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Preliminary screening of endophytic fungi hosted some wild plants in North Sinai for biogenic production of silver nanoparticles

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ABSTRACT

The biological synthesis of silver nanoparticles offers a promising alternative to traditional physical and chemical methods due to its inherent simplicity, non-toxic nature, and environmental friendliness. Within this study, endophytic fungi hosted six wild plants in Arish City, North Sinai, Egypt have been isolated by surface sterilization technique on different isolation media. Twelve species, based on their frequency of occurrence, out of thirty recovered taxa were tested for their capability to synthesize extracellular AgNPs. *Aspergillus terreus* (OQ119631) and *Aspergillus flavus* (OQ119633) recovered from *Hyoscyamus muticus* and *Zygophyllum album* respectively were found to be the best candidate for the production of mycogenic AgNPs among all examined species. Mycogenic AgNPs were characterized by UV-visible spectroscopy and Transmission Electron Microscopy for both taxa. The synthesized silver nanoparticles demonstrated antimicrobial efficacy against various pathogenic bacteria and fungi. Additionally, their cytotoxicity was assessed against the human normal fibroblast cell line (BJ) using a cell viability assay. The results revealed substantial growth inhibition with IC₅₀ concentration of 100 µg/mL, indicating effectiveness against diseased cells while maintaining relative safety towards healthy cells. This underscores the potential of eco-friendly AgNPs produced by native endophytic fungi for targeted cytotoxicity.

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Introduction

Plants in nature harbor a diverse array of symbiotic and non-symbiotic microorganisms within their tissues, and these microbes play a critical role in plant development, growth, and defense. This intricate relationship between microbes and plants is essential for their survival in challenging environments. Among these microorganisms, endophytic fungi, along with bacteria and yeast, establish symbiotic associations with plants (Rani et al. 2017; Gezaf et al. 2021).

The nomenclature "endophyte" was introduced by Hinrich Anton de Bary in 1884. Endophytic fungi are a unique group of microorganisms that stay within the

health tissues of plants, inhibiting inter- or intracellular spaces without causing any visible signs of disease or infections (Abdel-Azeem et al. 2016, 2018; Abo Nouh et al. 2021; Gezaf et al. 2023). These fungi establish a symbiotic relationship with their host plants, providing various benefits, including enhanced nutrient acquisition, stress tolerance, disease resistance, and improved growth (Baker et al. 2015; Sandhu et al. 2017).

Endophytic fungi, categorized into mycorrhizal, pasture, and non-pasture forms (Schulz 2006; AbuElsaoud et al. 2017), represent a variety of bioactive compounds with wide-ranging applications.

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These fungi synthesize a wealth of functional metabolites like alkaloids, terpenoids, amines, flavonoids, steroids, and phenolic compounds (Ding et al. 2008). Notably, they are recognized for their production of novel antimicrobial agents, anticancer compounds such as Taxol, and industrially valuable enzymes (Salem & Abdel-Azeem 2014; Netala et al., 2016; Mohamed et al. 2022; Moubasher et al. 2022).

Endophytic fungi, in addition to their production of various valuable metabolites, also exhibit the ability to synthesize silver nanoparticles. These silver nanoparticles hold great promise in various fields (Klasen 2000; AbuElsaoud et al. 2015; Rahman et al., 2019; Abdel-Azeem et al. 2020a).

Nanoparticles are microscopic particles of a metal or other material with a size ranging from 1 to 100 nanometers, which is one-billionth of a meter. They serve as a bridge between the properties of bulk materials and those of individual atoms and molecules (Soliman et al. 2023).

Nanotechnology has emerged as a transformative force in modern material science, offering a dynamic and innovative realm of research exploration (Khan & Rizivi 2014; El-Sayed & Kamel 2020; Meena et al. 2021). Its rapid advancements are revolutionizing the field of life sciences, particularly biotechnology and biomedical science. With its remarkable applications in pharmaceutical and biomedical science, nanotechnology is revolutionizing conventional drug delivery systems, ushering in a new era of enhanced treatment efficacy and patient outcomes (Bayda et al. 2020; Meena et al. 2021).

The world of metal nanoparticles, particularly silver nanoparticles (AgNPs), has captivated researchers in recent years, with a flurry of studies exploring their multifaceted potential (Kumari et al. 2011; Vannini et al. 2014; Srivastava et al. 2021). This surge in scientific interest stems from the remarkable properties of AgNPs, which hold promise for transforming various fields, including medicine, imaging, and electronics.

This research aims to identify and examine the endophytic fungi hosted medicinal and halophytic plants in North Sinai, Egypt, which produce AgNPs. Additionally, it seeks to analyse antimicrobial and the cytotoxic effects of these AgNPs on the human normal fibroblast cell line (BJ).

Materials and Methods

Sample collection

A total number of 120 samples representing six plant species (Fig.1) were collected in sterilized polyethylene bags from wild populations growing at coastal sites at Arish City, North Sinai (Fig.2) namely: *Zygophyllum*

album L., *Cakile maritima* Velen., *Datura metel* L., *Cleome amblyocarpa* Barratte & Murb., *Hyoscyamus muticus* L. and *Artemisia monosperma* L. Aerial parts (stems, leaves) were collected in sterilized polyethylene bags, secured with rubber bands, and transported to the laboratory for subsequent processing according to Abdel-Azeem et al. (2019) and Rosli et al. (2020). Plant samples were collected for scientific purposes, and no endangered or threaten species were involved in the study.

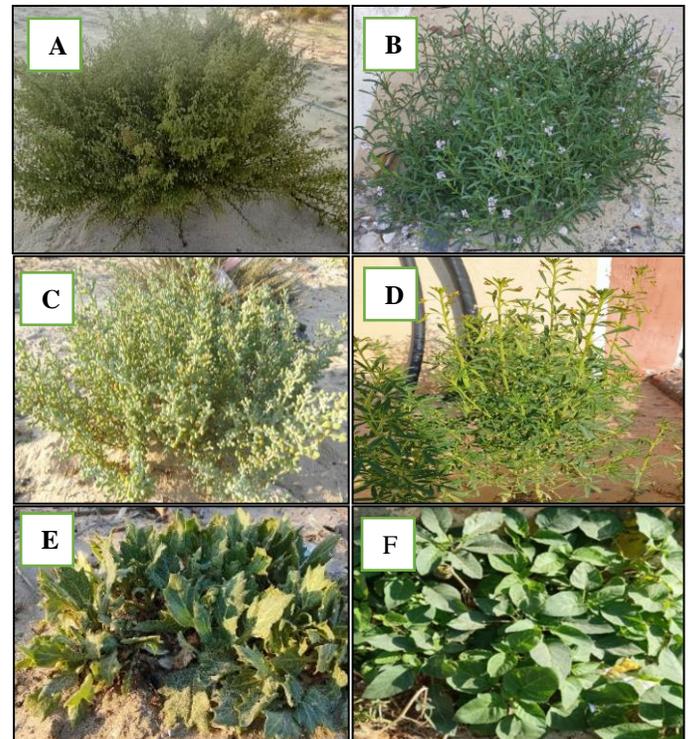


Fig 1. Plants under investigation (a) *Artemisia monosperma* (b) *Cakile maritima* (c) *Zygophyllum album* (d) *Cleome amblyocarpa* (e) *Hyoscyamus muticus* (f) *Datura metel*.

