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Mycosynthesis, Characterization, Antimicrobial and Antitumor Activity of Silver Nanoparticles Using Endophytic *Trichoderma harzianum* Isolated from Macroalgae *Ulva Lactuca*

Eman A. Attia
Department of Botany and Microbiology, Faculty of Science, University of Suez Canal, Ismailia 41522, Egypt.
*E. mail: eman_mohamed@science.suez.edu.eg

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ABSTRACT

Myco-synthesis of silver nanoparticles has garnered considerable interest and has been the subject of research due to their potential in medical diagnostics and treatments. Endophytic fungi that inhabit macroalgae are capable of producing numerous bioactive compounds. So, in this work, the cell-free aqueous filtrate of the endophytic fungus *Trichoderma harzianum* OR258299 isolated from the green macroalgae *Ulva Lactuca* to generate AgNPs. After being visually identified by a color shift, AgNPs were optimized and characterized by Fourier Transform Infrared Spectroscopy (FTIR), Transmission Electron Microscopy (TEM), UV-Vis spectroscopy, and X-ray diffraction (XRD). Antibacterial, antioxidant, and anticancer properties of the AgNPs were examined. The color changed from yellow to brown, suggesting the formation of biogenic AgNPs. FTIR spectra revealed various functional groups, including alcohols, alkanes, unsaturated ketones, aromatic, nitro, and fluorine compounds. Image analysis using TEM identified spherical and ovate-shaped AgNPs with 9.15–32.5 nm diameters. Among the seven separate deviations were observed in the crystalline nature of the AgNPs complex, seven values 1.35938, 1.96175, 2.04762, 2.35792, 2.62036, 2.77221, and 2.95877 were identified. AgNPs were more effective against *Proteus vulgaris* ATCC 13315, with an inhibition diameter zone of 19±0.577 mm. According to the DPPH assay, the IC_{50} values for Ascorbic acid and AgNPs are 10.21 ±0.77µg/ml and 108.07±3.83µg/ml respectively. Cytotoxicity assay demonstrated that AgNPs were highly cytotoxic against breast cancer cells (MCF-7 cell line), with a 50 % inhibitory concentration (IC_{50}) recording 85.97 ± 3.65 µg/ml. The findings demonstrated the encouraging therapeutic application of green synthetic AgNPs.

INTRODUCTION

Applications of compounds with unusual dimensions, typically on the order of 1–100 nm (Khan et al., 2019), are at the forefront of nanotechnology research. When compared to larger particles of the same type, nanoparticles exhibit extraordinary physiochemical features that result in high reactivity. As a result, both small and large nanoparticles are effective in their respective biological roles Dheyab *et al.* (2020).

Moderate operating conditions, low-cost techniques, and great efficiency are just a few benefits of green synthesis of silver nanoparticles.

Mie *et al*. (2014) Nanotechnology refers to the scientific field concerned with the creation and application of tiny particles. These particles' physical, chemical, and biological characteristics are easily modifiable and can be exploited in a variety of ways (Feynman 2008 **)**. They have drawn extensive research in a variety of disciplines, including biology, chemistry, agriculture, and electronics (Khan *et al.* 2019**)**. The production of food and medicine, as well as the transport of drugs and genes, are all significantly enhanced by the presence of these microscopic particles. Azadpour *et al*. (2022 **)**.

Gold, silver, zinc, and iron are all examples of pure metals that can be used to create nanoparticles with distinct morphologies, physical and chemical properties. AgNPs are the most significant because of the wide range of industries that can make advantage of their special features (K¨up *et al.*, 2020; Nguyen *et al.*, 2020 and Mehata *et al*. 2022).

