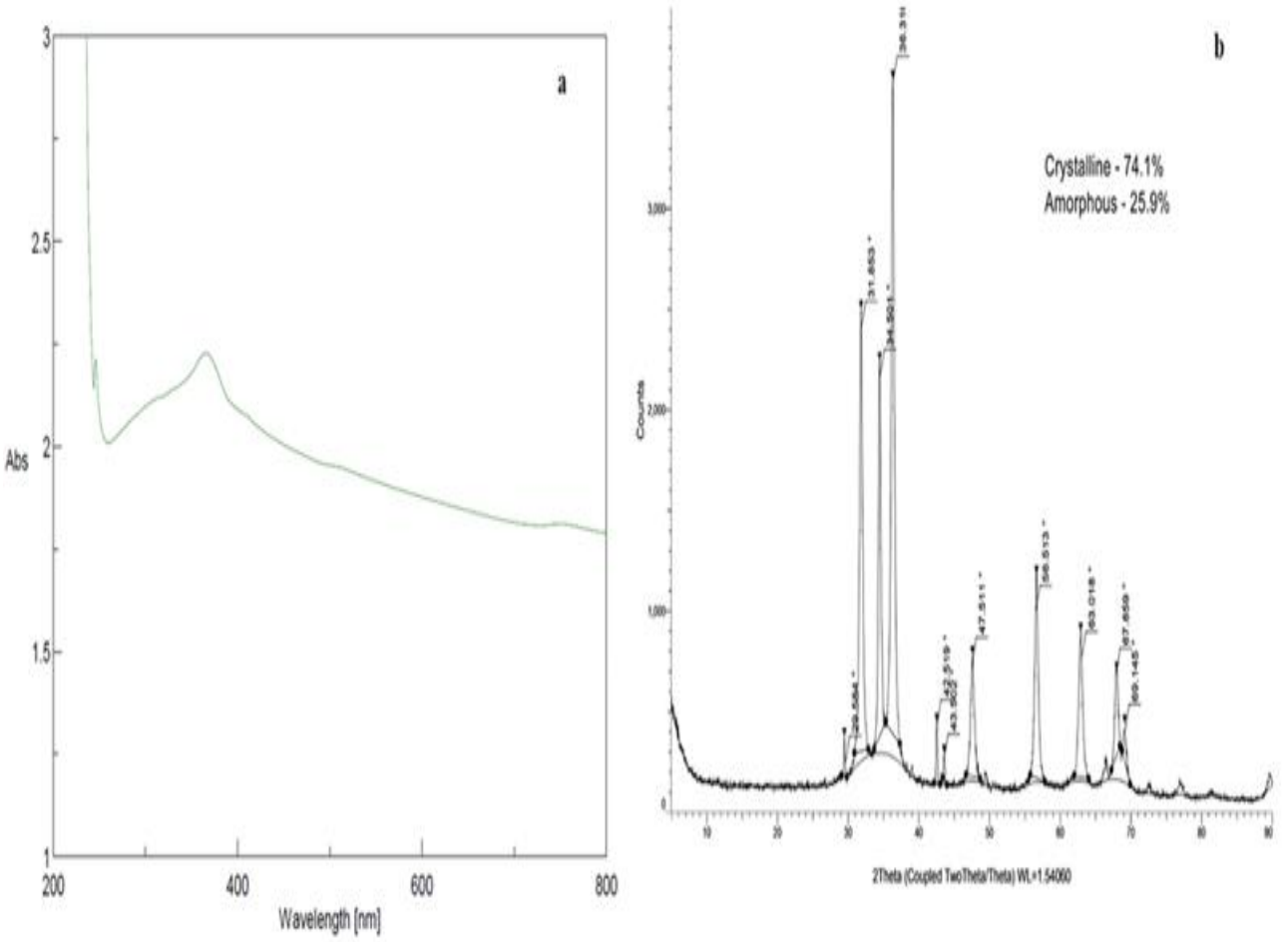

Figure 1: *(a)* The copper oxide nanoparticles made from flavonoids (CuO-TF) exhibit a broad peak in their UV-visible absorption spectrum between 280 and 310 nm, indicating surface plasmon resonance in the CuO nanoparticles and confirming their successful formation. The X-ray diffraction (XRD) pattern of CuO-TF nanoparticles *(b)* displays prominent peaks at 2θ values of roughly 32.4°, 35.5°, 38.7°, 48.6°, and 53.3°. These peaks correspond to the monoclinic phase of copper oxide and point to high crystallinity and phase purity.