Isolation of endophytic fungi

The aerial parts from each plant species under investigation were transversely cut into fragments of approximately five mm² in length and subjected to a 10-minute running water wash to remove adhering soil particles and debris. The fragments were then air-dried.

Plant material underwent a four-step surface sterilization process: 1 minute rinse with 70% ethanol, 3–5-minute dip in 2.5% sodium hypochlorite, 30 second rinse with 70% ethanol, and multiple rinses with sterile distilled water to remove residual sterilization agents (Fisher & Petrini 1990; Abdel-Azeem & Salem 2012; Singh et al. 2016; Hammad et al. 2023).

The sterilized plant tissue fragments (4 per plate) were gently pressed onto the surface of various isolation media (Abdel-Azeem et al. 2016). Czapek's yeast extract agar (CYA), potato dextrose agar (PDA), malt extract agar (MEA), potato carrot agar (PCA) and oatmeal agar (OA) were used as isolation media and supplemented with Rose Bengal (1/15,000) as a bacteriostatic agent and chloramphenicol (50 ppm) for the suppression of bacterial growth (Smith & Dawson 1944).

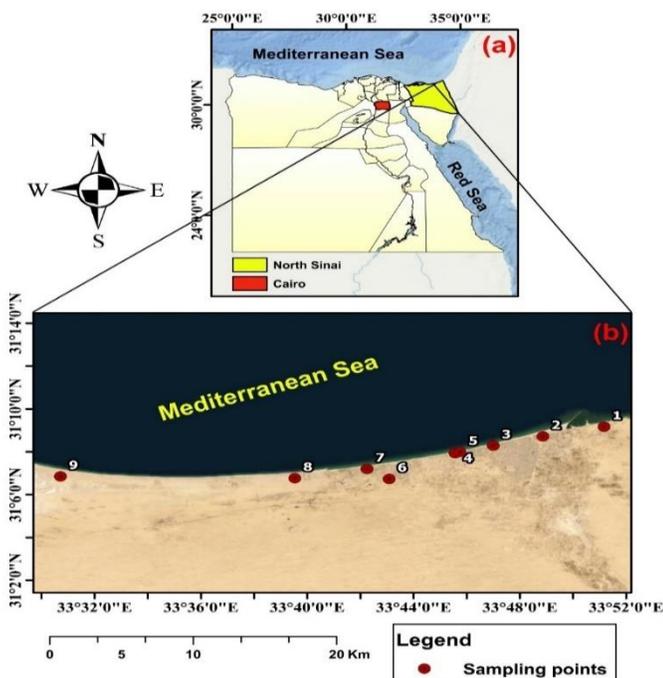


Fig 2. (a) Location of the study area (b) sampling points (1-9).

Cultures were incubated at 25°C for twenty-one days. Subsequently, to guarantee isolation of endophytic fungi exclusively from plant internal tissues, single fungal colonies emerging from the plant material were transferred to fresh purification media and incubated at 25°C.

Phenotypic identification of isolated taxa

Fungal isolates were morphologically identified to the species level based on standard media and phenotypic characteristics using relevant identification keys: for *Penicillium* (Raper & Thom 1949; Pitt 1980), Abdel-Azeem et al. (2020) for *Aspergillus*, for *Fusarium* (Booth 1971; Leslie & Summerell 2006) for ascomycetes (Guarro et al. 2012); for *Chaetomium* spp.

(Abdel-Azeem 2020b), for *Alternaria* spp (Simmons 2007).

Author names were abbreviated outlined by Kirk & Ansell (1992) and adopted the systematic classification provided in the 10th edition of Ainsworth and Bisby's Dictionary of Fungi (Kirk et al. 2008). The accuracy of names, authorities, and taxonomic assignments was further confirmed by cross-referencing with the Index Fungorum website (www.indexfungorum.org).

Abundance of endophytic fungi and frequency

Endophytic fungal abundance is categorized based on isolation frequency from plant hosts: High frequency (H): isolated or recorded 25 or more times indicating a dominant presence, moderate frequency (M): isolated or recorded 12-24 times representing a notable presence, low frequency (L): isolated or recorded 6-11 times suggesting an occasional occurrence and rare frequency (R): isolated or recorded less than 6 times signifying a sporadic presence. Additionally, colonization frequency is calculated using the method by Fisher & Petrini (1987):

Colonization Frequency (CF %) = (Number of segments colonized by fungus / Total number of segments) * 100

Screening fungal strains for biosynthesis of AgNPs

Based on their frequency of occurrence, twelve fungal species were chosen for examining their potential to biosynthesize AgNPs namely: *Alternaria alternata* (Fr.) Keissl, *A. solani* (Ellis & G. Martin) L.R. Jones & Grout, *A. tenuissima* (Kunze) Wiltshire, *Aspergillus flavus* Link, *A. niger* Tiegh, *A. terreus* Thom, *A. fumigatus* Fresen, *A. nidulans* (Eidam) G. Winter, *Trichoderma viride* Pers., *T. harzianum* Rifai, *Chaetomium globosum* Knuze and *Cladosporium Cladosporioides* Fresen.

Fungal taxa were cultured on PDA slants at a temperature of 25°C for two weeks (14 days) and stored at cold temperature (4 °C) for preservation. For biogenic synthesis of AgNPs, 100 µL (10⁶ conidia/ml) of each species conidia were inoculated in 250 mL Erlenmeyer flasks containing 100mL of broth media (potato dextrose broth). The cultures were incubated at 28 ± 2°C at 150rpm (round/min) for 5 days in an orbital shaker. After incubation, the fungal biomass was harvested through filtration and subsequently rinsed with sterile distilled water to remove any residual media components or impurities. Approximately 20g of biomass per species was then incubated in 200mL deionized water at 500 mL Erlenmeyer flask containing for 72 h in an orbital shaker at a speed of 150rpm and 28 ± 2°C. After incubation, the fungal filtrate was obtained

by passing it through double layers Whatman No.1 filter paper. For bio-synthesis of AgNPs was initiated by mixing 100 mL of 1 mM AgNO₃ solution with 100 mL of fungal filtrate in 500 mL Erlenmeyer flasks. The mixtures were shaken at 150 rpm and incubated in the dark at 28 ± 2°C. Conical flasks with fungal filtrate or AgNO₃ solutions were run as positive and negative controls. The colour change indicated the biosynthesis of silver nanoparticles (Abdel-Hadi et al. 2014; Elsharawy et al. 2023; Hammad et al. 2023).

