

Silver Nanoparticles Produced by *Alternaria tenuissima*: Antimicrobial and Anticancer Activity

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This study aimed to biosynthesize silver nanoparticles using the cell-free filtrate of the endophytic fungus *Alternaria tenuissima* and to evaluate their physicochemical properties, antimicrobial activity, and preferential in vitro cytotoxicity against cancer cell lines. The resulting *A. tenuissima*-mediated silver nanoparticles (At-AgNPs) were characterized using ultraviolet-visible spectroscopy, zeta-potential analysis, X-ray diffraction, transmission electron microscopy, and Fourier-transform infrared spectroscopy. Antimicrobial activity was assessed using agar well diffusion, minimum inhibitory concentration, minimum bactericidal concentration, and minimum fungicidal concentration assays. Cytotoxicity was evaluated using the MTT cell viability assay in MCF-7 breast cancer cells, HepG2 hepatocellular carcinoma cells, A549 lung carcinoma cells, and WI-38 normal fibroblasts. At-AgNP formation was confirmed by a surface plasmon resonance peak at 412 nm. Zeta-potential analysis showed negatively charged bimodal distributions at “32.7 and “25.7 mV, while X-ray diffraction confirmed a face-centered cubic crystalline structure. Transmission electron microscopy revealed predominantly spherical to quasi-spherical nanoparticles ranging from approximately 2 to 16 nm, with the highest frequency observed at 8–10 nm. At-AgNPs inhibited all tested bacterial strains and *Candida albicans*, with minimum inhibitory concentration values ranging from 7.80 to 31.25 µg/mL, but showed no measurable activity against *Aspergillus niger*. In the cytotoxicity assay, At-AgNPs reduced cancer cell viability in a concentration-dependent manner, with half-maximal inhibitory concentration values of 121.44 µg/mL for MCF-7, 112.63 µg/mL for HepG2, and 110.16 µg/mL for A549 cells, compared with 226.32 µg/mL for WI-38 normal fibroblasts. These findings demonstrate that At-AgNPs possess measurable in vitro antimicrobial activity and preferential cytotoxicity toward cancer cells, supporting further investigation of this biologically synthesized nanomaterial in antimicrobial and anticancer research.

Keywords: *Alternaria tenuissima*; Anticancer activity; Antimicrobial activity;
Green synthesis; Silver nanoparticles.

Antimicrobial resistance and cancer treatment-related toxicity remain two major challenges confronting contemporary biomedical science. ¹ The increasing loss of antimicrobial

effectiveness has strengthened the need for candidate therapeutics able to suppress pathogenic bacteria and fungi, particularly as resistant infections continue to compromise clinical

outcomes worldwide.² In parallel, despite substantial progress in cancer therapeutics, many conventional anticancer strategies remain limited by systemic toxicity, adverse effects, and insufficient selectivity toward malignant cells. These intersecting challenges have created a pressing demand for novel, biocompatible, and targeted therapeutic platforms that can address microbial infections while minimizing harm to normal mammalian cells.³ Nanotechnology has emerged as a transformative field in biomedical research because nanoscale materials exhibit physicochemical properties that differ substantially from their bulk counterparts. Silver nanoparticles (AgNPs) have attracted considerable attention owing to their broad antimicrobial potential, high surface-area-to-volume ratio, cellular interaction capacity, and relevance to drug delivery, diagnostics, and cancer therapy.⁴ Conventional physical and chemical methods for nanoparticle synthesis frequently necessitate significant energy input, high temperatures or pressures, costly instrumentation, and toxic chemical reagents. These factors raise important concerns about scalability, environmental safety, and biomedical compatibility. These limitations have heightened interest in green synthesis strategies that utilize biological systems as reducing and stabilizing agents.^{5,6}

Fungal endophytes stand out as a notably promising and underutilized resource for the biosynthesis of nanoparticles within biological platforms. These microorganisms reside within the internal tissues of plants without inducing visible disease symptoms. They are well recognized for producing diverse secondary metabolites with broad biological activities, enzymes, and bioactive compounds that exhibit antimicrobial, antioxidant, anti-inflammatory, and anticancer activities.^{7,8} Because fungal endophytes possess the ability to secrete extracellular biomolecules that can effectively reduce metal ions and stabilize nanoparticles. This characteristic presents an environmentally friendly and scalable and environmentally sustainable route for silver nanoparticle synthesis (AgNP).⁹ Medicinal plants are especially valuable reservoirs of endophytic fungi, as their associated microbial communities may reflect or complement the host plant's pharmacological potential.¹⁰ *Cissus*

rotundifolia is a medicinal plant reported to contain bioactive constituents¹¹ with antimicrobial,¹² anti-inflammatory¹³, antioxidant, and anticancer relevance.¹⁴ Its endophytic fungal community therefore represents a biologically plausible source of metabolites for green nanoparticle synthesis. Previous studies have demonstrated that endophytic fungi, including species of *Alternaria*, can mediate AgNP biosynthesis and generate nanoparticles with many pharmacological activities.¹⁵⁻¹⁷ However, empirical evidence regarding AgNPs biosynthesized from endophytic fungi associated with *Cissus rotundifolia* remains critically limited. There remains a substantial gap concerning the capacity of *Cissus rotundifolia*-derived fungal endophytes to produce structurally characterized AgNPs and the subsequent evaluation of these nanoparticles as dual antimicrobial and preferential anticancer agents.

Bridging this gap is important for advancing sustainable nanobiotechnology and identifying biologically derived nanomaterials with potential biomedical relevance. A green synthesis approach using fungal endophytes may reduce dependence on hazardous chemical synthesis while producing biofunctional nanoparticles capped by naturally derived fungal metabolites. Such nanoparticles may offer value in the search for safer antimicrobial agents and more selective anticancer strategies, particularly if their biological activity can be evaluated against pathogenic microbes, cancer cell lines, and normal cells within a single experimental framework. Therefore, this study sought to isolate and identify endophytic fungi associated with *Cissus rotundifolia* and to employ the selected isolate as a biological platform for the extracellular synthesis of silver nanoparticles. The biosynthesized AgNPs were characterized using established physicochemical techniques. Additionally, the study assessed their antimicrobial activity against specific bacterial and fungal pathogens, as well as their cytotoxic selectivity against cancer cell lines in comparison to normal fibroblast cells.