The benefits of green synthesis of nanoparticles are numerous, offering a rapid, non-toxic, environmentally friendly, cost-effective, and biocompatible alternative to physical and chemical methods (Ovais *et al*. 2018). Microalgae are a kind of living microorganisms that have the unique capability to synthesize a diverse array of valuable bioactive compounds through the utilization of sunlight, carbon dioxide, and water (Al-Hayali and Al-Katib, 2020). Green microalgae are a diverse group of phototrophic microorganisms that are widely recognized as a significant reservoir of chemical substances, such as minerals, lipids, and vitamins (Khorshed and Al-Katib, 2021 ; Yaqut *et al.*, 2022). Microalgae are widely recognized as highly abundant sources of bioactive chemicals derived from secondary metabolism, encompassing various phenolics, alkaloids, and carotenoids (Al-Taie and Al-Katib, 2020). In recent times, there has been a significant focus on utilizing environmentally friendly algal endophyte-mediated nanoparticles as potent antimicrobial agents to manage and control illnesses in both humans and plants. Previous studies have identified that silver nanoparticles (AgNPs) produced by green methods possess diverse biotechnological uses, including antibacterial, anticancer, and biosensing properties (Al-Hasso *et al*. 2022).

The production of nanoparticles can be achieved through three fundamental approaches : chemical, physical, and biological processes. The latter is also referred to as "green manufacturing," which is the preferred strategy according to Abbasi *et al*. (2016).

An endophyte is a microorganism that resides within the host's tissues without creating any visible signs of disease. According to Younis *et al*. (2022). They are regarded as a valuable source of abundant bioactive metabolites that possess significant promise in the fields of medicine, agriculture, and industry. Endophytes have not been extensively studied as a possible source for the manufacturing of silver nanoparticles (Ag NPs).

Various scientific articles have identified bacteria, fungi, yeast, and microalgae as reliable sources of AgNPs with distinct physical sizes, shapes, and chemical properties (Conine and Frost, 2017; Paosen *et al.*, 2017). Fungi are highly regarded as a promising option for producing AgNPs due to their diverse metabolic capabilities. They possess the ability to release various extracellular enzymes that can both reduce and cap the nanoparticles.

AgNPs are essential in medicine, principally as antifungal and antibacterial in a field of drug resistance, they showed better antimicrobial agents against

Escherichia coli, Pseudomonas aeruginosa, and *Candida spp,* (Adriana *et al*. 2024). *Trichoderma reesei* was previously regarded as a non-pathogenic fungus and could generate substantial quantities of extracellular compounds, particularly enzymes, (Oksanen *et al*. 2000). *Trichoderma reesei* was identified for its ability to produce reductase enzymes that convert harmful silver ions (Ag+) into harmless silver nanoparticles (AgNPs) (Vahabi and Dorcheh, 2014). The fungus is renowned for its ease of manipulation in large-scale manufacturing and its rapid growth rate. Research on the utilization of *Trichoderma sp* for the production of silver nanoparticles (AgNPs) is currently restricted in scope. (Saravanakumar and Wang, 2018).

Modifying the reaction parameters, such as adjusting the concentrations of the extracts, controlling the intensity of light, regulating the temperature, stirring the mixture, and managing the pH level, can have a significant impact on the synthesis, characterization, and use of AgNPs (Alharbi et al., 2022). To achieve high-quality synthesis of AgNPs, it was necessary to optimize the reaction conditions, ensuring the nanoparticles had precise sizes and morphologies. (Ahmed and Mustafa, 2020) ; Numan *et al.* (2022).

This research focused on optimizing the production of silver nanoparticles (AgNPs) using a metabolite derived from the endophytic fungus *Trichoderma harzianum*, isolated from the green macroalga *Ulva lactuca*. The primary goal was to enhance the efficacy of these biogenic AgNPs for diverse biological applications as antimicrobial activity, antioxidant activity and anti-cancer activity

MATERIALS AND METHODS

Seaweed Collection :

Seaweed Ulva *Lactuca* specimen was collected from Elfayrouez beach, Ismailia, Egypt, in December 2022. The samples were rinsed thoroughly with seawater to remove epiphytes and other extraneous materials before being transported to the laboratory for endophytic fungi isolation **(**Samae *et al.* ,2015**).**