Characterization of AgNPs

The preliminary test of biogenic AgNPs synthesis relied on visual observation of a color change in the fungal filtrates. Subsequently, UV-Visible spectrophotometry (Phonix 721, China) was employed to further characterize the nanoparticles, with readings taken at wavelengths between 300 and 500 nm at room temperature. Transmission Electron Microscopy (TEM, HRTEM on JEOL JEM 2100 HR made in Japan at an accelerating voltage of 200 Kv) in the Electron Microscope Unit, National Research Center (NRC), Egypt. Image of the AgNPs (shape and size of green synthesized) was investigated according to (Yu et al. 2019). For analyze the samples, two drops of each AgNP solution were placed onto carbon-coated copper grids. The grids were then left to dry at room temperature. The presence and elemental composition of any metals within the samples were then determined using energy-dispersive spectroscopy (EDS) on an INCA Energy TEM 200 transmission electron microscope (JEOL) operated at an accelerating voltage of 80 kV (Elbahnasawy et al. 2021; Mossa et al. 2023).

Molecular confirmation of potent endophytic taxa

For promising fungal taxa producing high amount of AgNPs, molecular confirmation was carried on. DNA extraction and purification utilized DNeasy Tissue Kits according to the manufacturer protocol (QIAGEN, Germany). The oligonucleotide primers described by White et al. (1990) were used for amplification and sequencing of the ITS regions. ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') AND ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Bioneer Corporation, South Korea) were selected for the present study.

Amplification reactions of the internal transcribed spacer (ITS) regions using ITS4 (5'- PCR amplification was performed on a Perkin-Elmer/GeneAmp® PCR System 9700 with 40 cycles following an initial denaturation step (5 min, 94°C) according to Khan & Bhadauria (2019). Sanger sequencing of PCR products

using forward primers was performed by FazaPazhouh Co. on an ABI 3730 xl DNA analyzer.

The nucleotide sequence data of the *Aspergillus terreus* and *Aspergillus flavus* isolates of the present study was deposited in the NCBI GenBank nucleotide sequence database under accession numbers OQ119631 and OQ119633 respectively.

Antimicrobial activity

The antibacterial and antifungal activity of silver nanoparticles synthesized by *Aspergillus flavus* (F) and *Aspergillus terreus* (M) were assessed against two bacteria and two fungi. Bacterial taxa namely: *Listeria monocytogenes* and *Pseudomonas aeruginosa*, while the fungal taxa namely: *Candida albicans* and *Aspergillus niger* the clinical samples were provided kindly from Suez Canal University Fungarium (<https://ccinfo.wdcm.org/details?regnum=1180>).

Bacteria and fungi were cultured on Mueller-Hinton agar (MHA) and Sabouraud dextrose agar (SDA), respectively. In the disc diffusion assay, 6 mm filter paper discs loaded with 50 µL of a 100 mg/ml stock solution were placed on the agar medium, and for the well-diffusion assay, 6 mm wells were created using sterile gel punctures, with 50 µL of the nanomaterial stock solution added to the wells (Abou Hammad et al. 2020; El Nahrawy et al. 2021). The plates were incubated at 37°C for bacteria and 28°C for fungi. After incubation, the diameter of the inhibition zone (IZ) around wells and discs was measured in mm, indicating the antimicrobial activity of the nanomaterials.

The minimum inhibitory concentration (MIC) values for the tested materials (F and M) and the survival of microbial species post-exposure were determined through the broth dilution method according to Elsherbiny et al. (2022).

Cytotoxicity of AgNPs on human normal fibroblast cell line (BJ).

The 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay was used to evaluate cell viability, measuring the conversion of the yellow MTT reagent to a purple formazan product by metabolically active cells (Mosmann 1983). Cells were cultured in DMEM-F12 medium with supplements and incubated at 37°C with 5% CO₂. After a 10-day batch culture, cells were seeded onto 96-well plates and subjected to different conditions, including a negative control and sample treatment with two samples at a final concentration of 100 µg/ml each. Following a 48-hour incubation, MTT solution was added, and the reaction was stopped with Sodium dodecyl sulfate

(SDS) solution (Thabrew et al. 1997). The absorbance at 595 nm was measured, and statistical analysis was performed using an independent t-test. The formula $((\text{Reading of AgNPs} / \text{Reading of negative control}) - 1) \times 100$ was used. To determine IC₅₀ and IC₉₀, a probit analysis was conducted with the SPSS 11 software. Doxorubicin at 100 µg/ml served as a positive control with known 100% lethality.

Results

Identification, taxonomy and abundance of recovered endophytic fungi

Thirty species of endophytic fungi belonging to 14 genera hosted six plant species were recovered during this study. The isolated taxa were taxonomically assigned into one phylum, three classes, five orders, and eight families. Orders Eurotiales and Pleosporales exhibited the highest species diversity, each containing ten different species. Five species belonged to the Hypocreales order, while all other orders harbored between one and three species each.

Preliminary observations on host plant richness revealed that the *Zygophyllum album* first hosted 15 species out of 30, followed by *Hyoscyamus muticus* (13 species) and *Artemisia monosperma* (11 species). The study revealed varying degrees of host specificity among the isolated endophytic fungi. While some species, like *Aspergillus terreus*, *A. niger*, *A. flavus*, and *Alternaria alternata*, appeared capable of colonizing multiple plant hosts, others exhibited a strong preference for specific plants. (Fig. 3) illustrates the most commonly isolated species and their respective host plants.

Table (1) presents abundance (A) and colonization frequency (CF) of the isolated fungal taxa on media incubated at 25 °C. Additionally, the table depicts the frequency class assigned to each taxon based on its isolation frequency. Among the isolated fungal endophytes, *Aspergillus* emerged as the most diverse genus, with 9 species identified out of 30 species. Other genera displayed lower species richness, where *Alternaria* represented by 3 species other remaining taxa typically having 1-2 species each.

Interestingly, *Alternaria alternata* was the most abundant species, accounting for 51.5% of the total colony-forming units (CFUs) isolated. Other prevalent *Aspergillus* species included *A. terreus* (18.5%), *A. flavus* (17%), and *A. niger* (13.5%).

Table (2) summarizes the taxonomic classification of the isolated fungal species. All species belong to a single phylum, three classes, five orders, and eight families. Where Eurotiales and Pleosporales exhibited the highest species diversity (10 species each), Hypocreales came

second by accommodating five species only. The remaining orders contained a smaller range of species, typically one to three each. On the terms of family-level diversity, Aspergillaceae stood out with the highest contribution, encompassing ten out of the total 30 isolated species. Pleosporaceae came second with eight species, while all other families had only one or three representatives.

For the species-to-genus (S/G) ratio for each fungal family, Aspergillaceae displayed the highest taxonomic diversity, boasting a ratio of 5 species per genus. This was followed by Pleosporaceae with a ratio of 2.7 species per genus (Table 3).

