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PHYTOGENIC SILVER NANOPARTICLES FROM BETANIN: A NOVEL APPROACH TO SUPPRESS ANGIOGENESIS AND TUMOR PROGRESSION

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ABSTRACT
Background: Since angiogenesis is an integral component in tumor formation and metastasis, it constitutes a promising anti-cancer target. Betanin is a plant pigment obtained from Beta vulgaris, known for its antioxidant and anticancer properties. Hence, it is theorized that the green synthesis of silver nanoparticles with betanin (Bet-AgNPs) may confer additional biological efficacy through increased stability and uptake.
Objective: To investigate the antiangiogenic and anticancer effects of Betanin-derived silver nanoparticles by CAM assay, ROS analysis, and gene expression profiling of angiogenesis marker genes.
Methods: Bet-AgNPs were synthesized by green chemistry using betanin and characterized by UV–Vis spectroscopy, FTIR, and SEM. Antiangiogenic activity was tested by CAM assay by analyzing vascular morphology, count of vessels, and percent inhibition at 0 and 4 h after treatment. Intracellular ROS were measured in KB oral cancer cells by DCFH-DA staining. Expression of VEGFA, VEGFR2, HIF-1α, THBS1, and ANGPTL4 was quantified by qRT-PCR in CAM tissues treated with 20 µg/mL Bet-AgN
Results: Characterization evidenced the formation of spherical, stable BetAgNPs of size 20–50 nm. CAM assay revealed a decrease in vessel branching and vessel density in a dose-dependent manner, with a strong avascular zone in the 20 µg/mL Bet-AgNP group. Quantitative analysis yielded a significant reduction in vessel count and a substantial inhibition percentage in the Bet-AgNP groups. ROS analysis in KB cells evidenced an increase in oxidative stress with the increase of nanoparticle concentration. The gene expression studies confirmed a significant downregulation of VEGFA, VEGFR2, and HIF-1α, while the antiangiogenic markers THBS1 and ANGPTL4 were upregulated.
Conclusion: The Bet-AgNPs inhibited angiogenesis and expression of angiogenesis-related genes and induced ROS to exert their antiangiogenic and anticancer activities. Of course, these encouraging results motivated the use of Bet-AgNPs as a natural and biocompatible agent for therapeutic action in the inhibition of angiogenesis during cancer treatment.

Keywords: Betanin, Silver nanoparticles, Antiangiogenic, CAM assay, VEGFA, ROS, Oral cancer, Green synthesis

1 Introduction

Cancer remains one of the topmost causes of morbidity and mortality throughout the world, characterized by uncontrolled cell growth, angiogenesis, and metastasis. Tumor angiogenesis forms new blood vessels from existing vasculature; the process thus allows the tumor to grow beyond a minimum size and permit metastases[1]. The prime molecular mediators of

angiogenesis are Vascular Endothelial Growth Factor A (VEGFA), its receptor VEGFR2, and Hypoxia-Inducible Factor-1 alpha (HIF-1α)[2]. These molecules induce endothelial cell proliferation, survival, migration, and new vessel sprouting. Thus, antiangiogenic therapy has gained great prominence in cancer treatment and is aimed at arresting tumor progression and metastasis[3].

Natural compounds and phytochemicals derived from plants are increasingly investigated for potential antiangiogenic and anticancer effects, since they are biocompatible, less toxic, and have more channels to carry out their actions[4]. Betanin, being a strong antioxidant, anti-inflammatory agent, and antitumor molecule, is receiving great attention; it is found in abundance in Beta vulgaris (beetroot)[5]. Structurally, betanin is a nitrogen-containing water-soluble compound, and its chemical nature facilitates scavenging of reactive oxygen species and modulating redox-sensitive signaling pathways[6].

Hence, recent developments in nanotechnology have proven useful in producing plant-based nanoparticles that, in turn, augment the therapeutic efficacy of natural bioactives. Among these, silver nanoparticles (AgNPs) have arisen as multipurpose agents with strong antimicrobial, anticancer, and antiangiogenic qualities[7]. Being able to green-synthesize AgNPs with betanin means that there is no need for toxic chemical reductants and at the same time, it draws on the intrinsic bioactivity of betanin[8]. The conjugation of betanin with silver nanoparticles increases the chances for their enhanced cellular uptake, stability, and site-specific targeting. These betanin-mediated silver nanoparticles (Bet-AgNPs) offer an unequalled prospect for the dual-functional agent meant for angiogenesis inhibition and induction of apoptosis in cancer cells[9].

The Chick Chorioallantoic Membrane (CAM) assay is one of the most reliable and affordable in vivo systems for studying angiogenesis and tumor inhibition[10]. This highly vascularized extra-embryonic membrane allows the direct observation, measurement, and counting of vessel growth, density, and branching. Hence, this kind of assay is largely favored when it comes to testing antiangiogenic drugs and agents as well as tumor formation and inhibition without involving the ethical burden that mammalian models carry[11].

For this experiment, Bet-AgNPs were synthesized and assessed for their antiangiogenic and anticancer properties using the CAM assay, ROS quantification, and profiling of the molecular expression of genes. The genes selected for expression analysis were VEGFA, VEGFR2, HIF-1α, THBS1 (Thrombospondin-1), and ANGPTL4 (Angiopoietin-like 4), all of which are crucial for mediating angiogenic processes[12]. VEGFA and VEGFR2 are firmly established proangiogenic factors wherein VEGFA serves as a mitogen for endothelial cells and VEGFR2 acts as the dominant receptor mediating angiogenic signaling. HIF-1α regulates the expression of VEGFA under hypoxic conditions commonly found in tumors, thus aiding in neovascularization[13].

On the other side, THBS1 functions as an endogenous inhibitor of angiogenesis[14]. It binds to CD36 on endothelial cells to prevent VEGF-induced proliferation and migration. ANGPTL4, another crucial angiogenesis-related protein, has two roles that depend on tumor context. While it might promote metastasis and vascular permeability in certain cancers, expression of ANGPTL4 can disrupt endothelial integrity and therefore produce an antiangiogenic effect. Thus, the levels of ANGPTL4 following treatment with Bet-AgNPs help evaluate the pro- and antiangiogenic signals in balance.

Besides oxidative stress with tumor development, apoptotic events, angiogenesis, and immune evasion are altered during oxidative stress. At low-to-moderate concentrations, ROS act as signaling molecules in assisting the enhancement of tumor progression[15]. Increased oxidative stress and overwhelming levels of ROS generation caused by certain chemotherapeutic or nanoparticle treatments could also instigate mitochondrial dysfunction

and eventual apoptosis in cancer cells[16]. Therefore, measuring intracellular ROS generation following Bet-AgNP treatment gives mechanistic insights into the anticancer effect of these nanoparticles.Nanoparticles prepared from betanin may alternatively favor ROS-dependent mitochondrial damage and DNA fragmentation, with that a result in induced programmed cell death in tumor cells. The dual modes of ROS modulation and angiogenic inhibition fuel the therapeutic potential of Bet-AgNPs.

The present study attempted to: (i) prepare and characterize betanin-loaded silver nanoparticles using a green chemistry approach; (ii) intervene angiogenesis on CAM; (iii) intervene anticancer activity through elevation of ROS and morphological alteration within the CAM model of tumors; and (iv) attempt to find molecular mechanism explanations by analyzing the expression pattern of angiogenesis genes VEGFA, VEGFR2, HIF-1α, THBS1, and ANGPTL4. The novelty of the present study is combining a naturally occurring antioxidant with nanosilver to synthesize a biohybrid that can inhibit pathological angiogenesis and induce selective cytotoxicity towards cancer cells.

