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Utilizing endophytic fungus *Alternaria tenuissima* for silver nanoparticles biogenic formation and their antibacterial efficacy

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ABSTRACT

Due to the growing issue of antibiotic-resistant microorganisms, it is imperative that new antimicrobial medications be developed. Silver nanoparticles give a satisfactory solution for antibiotic resistance that has proven to be a serious impediment to widespread usage of antibiotics. The production of these nanoparticles by biological processes presents a promising avenue for sustainable nanotechnology. The potential of *Alternaria tenuissima*, an endophytic fungus, as a bio-mediator in the production of silver nanoparticles (AgNPs) is investigated in this work. The fungal biomass filtrate functioned as a natural reducing and capping agent, enabling the rapid and stable formation of AgNPs under ambient conditions. The formed AgNPs were characterized via UV–Visible spectrophotometer, which revealed the high peak at 435 nm, and with the field-emission scanning microscope (FE-SEM), which confirmed the bio-production of predominantly spherical nanoparticles with a mean average size of 17.22 nm. Antibacterial efficacy of biosynthesized AgNPs was evaluated against specific Gram-positive *Staphylococcus aureus* and Gram-negative bacteria *Escherichia coli* and *Serratia marcescens* using the agar well diffusion method. The result revealed significant antibacterial activity from 20 nm to 15 nm, surpassing those of commonly used antibiotics such as novobiocin and tetracycline. The findings underscore the efficacy of *A. tenuissima* in silver nanoparticle fabrication and reinforce the promise of fungal biogenic AgNPs as eco-friendly antimicrobial agents for biomedical and pharmaceutical applications.

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Introduction

Nanotechnology is developing as a crucial imperative tool in contemporary agriculture and is anticipated to become a significant contributor to economic growth in the near future (Abobatta 2018, Abdel-Azeem et al. 2020, Srivastava et al. 2021, Gezaf et al. 2022, Mossa et al. 2024). Silver nanoparticles (AgNPs) are defined as diminutive particles composed of silver that generally measure less than 100 nm in size. These nanoparticles possess distinctive features that render them advantageous for diverse applications, such as in medicine and bioremediation (Lee & Jun, 2019; Mughal & Hassan, 2022). The biosynthesis of AgNPs has garnered considerable interest owing to its environmentally sustainable approach. Recently, researchers have been exploring various biological entities as excellent candidates for AgNPs production, including bacteria, yeast, fungi, algae, plants, and actinobacteria (Dhaka et al., 2023). Furthermore, scientific literature reported fungal

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extracts' potential to synthesize nanoparticles, including *Aspergillus flavus* (Vigneshwaran et al., 2007), *Trichoderma longibrachiatum* (Elamawi et al., 2018), *Alternaria* sp. (Win, 2020), and *Penicillium cyclopium* (Wanarska & Maliszewska, 2019).

It is possible to produce nanoparticles biogenically using fungi either with an extracellular or intracellular method. In the extracellular method, nanoparticles are produced after the addition of a metal precursor to the aqueous filtrate that only contains the fungal macromolecules. Since no steps are necessary to extract the nanoparticles from the cells, this final technique is the most popular (Tyagi et al., 2019; Murillo-Rábago et al., 2022). In this regard, fungal bioconversion is considered a simple, cost-effective, and energy-efficient biological approach that could effectively facilitate the synthesis of AgNPs. Fungi have extracellular enzymes that function as both reducing and capping agents to generate stable and shape-controlled AgNPs (Zhao et al., 2017). Endophytic fungi provide numerous benefits as a biogenic source of AgNPs, particularly their quantity, diversity, and capacity to synthesize nanoparticles with favorable physicochemical characteristics (Abdel-Azeem et al., 2020; Baron & Rigobelo, 2022). Studies conducted by mycologists highlight the promise of endophytic fungi as dependable sources of biologically significant nanomaterials (Manjunatha et al., 2023; Nassar et al., 2023). For the synthesis of novel bioactive compounds with a high level of biological and structural diversity, endophytic fungi are regarded as microbial bifactors (Singh et al., 2020).