The presence or absence-based distribution patterns of the identified endophytic fungi within the examined plants are depicted in Figure 4. Three distinct groups emerge from the analysis: **Group 1**; Composed of 16 species, including *Acrophialophora fusispora*, *Alternaria atrium*, *Aspergillus candidus*, and *Epicoecum nigrum*, exhibiting exclusive association with a single plant species each. **Group 2**; Containing 7 species, such as *Acremonium alternatum*, *Alternaria botrytis*, *Alternaria tenuissima*, *Aspergillus terreus*, *Chaetomium globosum*, *Didymella glomerata*, and *Trichoderma viride*, demonstrating occurrence in two or more plant species. **Group 3**, Encompassing 7 species, including *Aspergillus niger*, *Alternaria alternata*, *Aspergillus fumigatus*, *Alternaria solani*, *Aspergillus flavus*, *Cladosporium cladosporioides*, and *Penicillium brevicompactum*, characterized by widespread presence across almost all plant hosts.

Surveying of AgNPs producing Taxa

Twelve fungal species were specifically selected based on their frequency of occurrence and surveyed for their ability to biosynthesis of AgNPs species included: *Alternaria alternata*, *Alternaria solani*, *Alternaria tenuissima*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus fumigatus*, *Aspergillus nidulans*, *Trichoderma viride*, *Trichoderma harzianum*, *Chaetomium globosum* and *Cladosporium cladosporioides*.

Out of the 12 species examined, only five species gave positive results (colour change of fungal filtrate). Positive taxa namely: *Aspergillus terreus*, *Aspergillus flavus*, *Aspergillus niger*, *Chaetomium globosum* and *Alternaria alternata* (Table 4, Figure 5). By using UV-Visible spectroscopy, the production of AgNPs by five different species of fungi was measured. *Aspergillus terreus* and *Aspergillus flavus* came first by producing a high amount of a-NADPH-dependent nitrate reductase mediate silver-nano. Both species were recovered from *Hyoscyamus muticus* and *Zygophyllum album* as host plant respectively.

Table 1. Frequency and frequency class of taxa

Species	TC	NCI	CF%	A
Sordariomycetes				
<i>Acremonium alternatum</i> Link	7	5	1.75	L
<i>Acrophialophora fusispora</i> S.B. Saksena	10	5	2.5	L
<i>Chaetomium atrobrunneum</i> L.M. Ames	19	10	4.75	M
<i>Chatomium globosum</i> Knuze	29	16	7.25	H
<i>Fusarium Solani</i> (Mart.) Sacc.	9	7	2.25	L
<i>Trichoderma harzianum</i> Rifai	23	14	5.75	M
<i>Trichoderma viride</i> Pers.	18	12	4.5	M
<i>Trichothecium roseum</i> Pers.	16	10	4	M
Dothideomycetes				
<i>Alternaria alternata</i> (Fr.) Keissl	206	100	51.5	H
<i>Alternaria atrium</i> (Preuss)Woudep & Crous	5	4	1.25	R
<i>Alternaria botrytis</i> Preuss	11	6	2.75	L
<i>Alternaria chlamydospora</i> Mouch.	11	7	2.75	L
<i>Alternaria solani</i> (Ellis & G. Martin) L.R. Jones & Grout	30	17	7.5	H
<i>Alternaria tenuissima</i> (Kunze) Wiltshire	13	7	3.25	M
<i>Cladosporium Cladosporioides</i> Fresen	28	13	7	H
<i>Cladosporium sphaerospermum</i> Penz	27	15	6.75	H
<i>Didymella glomerata</i> (Cord) Qian Chen & L. Cai	9	4	2.25	L
<i>Epicoccum nigrum</i> Link	6	5	1.5	L
<i>Exserohilum rostratum</i> (Drechsler) K.J. Leonard & Suggs	5	4	1.25	R
<i>Stemphylium botryosum</i> Wallr.	8	7	2	L
Eurotiomycetes				
<i>Aspergillus candidus</i> Link	11	6	2.75	L
<i>Aspergillus flavus</i> Link	68	32	17	H
<i>Aspergillus fumigatus</i> Fresen	48	27	12	H
<i>Aspergillus japonicus</i> Saito	8	6	2	L
<i>Aspergillus nidulans</i> (Eidam) G. Winter	14	11	3.5	M
<i>Aspergillus niger</i> Tiegh	54	33	13.5	H
<i>Aspergillus niveus</i> Blochwitz	7	6	1.75	L
<i>Aspergillus terreus</i> Thom	74	38	18.5	H
<i>Aspergillus tubingensis</i> Mosseray	7	6	1.75	L
<i>Penicillium brevicompactum</i> Dierckx	12	8	3	M
Total Species = 30	793	441		

Where: Total count = (TC, colonies/cut), Number of cases of isolation= (NCI, out of 400 plant segments/ 100 plates for each plant), Colony forming unit = CFU.

Table 2. Taxonomic assignment of isolated species

Classes	Orders	Families	Genera	Species
		Incertae sedis	1	1
Sordariomycetes	Hypocreales	Hypocreaceae	2	3
		Nectriaceae	1	1
	Sordariales	Chaetomiaceae	2	3
Dothideomycetes	Capnodiales	Davidiellaceae	1	2
		Pleosporaceae	3	8
	pleosporales	Didymellaceae	2	2
Eurotiomycetes	Eurotiales	Aspergillaceae	2	10
Total	5	8	14	30

Table 3. Species Genus Ratio (S/G)

Families	Genera	Species	S/G
Incertae sedis	1	1	1
Hypocreaceae	2	3	1.5
Nectriaceae	1	1	1
Chaetomiaceae	2	3	1.5
Davidiellaceae	1	2	2
Pleosporaceae	3	8	2.7
Didymellaceae	2	2	1
Aspergillaceae	2	10	5
Total= 8	14	30	15.7