Isolation of Endophytic Fungi Associated with *Ulva Lactuca:*

The seaweed *Ulva Lactuca* was thoroughly washed in running faucet water and then cut into approximately 0.5 cm2 pieces, for the presence of endophytes. Each fragment was dipped in 70% ethanol for 5 seconds, followed by 10 seconds of immersion in a sterile 1% NaCl solution, as this was found to be sufficient for sterilizing the surface of the soft algal thallus (Suryanarayanan *et al.*, 2010 ; Thirunavukkarasu *et al*., 2011). Following the sterilizing process, samples of Ulva Lactuca were inoculated into Potato Dextrose Agar (PDA) containing a supplement of streptomycin antibiotic (4-5 segments of seaweed per Petri dish). The medium, consisting of 200 grams of potato, 20 grams of D-glucose, 15 grams of agar, per liter, and 1 percent NaCl, Petri dish was securely closed with parafilm and placed in a controlled environment at room temperature for a duration of 2 to 3 weeks. Subsequently, the fungal hypha was extracted from the periphery of the seaweed and transplanted onto the fresh PDA plate, allowing for its cultivation. Following incubation, the samples of fungi were subjected to subculturing and subsequently preserved for additional research purposes.

Identification of Endophytic Fungi by Morphological Characteristics :

The identification of endophytic fungi was primarily based on morphological characteristics and molecular identification. The fungus was recognized visually by lactophenol cotton blue mounting and molecularly using ITS sequencing (Selim *et al*. 2012).

Identification by Molecular Characteristics of Endophytic Fungi :

The identification of the fungus was confirmed as *T. harzianum* using genetic sequencing. The GenBank accession number signifies the deposition of the sequence at NCBI, the National Centre for Biotechnology Information.

The endophytic fungus *Trichoderma harzianum* was selected for molecular identification. The E.Z.N.A. Forensic DNA isolation Kit (Omega Bio-Tek) was utilized for the extraction of genomic DNA from recently harvested mycelia. Omega Bio-Tek Inc. (2013). The ITS region was amplified using 1U of Taq DNA polymerase in a reaction volume of 50µl, using 1× buffer, 2.5 mM MgCl2, 0.2 mM dNTPs, and 0.2 µM of each ITS1 and ITS4 primer. The PCR process commenced with a denaturation step lasting 2 minutes at a temperature of 96°C. This was succeeded by 35 cycles, each consisting of a 1-minute step at 96°C, a 1-minute step at 53°C, and a step at 72°C lasting between 1 and 30 minutes. The ultimate elongation occurred during a duration of 10 minutes at a temperature of 72°C. The PCR procedures for sequencing the β-tubulin gene consisted of 50µl volume and contained the following components : 1× buffer, 2.5 mM MgCl2, 0.2 mM dNTPs, 0.2 µM of each primer (βt2a and βt2b), and 1U Taq DNA polymerase.

The PCR process commenced with a denaturation step lasting 3 minutes at a temperature of 96°C. This was then followed by 35 cycles consisting of 1 minute at 53°C and 2 minutes at 72°C. The ultimate elongation lasted for a duration of 10 minutes at a temperature of 72°C. The PCR results were evaluated by running them on a 1% agarose gel electrophoresis and staining with ethidium bromide. The gel was then visualised under a UV transilluminator. The PCR products were sequenced in both directions using an automated DNA sequencer from Macrogen lnc., Korea. The Cap contig assembly, a supplementary tool in the BioEdit Programme (Biological sequences alignment editor), compiled all primer nucleotide sequences. The sequences were cross-referenced with the Genbank nucleotide databases (Hall's 1999).