MATERIALS AND METHODS

Isolation of Endophytic Fungi from plant

Fresh, healthy leaves and stems of *Cissus rotundifolia** were collected from Al-Khitan

Valley, Al-Baha region, Saudi Arabia. The plant tissues were washed thoroughly with distilled water, cut into approximately 1 × 1 cm segments, and surface-sterilized by immersion in 70% ethanol for 30–60 s, followed by treatment with 5% sodium hypochlorite for 5 min. The segments were subsequently rinsed several times with sterile distilled water, and the final rinse water was plated as a sterility control to verify the effectiveness of the surface-sterilization procedure. The sterilized tissue segments were placed on potato dextrose agar (PDA) supplemented with kanamycin sulfate and ampicillin (50 mg/L each) and incubated at 20–25 °C for 2–14 days. Emerging fungal colonies were transferred to fresh PDA and repeatedly subcultured until pure cultures were obtained. A total of eight endophytic fungal isolates were recovered. The dominant representative isolate was selected for further investigation based on its high recovery frequency and distinct macroscopic and microscopic characteristics consistent with the genus *Alternaria*. Molecular identification subsequently confirmed the isolate as *Alternaria tenuissima*. The confirmed isolate was selected for extracellular biosynthesis of *A. tenuissima*-mediated silver nanoparticles (At-AgNPs).

Morphological and Molecular Identification of the Fungal Isolate

Fungal isolates were initially characterized on the basis of colony morphology, pigmentation, texture, growth pattern, and microscopic features following lactophenol blue staining.¹⁸ Genomic DNA was subsequently extracted from purified fungal mycelia using the DNABler-Cells and Tissue Kit (Cat. No. DE95050; Haven Scientific, Saudi Arabia) in accordance with the manufacturer's instructions. Unless otherwise specified, centrifugation was performed at 10,000 × g for 1 min at room temperature. Briefly, fungal mycelia were transferred to RNase-free 2-mL tubes containing five 3-mm zirconia beads, chilled on dry ice for 2 min, and homogenized twice at 4,260 rpm for 30 s using a Bioprep-24 homogenizer. The disrupted biomass was incubated with 200 µL of digestion buffer and 20 µL of proteinase K at 60 °C for 40 min, followed by the addition of 200 µL of lysis buffer and centrifugation at 17,000 × g for 5 min. Approximately 400 µL of clarified supernatant was combined with 200 µL of absolute ethanol, vortexed for 60 s, and loaded onto a silica

spin column. The column was washed once with 500 µL of Wash 1 buffer and twice with 500 µL of Wash 2 buffer. DNA was eluted in 50 µL of elution buffer after a 2-min incubation at room temperature and stored at “20 °C until analysis. The concentration and purity of the extracted genomic DNA were evaluated using a NanoDrop One spectrophotometer.

The ITS1/ITS4 and NL1/NL4 primer pairs were employed to amplify the internal transcribed spacer region and the D1–D2 domain of the large-subunit ribosomal DNA, respectively. The primer sequences were as follows: ITS1, 52 -TCCGTAGGTGAACCTGCGG-32 ; ITS4, 52 -TCCTCCGCTTATTGATGATGC-32 ; NL1, 52 -GCATATCAATAAGCGGAGGA-32 ; and NL4, 52 -TTGGTCCGTGTTTCAAGACG-32 . The primers were synthesized by Haven Scientific (Thuwal, Saudi Arabia). For each 50-µL PCR mixture, 2 µL of genomic DNA, 25 µL of EverGreen Universal qPCR Master Mix (Lot No. 55.0019; Haven Scientific), each primer at a final concentration of 500 nM, and RNase-free water were used. Reactions were conducted in 0.2-mL semi-skirted 96-well plates (Cat. No. PCR-SSP-02; Haven Scientific), sealed with optical adhesive film (Cat. No. PCR-OS-0011), and amplified using a QuantStudio 5 Real-Time PCR System (Cat. No. A28139; Applied Biosystems). The amplification program began with denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 90 s. The final extension was performed at 72 °C for 5 min. To verify the specificity of the amplification and the presence of a single amplicon, agarose gel electrophoresis and melt-curve analysis were performed. MagPure A4 XP magnetic nanoparticles (Magen Biotechnology, China) were employed to purify the amplicons. A mixture of 100 µL of magnetic beads and approximately 50 µL of each PCR product was incubated at room temperature for 5 min. The beads were rinsed twice with 100 µL of 70% ethanol, air-dried for 5 min, and eluted in 20 µL of RNase-free water after magnetic separation.

The BigDye Terminator v3.1 chemistry (Sanger Sequencing Kit, Cat. No. A38073; Applied Biosystems) was employed to conduct Sanger sequencing. The corresponding sequencing primer (2.3 pmol) was added to 1 µL of 5× sequencing buffer, 2 µL of BigDye Terminator v3.1 Ready

Reaction Mix, and 20–80 ng of purified amplicon in each 15- μ L sequencing reaction. The reactions were conducted using a VeritiPro 96-well Thermal Cycler (Cat. No. A48141; Applied Biosystems). Denaturation was initiated at 96 °C for 1 min, and the reaction was then subjected to 25 cycles of 96 °C for 10 s, 55 °C for 5 s, and 72 °C for 4 min. Sequencing products were purified using 45 μ L of SAM Solution and 10 μ L of BigDye XTerminator Bead Solution, vortexed at 1,800 rpm for 20 min, centrifuged at $1,000 \times g$ for 2 min, and analyzed by capillary electrophoresis using a 3730xl DNA Analyzer (Cat. No. A41046; Applied Biosystems). Chromatogram quality was evaluated using Sequence Scanner Software v2.0.¹⁹

Biosynthesis and Physicochemical Characterization of At-AgNPs

Alternaria tenuissima was cultivated in potato dextrose broth at 28 ± 2 °C under agitation at 150 rpm for 5 days to facilitate the extracellular production and release of reducing and stabilizing metabolites. Following incubation, the fungal biomass was removed by filtration through Whatman No. 1 filter paper to obtain a cell-free culture filtrate. The filtrate was subsequently supplemented with silver nitrate (AgNO₃) to achieve a final concentration of 1 mM. The reaction mixture was incubated under static conditions in the dark at 28 ± 2 °C. The formation of a dark-brown coloration was observed as an initial visual indication of the reduction of Ag⁺ ions and the formation of silver nanoparticles.²⁰ The biosynthesized *A. tenuissima*-mediated silver nanoparticles (At-AgNPs) were recovered by centrifugation at $15,000 \times g$ for 15 min. The resulting nanoparticle pellets were washed repeatedly with deionized water to remove residual cellular and medium components and were subsequently subjected to physicochemical characterization. The formation of At-AgNPs was initially monitored by UV–visible spectroscopy using a Shimadzu UV-2600 spectrophotometer over a wavelength range of 300–500 nm. The crystalline structure and phase composition of the nanoparticles were determined by X-ray diffraction (XRD) using a Rigaku RINT2000 diffractometer operated at 40 kV and 20 mA with Cu K α radiation over a 2θ range of 10° – 70° . The surface charge and colloidal stability of the synthesized nanoparticles were evaluated using a Malvern Zetasizer Nano