According to the World Health Organization (2023), antibiotic-resistant bacteria will rank among the top ten dangers to world health in the 21st century. Methicillin-resistant *Staphylococcus aureus* (MRSA) causes the deaths of almost 120,000 people a year (Adedeji-Olulana et al., 2024). The most prevalent Gram-negative bacterial pathogen, *E. coli*, poses a clinical and epidemiological problem. Several high-risk, multidrug-resistant strains have been successful in the past ten years, including the strain *Escherichia coli* ST131. The evolution is primarily attributable to the increasing selective pressure of antibiotic usage (Paitan, 2018). Therefore, it is crucial to introduce new antibiotics into the market owing to the proliferation of bacterial strains that are resistant to antibiotics (Serwecińska, 2020).

This study aimed to use the endophytic fungus *Alternaria tenuissima* as a biological mediator to produce AgNPs and to evaluate their antibacterial efficacy against selected pathogenic bacteria in comparison to conventional antibiotics.

Materials and Methods

Chemicals

Chemicals: Potato dextrose agar, potato dextrose broth, $AgNO_3$ powder (99.9%), nutrient agar, nutrient broth, tetracycline "TE" (30 µg/ml), oxacillin "OX" (1U), and novobiocin "NO" (30 µg/ml). All media used in this study were autoclaved at 121°C and 15 lbs. pressure for 15 min.

Isolation and identification of endophytic fungal isolate

The *Alternaria tenuissima* (LD2_2) was previously isolated from *Lavandula dentata* L. leaves from Al Baha region, KSA, in January 2025. The endophytic isolate (LD2_2) was subcultured on a PDA plate for 7 days at 25°C and examined under a microscope at 40X using lactophenol cotton blue stain for morphological identification. The internal transcribed spacers ITS1 and ITS4 were amplified by PCR after DNA was extracted using the procedure outlined by Weiland (1997). Maximum Likelihood was employed after the BLAST analysis, and the phylogenetic tree was constructed according to Tamura and Nei (1993).

Pathogenic bacteria

Escherichia coli (ATCC 35218), *S. aureus* (ATCC 33591), and *S. marcescens*—was kindly provided by King Abdulaziz University.

AgNPs production

Fungal biomass production

Small discs were taken from the fresh culture using a sterilized cork borer and then were transferred using a sterile scalpel to 250 ml of sterile potato dextrose broth in an Erlenmeyer flask (500 ml). The flask was then covered with thin foil and Parafilm and incubated at 28°C and 120 rpm for 7 d.

Cell-Free Filtrate (CFF) preparation

The fungal biomass was collected after incubation using a filter paper (Whatman no. 1). Afterward, the biomass was rinsed with sterile distilled water to eliminate the remaining broth constituents. Subsequently, 20 g of the fresh biomass was suspended in 200 ml of sterile distilled water in a 250 ml flask and incubated at 25 ± 2°C in a shaker at 120 rpm for 72 h. The CFF was obtained by filtration via Whatman no. 1 and utilized for the AgNPs biosynthesis (Javed et al., 2010).

Biosynthesis of AgNPs

The CFF was mixed with 1 mM $AgNO_3$ in a 1:1 ratio and kept at room temperature till the color changed. In an experimental flask, the positive control (CFF) and negative control (1 mM $AgNO_3$) were incubated. The production of

AgNPs was visually detected and then confirmed using UV–vis analysis (Raza et al., 2021).

AgNPs characterization

Ultraviolet–visible spectrophotometry (UV-vis)

The confirmation of AgNPs formation in the mixture was detected via UV-vis spectrophotometer (Shimadzu, Japan). The absorption was measured at a resolution of 1 nm in the 200–600 nm wavelength range by utilizing a quartz cuvette to contain 3 ml of the honey brown mixture. The appearance of a distinct and well-defined LSPR band around 400 nm signifies the synthesis of AgNPs (Alves & Murray, 2022).

Field emission scanning electron microscopy (FE-SEM)

The AgNPs solution was subjected to FE-SEM, which offers topographic information of the surface of AgNPs. The average diameter of the produced nanoparticles was drawn with OriginLab.