Mycosynthesis of Silver Nanoparticles (AgNPs) :

The cultivation of the selected fungus *Trichoderma harzianum* accession number OR258299 was conducted to synthesize AgNPs. This was done by employing modified versions of the protocols described by Xue *et al*. (2016). The fungus was grown in an environment with an ample supply of oxygen. for 10 days at a temperature of 28 °C in a 500 mL Erlenmeyer flask containing 200 mL of Potato Dextrose Broth (PDB). The fungal biomass was collected using filtration and then washed many times with deionized water. A 100 ml sterile deionized water solution was mixed with 10g (wet weight) of fungal mycelia. The mixture was thereafter placed in an incubator set at a temperature of 28°C and subjected to agitation using an orbital shaker working at a speed of 120 revolutions per minute for a length of 48 hours. After incubation, the mixture was filtered using Whatman filter paper No. 1. To promote the formation of AgNPs, a solution of silver nitrate (AgNO3) was introduced into the filtrate at a concentration of 1 mM. The reaction mixture was kept at a temperature of 28°C for 1 hour, with the ratio of cell filtrate to AgNO3 maintained at 1:1 (volume/volume). Controls were employed without the addition of AgNO3. The synthesized AgNPs were obtained and purified using centrifugation at a speed of 15,000 revolutions per minute for 15 minutes, after a color shift to brown after a suitable incubation period. The generated nanoparticles were subsequently rinsed with sterile distilled water to eliminate any remaining contaminants.

UV-Vis Spectroscopy Measurements :

The colour turning brown upon visual observation indicated the existence of silver nanoparticles (AgNPs) in the reaction media. Subsequently, the confirmation of metal ion reduction was achieved by quantifying the absorption using a T 90 + UV/VIS spectrophotometer within the wavelength range of 200-900nm. The dimensions and distribution of the generated AgNPs were assessed based on

the peak of surface plasmon resonance. Through 24 to 96 hours of the reaction, scanning of the absorption was accomplished (Xue *et al.*, 2016 ; Ruby *et al*., 2022).

Optimization of Green Synthesis of AgNPs.

The optimization of processing parameters, such as the AgNO3 concentration, reaction pH, temperature, and duration, was carried out to enhance the caliber of the AgNPs. The study examined several factors, such as pH levels of 4, 8, and 10, temperatures ranging from 30 to 70 °C, substrate concentrations of 1, 2, and 5 mM AgNO3, and reaction durations of 24, 48, 72, and 96 hours. The pH of the mixture was modified by adding either 0.1N HCl or 0.1N NaOH solution. The optimal parameters were employed to synthesize AgNPs of superior grade. The procedure was described in previous research conducted by Devanesan *et al.* (2018). A certaine proportion of the mycelial free-fungal filtrate was introduced into a 1 mM AgNO3 solution, and the process of green synthesis was studied over some time. The hue of the solution transitioned from a light shade to reddish-brown following the reduction of silver ions into AgNPs, thereby validating the reduction process. The reason for the dark brown color of AgNPs in a water-based solution is the stimulation of surface plasmon vibrations within the metal nanoparticles. (Mulvaney, 1999 ; Saware and Venkataraman, 2014). Furthermore, it was asserted that the presence of a consistent dark brown hue provided evidence that each silver ion had undergone complete reduction into AgNPs (Sharma, *et al.* 2018). The absorbance of the colored solution was measured using a UV-vis spectrophotometer.

Characterization Techniques of Silver Nanoparticles (AgNPs):

The AgNPs generated during biosynthesis were examined using ultraviolet-visible spectroscopy (UV-Vis), Fourier-transform infrared (FTIR) spectroscopy, Transmission electron microscopy, and X-ray diffraction (XRD) analysis. The morphology, size, and shape of AgNPs have a substantial impact on their biological production. These factors also have a crucial role in defining the biological activity and toxicity of nanoparticle systems (Hashemi *et al.*, 2020).

Infrared (FTIR) Fourier Transform Spectroscopy:

The formed AgNPs were collected, dried by lyophilization, and then analyzed by Fourier transform infrared (FT-IR). To identify the presence of functional groups, the FT-IR spectra of AgNPs was monitored in the 4000-400/cm $^{-1}$ region using FTIR spectroscopy (Bruker .. Alpha II, with platinum ATR, Germany) via KBr pellet technique (Balakumaran *et al*., 2016).