ZS. Particle morphology and size distribution were examined by transmission electron microscopy (TEM) using a Hitachi H-800 microscope operated at an accelerating voltage of 200 kV. In addition, the surface-associated functional groups and potential biomolecules involved in nanoparticle stabilization were identified by Fourier-transform infrared (FTIR) spectroscopy using a PerkinElmer Spectrum Two spectrometer.²¹

Antibacterial and Antifungal Activity

The antimicrobial activity of the biosynthesized silver nanoparticles (AgNPs) was evaluated against a panel of bacterial and fungal pathogens, including *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Candida albicans*, and *Aspergillus niger*. The agar well diffusion method was employed to determine the zones of inhibition. Briefly, 100 μ L of the AgNP suspension was introduced into wells prepared in the inoculated agar plates under aseptic conditions. Gentamicin was used as the positive control for bacterial strains, whereas fluconazole was used as the positive control for fungal strains.

Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of the biosynthesized AgNPs was determined using the broth microdilution method. Microbial suspensions were standardized to approximately 2×10^6 CFU/mL for both bacterial and fungal assays. Serial two-fold dilutions of the AgNPs were prepared to obtain final concentrations ranging from 0.125 to 64 μ g/mL. Nutrient broth was used for bacterial cultures, while Czapek's yeast broth was used for fungal cultures. In sterile 96-well microplates, 100 μ L of the standardized microbial suspension was mixed with 100 μ L of each AgNP dilution. The bacterial and fungal plates were incubated separately at 37 °C and 28 °C, respectively. Following incubation, MIC values were determined as the lowest concentration of AgNPs that markedly inhibited visible microbial growth after 24 h for bacteria and 72 h for fungi. Gentamicin and fluconazole were included as positive controls, while silver nitrate was used as a comparative control.

In Vitro Cytotoxicity Assay

The cytotoxicity of At-AgNPs was assessed using the MTT assay in MCF-7 human

breast cancer cells, HepG2 hepatocellular carcinoma cells, A549 lung carcinoma cells, and WI-38 normal human fibroblasts, all obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). To prepare the 96-well tissue culture plates for monolayer formation, cells were seeded at a density of 1×10^4 cells/mL, with 100 μ L added to each well, and then incubated at 37 °C for 24 hours. Following the incubation period, the growth medium was carefully removed, and the cell monolayer was washed twice with washing medium. RPMI medium mixed with 2% serum was used to prepare two-fold serial dilutions of the biosynthesized AgNPs. Wells that were not treated received only maintenance medium, while treated wells received 100 μ L of each dilution. Cells were analyzed microscopically for morphological signs of cytotoxicity, such as partial or total monolayer loss, rounding, shrinkage, and granulation, after the plates were incubated at 37 °C. The MTT solution was prepared at a concentration of 5 mg/mL in phosphate-buffered saline, with MTT reagent sourced from Bio Basic Canada Inc. (Markham, ON, Canada). Twenty microliters of MTT solution were added to each well, and the plates were placed on a shaking table at 150 rpm for 5 minutes to achieve complete mixing. The plates were subsequently incubated at 37 °C in 5% CO₂ for 4 hours to facilitate MTT metabolism and the production of formazan crystals. The medium was removed, and the formazan crystals were solubilized in 200 μ L of dimethyl sulfoxide. The plates were agitated at 150 rpm for 5 min, and optical density was measured at 560 nm with background correction at 620 nm. Untreated cells maintained in culture medium without At-AgNPs were used as the negative control and were considered to represent 100% cell viability. No separate vehicle control was required because At-AgNPs were diluted directly in maintenance medium without an additional solvent vehicle. Cell viability was calculated relative to the untreated control wells using the equation: cell viability (%) = (OD of treated cells / OD of untreated control cells) $\times$ 100, where OD represents optical density. The selectivity index was calculated as the IC₅₀ of WI-38 normal fibroblasts divided by the IC₅₀ of each cancer cell line.

Statistical Analysis

Data were analyzed using IBM SPSS

Statistics version 20.0 (IBM Corp., Armonk, NY, USA) and are presented as mean $\pm$ standard deviation (SD) of triplicate measurements ($n = 3$). Pairwise comparisons were performed between At-AgNP-treated groups and their corresponding positive controls for antimicrobial assays, and between each cancer cell line and WI-38 normal fibroblasts at the same At-AgNP concentration for cytotoxicity assays. Because multiple comparisons were involved, the statistical interpretation was moderated, and this limitation was acknowledged. Half-maximal inhibitory concentration (IC₅₀) values were calculated from concentration–viability data using dose–response curve fitting in IBM SPSS Statistics version 20.0 (IBM Corp., Armonk, NY, USA). Confidence intervals for IC₅₀ values were not available from the original analysis.²²

RESULTS

Morphological and Microscopic Identification of the Endophytic Fungi

Eight endophytic fungal isolates were obtained from the aerial tissues of the medicinal plant *Cissus rotundifolia*. The recovered isolates were purified by culturing them on potato dextrose agar (PDA) and incubating the plates at 28 °C for 7 days. Following incubation, preliminary identification and characterization of the fungal isolates were performed based on their macroscopic colony characteristics and microscopic morphological features.

Molecular Identification of the selected Endophytic Fungal Isolate

Molecular analysis identified the selected endophytic fungal isolate as *Alternaria tenuissima*.