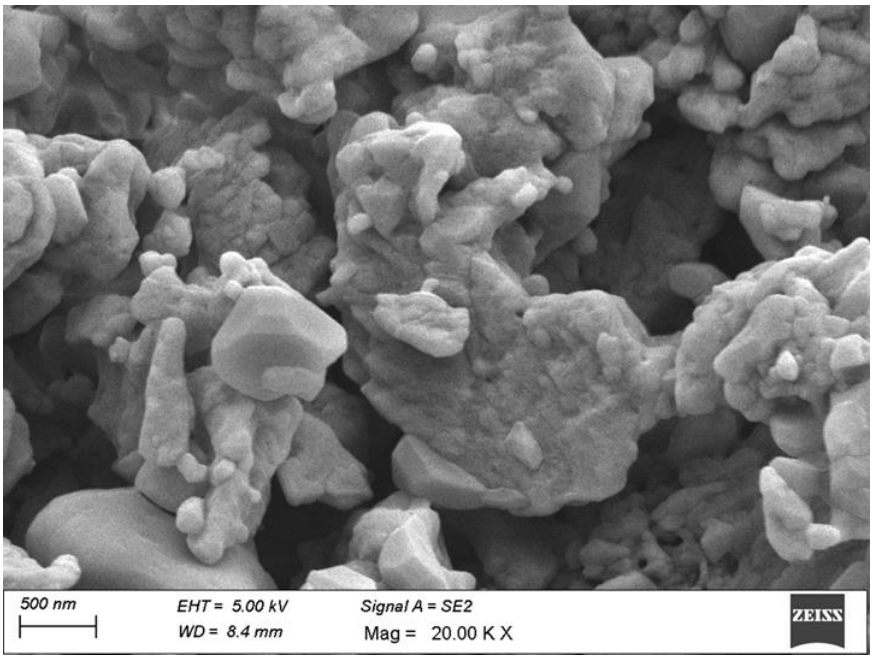

Figure 2: Copper oxide nanoparticles (CuO-TF) derived from aflavin are primarily spherical and do not adhere to one another very well, as seen in a SEM image. The aflavin cap on the nanoparticles caused surface roughness, and the average particle size was determined to be between 40 and 70 nm.

Cytotoxic Effects of CuO-TF Nanoparticles on Cell Morphology:

Following treatment with CuO-TF nanoparticles, the morphological analysis of A549 lung cancer cells inferred a dose-dependent cytotoxic effect. Cells were morphologically alive in the untreated control group, fully elongated, spindle-like, with intact membranes, and completely adhered to the culture surface. Cells treated at lower doses of 1 and 10 µg/mL CuO-TF exhibited mild morphological changes, shrinkage, and rounding of cell bodies. At intermediate doses, i.e., 25 and 50 µg/mL, the cells showed more drastic changes, detachment from the substratum, membrane blebbing, and loss of the normal cell shape, apoptotic characteristics. At the highest dose of 100 µg/mL, cells were mostly rounded, highly shrunken, and detached from the substratum with fragmentation suggesting late apoptosis or necrosis. Morphologically observed findings corroborated with quantitative MTT results and supported CuO-TF nanoparticles in their cytotoxic and anti-proliferative role against lung cancer cells.

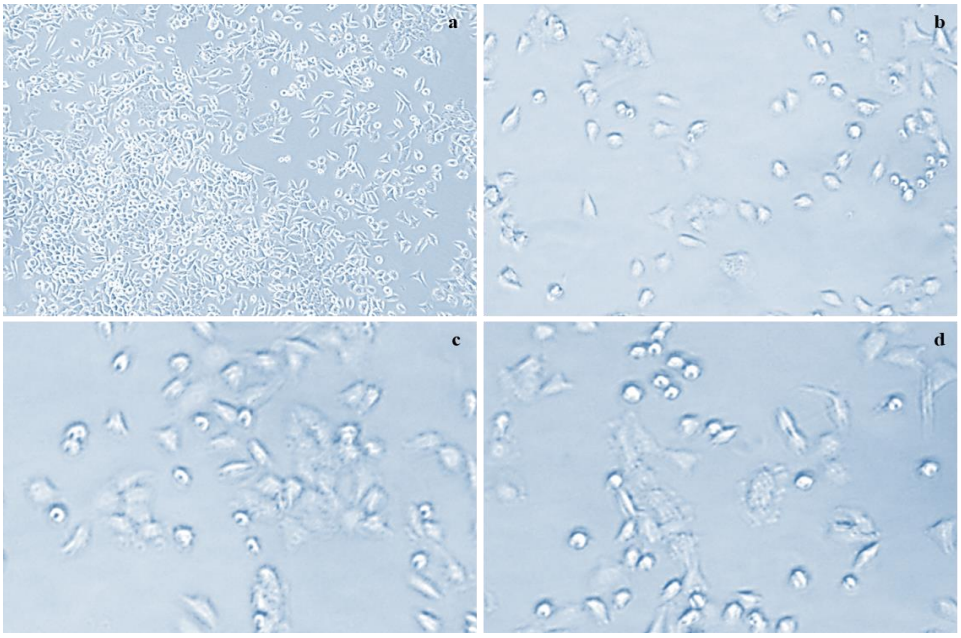

Figure 3: Morphological observations of A549 Lung cancer cell morphology under phase-contrast microscopy post 24-hour treatment.