Transmission Electron Microscopy Analysis:

The size and exterior structure of AgNPs were evaluated using transmission electron microscopy (TEM) at the Electron Microscope Unit of the Regional Center for Mycology and Biotechnology, Al AZHAR University, Egypt. An aliquot of approximately 10 microliters of the reaction solution containing silver nanoparticles (AgNPs) was deposited onto a copper grid coated with carbon. The grid was subsequently left at ambient temperature for the duration of the night. Afterward, the grid was inserted directly into the specimen container, using the procedure outlined by Sayed *et al*. (2022). The nanoparticles were visualized and their size was randomly assessed and documented in nanometers.

X-ray Diffraction (XRD) Measurement:

The nanoparticles were subjected to X-ray diffraction (XRD) utilizing Cuka radiation (k = 1.5406) through the X' Pert PRO Philips Analytical-PW 3040/60. The sample was scanned from 25° to 80° while being evaluated at 40 kV and 30 mA in a 2 h angle pattern. To determine the crystalline structure of nanoparticles, the micrograph produced by scanning was compared to the Joint Committee on Power

Diffraction Standards Library **(**Sun and Xia, 2002).

Antimicrobial Activity of Myco-Synthesized AgNPs:

The effectiveness of the synthesized silver nanoparticles (AgNPs) in killing microorganisms was assessed using the disc diffusion method against two types of bacteria, *Bacillus subtilis* and *Staphylococcus aureus*, which are classified as Gram-positive, as well as two types of bacteria, *Escherichia coli* and *Proteus vulgaris*, which are classified as Gram-negative. Additionally, the efficacy of the AgNPs was tested against two types of fungi that are harmful to humans, *Aspergillus fumigatus* and *Candida albicans*. The evaluation centered on studying the rate of growth and establishing the minimal concentration at which growth is inhibited (MIC). The research was carried out at the Regional Centre for Mycology and Biotechnology, which is affiliated with Al Azhar University, located in Egypt. The bacterial cultures were introduced into 5 mL of aseptic nutrient broth and subjected to incubation at a temperature of 37 ◦C until the degree of cloudiness corresponded to that of the 0.5 McFarland standard. The sterile Mueller Hinton agar (MHA) plates were inoculated with the bacterial broth to produce a culture that was free from contamination. The 6 mm sterile discs were submerged in a solution containing silver nanoparticles (0.25 mg/mL) overnight. The discs were thereafter organized in a consistent fashion on MHA plates and let to spread out at a temperature of 4 ◦C for 4-5 hours. The experimental positive control consisted of Gentamycin, a bactericidal agent, and Terbinafine hydrochloride, an antifungal agent, both at a dose of 25 µg/disc. On the other hand, the negative control was shown by the culture filtrate. Subsequently, the plates were placed in an incubator adjusted at a temperature of 37 ◦C. The following day, the size of the zone of inhibition was assessed. The experiments were conducted three times, and the average data were recorded (Abdel-Kareem and Zohri, 2018 ;(Sumitha and Senthil, 2020).

Antioxidant Assay:

The extract's antioxidant activity was assessed at the Regional Centre for Mycology and Biotechnology (RCMB) at Al-Azhar University. The measurements were conducted three times, and the average values were considered**.**

DPPH Radical Scavenging Activity:

The assessment of antioxidant activity was performed using the DPPH technique. The protocols of Adebayo *et al*. (2019) and Jahan *et al*. (2021**)** were implemented with minor modifications. The efficacy of AgNPs in scavenging DPPH was evaluated using the spectrophotometric method **(**Mahdi ***et al.,* 2021).** For each ascorbic acid and AgNPs, a series of methanol-based solutions was prepared with different concentrations ranging from 0.5 to 1000 µg/ml. 0.36 grams of DPPH were dissolved in 4 milliliters of methanol. A 0.15 mL volume of the DPPH solution was mixed with 3 mL of each of the prepared concentrations, while deionized water was used as the control. The tubes were kept undisturbed in a lightless atmosphere for a period of 30 minutes. Afterward, the measurement of absorbance was taken for each tube at a wavelength of 517 nm. The results obtained for each extract were compared to the positive control. The antioxidant capacity was quantified by determining the percentage of blockage of the DPPH using the approach outlined by Lateef *et al.* (2018).