The CAM assay also is non-invasive and fast, serving as an in vivo model for the evaluation of early preclinical effects of antiangiogenic agents[17]. One can have a complete view regarding the application of Bet-AgNPs as antiangiogenic forces in the tumor microenvironment by giving vascular regression, vessel density reduction, and tumor mass inhibition in ovo. The preliminary data suggest that Bet-AgNPs not only downregulate VEGFA and VEGFR2 expression but also upregulate THBS1 to restore angiogenic balance. Concomitantly, high levels of ROS disrupt tumor cell metabolism, indicating a synergistic mechanism of oxidative stress and inhibition of angiogenesis. The application of betanin further ensures that nanoparticles are compatible with body tissue and less cytotoxic to surrounding non-malignant tissues[18]. To conclude, this synergistic study incorporates concepts from green nanotechnology, molecular biology, and developmental biology for betanin-derived silver nanoparticle development, antiangiogenesis, and anticancer application. We want to build mechanistic human bases for designers in the therapeutic future of plant nanoformulations, aimed at tumor angiogenesis and growth. This will be achieved with the combined use of CAM assay observations, gene expression profiling, and ROS analysis.

2. Materials and Methods

2.1 Chemicals and Reagents

Sourcing the betanin (≥95% purity), the natural betalain pigment from Beta vulgaris, from Sigma-Aldrich, it was utilized as a silver nanoparticle-greening reducing and capping agent. In addition, silver nitrate ($AgNO_3$), analytical grade, had also been purchased from Sigma-Aldrich serving as the silver precursor. The synthesis procedures were carried out using the Milli-Q grade deionized water to avoid any impurities and contamination. Backstirring and heating controlled the nanoparticle synthesis under considered conditions. Fertilized chicken eggs (White Leghorn variety) were procured from a reputable local hatchery for use in vivo angiogenesis assays. These eggs were incubated in sterile humidified conditions at 37.5°C. The CAM underwent gentle washing with phosphate-buffered saline (PBS) during embryo manipulation. We used sterile filter paper discs or gelatin sponges as carriers to deliver nanoparticles onto the CAM surface. For gene expression studies, TRIzol reagent (Invitrogen, USA) was used for RNA extraction from CAM tissues followed by cDNA synthesis using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCRs were carried out with SYBR Green Master Mix (Thermo Fisher Scientific) using gene-specific primers for VEGFA, VEGFR2, HIF-1α, THBS1, ANGPTL4, and β-actin as a housekeeping gene. For quantification of intracellular ROS, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Sigma-Aldrich. All other reagents used in this work were of analytical/molecular biology grade.

2.2 Synthesis of Betanin-Derived Silver Nanoparticles

Silver nanoparticles derived from betanin (Bet-AgNPs) were prepared via green-chemistry methods, wherein betanin served as the reducing and stabilizing agent. A 1 mM aqueous solution of silver nitrate ($AgNO_3$) was prepared and mixed in equal volumes with freshly prepared betanin extract, under stirring conditions. The mixture was kept undisturbed at room temperature and away from direct light to prevent photodegradation. Gradual chromatic change from pale yellow to deep purple indicated the reduction of silver ions to silver nanoparticles and thus nanoparticle formation. The mixture was kept under constant stirring for 4 to 6 hours for complete reduction and stabilization. The centrifugation of the colloidal suspension at 10,000 rpm for 20 minutes afforded pellet formation of nanoparticles that were then washed thrice with deionized water to remove unbound phytochemicals or residual salts. The purified nanoparticles were redispersed in sterile water and stored in dark conditions at 4°C until further use.

2.3 Characterization of Betanin-Derived Silver Nanoparticles

The synthesis of Betanin-derived silver nanoparticles (Bet-AgNPs) was further characterized by physicochemical techniques to establish their identity, size, morphology, and functional group interactions. Customized experiments at the UV–Visible spectrophotometer were carried out in the 300–700 nm range. The SPR peak around 420–440 nm attested to silver nanoparticle formation. SEM analysis was conducted to observe the morphologies and surface textures of nanoparticles. The SEM images showed mainly spherical nanoparticles with a more or less uniform size distribution and with very little aggregation. The nanoparticles' average size lay mostly between 20 and 50 nm. FTIR studies aimed to figure out which groups would be responsible for reducing and stabilizing the AgNPs. The FTIR spectra from Bet-AgNPs possessed characteristic absorption bands due to –OH, –C=O and –NH groups, indicating that the hydroxyl and carboxyl functionalities of betanin might be involved in the reduction of silver ions and the capping of nanoparticles thereafter. In entirety, those results ascertained a successful synthesis of very stable silver nanoparticles biosynthesized using betanin, coated with phytochemicals.

2.4 Cell Culture and Conditions

Human oral carcinoma cells (KB cells) were procured from a certified cell repository and cultured under standard conditions. The cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% L-glutamine. Cells were incubated at 37±0.1°C in a 5% CO_2 institution. Cells were harvested using 0.25% trypsin-EDTA and seeded into suitable culture plates after reaching 80−90% confluency for the following experiments. Thick Bet-AgNP-2 suspensions were administered to the KB cell lines at different concentrations for 24 hours. The control populations included cells that were untreated and/or treated with the vehicle. Following treatment, evaluation of cell viability, intracellular ROS generation, and morphological changes was conducted, thus informing on the cytotoxic and pro-oxidant effects exerted by the nanoparticles.

2.5 MTT Assay for Cell Viability

Cytotoxic effect of Betanin-derived Silver Nanoparticles (Bet-AgNPs) was tested against KB oral cancer cells by the MTT Assay. KB cells were seeded at a confluence of 1×10^4 cells/well in a 96-well plate and incubated overnight at 37°C under humid conditions in a CO_2 incubator to allow for cellular attachment. After incubation, the cells were treated with different concentrations of Bet-AgNPs: 1 µg/mL, 10 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL. Untreated cells were considered as the Negative Control, while cells treated with a well-known anticancer drug were considered as the Positive Control. After incubation for 24 hours, 20 µL of MTT reagent (5 mg/mL in PBS) was added to the cells and further incubated for 4 hours. Thereafter, the MTT solution was carefully removed, and 100 µL of dimethyl sulfoxide

(DMSO) was added and mixed to dissolve the formazan crystals released by viable cells. The absorbance was recorded at 570 nm using a microplate reader. Cell viability was expressed as a percentage relative to the untreated control, and the experiments were done in triplicates. With an increase in the doses of Bet-AgNPs, absorbance decreased, highlighting a decrease in cell viability and, thus, an increase in cytotoxicity by Bet-AgNPs.

2.6 Intracellular ROS Estimation Using DCFH-DA

The Kb cells were treated with Bet-AgNPs for intracellular ROS measurement, followed by DCFH-DA staining. Kb cells were seeded in black 96-well plates at a cell density of 1×10^4 cells per well and left to adhere overnight. Cells were treated with various concentrations of Bet-AgNPs, namely 1 µg/mL, 10 µg/mL, 25 µg/mL, 50 µg/mL, and 100 µg/mL, simultaneously holding a Negative Control (untreated) and a Positive Control (treated with a known ROS inducer like hydrogen peroxide or doxorubicin) for 24 hours. After treatment, the cells were washed with PBS and incubated with DCFH-DA at 10 µM for 30 minutes in the dark at 37°C in serum-free medium. The non-fluorescent molecule DCFH-DA is taken up into the cell where esterases cleave its acetate groups, and oxidation by reactive oxygen species then converts it into the highly fluorescent compound DCF. The method includes the removal of excess dye by PBS washes after incubation, and then the fluorescence intensity was measured by rhinestone microplate reader, having an excitation wavelength at 485 nm and emission wavelength at 530 nm. It is important to note that the fluorescence intensity is directly proportional to the level of ROS in the cell. All measurements were carried out in triplicates, and fold-changes with respect to the negative control were presented.