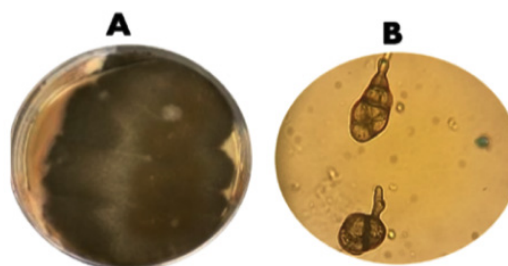


Fig. 1. (A) Macroscopic morphology and (B) microscopic characteristics of the selected endophytic fungal isolate

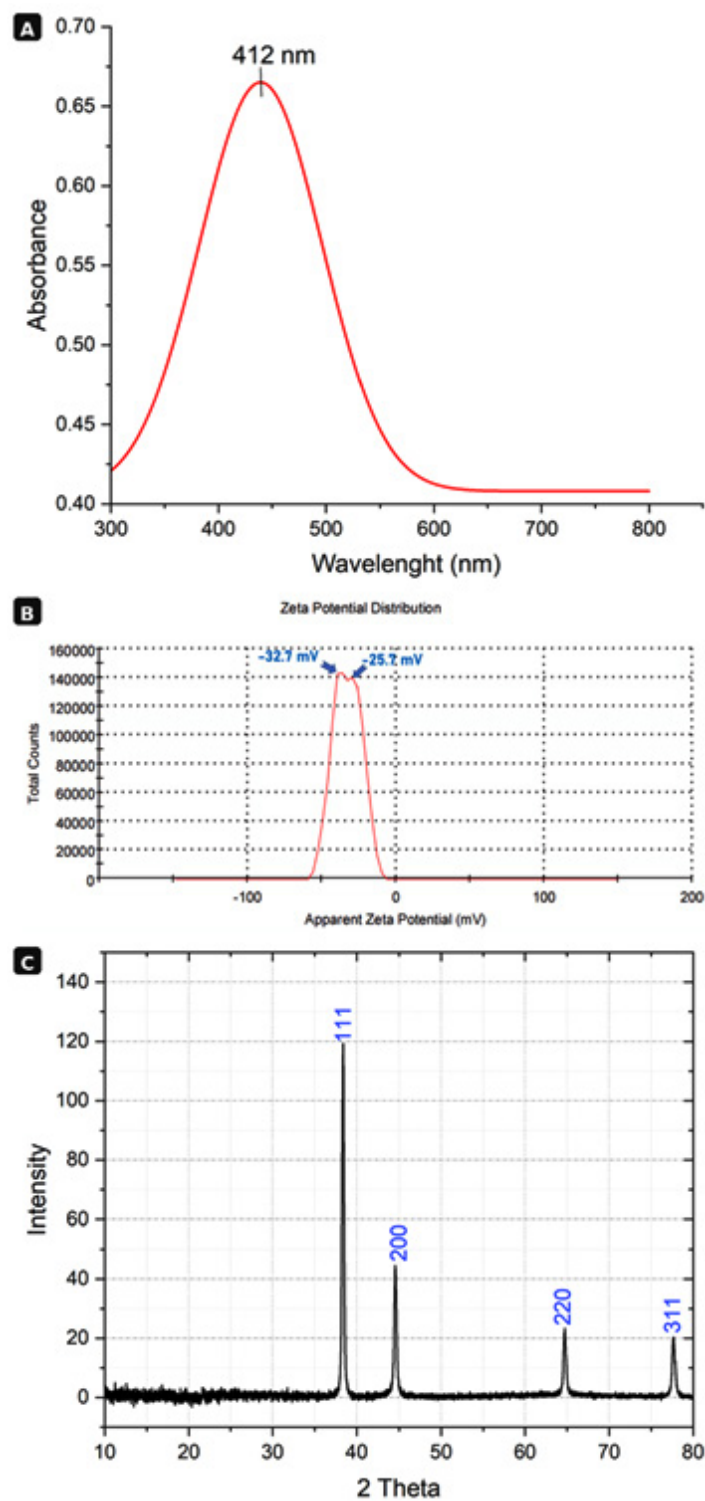


Fig. 2. Physicochemical characterization of *Alternaria tenuissima*-mediated silver nanoparticles (At-AgNPs). (A) UV-Vis absorption spectrum showing the characteristic surface plasmon resonance peak at 412 nm. (B) Zeta potential distribution of the biosynthesized AgNPs. (C) X-ray diffraction pattern.

Sequence analysis of the amplified ribosomal DNA region corroborated the morphological identification, with BLAST analysis revealing 99–100% sequence similarity to reference sequences of *A. tenuissima*. The obtained

nucleotide sequence was deposited in GenBank under accession number PZ679418. Collectively, the macroscopic, microscopic, and molecular characteristics confirmed the identity of the isolate as *A. tenuissima*, which was subsequently

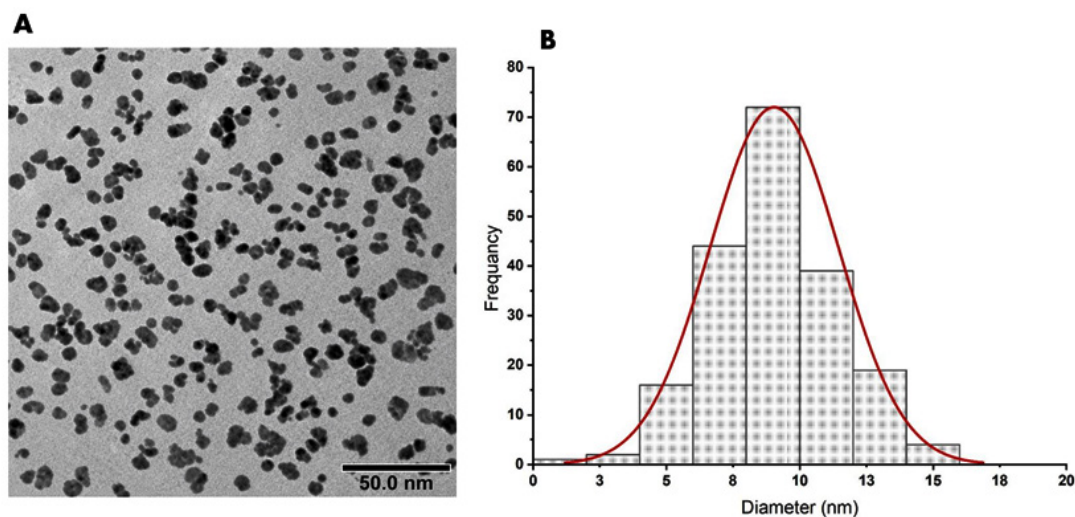


Fig. 3. Morphological and size characterization of *Alternaria tenuissima*–mediated silver nanoparticles (At-AgNPs). (A) Representative transmission electron microscopy image of At-AgNPs. (B) Particle-size distribution derived from transmission electron microscopy measurements.