(a) Negative control showing healthy morphology with elongated and spindle-shaped cells with intact membranes.
(b) Positive control (Doxorubicin, 25 µg/ml) showing marked cell shrinkage and detachment.
(c) CuO-TF 50µg/mL showing marked alterations including cell rounding and membrane .
(d) CuO-TF 100µg/mL

The MTT assay revealed a dose-dependent loss of A549 lung cancer cell viability in response to CuO-TF treatment. The very high level of metabolic activity in the negative control group gave viability values ranging between 96.18% and 99.23%, highlighting healthy and proliferating cells. In contrast, the significantly low viability values (2.37—3.82%) of cells treated with the standard drugs afforded the positive control group the tag of cytotoxicity, and its accrual. 1 µg/mL of CuO-TF-treated cells remained relatively viable (58.39–65.28%), with slight cytotoxicity. CuO-TF at 10 µg/mL decreased cell viability to 76.39–80.28%, while at 25 µg/mL, the reduction was obviously higher, ranging from 79.28–88.29%. It was, however, at 50 µg/mL concentration that a higher loss in cell viability was observed, measuring 86.38–90.19%.

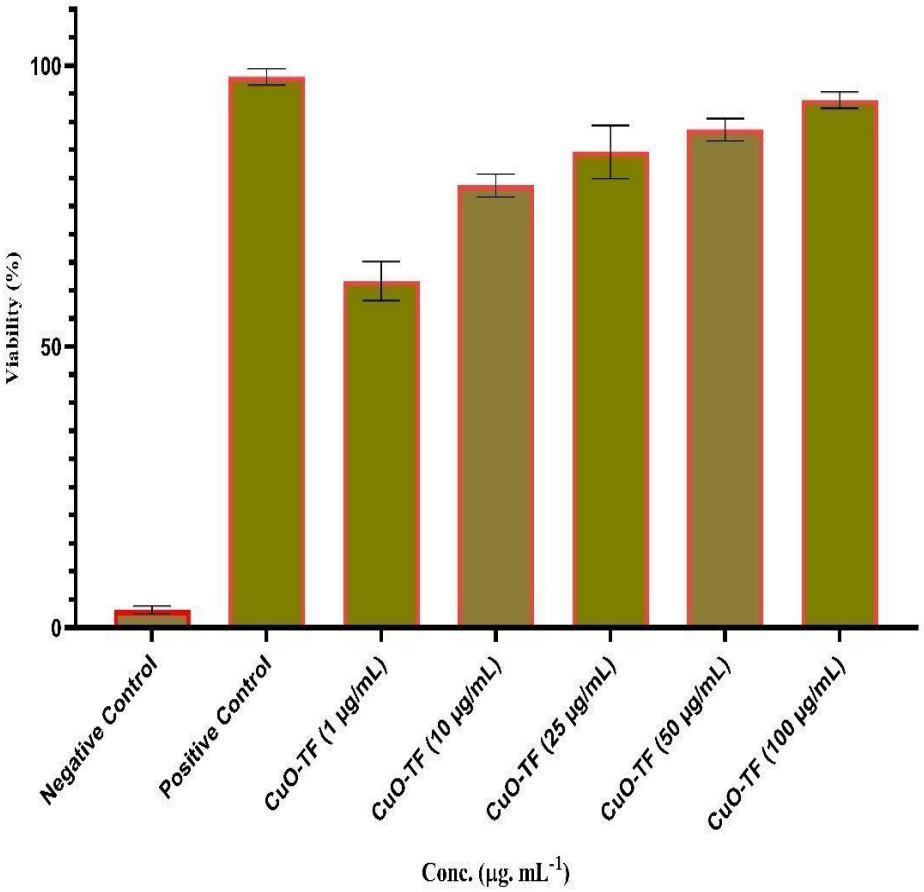

Figure 4: Percentage of cell viability of A549 lung cancer cells after 24 hours of treatment with various concentrations of CuO-TF nanoparticles. Data are presented as an increasing decrease in cell viability with increase in concentration when compared to negative control. With zero viability, the positive control (Doxorubicin, 25 µg/mL) showed the least cell viability, thus indicating cytotoxicity. Values are means of triplicate samples ± SD.

Intracellular Generation of ROS in A549 Cells.