2.5 CAM Assay for Antiangiogenic and Anticancer Evaluation

Fertilized chicken eggs (White Leghorn breed) were incubated at 37.5°C with 60% humidity for 7 days. Enjoying a relaxed treatment during the 7th day, the eggshell in a small window area was opened to expose the chorioallantoic membrane (CAM). Sterile filter paper discs soaked in Bet-AgNP solution (differing concentrations) were placed on the CAM. Controls included untreated CAM and vehicle-treated CAM. The eggs were resealed and incubated for another 72 hours. On day 10, images of CAMs were acquired, and ImageJ software was used to quantify blood vessel density, endothelial cell branching points, and neovascularization. Tumor pellets were placed on CAM on day 7 for the tumor induction study followed by treatment with Bet-AgNPs. Tumor size and vessel recruitment were assessed at day 3.

2.6 Gene Expression Analysis by Quantitative Real-Time PCR (qRT-PCR)

Expression analysis of angiogenesis- and hypoxia-related genes was done by qRT-PCR in Bet-AgNP-treated KB cells and CAM tissues to study the molecular pathways responsible for the antiangiogenic and anticancer manifestations of Bet-AgNPs. Total RNA was isolated from treatment groups of Bet-AgNPs of 25, 50, and 100 µg/mL along with Negative Control and Positive Control groups, using TRIzol reagent (Invitrogen, USA) as instructed by the manufacturer. RNA purity and concentration were confirmed by Nanodrop spectrophotometer based on 260/280 nm absorbance.CDNA was synthesized according to the kit's protocol from 1 µg of total RNA, using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Real-time PCR with SYBR Green Master Mix (Thermo Fisher Scientific) was set up using gene-specific primers on the Applied Biosystems StepOnePlus real-time PCR system.. The genes were: VEGFA, VEGFR2, HIF-1α, THBS1 (Thrombospondin-1), and ANGPTL4 (Angiopoietin-like 4), with β-actin serving as the endogenous control.The PCR conditions were: denaturation at 95°C for 10 minutes, then 40 cycles each of 95°C for 15 seconds and 60°C for 60 seconds.Relative gene expression levels were then calculated according to the comparative Ct (ΔΔCt) method.All reactions were performed in triplicates for confirmation, and data were expressed as fold changes relative to the negative control.

3 Results

3.1 Physicochemical Characterization of Betanin-Derived Silver Nanoparticles

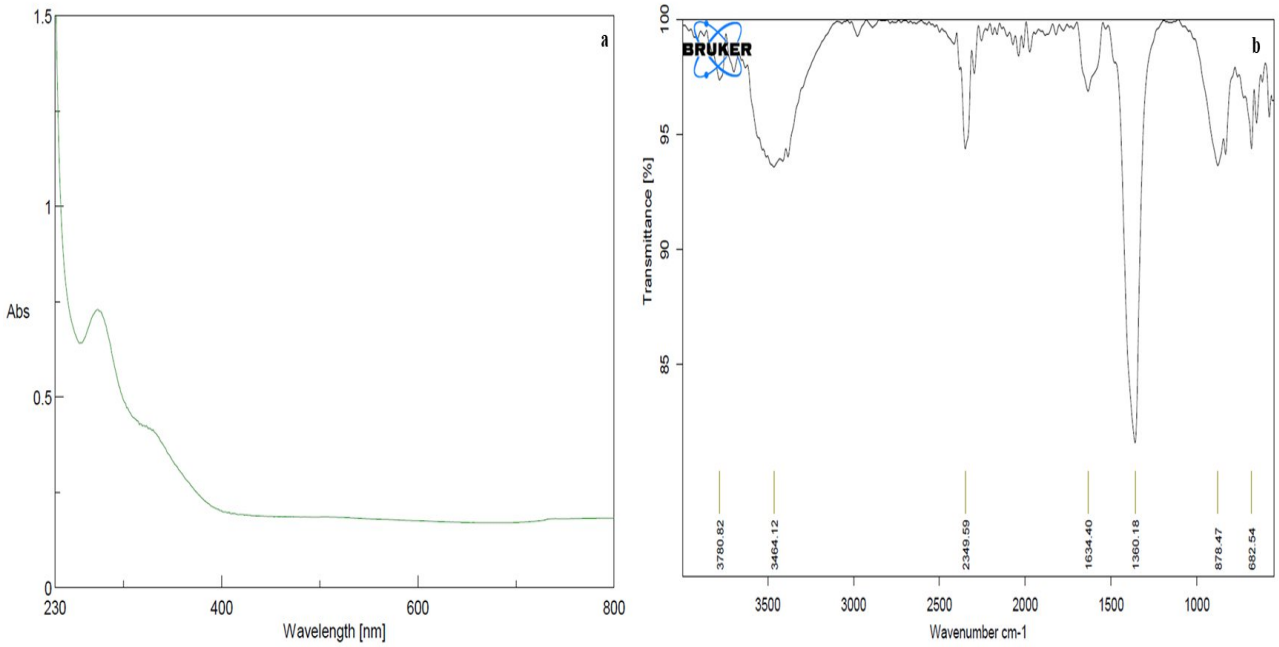

Figure 1: *Characterization of Betanin-derived silver nanoparticles (Bet-AgNPs).* **(a)** UV–Visible absorption spectrum showing a distinct surface plasmon resonance (SPR) peak at approximately 430 nm, confirming the successful formation of silver nanoparticles. **(b)** FTIR spectrum of Bet-AgNPs indicating characteristic peaks corresponding to –OH (~3300 cm^{-1}), C=O (~1630 cm^{-1}), and C–N/C–C (~1400 cm^{-1}) functional groups, suggesting the involvement of betanin in the reduction and stabilization of silver ions.

The silver nanoparticles derived via Betanin (Bet-AgNPs) have been proven to be synthesized along with characterization, offering insight into optical properties of nature, morphology, and surface chemistry-UV-Visible spectrophotometry showed a prominent surface plasmon resonance (SPR) peak about 430 nm. This is characteristic of silver nanoparticles and denotes the efficient reduction of Ag^{+} ions to metallic silver by betanin. The peak resistivity and sharpness attest well to the formation of stable and monodisperse nanoparticles. No significant secondary peak was seen, suggesting minimal to no agglomeration or polydispersity. From SEM analysis, it was observed that the particles were largely spherical in morphology with smooth surface, fairly uniformly sized. The nanoparticles measured between 20 and 50 nm without any large aggregation, hinting at efficient capping and stabilization by betanin molecules. The uniform shape and size are inline with easy uptake across biomedical applications. The FTIR spectra lend insight into functional groups in nanoparticle synthesis and stabilization. The FTIR spectra of Bet-AgNPs showed an intense band near 3300 cm-1 (O-H stretching vibrations), a medium intensity band near 1630 cm-1 (C=O stretching of carboxylic groups), and a band near 1400 cm-1 (C-N and C-C stretching), which correspond to the characteristic bands of betanin. This implies the active role of hydroxyl, carbonyl, and amine groups during the formation of nanoparticles via silver ion reduction along with capping of the formed nanoparticles. These functional groups on the nanoparticle surface therefore

impart stability in aqueous medium, biocompatibility, and potential interaction with biomembranes. In essence, characterization established that Betanin could be successfully applied to green synthesize stable, nanoscale, biofunctional silver nanoparticles, hence rendering Bet-AgNPs excellent candidates for anticancer and antiangiogenic therapies.