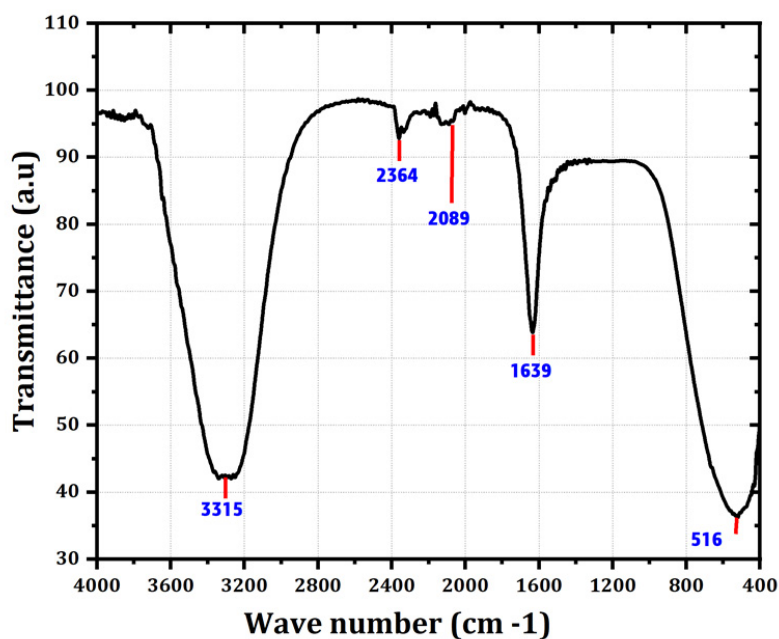


Fig. 4. Fourier-Transform Infrared Spectral Analysis of *Alternaria tenuissima*–Mediated Silver Nanoparticles (At-AgNPs)

employed for the extracellular biosynthesis of At-AgNPs.

Characterization of Biosynthesized At-AgNPs

UV–Vis spectroscopy of At-AgNPs displayed a distinct surface plasmon resonance band at 412 nm, confirming nanoparticle formation (Fig. 2A). Zeta-potential analysis demonstrated a negatively charged bimodal distribution, with peaks at “32.7 and “25.7 mV. These negative values

support electrostatic repulsion among nanoparticles, whereas the bimodal profile indicates surface-charge heterogeneity, likely reflecting differences in biomolecular capping or particle populations rather than a single homogeneous colloidal fraction (Fig. 2B). X-ray diffraction analysis revealed four distinct reflections at 2θ values of 38.262° , 44.473° , 64.714° , and 77.737° , corresponding to the (111), (200), (220), and (311) crystallographic planes,

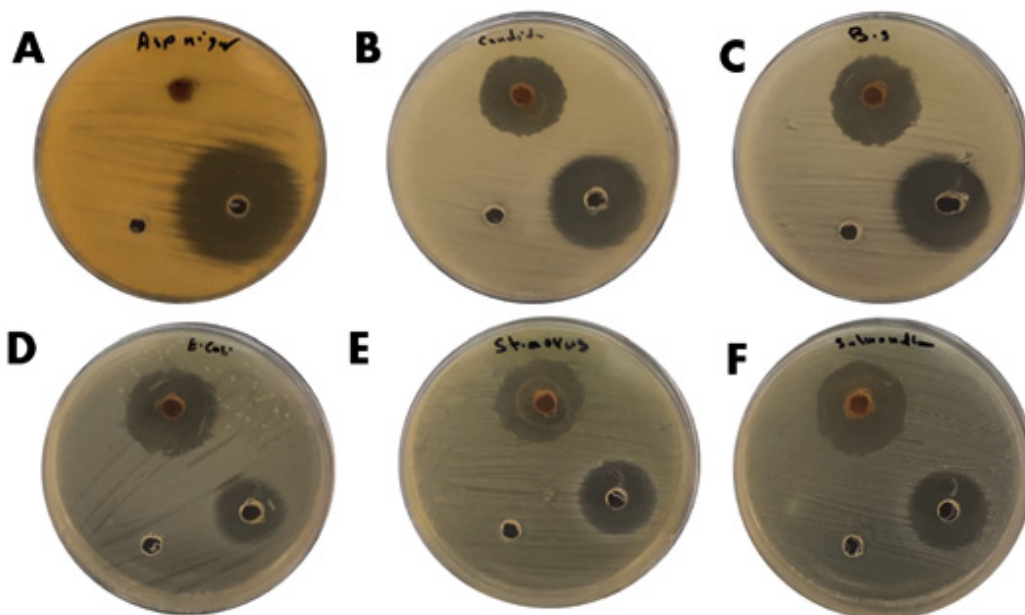


Fig. 5. Antimicrobial efficacy of biosynthesized silver nanoparticles (AgNPs) derived from the isolated endophytic fungus *Alternaria tenuissima* was evaluated using agar well diffusion assay. (A) *Aspergillus niger*, (B) *Candida albicans*, (C) *Bacillus subtilis*, (D) *Escherichia coli*, (E) *Staph. aureus*, (F) *Salmonella typhi*.

Table 1. Antimicrobial Activity of Biosynthesized Silver Nanoparticles (At-AgNPs) Against Selected strains of bacterial and fungal Pathogens

Pathogen	Zone of inhibition (mm)		MBC/MFC	MIC
	Sample	Control		
<i>Bacillus subtilis</i> (ATCC 6633)	26.0±0.1	28.0±0.2 ^{ns}	62.50	31.25
<i>Staph. aureus</i> (ATCC 6538)	25.0±0.2	24.0±0.2 ^{ns}	31.25	15.62
<i>Escherichia coli</i> (ATCC 8739)	28.0 ±0.2	22.0±0.1 *	31.25	15.62
<i>Salmonella typhi</i> (ATCC 6539)	25.0±0.2	23.0±0.2 ^{ns}	31.25	7.80
<i>Candida albicans</i> (ATCC 10221)	26.0±0.2	29.0±0.2*	125.00	31.25
<i>Aspergillus niger</i> (ATCC 16888)	NA	37.0±0.1 ^{ns}	NA	NA

Data are presented as mean ± SD from three technical replicates (n = 3).. Inhibition zones are expressed in mm, and MIC/MBC/MFC values in µg/mL. $p < 0.05$ was considered significant; ns, not significant. Gentamicin was used as the positive antibacterial control, whereas fluconazole was used as the positive antifungal control., respectively. MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MFC, minimum fungicidal concentration; NA, not applicable.