PI = [{(AC- AT)/ AC} x 100] (1)

Where AC = Absorbance of the control at t = 0 min and AT = absorbance of the sample+DPPH.

The 50% inhibitory concentration (IC50), which indicates the concentration required to achieve 50% DPPH radical scavenging activity, was calculated by evaluating the dose-response curve using GraphPad Prism software (San Diego, CA, USA).

Evaluation of Cytotoxicity against MCF-7 Cell Line :

Cell Lines and Cell Cultures:

The MCF-7 cells, a cell line derived from human breast cancer, were acquired from the American Type Culture Collection (ATCC) located in Rockville, MD. The ingredients Dimethyl sulfoxide (DMSO), MTT, and trypan blue dye were acquired from Sigma (St. Louis, Mo., USA). The subsequent items were acquired from Lonza (Belgium): Foetal Bovine serum, DMEM, RPMI-1640, HEPES buffer solution, L-glutamine, gentamycin, and 0.25% Trypsin-EDTA. The experiment was conducted at the Regional Centre for Mycology and Biotechnology (RCMB) of Al-Azhar University.

***In Vitro* Assay for Cytotoxicity Activity (MTT Assay):**

Using the MTT assay, the cytotoxicity of samples containing green-synthesized AgNPs dissolved in 0.3 DMSO (AgNPs) was determined. A prior study by Mosmann (1983) was followed to conduct an MTT experiment. The cells were seeded in 96-well plates at a density of 1×10^4 cells per well, using DMEM as the medium. The cells were let to adhere to the plates by incubating them for 24 hours at 37°C in an atmosphere with 5% CO2. Following a 24-hour incubation period, two sets of cell cultures were subjected to various concentrations of treatments (3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, and 500 µg/mL), while the control cell cultures remained untreated. After incubation, the current media was removed and 100 L of MTT solution was introduced into each well. Subsequently, the 96-well plates were incubated for an additional duration of 3-4 hours, sufficient for the production of formazan crystals. Substituting the medium with 100 L of a DMSO solution, the optical density (OD) was subsequently determined at 570 nm using an ELISA reader. The percentage of viable cells following treatment with one of the three pharmaceuticals employed for breast cancer was determined using the subsequent mathematical expression.

$$\% \text{ Cell Viability} = \frac{\text{Absorbance treated cells} - \text{Absorbance blank}}{\text{Absorbance control cells} - \text{Absorbance blank}} \times 100$$

Blank refers to the background, which means the medium, MTT solution, and DMSO. (ii) Control cells refer to untreated cells.

Statistical Analyses :

The statistical analysis was conducted using SPSS. The data underwent one-way analysis of variance (ANOVA), and Tukey's post hoc test was utilized to evaluate statistical significance at a significance level of $P < 0.05$.

RESULTS AND DISCUSSION

Seaweeds as green alga *Ulva Lactuca* are considered a source of bioactive compounds due to their ability to generate a wide range of secondary metabolites, including antiviral, antifungal, antibacterial, anticancer, and antioxidant compounds. (Moubayed *et al*., 2017). Many endophytic fungal species were isolated from green algal thalli. Endophyte-host interactions are affected by environmental factors as well as plant and fungal genetics and nutrition. This may explain endophyte diversity from some algae species (Manomi *et al.*, 2015).

Identification of the Endophytic Fungi :

Classical mycology describes the majority of endophytic fungi based on their morphological characteristics, such as ascospore and peridium morphology, and finally odor (Fig.1). In this study, the fungal isolates could be identified at the genus level utilizing conventional morphological techniques. There were four endophytic fungi isolated from *Ulva Lactuca* such as *Penicillum spp, Aspergillus fumigatus, Aspergillus niger,* and *Trichoderma harzianum.* Our findings indicated that *Trichoderma harzianum* was the most common isolated fungal endophyte. Therefore, the *Trichoderma harzianum* isolate was chosen for further study. Based on molecular identification *Trichoderma sp* was identified as a *Trichoderma harzianum* with accession number (OR258299).