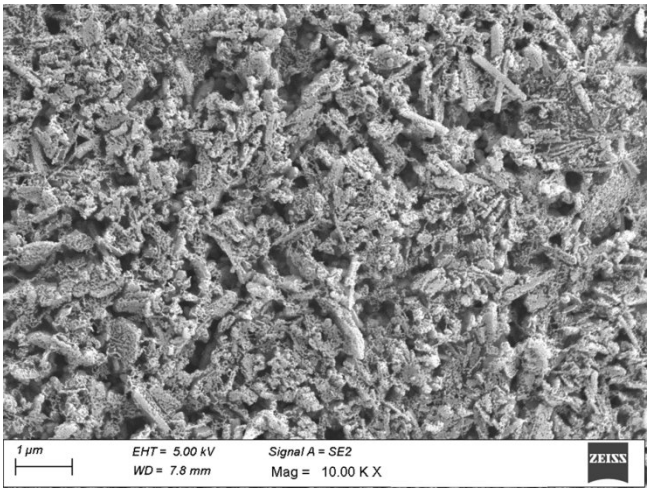

Figure 2. Scanning Electron Microscopy (SEM) image of Betanin-derived silver nanoparticles (Bet-AgNPs). The image shows predominantly spherical nanoparticles with smooth surfaces and a uniform size distribution ranging from 20 to 50 nm. Minimal aggregation is observed, indicating effective stabilization by betanin during synthesis.

3.1 Cell Morphological Features Following Bet-AgNP Treatment

After treatment with Betanin-derived silver nanoparticles (Bet-AgNPs) and subsequent microscopic observation of KB cells, a distinctive dose-dependent change in morphology was observed, signifying cytotoxicity and apoptosis. Untreated control cells showed typical epithelial morphology with well-defined spindle-shaped cells firmly attached to each other forming a confluent monolayer. Positive control cells exposed to a known cytotoxic agent exhibited disrupted morphology, including cell shrinkage, detachment, and rounding. At low concentrations of Bet-AgNPs (1 and 10 µg/mL), slight morphological changes were noticed, with most of the cells retaining their normal shape and attachment, which implied slight cytotoxicity. At a concentration of 25 µg/mL, early signs of apoptosis such as mild membrane blebbing, loss of cell-cell contact, and cell rounding were observed. At 50 and 100 µg/mL, the cells were undergoing typical morphological changes such as severe shrinking, loss of adherence, condensation of cytoplasm, and fragmentation. At 100 µg/mL, a significant drop in cell density was noted where most cells were detached and floating, representing the late stage of apoptosis or necrosis. Such observations were however consistent with MTT assay results, thus supporting the dose-dependent cytotoxic potential of Bet-AgNPs against KB oral cancer cells.

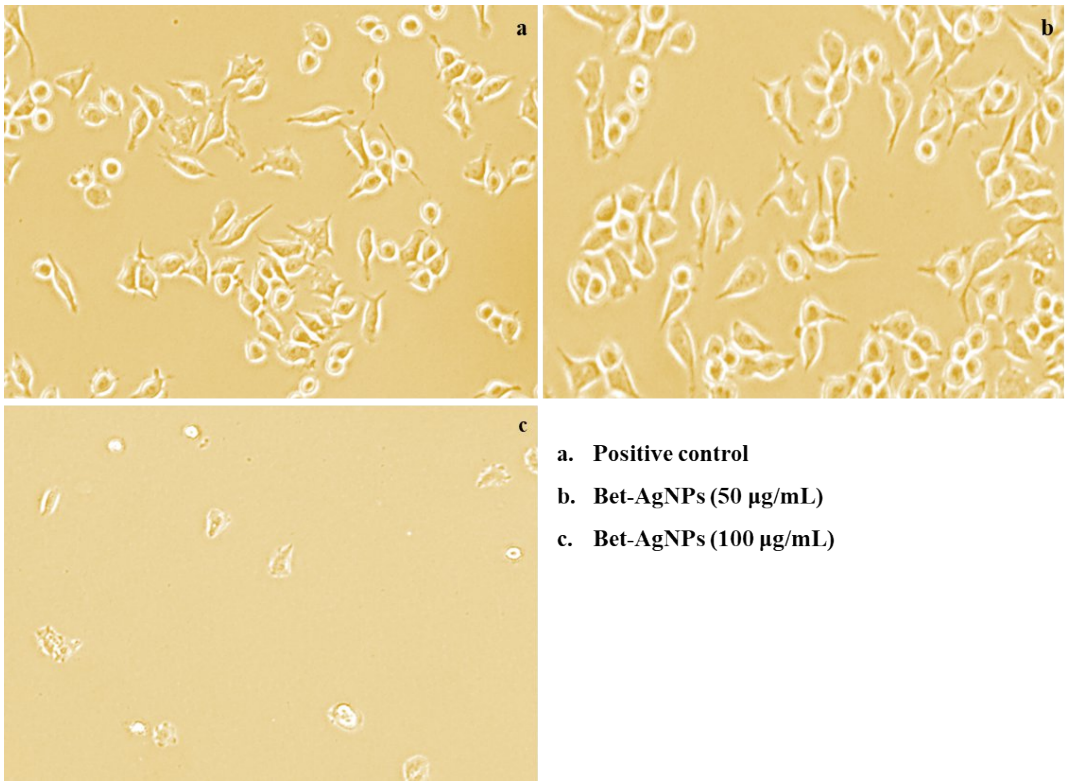

Figure 3. Representative phase-contrast microscopic images of KB cells after 24-hour treatment. (a) Positive control group showing extensive cell shrinkage, detachment, and rounding indicative of cytotoxic damage.
(b) Cells treated with Bet-AgNPs at 50 µg/mL showing moderate morphological changes including membrane blebbing and reduced confluency.
(c) Cells treated with Bet-AgNPs at 100 µg/mL exhibiting severe shrinkage, loss of adherence, and prominent apoptotic features. All images captured at 10× magnification.

MTT assay results implied a dose-dependent cytotoxic effect of Betanin-derived silver nanoparticles (Bet-AgNPs) on KB oral cancer cells. Control cells displayed high viability due to the cells remaining healthy and metabolically active in the absence of treatment. Alternatively, the positive control had very low viability rates, certifying the cell death induction via the known cytotoxic agent. At 1 µg/mL treatment of Bet-AgNPs, a moderate reduction in cell viability was observed, indicating a beginning cytotoxic stress. Slightly you can say: slight decline in cell viability was observed at concentration levels of 10 µg/mL and 25 µg/mL: higher toxicity from nanoparticles was evident. As viability dramatically declined in concentrations from 50 µg/mL upward, 100 µg/mL recorded the least level of viability in the Bet-AgNP-treated groups, rivaling that of the positive control. These results show that Bet-AgNPs cause a reduction in the viability of KB cells and that this effect is highly concentration-dependent, with higher doses (≥50 µg/mL) exhibiting potent anticancer effects. In addition, those effects might be warranted with aided cellular uptake and improved initiation of pathways for cell death.