Table 2. Comparative Viability of Experimental Cancer Cell Lines and the Normal Control Cell Line Following At-AgNPs Treatment

At-AgNP concentration (µg/mL)	Experimental cancer cell lines			Control cell line WI-38 viability (%)
	MCF-7 viability(%)	HepG2 viability (%)	A549 viability (%)	
1000	2.60 ± 0.07*	2.91 ± 0.15*	2.98 ± 0.09*	15.74 ± 0.48
500	2.69 ± 0.06*	3.15 ± 0.12*	4.49 ± 0.18*	17.11 ± 0.82
250	2.92 ± 0.15*	5.78 ± 0.59*	7.26 ± 0.29*	41.07 ± 0.49
125	47.42 ± 0.34*	40.59 ± 0.92*	38.43 ± 1.02*	99.53 ± 0.22
62.5	98.65 ± 0.29 ^{ns}	89.97 ± 0.69 ^{ns}	93.14 ± 0.38 ^{ns}	99.77 ± 0.12
31.25	99.91 ± 0.19 ^{ns}	99.81 ± 0.15 ^{ns}	98.69 ± 0.26 ^{ns}	99.86 ± 0.25

Data are presented as mean ± SD from three technical replicates (n = 3). At each At-AgNP concentration, viability of each cancer cell line was compared with WI-38 normal fibroblasts using an independent-samples *t* test. *p* < 0.05 was considered statistically significant; ns, not significant.

Table 3. IC₅₀ Values and Selectivity Indices of At-AgNPs

Cell line	Cell type	IC ₅₀ (µg/mL)	Selectivity index
MCF-7	Breast cancer cells	121.44	1.86
HepG2	Hepatocellular carcinoma cells	112.63	2.01
A549	Lung carcinoma cells	110.16	2.05
WI-38	Normal fibroblasts	226.32	—

respectively. The corresponding d-spacings were 2.350, 2.036, 1.439, and 1.228 Å, with relative intensities of 100.0%, 46.7%, 25.5%, and 27.1%. The calculated lattice constant was 4.071 Å, and the diffraction pattern closely matched the COD 1509146 reference, confirming the face-centered cubic crystalline structure of metallic silver. The sharp diffraction peaks and absence of prominent additional reflections supported the crystalline nature of the At-AgNPs, while the dominant (111) reflection suggested preferential orientation along this crystallographic plane (Fig. 2C).

The At-AgNPs were characterized by a uniform nanoscale distribution, particle clustering to a certain extent, and a spherical to quasi-spherical shape, according to transmission electron microscopy (Fig. 3A). Particle concentration was highest in the 8–10 nm interval, suggesting that the majority of particles were located in the lower nanometer range, according to the TEM-derived size distribution, which varied from 2 to 16 nm (Fig. 3B). FTIR examination of the At-AgNPs exhibited a large absorption band at 3315 cm⁻¹, primarily ascribed to O–H and N–H stretching vibrations of hydroxyl and protein-related functional groups.

The significant band at 1639 cm⁻¹ was attributed to amide I C=O stretching and/or H–O–H bending vibrations, signifying the interaction of fungal biomolecules with the nanoparticle surface. Minor bands at 2364 and 2089 cm⁻¹ corresponded to weak ambient CO₂ absorption and the stretching vibrations of unsaturated functional groups, respectively. The low-frequency band at 516 cm⁻¹ was ascribed to metal–ligand vibrations linked to silver nanoparticles. The spectral characteristics collectively showed that extracellular fungal metabolites facilitated the reduction, capping, and stabilization of the At-AgNPs (Fig. 4).

Antimicrobial Activity of At-AgNPs

At-AgNPs exhibited measurable antimicrobial activity against all tested bacterial strains and *Candida albicans*, as demonstrated by clear inhibition zones in the agar well-diffusion assay, whereas no inhibitory activity was observed against *Aspergillus niger* (Fig. 5A–F; Table 1). The largest inhibition zone was recorded against *Escherichia coli* (28.0 ± 0.2 mm), followed by *Bacillus subtilis* and *C. albicans* (26.0 ± 0.1 and 26.0 ± 0.2 mm, respectively), while *Staphylococcus aureus* and *Salmonella typhi* each showed

inhibition zones of 25.0 ± 0.2 mm. Compared with the corresponding positive antimicrobial controls, At-AgNPs produced significantly greater inhibition of *E. coli* (28.0 ± 0.2 vs. 22.0 ± 0.1 mm; $p < 0.05$), whereas inhibition of *C. albicans* was significantly lower than that of the antifungal control (26.0 ± 0.2 vs. 29.0 ± 0.2 mm; $p < 0.05$). No statistically significant differences were observed for *B. subtilis*, *S. aureus*, or *S. typhi*. MIC values ranged from 7.80 to 31.25 $\mu\text{g/mL}$, with *S. typhi* showing the greatest susceptibility (MIC, 7.80 $\mu\text{g/mL}$), followed by *S. aureus* and *E. coli* (15.62 $\mu\text{g/mL}$ each), and *B. subtilis* and *C. albicans* (31.25 $\mu\text{g/mL}$ each). The corresponding MBC/MFC values were 31.25 $\mu\text{g/mL}$ for *S. aureus*, *E. coli*, and *S. typhi*, 62.50 $\mu\text{g/mL}$ for *B. subtilis*, and 125.00 $\mu\text{g/mL}$ for *C. albicans*.

Data are presented as mean $\pm$ SD from three technical replicates ($n = 3$). Inhibition zones are expressed in mm, and MIC/MBC/MFC values in $\mu\text{g/mL}$. $p < 0.05$ was considered significant; ns, not significant. Gentamicin was used as the positive antibacterial control, whereas fluconazole was used as the positive antifungal control, respectively. MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MFC, minimum fungicidal concentration; NA, not applicable.