Antibacterial activity of AgNPs

The chosen isolates were employed to assess the antibacterial efficacy of AgNPs, comprising one Gram-positive (MRSA) and two Gram-negative bacteria, *E. coli* and *S. marcescens*, using the agar well diffusion technique (Wayne, 2020). Overnight bacterial suspensions (approximately 10^8 CFU/ml) were lawned on sterile NA plates (100 µl) uniformly using a sterile cotton swab and kept for drying. After 5 min, wells of 6 mm diameter were aseptically punched into the agar using a sterile cork-borer. Each well was loaded with 100 µl of each AgNPs solution and distilled water (as a negative control). As positive controls, standard antibiotic discs of novobiocin or tetracycline were also placed, and NA plates were incubated for 24 h at 37°C. After incubation, the inhibition zone was measured in mm, and the zone diameter for antibiotics was recorded.

Statistical analysis

All measurements were repeated in triplicate to guarantee reproducibility, and all data were expressed as mean ± standard deviation (SD). With Prism version 10.3.1, the differences in means of the different groups were compared (ANOVA). P-values of ≤ 0.05 were considered significant.

Results and Discussion

Endophytic fungus isolate identification

The endophytic isolate LD2_2 was morphologically identified according to its appearance on PDA (Fig. 1A) and via the light microscope. The results of Blastn, pairwise, and multiple sequence alignment revealed 100% identity with *A. tenuissima* strains. Based on maximum likelihood, a phylogenetic tree was constructed, and the branches are accompanied by the percentage of trees in which the related taxa clustered together in Fig. 1B.

AgNPs mycosynthesis

The collected biomass, which was washed and immersed in distilled water for three days. After incubation, the CFF mixture with $AgNO_3$ developed a honey-brown color, indicating that silver ions were bio-reduced and that AgNPs were formed (Fig. 2). Researchers have reported that AgNPs can produce a variety of shades and tones, ranging from yellow (Chan & Don, 2013) and honey to deep brown (Taha et al., 2024).

UV-vis

The biosynthesized AgNPs exhibited a maximum peak at 435 nm with 1.6 a.u., as represented in Fig. 3. This result confirmed the production of bio-AgNPs that attributable to the surface plasmon resonance of the particles (Vahabi et al., 2011). This result is consistent with the maximum absorbance of 435 nm for AgNPs generated by *Alternaria* sp. (Win et al., 2022) and 430 nm for AgNPs manufactured by *Aspergillus niger* IPT856 (Ottoni et al., 2017). The absorption peak of AgNPs may fluctuate depending on the microorganism used.

FE-SEM of AgNPs

FE-SEM analysis was used to investigate the morphological shape and size of nanoparticles. The AgNPs exhibit well-defined nanoscale structures with recognizable spherical forms with an average diameter of 17.22 nm (Fig. 4). We found good agreement with the previously reported by Win et al. (2020) from *Alternaria* sp., which ranged from 3 to 10 nm. Furthermore, it was shown that chemically synthesized small spherical-shaped AgNPs exhibited superior antibacterial efficacy against *Pseudomonas aeruginosa* and *E. coli* in comparison to larger AgNPs and triangular-shaped AgNPs (Raza et al., 2016).