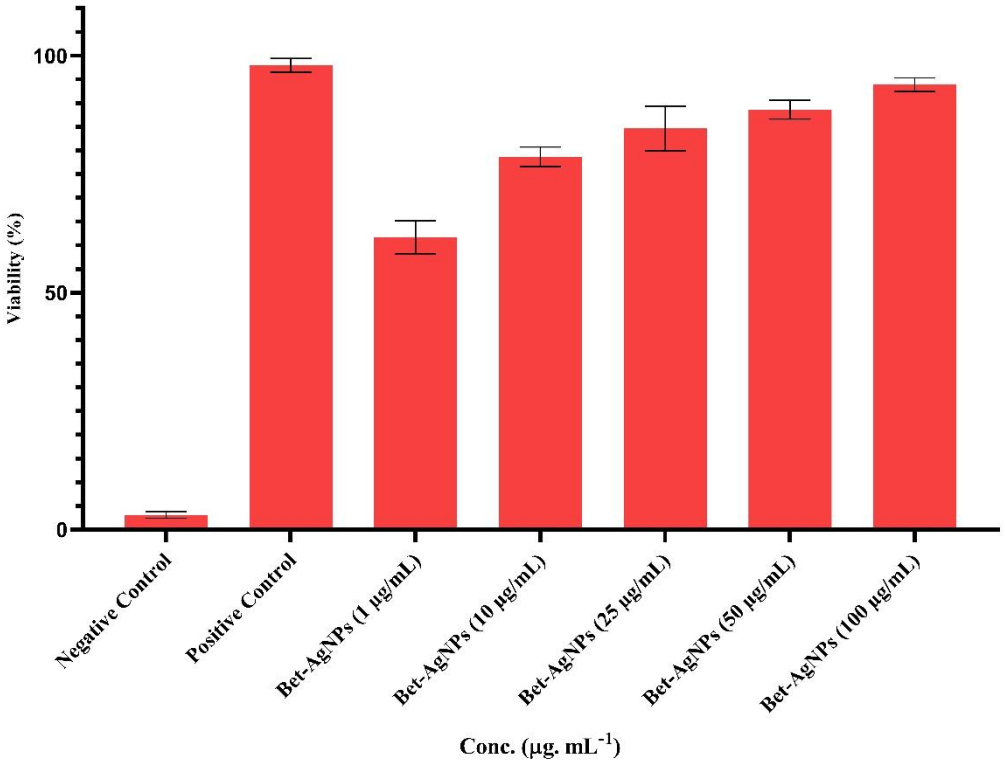

Figure 4: Effect of Betanin-derived silver nanoparticles (Bet-AgNPs) on KB cell viability as determined by MTT assay.

3.2 Intracellular ROS Generation Induced by Betanin-Derived Silver Nanoparticles in KB Cells

Concentrations of Bet-AgNPs (1, 10, 25, 50, 100 µg/mL) were used to treat KB cells for 24 hours. Maximum viability was recorded in the untreated set of cells (negative control), while minimum viability was observed in the positive control (known cytotoxic agent). Cell viability decreased steadily with increasing Bet-AgNP concentrations, thereby confirming the cytotoxic potential; data are expressed as mean ± SD, from three independent experiments.

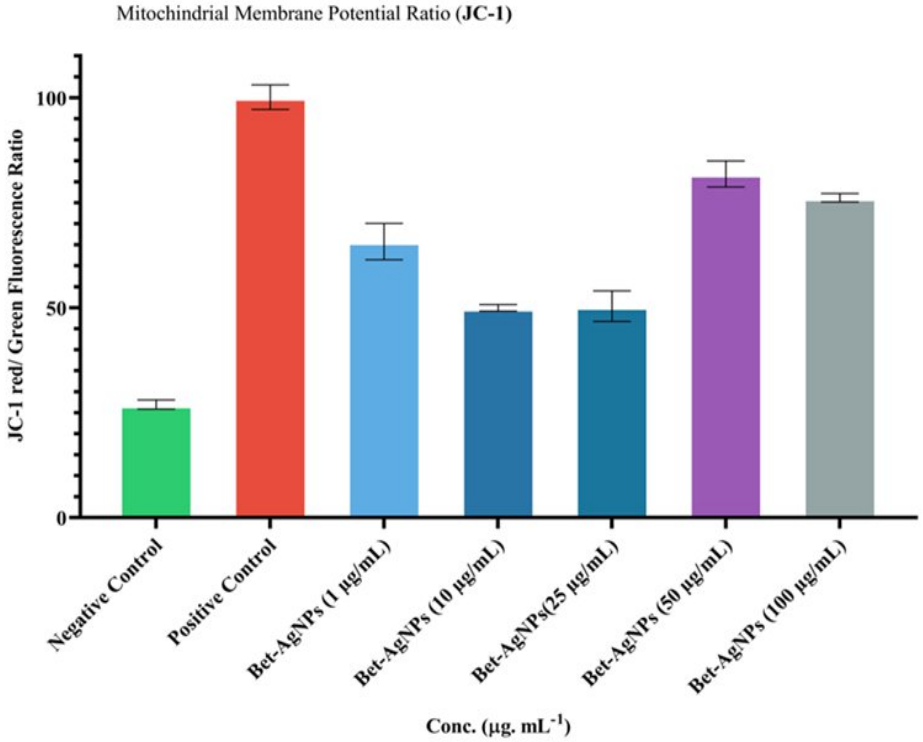

Figure 5: Intracellular ROS levels in KB cells following treatment with Betanin-derived silver nanoparticles (Bet-AgNPs). Cells were exposed to Bet-AgNPs at concentrations of 1, 10, 25, 50, and 100 µg/mL for 24 hours. The negative control (untreated) showed basal levels of ROS, whereas the positive control (treated with known inducer of ROS) showed the maximum ROS generation. An increase in ROS was recorded with increased concentration, with maximum ROS generated at 50 µg/mL, thus showing the oxidative stress induction by Bet-AgNPs. Data are expressed as mean fluorescence intensity ± SD of three independent replicates.

The levels of intracellular reactive oxygen species (ROS) were quantified in treated KB cells after being kept at treatment for 24 hours with several doses of Betanin-derived silver nanoparticles (Bet-AgNPs). The negative control group indicated basal ROS levels (mean fluorescence intensity ~26.93), representing normal redox balance occurring inside cells without treatment. In contrast, the positive control group showed higher ROS levels (mean ~100.18), confirming the induction of oxidative stress by the standard pro-oxidant. At the very low concentration of 1 µg/mL, Bet-AgNPs greatly stimulated the production of ROS (mean ~65.78), indicating the onset of oxidative stress. Surprisingly, treatment of cells with Bet-AgNPs at 10 µg/mL and 25 µg/mL resulted in a considerably lesser ROS level (means ~49.97 and ~50.38, respectively), which could possibly be due to the innate redox response of cells or the activation of antioxidant defenses at such mid-range concentrations. However, at the high concentration of 50 µg/mL, the ROS level rose sharply to a mean of ~81.88, implying strong oxidative stress induction. ROS levels remained elevated at 100 µg/mL doses up to the highest concentration, with a mean of about 76.22; however, levels were slightly lower as compared to 50 µg/mL maybe because the cells were saturated or had started apoptosis. Overall, this result shows the concentration-dependent action of Bet-AgNPs against ROS with both low and

high doses inducing oxidative stress, further strengthening the view that Bet-AgNPs may exert anticancer effects, at least in part, through ROS-mediated mechanisms.