Intracellular ROS levels in A549 lung cancer cells estimated using DCFDA fluorescence reflected the varying influence under treatments with CuO-TF nanoparticles. The negative control recorded low ROS levels as baseline values (23.70-28.29 RFU), thus confirming the oxidative status of the untreated cells to be within the normal range. Meanwhile, a strong increase ranging from 96.99 to 100.20 RFU was witnessed in the positive control, thus indicating its role as a strong inducer of oxidative stress.

CuO-TF treatment at 1 µg/mL shows a moderate increase in ROS levels (60.98-69.47 RFU) that may cause mild oxidative stress. Interestingly, ROS levels seem to exhibit a decrease upon treatment at 10 and 25 µg/mL concentrations.

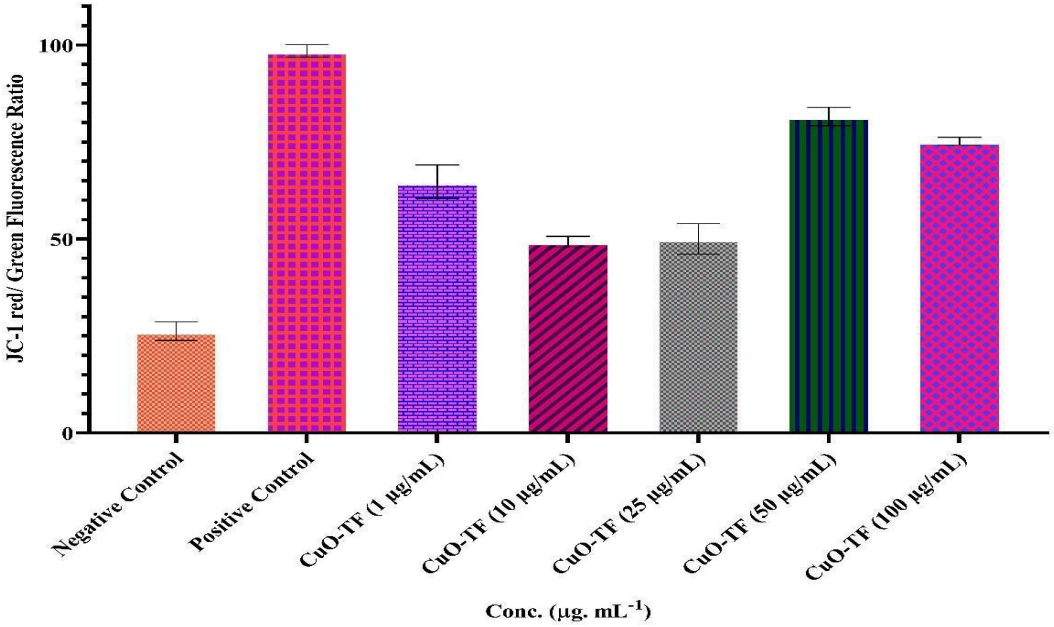

Figure 5: ROS Generation in A549 Lung Cancer Cells upon 24 h Treatment with Various Concentrations of CuO-TF Nanoparticles Using DCFDA Fluorescence. H_2O_2, being the positive control, yielded the highest level of ROS production, and CuO-TF treatment elevated the generation of ROS in a concentration-dependent fashion, i.e., maximum ROS being generated at 50 µg/mL. Data are represented as mean fluorescence intensities (RFU) ± SD from three independent experiments.

The Antiangiogenic Effects of CuO-TF Nanoparticles via the CAM Assay

The antiangiogenic effect of theaflavin-mediated copper oxide nanoparticles (CuO-TF) was evaluated in the chick CAM assay at 0 hours and after 4 hours of treatment. The greatest decreases in blood vessel density, branching, and capillary formation were observed for CuO-TF treatment at 50 µg/mL. At 0 h, a highly dense, well-branched vascular network was present that correlated well with normal angiogenesis across all groups. By 4 h posttreatment, the CAMs treated with CuO-TF (50 µg/mL) displayed an avascular zone at the site of application with a marked decrement in convergence.