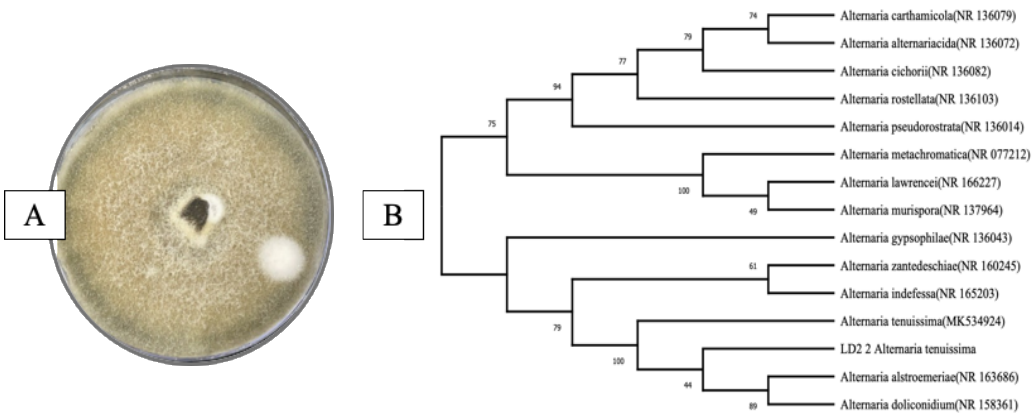

Fig 1. (A) *A. tenuissima* (LD2-2) colony on PDA incubated at 25°C for 7 days. (B) Neighbor-joining tree demonstrating connection between results from NCBI database and LD2_2 gene.

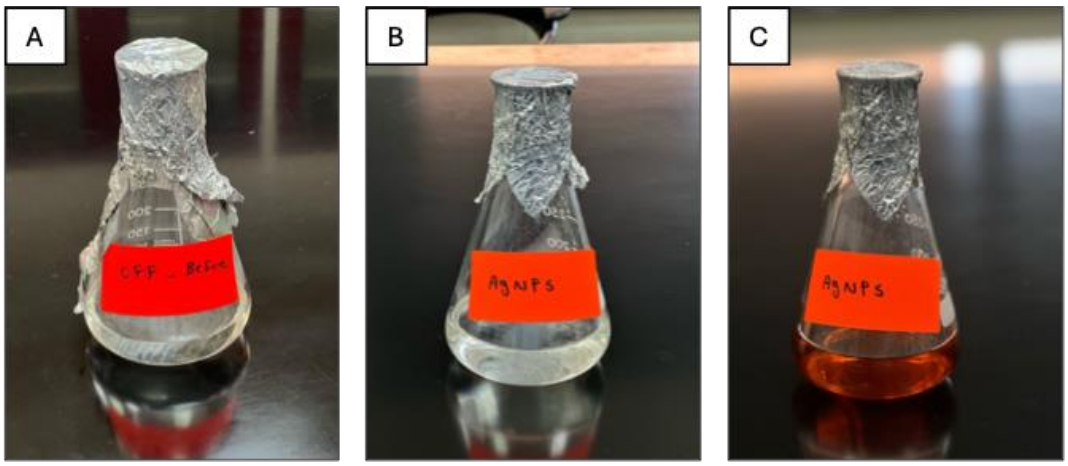

Fig 2. (A) The CFF of *A. tenuissima*, (B) the mixture of CFF with 1 mM $AgNO_3$, and (C) the visual detection of AgNPs formation after 1 h of reaction.

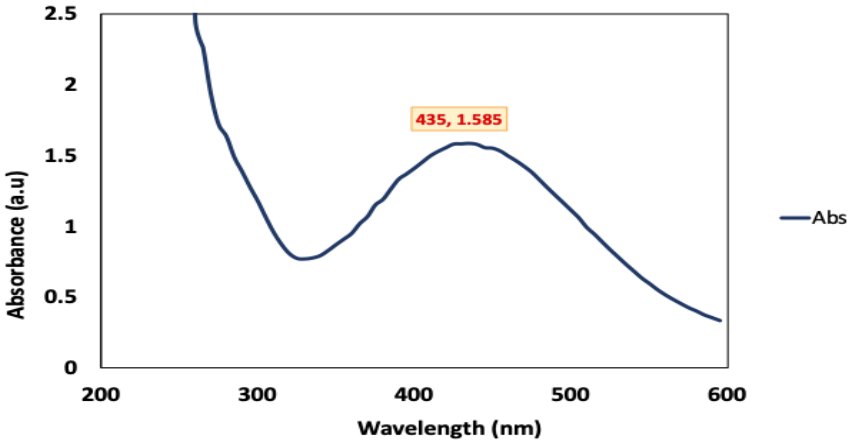

Fig 3. UV-vis absorption peak of silver nanoparticles (AgNPs) from *A. tenuissima.*