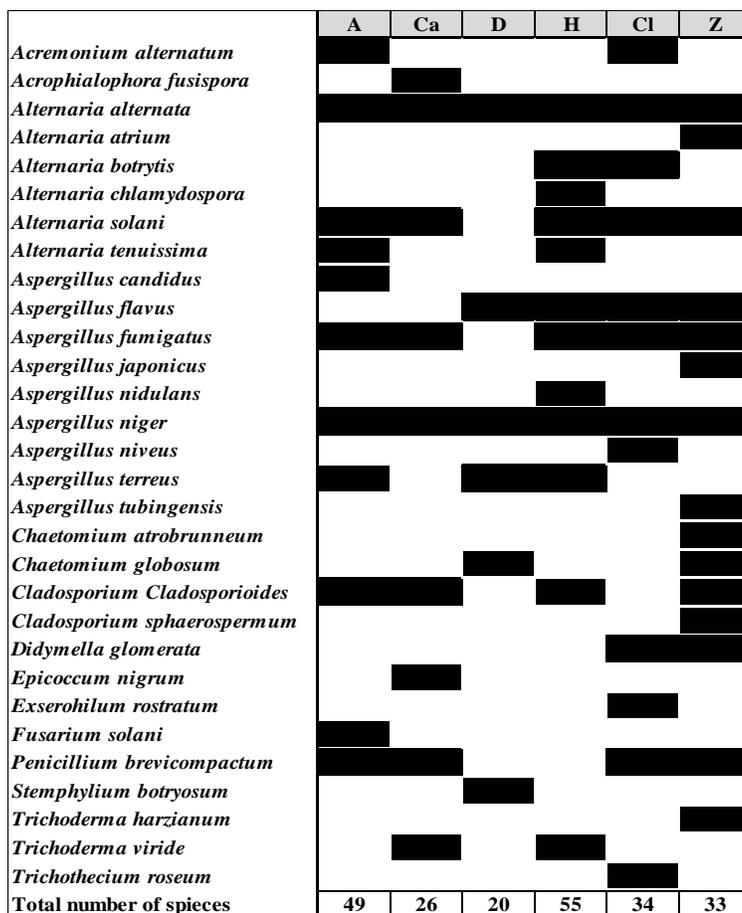
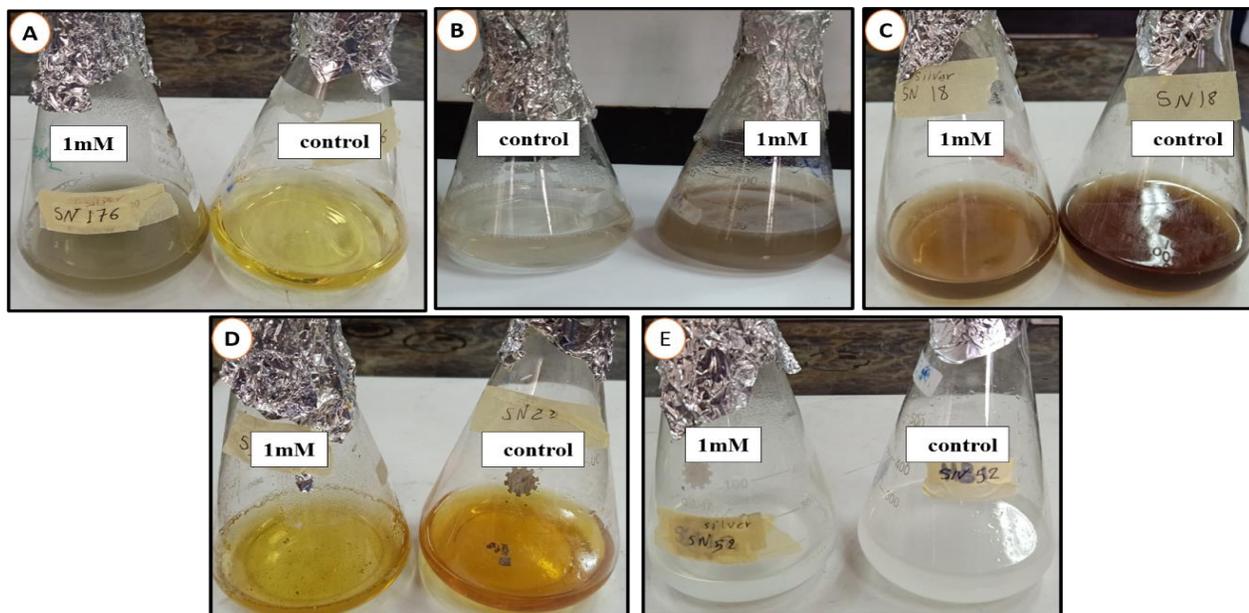


Fig 4. Distribution of endophytic fungi in different host plants, where, A= *Artemisia monosperma*, Ca= *Cakile maritima*, D= *Datura metel*, H= *Hyoscyamus muticus*, cl= *Cleome amblyocarpa*, and Z= *Zygophyllum album*.

Table 4. Degree of AgNPs production by twelve selective species under investigation.

Taxa	No activity	Low	Moderate	High
<i>A. flavus</i>				+++
<i>A. fumigatus</i>		+		
<i>A. nidulans</i>	–			
<i>A. niger</i>			++	
<i>A. solani</i>	–			
<i>A. tenuissima</i>	–			
<i>A. alternata</i>		+		
<i>A.terreus</i>				+++
<i>C. cladosporioides</i>		+		
<i>C. globosum</i>			++	
<i>T. harzianum</i>		+		
<i>T. viride</i>			++	

**Fig 5.** Mycogenic production of AgNPs by five promising taxa where, (A) *Aspergillus terreus* (B) *Aspergillus flavus* (C) *Aspergillus niger* (D) *Chaetomium globosum* and (E) *Alternaria alternata*.

Characterization of AgNPs

UV-Visible spectroscopy revealed distinct peak absorption wavelengths for AgNPs biosynthesized by different endophytic fungi. AgNPs from *Aspergillus terreus* displayed a peak absorbance at 420 nm, while those from *Aspergillus flavus* peaked at 400 nm. Notably,

Aspergillus terreus (OQ119631) and *Aspergillus flavus* (OQ119633) exhibited the strongest absorption intensities, suggesting higher AgNPs concentrations compared to other analyzed fungi (Fig. 6). Conversely, *Alternaria alternata* demonstrated minimal spectral absorption, indicating potentially lower AgNPs production.

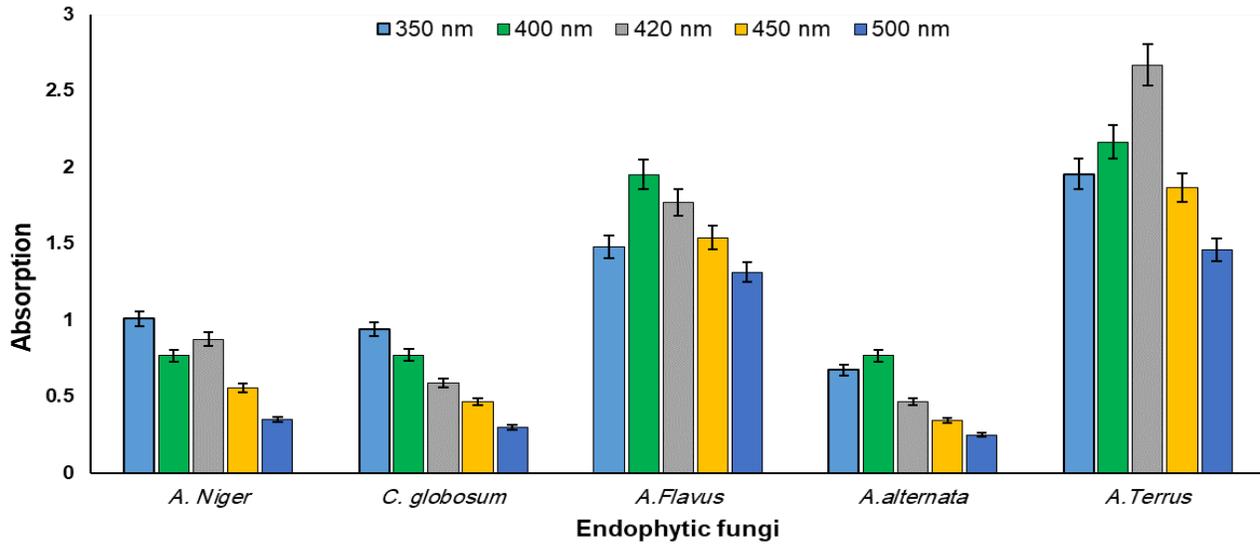


Fig 6. UV-Visible spectrum showing the absorbance of AgNPs at different wavelength of taxa under investigation.