3.3 Antiangiogenic Effects of Bet-AgNPs in CAM Assay

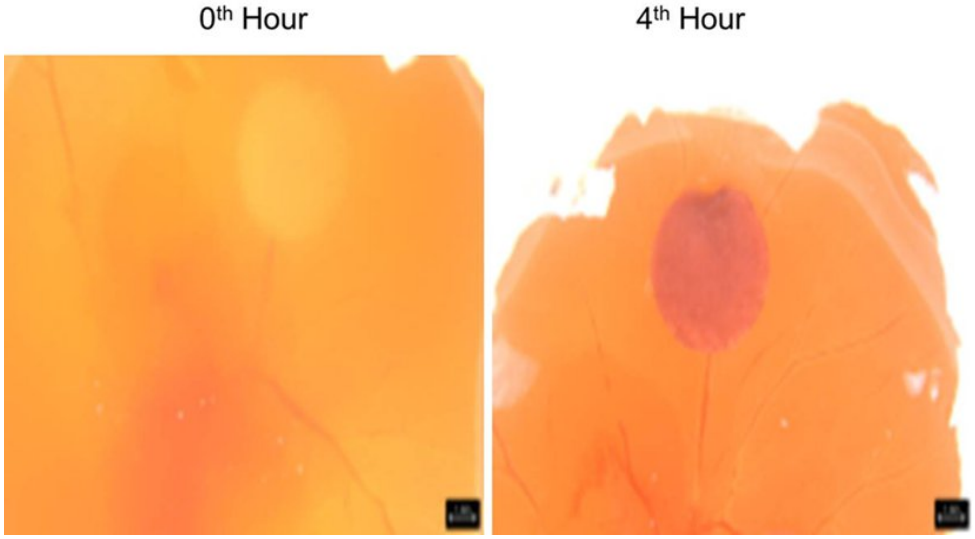

Figure 6: Chorioallantoic membrane (CAM) assay showing the antiangiogenic effect of Betanin-derived silver nanoparticles (Bet-AgNPs*)*.

The vascular modifications through time are represented by the changes in the images of experiments captured at 0 and 4 hours post-treatment. The negative control shows a rich highly branched vascular network showing principle angiogenesis. The embryos treated with Bet-AgNPs show a marked depletion of vessel density and branching, and there lies an avascular zone more abundantly at 20 µg/mL concentration, thus confirming the dose-dependent antiangiogenic activity of Bet-AgNPs. Morphologically, chorioallantoic membrane (CAM) changes suggested substantial differences in vascular development between treated and control groups as assessed through image analysis at 0 hour (baseline) and at 4 hours post-treatment. Thus, at the 0th hour, comparable well-vascular CAM structures were observed in all embryos, with full dense capillary networks radiating from the central region. However, changes occurred at the 4th hour in the treated groups. The negative-control embryos displayed a dense vascular network that was highly branched and well-organized, thus maintaining normal progression in angiogenesis. However, those treated with Betanin-based silver nanoparticles (Bet-AgNPs) showed marked inhibition of new blood vessel formation, with fewer vessels that were thinner and less branched. The effect of inhibition was most significant at 20 µg/mL, at which highest concentration a clear expansive zone of avascularity was observed surrounding the site of nanoparticle application. Thus, this temporal comparison confirmed the rapid manifestation of the antiangiogenic action of Bet-AgNPs, proving to exhibit strong dose-dependent inhibition on neovascularization in the developing CAM.

3.4 Quantitative Assessment of Vessel Count in CAM Assay

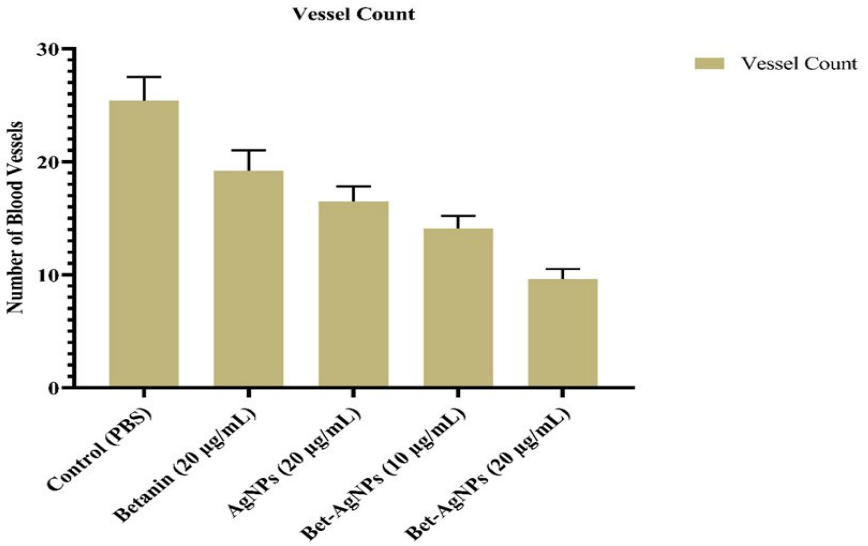

Figure 7. Quantitative analysis of blood vessel count in the CAM assay following treatment with different test groups.

The groups were as follows: Control (PBS), Betanin (20 µg/mL), AgNPs (20 µg/mL), Bet-AgNPs (10 µg/mL), and Bet-AgNPs (20 µg/mL). The control group showed the highest vessel densities, whereas the other treatments with Bet-AgNPs were capable of reducing vessel presence in a dose-dependent manner. Highest inhibition of angiogenesis was noted with Bet-AgNPs at 20 µg/mL, thus confirming the enhanced efficacy of the combined formulation under investigation. Data are expressed as mean ± SD of three independent experiments. Values of $p < 0.05$ were considered significantly different from the control. Quantitative analysis of vessel counts in the CAM assay revealed a visible disparity in neovascularization among the groups treated. The control group, or PBS group, had the highest number of visible blood vessels, consistent with normal angiogenic embryonal vessels formation. Similarly, the Betanin 20 µg/mL group showed slight non-significant reduction just like inadequate antiangiogenesis activity when betanin was used alone. AgNPs 20 µg/mL treatment showed almost moderate inhibition or suppression in vessel formation, thus revealing that silver nanoparticles have their own antiangiogenic property. On the contrary, the treatment of embryos with Betanin-derived silver nanoparticles (Bet-AgNPs) showed significant and dose-dependent inhibition of angiogenesis. Bet-AgNPs at 10 µg/mL clearly suppressed vessel number with respect to the control, whereas, at 20 µg/mL, the vessel count was the lowest when compared to all other treatment groups exhibiting a synergistic antiangiogenic effect of betanin and silver nanoparticles. Thus, cumulatively, these findings confirm that Bet-AgNPs essentially inhibit blood vessel formation in a concentration-dependent manner and do so more efficiently than betanin and silver nanoparticles in isolation.

3.5 Assessment of Vessel Inhibition Percentage in CAM Assay

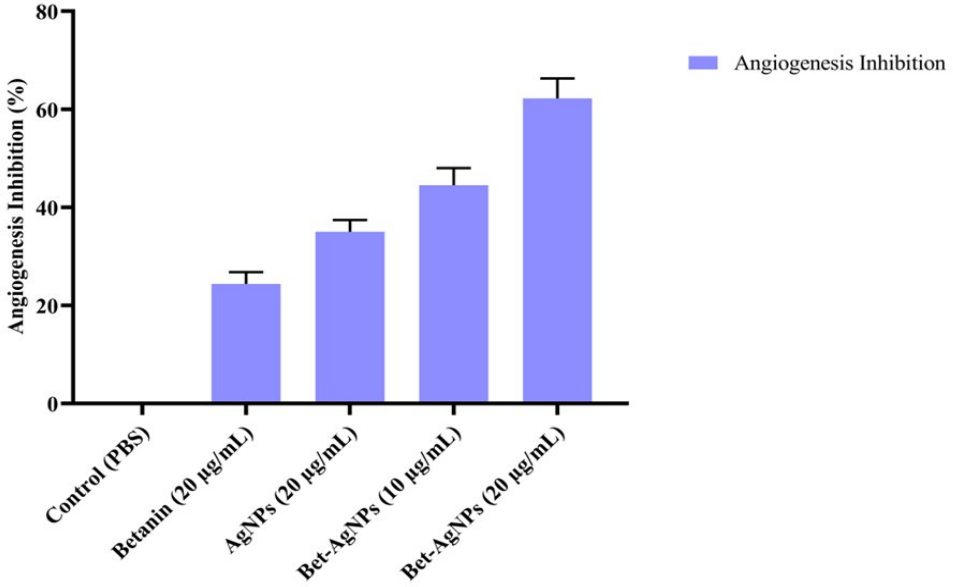

Figure 8: Percentage of blood vessel inhibition in CAM assay following treatment with different test groups.