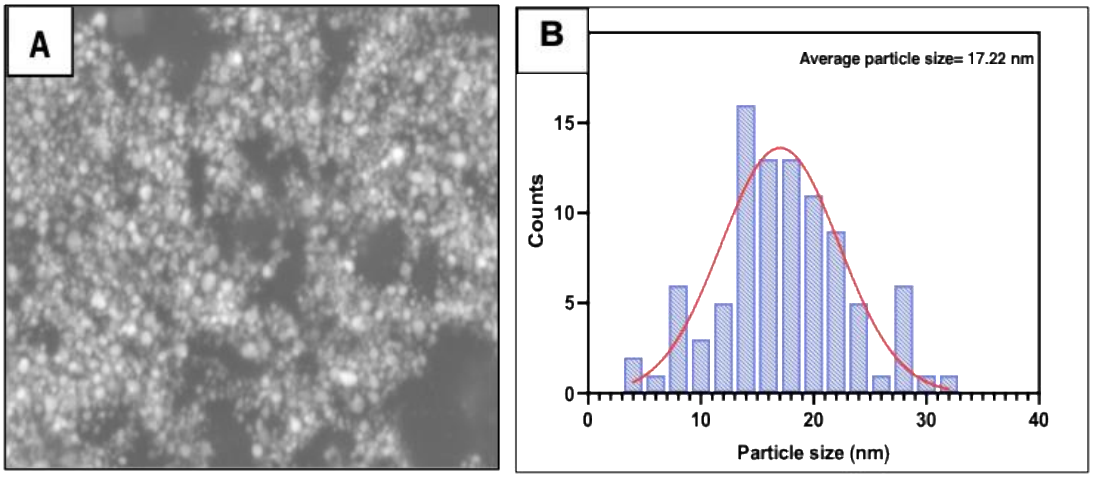

Fig 4. (A) FE-SEM image of AgNPs (B) Histogram of average particle size of AgNPs.

Antibacterial activity of AgNPs

The antibacterial efficacy of AgNPs was investigated against *S. aureus, S. marcescens*, and *E. coli* using agar-well diffusion (Fig. 5). The mean colony diameter calculated in mm ± SD is represented in Table 1. AgNPs showed prominent antibacterial properties against all strains with the standard antibiotic. Overall, the AgNPs exhibited significant inhibitory effects to different degrees against the species of bacteria tested. Our findings indicate that the antibacterial activity of AgNPs was not associated with the bacterial type, whether Gram-positive or Gram-negative, but rather exhibited a species-dependent effect. The highest zone of inhibition was found against *E. coli* (20 ± 0.3 mm), followed by *S. aureus* (15.3 ± 0.6 mm) and *S. marcescens* (15 ± 0.0 mm). According to the data shown in Fig 6, *E. coli* is more susceptible to AgNPs than *S. aureus* and S. *marcescens.* These findings were consistent with the previous antibacterial activity results obtained by biogenic AgNPs from fungal extracts by War et al. (2022), which showed a zone of inhibition of 13.33 ± 0.57 mm against *E. coli* and 11.66 ± 0.57 mm against *S. aureus*. Similar results were obtained from AgNPs from endophytic fungus *Talaromyces purpureogenus* by Hu et al. (2019). Such an approach using *Aspergillus flavus* produced good results against different gram-positive and gram-negative bacteria (Al-Soub et al., 2022). Also, in accordance with the findings of a previous study that investigated the antibacterial effect of AgNPs produced by *Aspergillus niger* against *S. aureus* and *E. coli* (Ottoni et al., 2017). It is known that the antimicrobial process of AgNPs may entail the adhesion of AgNPs to external proteins, resulting in pore formation, which disrupts DNA replication or generates reactive oxygen species (ROS), including hydrogen peroxide, superoxide anions, and hydroxyl radicals. Or through the inactivation of sulfhydryl groups in the cell wall and the breakdown of membrane-bound enzymes and lipids, leading to cell lysis (Sameena & Thoppil 2022).

Table 1 Antibacterial activity of AgNPs against pathogenic bacteria using the agar-well technique

	Gram-*ve*		Gram+*ve*
	E. coli	***S. marcescens***	***S. aureus***
AgNPs	20±0.3	15±0.0	15.3±0.6
TE	9±0.0	7.0±0.0	9.0±0.0
NO	NA	5.0±0.0	NA
OX	NA	NA	0.0 ± 0.0^{R}
-ve	0.0	0.0	0.0

* NA = not applicable, R = resistant, and all the values are presented as mean ± SD.