Cytotoxicity of At-AgNPs Against Cancer Cell Lines

At-AgNPs reduced the viability of MCF-7, HepG2, and A549 cancer cells in a concentration-dependent manner, while WI-38 normal fibroblasts showed comparatively greater resistance at corresponding concentrations (Table 2). At 125 $\mu\text{g/mL}$, cell viability decreased to $47.42 \pm 0.34\%$ in MCF-7, $40.59 \pm 0.92\%$ in HepG2, and $38.43 \pm 1.02\%$ in A549 cells, whereas WI-38 viability remained at $99.53 \pm 0.22\%$. Further reductions in cancer-cell viability were observed at 250–1000 $\mu\text{g/mL}$, with viability values ranging from 2.60% to 7.26% in cancer cells, compared with 15.74%–41.07% in WI-38 cells. These differences were statistically significant at concentrations of 125–1000 $\mu\text{g/mL}$ ($p < 0.05$). By contrast, at 31.25 and 62.5 $\mu\text{g/mL}$, viability remained high across all cell lines, and no significant differences were detected relative to WI-38 cells. To further support preferential cytotoxicity, IC₅₀ values and selectivity indices were calculated for each

cancer cell line relative to WI-38 normal fibroblasts (Table 3). At-AgNPs showed lower IC₅₀ values against cancer cells than against WI-38 cells, with IC₅₀ values of 121.44 $\mu\text{g/mL}$ for MCF-7, 112.63 $\mu\text{g/mL}$ for HepG2, and 110.16 $\mu\text{g/mL}$ for A549, compared with 226.32 $\mu\text{g/mL}$ for WI-38 cells. The corresponding selectivity indices were 1.86, 2.01, and 2.05 for MCF-7, HepG2, and A549 cells, respectively. These findings indicate preferential in vitro cytotoxicity of At-AgNPs toward cancer cells compared with normal fibroblasts.

Data are presented as mean $\pm$ SD from three technical replicates ($n = 3$). At each At-AgNP concentration, viability of each cancer cell line was compared with WI-38 normal fibroblasts using an independent-samples *t* test. $p < 0.05$ was considered statistically significant; ns, not significant.

DISCUSSION

This study established an extracellular fungal platform for producing *Alternaria tenuissima*-mediated silver nanoparticles (At-AgNPs) and evaluated their physicochemical, antimicrobial, and anticancer properties. Convergent evidence from UV–Vis spectroscopy, zeta-potential analysis, X-ray diffraction (XRD), transmission electron microscopy (TEM), and Fourier-transform infrared spectroscopy (FTIR) supported nanoparticle formation, crystallinity, nanoscale dimensions, and fungal biomolecule-mediated surface functionalization. The At-AgNPs inhibited all examined bacterial strains and *Candida albicans*, whereas no significant activity was detected against *Aspergillus niger*. They diminished the metabolic viability of MCF-7, HepG2, and A549 cancer cells in a concentration-dependent manner, while demonstrating somewhat lower cytotoxicity towards WI-38 normal fibroblasts. Nonetheless, the results ought to be regarded as preliminary in vitro evidence rather than definitive proof of therapeutic efficacy, especially as nanoparticle-specific controls, mechanistic experiments, and in vivo validation were absent.

The surface plasmon resonance band observed at 412 nm provided strong evidence for the extracellular reduction of Ag⁺ to elemental silver and the subsequent formation of At-AgNPs. This finding was consistent with the absorption range commonly reported for biologically

synthesized AgNPs; Vijayabharathi *et al.* reported characteristic peaks at 413–417 nm, whereas *Alternaria alternata*–mediated AgNPs exhibited a peak at approximately 400 nm in the study by Kareem *et al.* The modest differences in peak position may reflect variations in particle size, morphology, aggregation state, surface coating, and the dielectric environment surrounding the nanoparticles. Thus, the single, well-defined band at 412 nm suggested the predominance of relatively small AgNPs without pronounced optical evidence of extensive aggregation.²³ The bimodal zeta-potential distribution, with peaks at “32.7 and “25.7 mV, indicated that the At-AgNPs possessed The negative zeta-potential values of At-AgNPs suggest electrostatic repulsion between particles, which may contribute to short-term colloidal stabilization. The bimodal distribution, however, indicates surface-charge heterogeneity rather than a single homogeneous nanoparticle population. This heterogeneity may reflect differential adsorption of fungal biomolecules, including proteins, peptides, polysaccharides, and ionized functional groups, onto the nanoparticle surface. Therefore, the zeta-potential data support electrostatic stabilization but do not independently confirm long-term colloidal stability, which would require time-dependent assessment of hydrodynamic size, polydispersity index, zeta potential, and aggregation under biologically relevant conditions.²⁴

The XRD reflections detected at 2θ values of 38.262° , 44.473° , 64.714° , and 77.737° were assigned to the (111), (200), (220), and (311) crystallographic planes, respectively, confirming the face-centered cubic structure of metallic silver. The calculated lattice constant of $4.071 \text{ \AA}$ and the close correspondence with COD 1509146 further supported this structural assignment. These findings agreed with previous studies of fungal- and biologically mediated AgNPs, in which comparable diffraction planes confirmed the formation of crystalline face-centered cubic silver.^{25,26} The dominant (111) reflection observed in the present study may indicate preferential growth or greater exposure of the thermodynamically stable (111) facet. Nevertheless, the absence of prominent additional reflections should be interpreted as evidence that crystalline silver was the predominant detectable phase, rather than definitive proof of complete phase purity, because minor amorphous

components or low-abundance impurities may remain below the detection limit of conventional XRD.

TEM revealed predominantly spherical to quasi-spherical At-AgNPs ranging from approximately 2 to 16 nm, with the highest particle frequency within the 8–10 nm interval. The formation of this predominantly small nanoparticle population may be attributed to rapid extracellular reduction of Ag⁺ and nucleation, followed by the adsorption of fungal proteins, peptides, polysaccharides, and other secreted metabolites onto the surfaces of newly formed silver nuclei. This biomolecular coating can restrict subsequent crystal growth and particle coalescence, thereby favoring the formation and stabilization of small nanoparticles. Experimental evidence has directly demonstrated the association of fungal proteins with biogenic AgNP surfaces and their contribution to nanoparticle stabilization.²⁷ Comparable findings demonstrate well-dispersed, spherical *Fusarium oxysporum*–mediated AgNPs measuring 5–13 nm under optimized biosynthetic conditions,²⁸ while other investigators reported fungal-derived AgNPs with an approximate size of 15 nm.²⁹ Thus, the predominance of At-AgNPs within the 8–10 nm range was consistent with efficient fungal-mediated nucleation and biomolecular capping by extracellular metabolites of *A. tenuissima*.