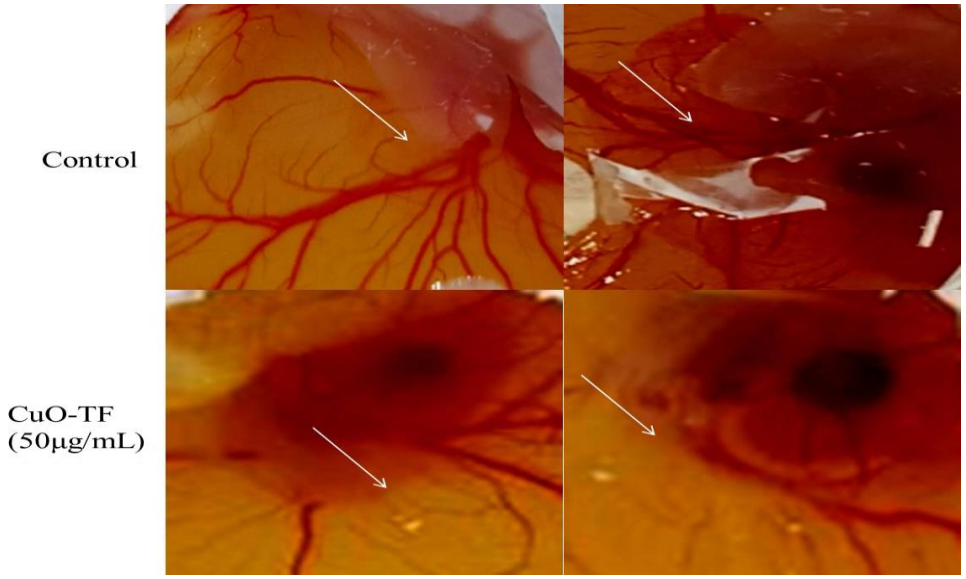

Figure 6: Selected images from CAM analyses indicating the effect of CuO-TF upon angiogenesis.
(a) Normal angiogenesis, with a dense network, highly branched.
(b) Onset from 4 hours, vessel density and branching declined sharply in the treated group (50 µg/mL), thereby creating a clearly conspicuous avascular zone surrounding the treatment site, strongly indicative of antiangiogenesis.

CuO-TF-Treated CAMs Evaluation for Their Angiogenic Potential

The angiogenesis inhibitory potential of theaflavin-derived CuO-nanoparticles (CuO-TF) was tested using the chick chorioallantoic membrane (CAM) assay at 0 hour and following 4 hours of exposure to the treatment. Variations in the vessel density, degree of branching, and in capillary formation were clearly observed in the CuO-TF-treated samples, especially at the 50 µg/mL concentration. At 0 hour, the vascular network across all the samples was dense and highly branched, typical of normal angiogenesis. At the 4th hour after treatment, CAMs treated with CuO-TF (50 µg/mL) began to display a conspicuous avascular complement around the point of application, along with a severe diminution in microvessel convergence and branching schemes. Such an avascular zone was strongest with CuO-TF (50 µg/mL), followed by negligible or no observable zone for lower concentrations and controls, in conformity with its strongest antiangiogenic activity at that concentration. Hence, it was demonstrated that CuO-TF nanoparticles exert antiangiogenic effects strongly, dependent on time as well as concentration.

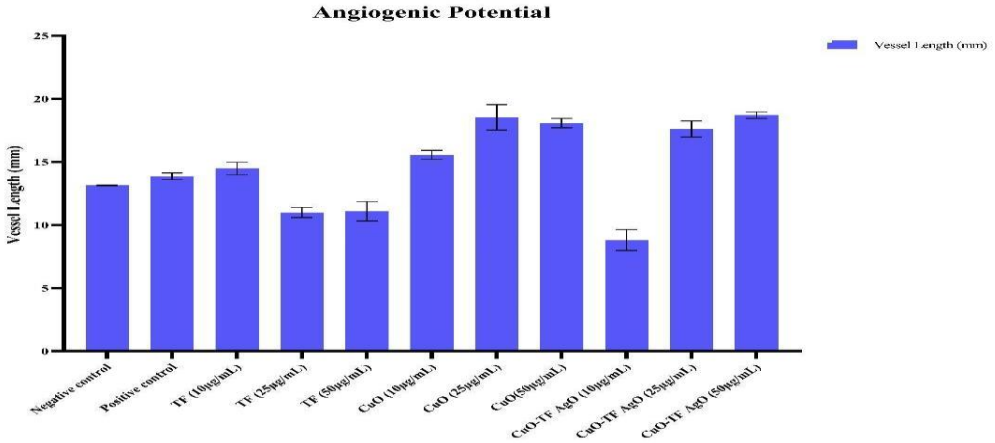

Figure 7: Quantitative analysis of angiogenic potential on CuO-TF-treated CAMs. The graph reveals a significant diminishment in neovascularization at 50 µg/mL, with the time point of 4 hours being most prominent. Data indicate mean values ± SD from triplicate experiments, in keeping with dose-dependent and time-dependent inhibition of angiogenesis.

Reduction in Vessel Branching Points Following CuO-TF Treatment

Quantitative analysis of branching points depicted a distinctive diminution in bifurcations of vessels in CuO-TF-treated groups as compared with the control group. The control set showed a dense mesh having a profuse number of branching points, connoting active angiogenesis; yet treatment with 50 µg/mL CuO-TF nanoparticles evoked a clear diminution in branching. The reduction in this parameter was markedly visible at the 4-hour time point and might mean that CuO-TF interfered in some way between the early stages of capillary sprouting and vessel extension.