Transmission electron microscopy was used to characterize the morphological and shape criteria of the AgNPs for *Aspergillus flavus* (OQ119633) and *Aspergillus terreus* (OQ119631) which had the highest absorption for UV. The TEM micrograph (Figs 7 and 8) showed that the AgNPs were spherical and well-dispersed, with no agglomeration. The size of the

AgNPs biogenic synthesized by the endophytic fungi *Aspergillus flavus* ranged from 2-33 nm and *Aspergillus terreus* ranged from 3-60 nm

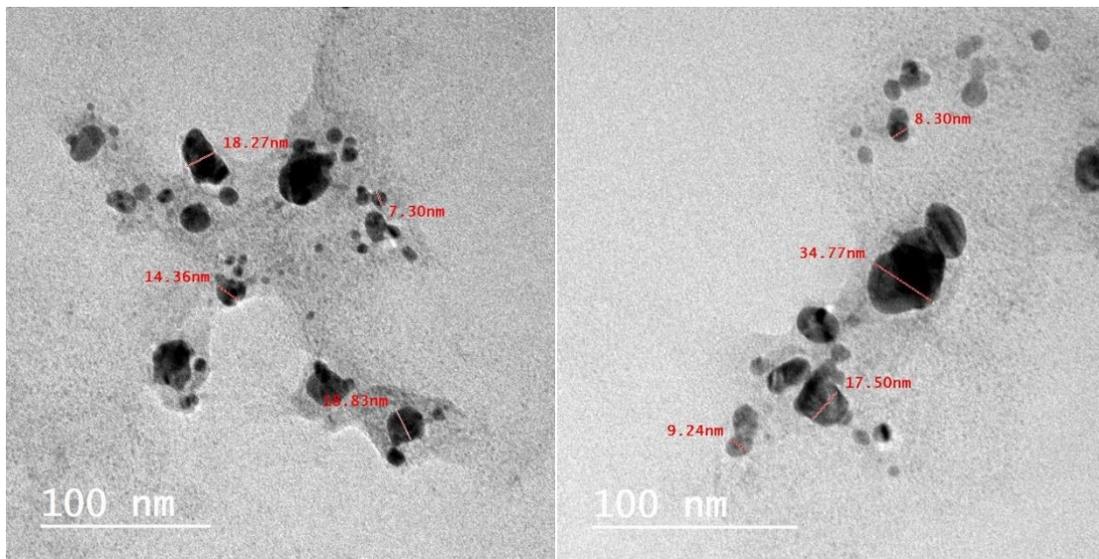


Fig 7. AgNPs produced by *Aspergillus flavus* (OQ119633).

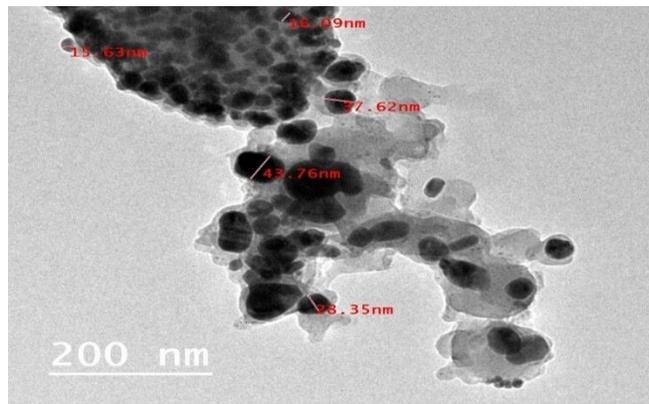


Fig 8. AgNPs produced by *Aspergillus terreus* (OQ119631).

Antimicrobial activity

Table 5 outlines the results of the disc and well diffusion methods, indicating the Zone of Inhibition (ZOI) widths in millimeters (mean \pm standard deviation) for two nanoparticle (NP) types generated through fungal-mediated synthesis. NPs, denoted as "F" (AgNPs of *A. flavus*), and "M" (AgNPs of *A. terreus*), were tested against various pathogenic microorganisms.

For *Listeria monocytogenes* (*L. monocytogenes*), ZOI widths were 13.8 ± 0.48 mm (disc) and 15.2 ± 0.24 mm (well) for " *A. flavus* " NPs, and slightly higher at 15 ± 0.35 mm (disc) and 16.6 ± 0.82 mm (well) for " *A. terreus* " NPs.

Pseudomonas aeruginosa (*P. aeruginosa*) showed ZOI widths of 14.3 ± 0.68 mm (disc) and 16 ± 0.75 mm (well) for " *A. flavus* " NPs, while " *A. terreus* " NPs exhibited 17 ± 0.39 mm (disc) and 18.6 ± 0.45 mm (well). For *Candida albicans* (*C. albicans*), ZOI widths were 12.7 ± 0.42 mm (disc) and 13.7 ± 0.83 mm (well) for " *A. flavus* " NPs, and 14.7 ± 0.19 mm (disc) and 15.3 ± 0.27 mm (well) for " *A. terreus* " NPs.

Aspergillus niger (*A. niger*) demonstrated ZOI widths of 12.1 ± 0.84 mm (disc) and 12.8 ± 0.63 mm

(well) for " *A. flavus* " NPs, and 13.5 ± 0.93 mm (disc) and 14.2 ± 0.37 mm (well) for " *A. terreus* " NPs.

The outcomes depicted in (Fig. 9) showcase the efficacy of compounds AgNPs of *A. terreus* and AgNPs of *A. flavus* against pathogenic microbes. The investigation aimed to establish the optimal dosage and killing time for these compounds, revealing that AgNPs of *A. terreus* exhibited superior efficacy compared to AgNPs of *A. flavus* across all targeted microbes.

For *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Candida albicans*, and *A. niger*, the Minimum Inhibitory Concentration (MIC) dosage for AgNPs of *A. flavus* at a killing time of 15 minutes was 100 mg/L, 125 mg/L, 150 mg/L, and 150 mg/L, respectively (Fig. 9-i). In contrast, AgNPs of *A. terreus* demonstrated a twofold better antimicrobial efficacy than AgNPs of *A. flavus* against all targeted microbes. The MIC dosage for AgNPs of *A. terreus*, with a killing time of 10 minutes for *Listeria monocytogenes* and 15 minutes for *Pseudomonas aeruginosa*, *Candida albicans*, and *A. niger*, was 125 mg/L (Fig. 9-ii).