The FTIR study suggested the possible involvement of fungal biomolecules in At-AgNP reduction, capping, and stabilization. The extensive band at 3315 cm^{-1} was ascribed to O–H and N–H stretching vibrations, indicating the presence of hydroxyl- and amine-containing macromolecules such as proteins, peptides, and polysaccharides. Simultaneously, the absorption band at 1639 cm^{-1} corresponded to amide I carbonyl stretching and/or protein-related vibrations, suggesting that fungal proteins or peptide residues were probably adhered to the nanoparticle surface. This analysis aligns with the findings of Jaidev and Narasimha, who identified FTIR bands at 3347.85 and 1636.17 cm^{-1} in *Aspergillus niger*–mediated AgNPs, attributing these to primary amine stretching and amide carbonyl groups of proteins, respectively; they also proposed that fungal proteins interact with AgNPs via amine or cysteine residues, serving as capping agents.³⁰ This effect may be explained by the presence of proteins and amino acid residues

in fungal extracts, which exhibit high affinity for nanoparticle surfaces and contribute to reduction, stabilization, reduced agglomeration, and surface-chemistry modulation.³¹ The low-frequency band detected at 516 cm⁻¹ might relate to metal–ligand vibrations linked to silver–biomolecule interactions, which is near the 548.38 cm⁻¹ band previously documented for fungal AgNPs.³⁰ Consequently, the FTIR results align with a biomolecule-capped AgNP framework wherein extracellular metabolites from *A. tenuissima* facilitated the synthesis and stabilization of nanoparticles. Nevertheless, as the spectra of untreated fungal filtrate and silver nitrate were not concurrently analyzed, the attribution of particular functional groups must be approached with caution; a comparative FTIR analysis would enhance the recognition of biomolecular alterations directly linked to Ag⁺ reduction and At-AgNP capping.

The antimicrobial results indicate that At-AgNPs displayed extensive inhibitory effects on both Gram-positive and Gram-negative bacteria, with inhibition zones measuring between 25.0 and 28.0 mm and minimum inhibitory concentration values ranging from 7.80 to 31.25 µg/mL. These results align with earlier research about fungal-mediated silver nanoparticles. Previous study revealed that AgNPs produced with *Fusarium oxysporum* and *Macrophomina phaseolina* shown antibacterial efficacy against *Staphylococcus aureus* and *Salmonella typhi*, hence affirming the antibacterial capabilities of mycogenic AgNPs.³² AgNPs derived from *Aspergillus flavus* have previously demonstrated broad antibacterial activity against several bacterial strains, including *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Bacillus coagulans*, and *Corynebacterium glutamicum*, with the largest reported inhibition zone of 25.16 ± 0.80 mm observed against *B. coagulans*. In the present study, the relatively greater inhibition observed against *Escherichia coli* may be attributed to enhanced interactions between nano silver and the bacterial envelope, leading to membrane disruption, silver-ion release, and oxidative damage. However, susceptibility did not consistently differ between Gram-positive and Gram-negative bacteria, suggesting that the antimicrobial activity of At-AgNPs was likely influenced by multiple factors, including nanoparticle size, fungal biomolecule-mediated

surface functionalization, silver-ion availability, microbial envelope composition, and species-specific stress responses.³³

At-AgNPs caused a concentration-dependent reduction in the viability of MCF-7, HepG2, and A549 cancer cells, while WI-38 normal fibroblasts showed higher viability at comparable concentrations, as shown in Table 2. This selectivity was most evident at 125 µg/mL, where viability decreased to 47.42%, 40.59%, and 38.43% in MCF-7, HepG2, and A549 cells, respectively, compared with 99.53% in WI-38 cells. The lower IC₅₀ values and selectivity indices presented in Table 3 further supported the preferential cytotoxicity of At-AgNPs toward cancer cells. Similar preferential effects have been reported for fungal-mediated AgNPs by Netala *et al.* and Baker *et al.*, who demonstrated cytotoxic activity against cancer cells with comparatively lower toxicity toward normal cells.³⁴ The slightly greater sensitivity of A549 cells in the present study may reflect cancer-cell-specific differences in membrane composition, nanoparticle uptake, mitochondrial activity, redox balance, and susceptibility to silver-induced oxidative stress. This interpretation is consistent with mechanistic evidence indicating that AgNPs can induce excessive intracellular reactive oxygen species generation, mitochondrial dysfunction, DNA damage, cell-cycle arrest, and apoptotic signaling in cancer cells.^{35,36} Isaq *et al.* reported that endophytic *Cladosporium oxysporum*-derived AgNPs induced cytotoxicity in HCT-116 colon cancer cells through increased intracellular reactive oxygen species generation, DNA damage, cell-cycle arrest, and apoptosis. Likewise, studies on A549 cells have linked AgNP exposure to mitochondrial injury, cell-cycle disruption, and apoptosis, providing a plausible mechanistic explanation for the sensitivity of lung cancer cells to nanosilver-based treatment.³⁷

A very straightforward biological platform for manufacturing surface-functionalized AgNPs with dual antibacterial and anticancer action could be an endophytic fungal filtrate, according to the study. The use of biologically produced coatings that affect colloidal behavior and cellular interactions is being considered as an alternative to harsh reducing agents. Further research into

nanomedicine, antimicrobial coatings, topical formulations, or localized treatment systems is warranted because of the combination of antimicrobial activity and preferential cytotoxicity toward cancer cells.

CONCLUSION

Silver nanoparticles mediated by *Alternaria tenuissima* (At-AgNPs) were successfully biosynthesized using a cell-free fungal filtrate and exhibited a crystalline face-centered cubic structure, nanoscale size distribution, predominantly spherical to quasi-spherical morphology, and surface-associated functional groups derived from fungal biomolecules. At-AgNPs showed broad antibacterial activity, measurable antifungal activity against *Candida albicans*, and concentration-dependent cytotoxicity toward MCF-7, HepG2, and A549 cancer cells. Their comparatively lower cytotoxicity toward WI-38 normal fibroblasts indicated preferential in vitro cytotoxicity toward cancer cells. However, this study was limited to in vitro assays, and fungal filtrate, silver nitrate, and nanoparticle-specific interference controls were not included in the cytotoxicity experiments. Therefore, further studies incorporating appropriate controls, mechanistic assays, long-term stability evaluations, and in vivo validation are required before biomedical applications can be considered. Overall, these findings support At-AgNPs as a promising biologically synthesized nanomaterial for further antimicrobial and anticancer investigation.

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This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

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Not Applicable.

Author Contributions

Sawsan Saeed Zarban: Contributed to experimental work, investigation, data collection, data curation; Sahar Abdulaziz Alshareef: Contributed to supervision, conceptual guidance, methodology review, validation, project administration, and manuscript review and editing; Hend Maarof Tag: Contributed to supervision, scientific guidance, validation, critical revision of the manuscript, and manuscript review and editing; Eman Anwar Attia: Contributed to experimental design, laboratory methodology, practical assay supervision, data analysis, interpretation of results, and manuscript review and editing.

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