Group test included Control (PBS), Betanin (20 µg/mL), AgNPs (20 µg/mL), Bet-AgNPs (10 µg/mL), and Bet-AgNPs (20 µg/mL). The control group showed 0% of inhibition, while the inhibition of the vessels by Bet-AgNPs varied with concentration. Highest inhibition was recorded in the Bet-AgNPs 20 µg/mL groups of treatment, thus enhanced antiangiogenic activity could be conferred by the synergistic effect of betanin and silver nanoparticals. Data are mean ± SD from three independent replicates. Considered statistically significant when $p < 0.05$ against control.

To measure the antiangiogenic potential of the treatment agents in the CAM assay, vessel inhibition was calculated. In the control group, angiogenesis went on to its full extent without any inhibition, laying down the baseline. Betanin at 20 µg/mL provided very little vessel inhibition, hence indicating weak antiangiogenic effect when used alone. AgNPs at 20 µg/mL showed moderate vessel inhibition, thereby showing an inherent antiangiogenic property of silver nanoparticles. Treatment with Betanin-derived AgNPs caused a significant increase in vessel inhibition with concentration dependency. The 10 µg/mL Bet-AgNPs group exhibited greater vessel inhibition than the control or any other treatment used individually, while the 20 µg/mL Bet-AgNPs group showed the highest vessel inhibition percentage by far, strongly suggestive of synergism between the two constituents. These observations confirm the stronger antiangiogenic behavior of Bet-AgNPs as compared to the two constituents individually and advance their candidacy as antiangiogenic agents.

3.6 Gene Expression Analysis of Angiogenesis-Related Markers

Gene expression analysis was performed on CAM tissues treated with Betanin-synthesized AgNPs (Bet-AgNPs) at 20 µg/mL to elucidate their molecular effects on angiogenesis. The embryo groups treated with Bet-AgNPs exhibited a very significant downregulation of several essential proangiogenic marker genes, namely VEGFA, VEGFR2, and HIF-1α, when compared with the control group (PBS-treated embryos). This suppression indicates that Bet-AgNPs inhibit the VEGF signaling axis and counteract hypoxia-driven angiogenic stimulation. Conversely, THBS1 (Thrombospondin-1), an endogenous antiangiogenic factor, was robustly

upregulated, implying the activation of a natural angiogenesis-inhibitory mechanism. Likewise, ANGPTL4, which regulates vascular permeability and remodeling, also increased substantially after Bet-AgNP exposure. From these findings, it can be interpreted that Bet-AgNPs exert their antiangiogenic effect at 20 µg/mL via both downregulation of genes conducive to angiogenesis and upregulation of those inhibiting vessel formation, thereby strongly contributing to a molecular underpinning of observed in vivo antiangiogenic function.

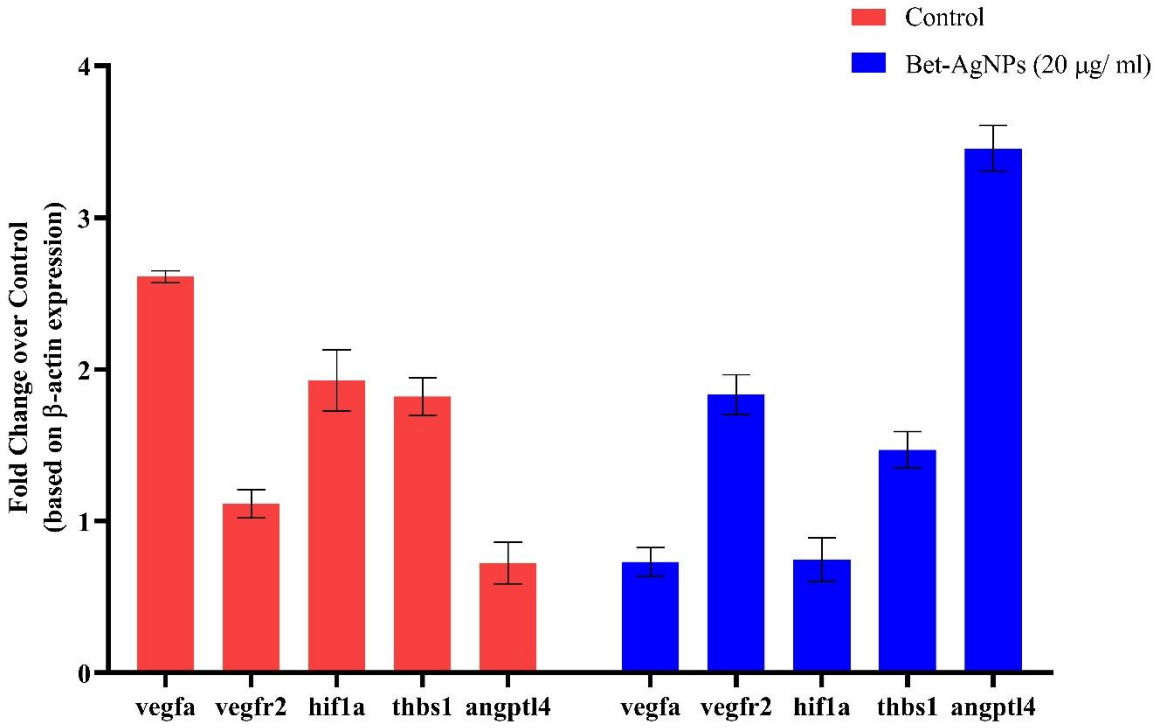

Figure 9: Relative gene expression levels of angiogenesis in CAM tissues upon treatment with Betaisomers silver nanoparticles (Bet-AgNPs, 20 µg/mL).

We have done quantitative real-time PCR for VEGFA, VEGFR2, HIF-1α, THBS1, and ANGPTL4 genes using β-actin as an internal standard. The treated group showed markedly suppressed expression of VEGFA, VEGFR2, and HIF-1α while showing amassed upregulation of THBS1 and ANGPTL4 than the control group treated with PBS. Data are represented as mean ± SD from three independent experiments, and $p < 0.05$ was considered statistically significant.

4 Discussion

Angiogenesis, or the building of new vessels out of preexisting vasculature, is involved in most of physiological and pathological significant processes, including wound healing and tumor progression[19]. In cancer, tumor-induced angiogenesis allows tumors to survive by supplying oxygen and nutrients, enable metastasis, and foster therapy resistance[20]. In that respect, inhibition of the angiogenic process is an important strategy for treating cancer. Therefore, our focus was to check the antiangiogenic and anticancer properties of Betanin-derived silver nanoparticles (Bet-AgNPs) by harnessing both the natural bioactivity of the betalain pigment betanin and the physicochemical types of AgNPs[21].