Table 5. The width of ZOI in mm of the two NPs against different kinds of pathogenic microbes using the disc and well diffusion method.

Tested microbes	Fungal-mediated synthesized NPs			
	F		M	
	Disc	Well	Disc	Well
<i>L. monocytogenes</i>	13.8 \pm 0.48	15.2 \pm 0.24	15 \pm 0.35	16.6 \pm 0.82
<i>P. aeruginosa</i>	14.3 \pm 0.68	16 \pm 0.75	17 \pm 0.39	18.6 \pm 0.45
<i>C. albicans</i>	12.7 \pm 0.42	13.7 \pm 0.83	14.7 \pm 0.19	15.3 \pm 0.27
<i>A. niger</i>	12.1 \pm 0.84	12.8 \pm 0.63	13.5 \pm 0.93	14.2 \pm 0.37

Cytotoxicity of the AgNPs on BJ

The MTT assay was used to assess cell viability across a range of sample concentrations (0.78 to 100 µg/ml) of mycogenic AgNPs (Fig. 10). The figure clearly demonstrates that increasing sample concentration from 0.78 µg/ml to 100 µg/ml leads to a gradual decrease in cell viability. Notably, significant cytotoxicity was observed at the highest concentration of 100 µg/ml, as further detailed in Table 6.

Table 6. Cytotoxicity of AgNPs on BJ cell line at 24 hours and 48 hours at 100 µg/ml concentration.

Code	LC50 (µg/ml)	LC90 (µg/ml)	Remarks
T	-----	-----	32.5% at 100ppm
F	-----	-----	23.4% at 100ppm
DMSO	-----	-----	1% at 100ppm
Negative control	-----	-----	0%

*LC50: Lethal concentration of the sample which causes the death of 50% of cells in 48 hrs.

*LC90: Lethal concentration of the sample which causes the death of 90% of cells in 48 hrs.

*DMSO=Dimethyl sulfoxide

Discussion

Current study has been designed to isolate of endophytic fungi from six dominant plant species growing in Arish city in North Sinai. Taxa of endophytic fungi were previously recovered by several researchers including Abdel-Azeem et al. (2018), and Abo-Nouh et al. (2023).

Four host plants (*Zygophyllum album*, *Datura metel*, *Hyoscyamus muticus* and *Artemisia monosperma*) hosted endophytic fungi were extensively studied by Abo-Nouh et al. (2023) while this research is the pioneer in isolation of endophytic fungi from *Cakile maritima* and *Cleome amblyocarpa* grown at Arish city in North Sinai.

From six plant species, a total number of 793 CFUs were isolated, belonging to 30 species. The taxa of endophytic fungi are similar to taxa recovered by Balbool & Abdel-Azeem (2020) and Abo-Nouh et al. (2023).

Out of the 12 species which were screened for the ability to synthesis AgNPs only five species showed positive capability to synthesize AgNPs. The most potent two species namely: *Aspergillus flavus* and *Aspergillus terreus*. The production of AgNPs were proved by colour change observation, UV-Visible spectrophotometric and by using TEM as recommended by various researchers

(Singh et al. 2016; Mohamed et al. 2018, Abdel-Azeem et al. 2020a).

The specific absorption peak of AgNPs typically falls within the range of 380-450 nm. This range can vary depending on many factors such as their shape, size, and interactions with each other (Lotfy et al. 2021).

The AgNPs synthesized by *Aspergillus terreus* exhibited peak absorption at 420 nm, which agrees with previous findings demonstrated by Verma et al. (2010) and Li et al. (2012). Similarly, the AgNPs from *Aspergillus flavus* displayed a maximum absorbance of 400 nm, consistent with the observations of Bhangale et al. (2018).

TEM showed that the AgNPs were spherical and well-dispersed, with no agglomeration. The size of the AgNPs biogenic synthesized by the endophytic fungi *Aspergillus flavus* ranged from 2-33 nm and *Aspergillus terreus* ranged from 3-60 nm. Several studies have reported the biosynthesis of AgNPs using various fungi. Mohamed et al. (2018) synthesis spherical and poly-disperse AgNPs ranging from 45.2 to 45.7 nm using endophytic fungus *Aspergillus terreus*. Farkanda et al. (2011) produced spherical and poly-disperse AgNPs ranging from 10 to 40 nm using the endophytic fungus *Pestalotia* sp. Afreen et al. (2011) employed *Rhizopus stolonifer* to synthesize spherical AgNPs ranging from 3 to 20 nm. Additionally, Sophiya et al. (2013) reported a method for synthesizing spherical AgNPs using two fungal species: *Alternaria solani* and *Penicillium funiculosum*, the resulting AgNPs exhibited spherical morphology with sizes ranging from 5 to 20 nm for *A. solani* and 5 to 10 nm for *P. funiculosum*. Sharanabasava et al. (2012) demonstrated the biogenic synthesis of spherical AgNPs with sizes ranging from 5 to 50 nm using the fungus *Penicillium diversum*, while while Ninganaqouda et al. (2014) found spherical AgNPs with sizes ranging from 20-55 nm using *Aspergillus niger*.

Our study shed light on the antimicrobial efficacy of AgNPs produced by native taxa (*A. flavus* and *A. terreus*). The discussion focuses on the Zone of Inhibition (ZOI) widths and Minimum Inhibitory Concentration (MIC) dosages obtained through disc and well diffusion methods, as well as the comparison of the antimicrobial effectiveness between the two types of AgNPs. The investigation suggests that "AgNPs of *A. terreus*" exhibit superior antimicrobial efficacy compared to "AgNPs of *A. flavus*" across all tested microbes. The MIC dosages of "AgNPs of *A. terreus*" were consistently lower, signifying enhanced potency and a more rapid killing effect. The results align with the ZOI widths, reinforcing the conclusion that "AgNPs of *A. terreus*" outperform "AgNPs of *A. flavus*" in terms of antimicrobial activity. These findings emphasize the potential of AgNPs synthesized through fungal-mediated synthesis, particularly those derived from *A. terreus*, as promising

agents for combating a range of pathogenic microorganisms. Further studies may delve into the underlying mechanisms contributing to the observed

differences in antimicrobial efficacy between the two types of AgNPs. This result agrees with Rani et al. (2017), Abdel-Azeem et al. (2020a) and Lotfy et al. (2021).