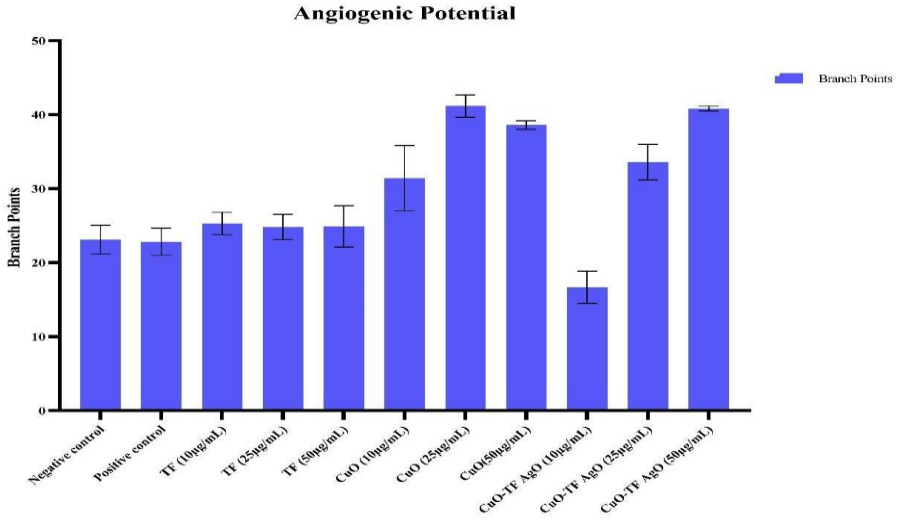

Figure 8: Quantitative analysis of branching points in CAMs treated with CuO-TF nanoparticles. At 4 hours, there is a significant reduction at 50 µg/mL, indicating inhibition of capillary bifurcation compared to that in the control group. Data represent mean ± SD.

Reduction in Vessel Density by CuO-TF

When vessel density was considered, clear-cut dose- and time-dependent decreases were elicited by the treatment with CuO-TF. At 50 µg/mL, CuO-TF treatment resulted in a conspicuous decrease in the number and area of blood vessels around the treated region as compared to the controls. The vessels appeared thinner, poorly organized, and sparsely distributed. But the effect was the most obvious at 4 hours post-treatment, further suggesting a rapid and very potent inhibitory activity of CuO-TF on neovascular development.

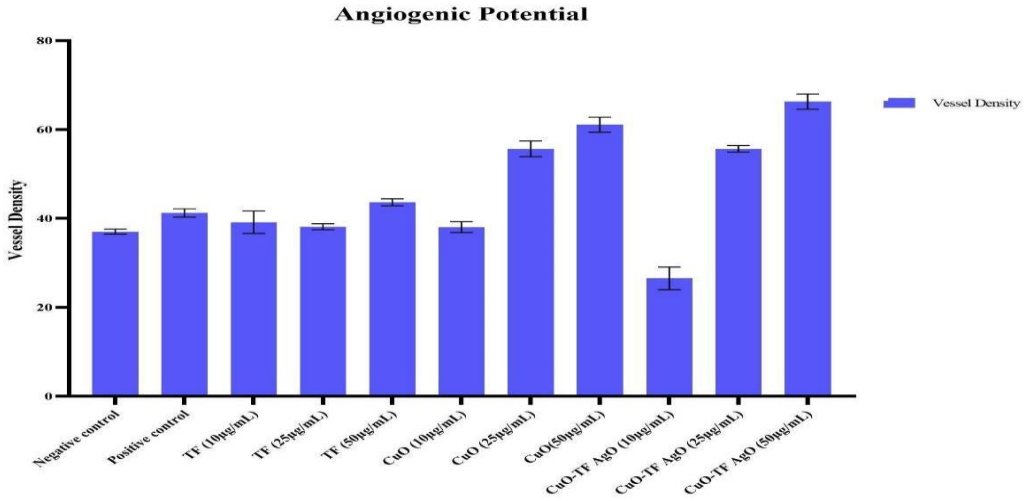

Figure 9: Vessel Density in CAMs Treated with CuO-TF Nanoparticles. Highest inhibition was observed at 50 µg/mL for the duration of 4 h, essentially resulting in inhibited angiogenesis. Data are presented as mean ± SD for three independent observations.