Betanin is an active metabolite of Beta vulgaris that has been extensively studied for its antioxidant, anti-inflammatory, and anticancer properties[22]. Nevertheless, its application as a drug is hampered due to the inability to remain stable and to the limited bioavailability[23]. The biosynthesis of nanoparticles through plant-based bioactives hence started to gain importance for its possibilities in green synthesis. Betanin was used here as a reducing and stabilizing agent in the synthesis of silver nanoparticles that were stable, spherical, and 20–50 nm in size. Green synthesis measures jointly assure biocompatibility and eco-friendliness while allowing synergistic therapeutic effects of betanin and silver ions. Characterization studies of these synthesized Bet-AgNPs with UV–Visible spectroscopy, SEM, and FTIR techniques demonstrated successful formation of nanoparticles. The sharp distinct silver SPR band at around 430 nm indicates the good evolution of silver nanostructures from silver ions. SEM showed nanostructures with uniform spherical morphology with less aggregation. Presence of hydroxyl and carbonyl functional groups, as indicated by FTIR spectra, are responsible for the capping and stabilization activities via betanin molecules[24]. These results surely prove the participation of phytochemical moieties in the synthesis and functionalization of nanoparticles.

The antiangiogenic potential of Bet-AgNPs was assessed using the chick chorioallantoic membrane (CAM) assay, a standard in-vivo procedure for the observation and evaluation of neovascularization[25]. Morphological assessment revealed the substantial diminishment of vessel density and vessel branching in Bet-AgNP-treated groups compared to the control. The greatest effect was observed from 20 μg/mL, where an avascular zone appeared to completely surround the site of application. This was supported by the vessel count and percentage vessel inhibition, both of which gave strong confirmation of concentration-dependent angiogenesis suppression. Moderate angiogenesis inhibition was observed for both betanin and AgNPs administered individually; however, the combined use of the two to formulate Bet-AgNPs was far more effective, thereby supporting the hypothetical synergism.

ROS levels act synergistically; in moderation, ROS can induce cell proliferation and angiogenesis, whereas high quantities of ROS lead to oxidative stress, mitochondrial dysfunction, apoptotic processes, and inhibition of angiogenic signaling[26]. In this study, intracellular ROS levels in KB oral cancer cells were greatly elevated on treatment with Bet-AgNPs, with a pronounced effect at 50 μg/mL and 100 μg/mL. These results unequivocally indicate that Bet-AgNPs can induce oxidative stress in these tumor cells, which may contribute to their death and inhibition of tumor-associated angiogenesis[27]. To understand the molecular mechanism involved in Bet-AgNP-mediated antiangiogenesis, gene expression analysis was conducted using CAM tissues treated with 20 μg/mL of Bet-AgNPs. One key angiogenic marker, VEGFA, and VEGFR2 and HIF-1α, were notably suppressed. VEGFA is a principal mediator of angiogenesis and serves as a signal for endothelial cell proliferation, migration, and new vessel formation. VEGFR2 is its main effector receptor transducing the proangiogenic signals. HIF-1α is a transcription factor whose expression is elevated under conditions of hypoxia; it enhances the production of VEGF and thus serves to link hypoxia to angiogenesis in tumors[28]. Inhibition of gene expression for VEGFA, VEGFR2, and HIF-1α by Bet-AgNPs indicates that these nanoparticles act on both the angiogenic pathways and tumor hypoxia-dependent neovascularization.

Interestingly, the treatment of cells with Bet-AgNP has also caused the upregulation of THBS1 (Thrombospondin-1) and ANGPTL4 (Angiopoietin-like 4). THBS1 is a major endogenous inhibitor of angiogenesis, working through interaction with CD36 that blocks endothelial cell proliferation and migration[29]. There is suggestive proof that the increased expression of THBS1 is connected to Bet-AgNPs activating natural antiangiogenic pathways. ANGPTL4 sometimes acts as an angiogenesis enhancer while under different conditions inhibiting

vascular permeability. In the present study, the upregulation of this gene may contribute to endothelial barrier stabilization and the inhibition of abnormal vascular sprouting. Our results on gene expression fit well into the working model, where the action mechanism of Bet-AgNPs would be the inhibition of proangiogenic drivers and means of activation of antiangiogenic regulators.

Molecular and phenotypic findings integrate to provide a panoramic view of the antiangiogenic and anticancer mechanism of Bet-AgNPs. The synergy of betanin and silver produces multifunctional nanoparticles capable of targeting tumor vasculature through multiple mechanisms; that is, through direct cytotoxicity by ROS generation, disruption of VEGF-mediated pathways, inhibition of hypoxia-responsive elements, and promotion of natural angiogenesis inhibitors. Such multimodal activity should prove especially useful for dealing with the complexity and redundancy of tumor angiogenesis. Besides these mechanistic insights, the use of the CAM assay model presents a rapid, inexpensive, and ethically acceptable alternative to mammalian assays for early-stage screening of antiangiogenic compounds. The avian embryo system allows researchers to capture real-time images of vascular changes and, as such, paves the way for the dynamic evaluation of nanoparticle-based therapeutics. The fact that Bet-AgNPs produce an avascular zone in just 4 hours after application attests to their rapid onset of action and an opportunity for localized therapeutic use. Studies conducted in the past have confirmed that both silver nanoparticles and betanin have anticancer potential[30]. Silver nanoparticles induce apoptosis, disrupt mitochondrial function, and cause DNA damage in various cancer cell lines. Betanin, on the contrary, functions as a free radical scavenger; it modulates redox-sensitive transcription factors and inhibits tumor cell proliferation[31]. This study provides further evidence that shows that Bet-AgNPs exhibit better efficacy than the individual components, probably through enhanced bioavailability, cellular uptake, and synergistic modes of action.

Although other studies are required to truly ascertain the long-term biocompatibility, biodistribution, and to evaluate the possible toxicity of Bet-AgNPs in mammalian systems, it could be speculated that investigations of their effects on other angiogenic pathways (like Notch, PI3K/AKT, MAPK) and their application in solid tumor models could be important from a translational standpoint[32]. Further studies might also consider combining Bet-AgNPs with standard chemotherapeutics for better efficacy and reduced drug resistance.

From this study, Betanin-synthesized silver nanoparticles have shown pronounced antiangiogenic and anticancer effects, mainly through ROS generation, inhibition of VEGF signaling, and upregulation of antiangiogenic genes[33]. Their rapid action in the CAM model and effect at the molecular level offers some potential for their application as a natural, green-synthesized nanotherapeutic against tumor angiogenesis. Such findings imply a novel route for phytochemical-based nanomedicine development in cancer therapy.

Conclusion

This study thus demonstrates the antiangiogenic and anticancer potential of Bet-AgNPs synthesized in an environmentally friendly and green protocol. Characterization confirmed stable spherical nanoparticles having good properties for biological applications. The CAM assay revealed a dose-dependent antiangiogenesis effect bearing the highest degree of avascular response exhibited by the 20 μg/mL concentration. ROS analysis indicated that cytotoxicity induced by Bet-AgNPs was due to oxidative stress in cancer cells. Gene expression analyses were consistent, showing downregulation of proangiogenic genes (VEGFA, VEGFR2, HIF-1α) and upregulation of antiangiogenic genes (THBS1, ANGPTL4). Therefore, the results emphasize the double action of Bet-AgNPs in suppressing angiogenesis and promoting cancer cell death. The meld of natural bioactivity and nanotechnology gives Bet-

AgNPs a promising potential in future antiangiogenic cancer therapies that need to be further explored in advanced in vivo models as well as clinical studies.

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