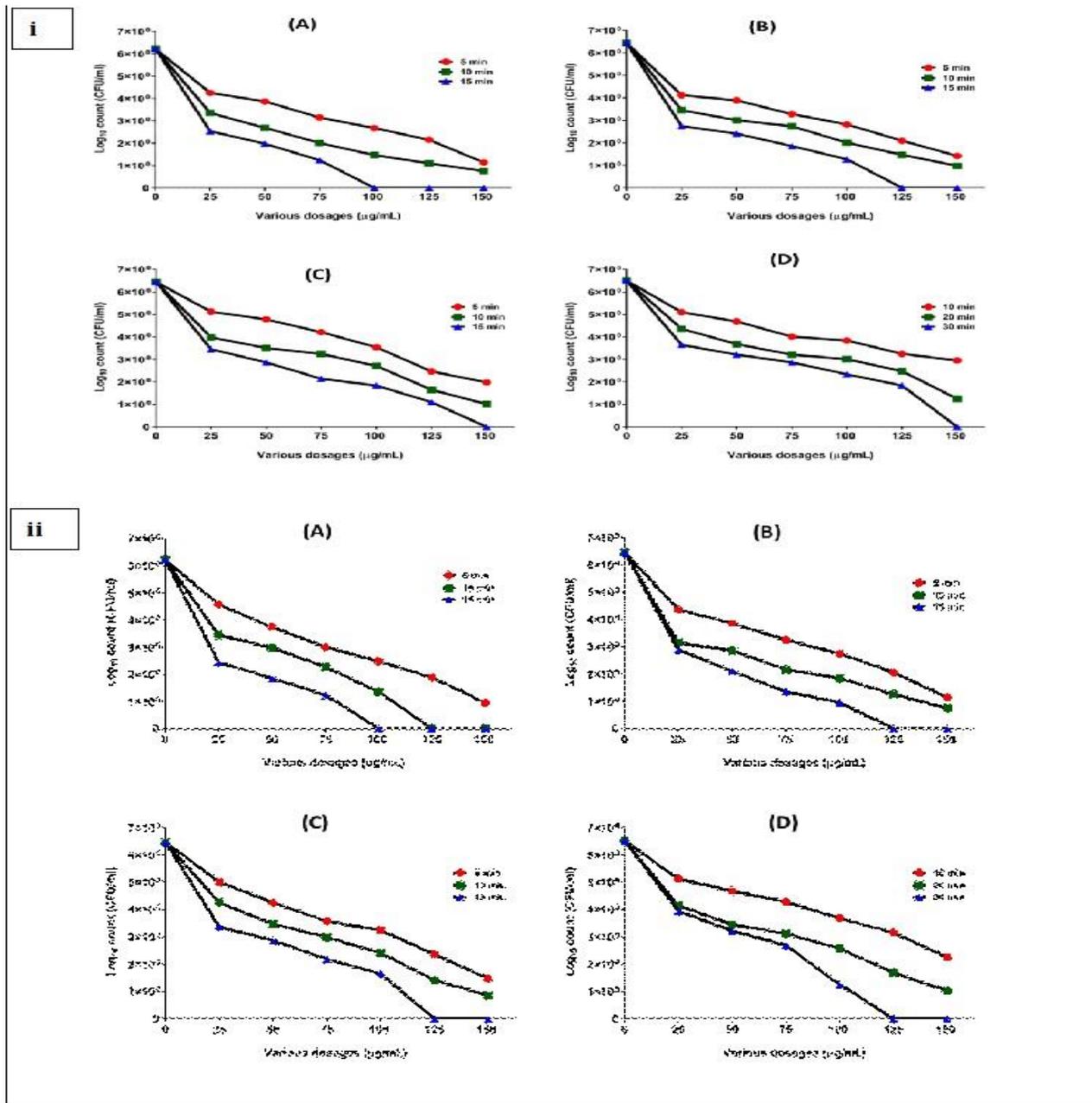


Fig 9. The effect of different time intervals (5, 10, and 15 min) and various dosages (25-150 mg/L) killing kinetics effect of (i) AgNPs of *A. flavus* and (ii) AgNPs of *A. terreus*, against (A) *L. monocytogenes*, (B) *P. aeruginosa*, (C) *C. albicans* and (D) *A. niger*.

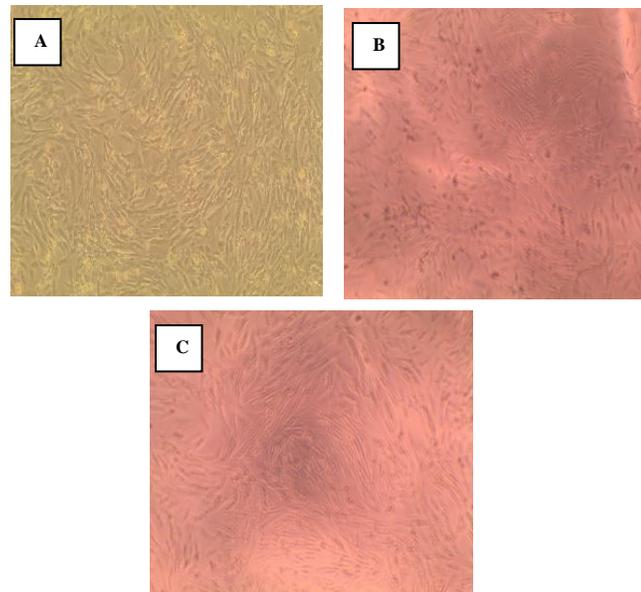


Fig 10. Cytotoxicity of AgNPs of *A. terreus* and *A. flavus* on BJ cells (A) Control of BJ cell line (B) 100 µg/ml of AgNPs synthesized by *A. terreus* (C) 100 µg/ml of AgNPs synthesized by *A. flavus*.

Cytotoxicity of nanoparticles is extensively investigated in vitro due to its ease, control, and interpretation, serving as a preliminary study which mimic in vivo conditions. While qualitative and quantitative tests exist, the latter are preferred for their ability to precisely quantify the number of viable cells (Hanks et al. 1996). Han et al. (2014) demonstrated the enhanced cytotoxicity of biosynthesized AgNPs compared to their synthetic counterparts. Their study revealed that biosynthesized AgNPs had an IC₅₀ value of 20 µg/ml against human lung epithelial adenocarcinoma cell lines, whereas synthetic AgNPs required a higher concentration of 70 µg/ml to achieve the same effect. This finding suggests that biosynthesized AgNPs possess greater efficacy at lower doses compared to synthetic ones.

This study evaluated the cytotoxicity of AgNPs synthesized by *Aspergillus flavus* and *Aspergillus terreus* against human normal fibroblast cells (BJ). While neither AgNP type achieved 50% inhibition (LC₅₀) at the highest tested concentration (100 µg/ml), *A. flavus* AgNPs exhibited 23.4% inhibition at this concentration, compared to 32.5% for *A. terreus* AgNPs. These findings suggest that concentrations below 100 µg/ml could be effective against diseased cells while minimizing harm to healthy cells. This agrees with observations of Magdi et al. (2014), who also reported increasing cytotoxicity of AgNPs with increasing concentration against normal human cells.

Conflict of interest

The authors declare that they have no conflict of interest.

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