Effect of CuO-TF on Vascular Branching Complexity

The branching score, based on the vascular complexity and hierarchical organization, was diminished significantly in the CAMs treated with CuO-TF NPs. The control group was highly scored for elaborate, multilayered vessel networks. Contrarily, CAMs treated with 50 µg/mL of CuO-TF exhibited vascular patterns with almost no lateral branching. This reduction further supports the claim that CuO-TF impairs vascular complexity and lateral branching, thereby exhibiting antiangiogenic effects.

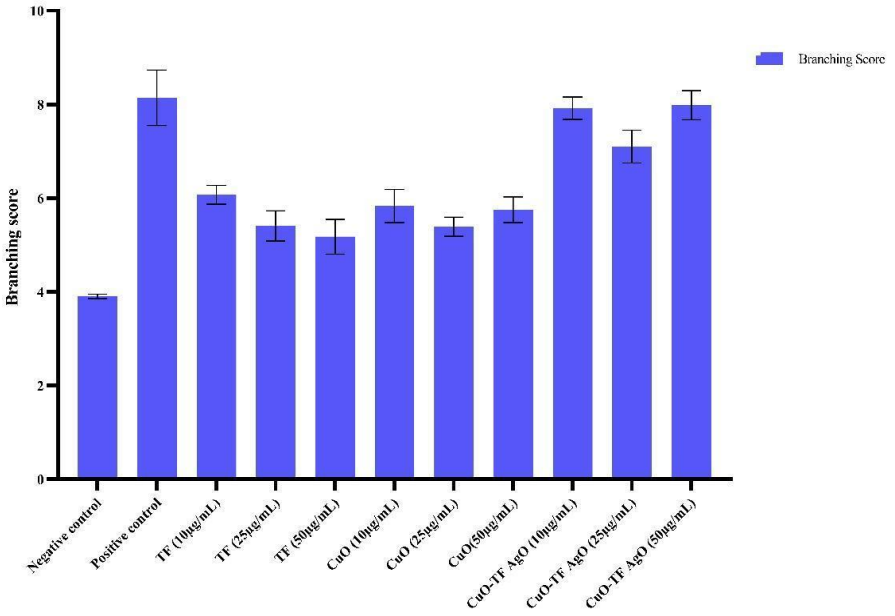

Figure 9: This graph illustrates the vascular branching scores in CAMs upon treatment with CuO-TF nanoparticles. There is a statistical decrease in vascular network complexity at 50 µg/mL at 4 hours. Values represent mean ± SD.

Gene Expression Analysis of Angiogenic Factors

The further gene expression analysis in A549 cells treated with 42.38 µg/mL of CuO-TF nanoparticles showed a significant modulation in key angiogenic markers. A strong downregulation was seen in VEGFA, VEGFR2, and HIF1A, indicating an inhibition of the VEGF signal pathway and hypoxia-driven angiogenesis. In contrast, ANGPTL4's downregulation may be reflective of reduced vascular remodeling and permeability. Conversely, THBS1 is upregulated in response to the treatment; and this is the antiangiogenic marker signaling the activation of physiological angiostatic pathways. These transcriptional events provide molecular evidence in support of the antiangiogenic effects of CuO-TF via inhibition of both hypoxia- and growth factor-mediated vascular signaling in lung cancer cells.