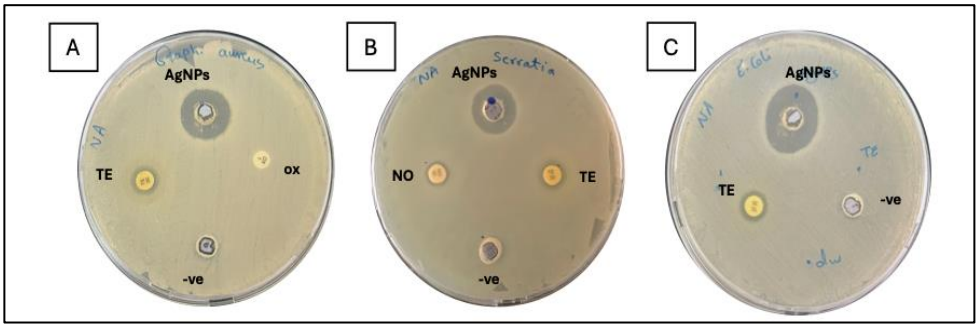

Fig 5. Antibacterial activity of AgNPs against pathogenic bacteria: (A) *S. aureus*, (B) *S. marcescens*, and (C) *E. coli*. "-ve" represents the negative control, while positive controls are "TE" tetracycline (30 µg/ml), "OX" oxacillin (1 u/ml), and "NO" novobiocin (30 µg/ml).

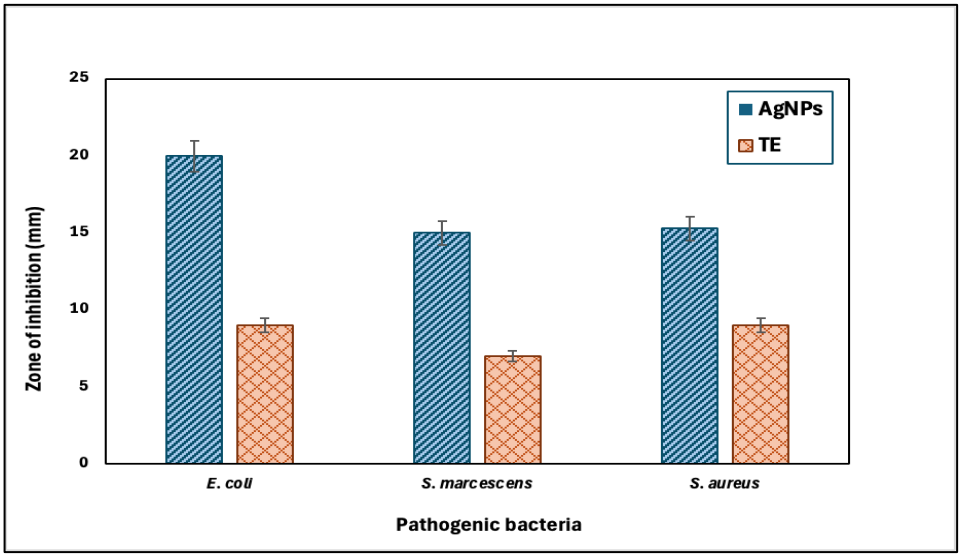

Fig 6. Antibacterial activity of AgNPs against *E. coli*, *S. marcescens*, and *S. aureus* compared to the broad-spectrum antibiotic "TE" tetracycline (30 µg/ml).

Conclusion

In conclusion, the study succeeded in biosynthesizing AgNPs using the endophytic fungus *A. tenuissima* as a bio-mediator. The study found that these AgNPs with the size of 17.22 nm have substantial antibacterial activity against MRSA and gram-negative bacteria. In light of these findings, fungal bio-AgNPs can serve as alternative sources of biocontrol agents, which could be an option for controlling certain pathogenic microbes in a variety of ways.

Conflict of Interest:

The author declares that they have no conflict of interest.

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