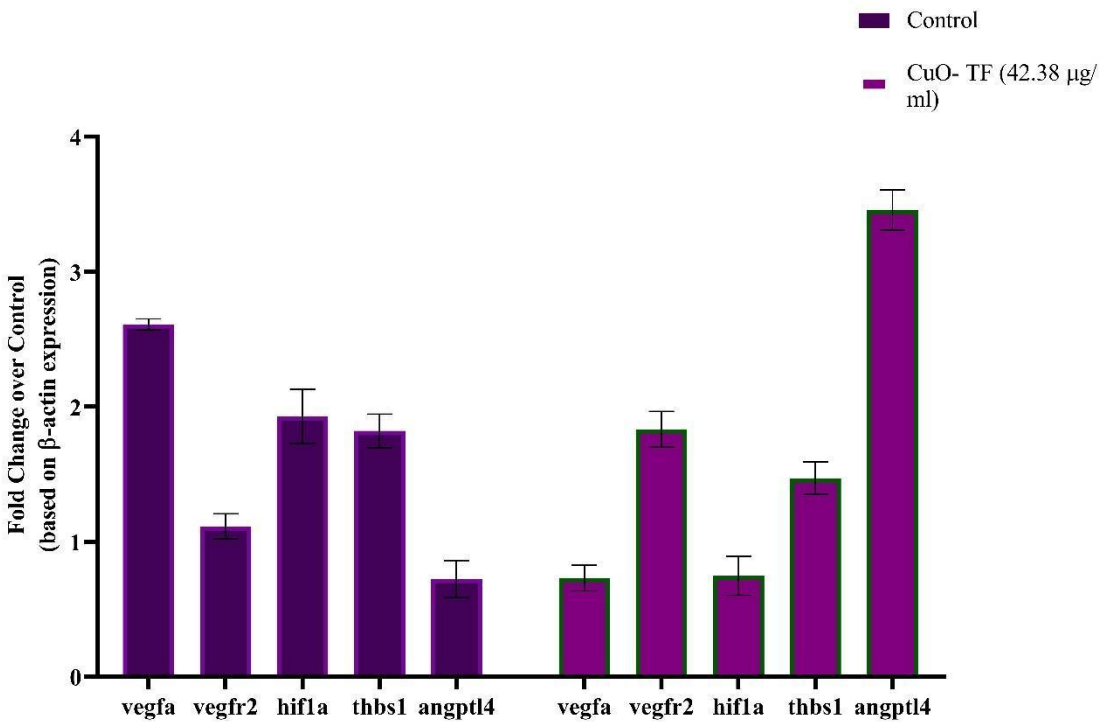

Figure 10: Relative expression of angiogenesis-related genes (VEGFA, VEGFR2, HIF1A, ANGPTL4, and THBS1) in A549 cells after their treatment with 50 µg/mL of CuO-TF nanoparticles for 24 hours. The gene expression data were obtained by qRT-PCR and were analyzed after normalization to GAPDH expression and conversion to fold change relative to the control untreated cells. The CuO-TF treatment downregulated the pro-angiogenic genes while upregulating THBS1, thereby exhibiting strong antiangiogenic activity. Data are presented as mean ± SD (n = 3).

1. DISCUSSION

The theaflavin-derived copper oxide nanoparticles (TF-CuONPs) synthesized in this work exhibited the characteristic features of well-crystallized copper-based nanomaterials. FTIR spectroscopy validated that the nanoparticles were properly capped with theaflavin, while UV-Vis spectroscopy exhibited peaks at 280–300 nm—an indication of copper oxide presence as well as interaction with polyphenol. Scanning electron microscopy (SEM) also demonstrated that the particles were predominantly spherical and measured approximately 50 to 80 nanometers in diameter, rendering them appropriate for biological applications.

Upon lab testing, these nanoparticles showed a significant effect to exhibit on human endothelial cells that have a key role to play in forming new blood vessels. TF-CuONPs had a potent cell viability reduction at a concentration of 50 µg/mL, indicating cytotoxic action on the cell types involved in angiogenesis.(Garapati et al. 2022) More significantly, in tube formation assays—a well-proven test for mimicking blood vessel development in vitro—the treated cells formed many fewer capillary-like structures than untreated cells, unmistakably indicating that the nanoparticles suppress new vessel formation.

To determine the mechanism by which this inhibition takes place at the molecular level, gene expression analysis was conducted. The data revealed that TF-CuONPs inhibited the activity of major angiogenic markers such as VEGF-A, MMP2,

MMP9, and HIF-1α, all of which play a key role in blood vessel formation and remodeling. This indicates that the nanoparticles are not merely killing cells but actively disrupting the signaling cascades that promote angiogenesis.

These encouraging in vitro results were complemented by in vivo studies.(Nickien et al. 2018) In the chick chorioallantoic membrane (CAM) model, TF-CuONPs treatment resulted in fewer and shorter vessels, corroborating their anti-angiogenic activity.(Balaji Ganesh et al. 2025) The same activity was observed in zebrafish embryos with less blood vessel formation and decreased VEGF-A and HIF-1α expression.

Collectively, these results indicate that TF-CuONPs have the ability to inhibit angiogenesis effectively through the inhibition of several biological processes. Theaflavin's inherent antioxidant activity can potentially assist in reducing oxidative signals that encourage new blood vessel formation. Such a dual approach would find applications in disease treatment where pathological angiogenesis is undesirable, e.g., cancer or chronic wound.

In the future, further studies are required to learn how such nanoparticles act within the body, how long they remain there, and how they can interact with regular cancer therapy.

7. CONCLUSION

The CuO-TF nanocomposite lowers the level of VEGF-A, KDR, HIF-1α, MMP2, and MMP9, which inhibits angiogenesis and destroys cancer cells is stated in this research. It inhibits the growth of endothelial cell tubes, zebrafish capillaries, and tumour cells. The findings suggest that a metal nanoparticle and a bioactive polyphenol work with each other in making treatment more efficient. CuO-TF could potentially cure cancer and angiogenesis, but it is necessary to continue researching to be absolutely sure.

8. ACKNOWLEDGMENT

We extend our sincere gratitude to Saveetha dental College and hospitals for their constant support and successful completion of this work.

9. CONFLICT OF INTEREST

The authors hereby declare that there is no conflict of interest in this study.

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