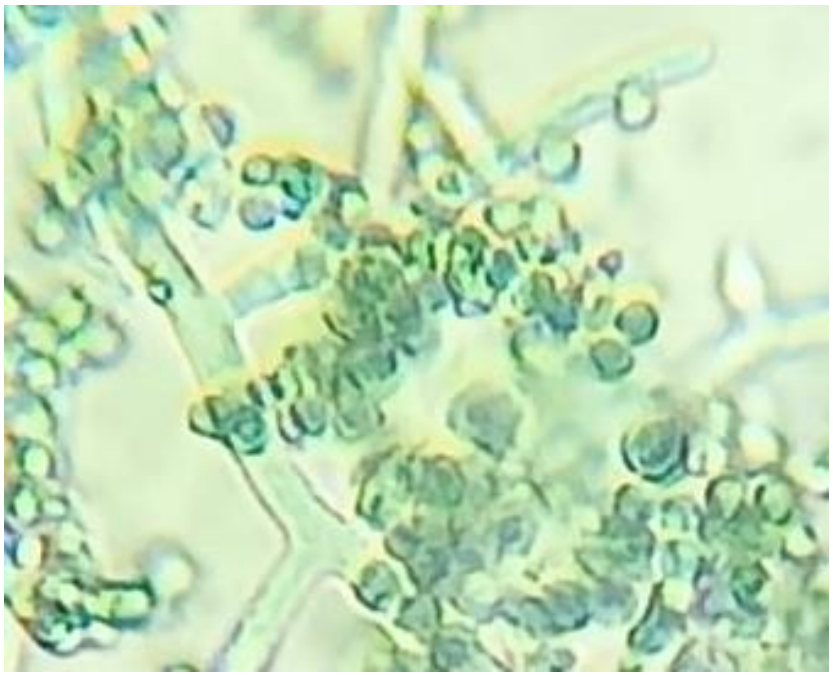

Fig .1 ***Trichoderma harzianum*** **X40**

Mycosynthesis, Optimization, and characterization of AgNPs using *Trichoderma harzianum* extract **:**

It has been discovered that the fungal system is a flexible biological system with the capacity to synthesize metal nanoparticles outside of the cells as reported by Rai ***et al.*** **(2021).** By observing visually, the color was changed over time from light yellow to dark brown after 24 h incubation (Fig.2). The present work successfully created AgNPs from an aqueous solution of AgNO3 employing *Trichoderma harzianum* cell filtrate.

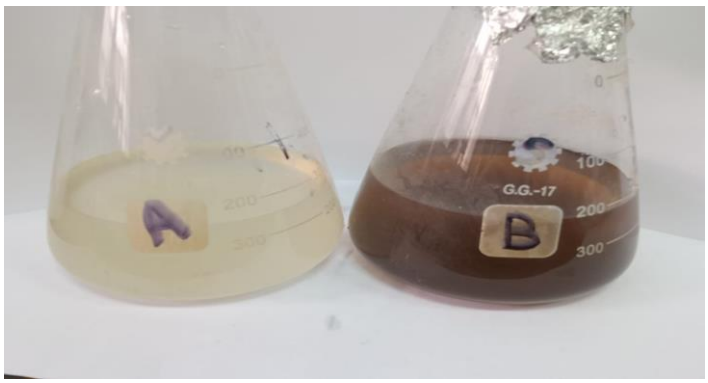

Fig. 2**.** Biosynthesis of AgNPs by *Trichoderma harzianum* A: Aqueous fungal extract. B: AgNO3 solution with aqueous fungal extract after incubation

The same observation was reported by Gemishev *et al*. (2019), who confirmed the AgNPs biosynthesis from *Trichoderma reesei* which can be observed visually by the color changing, which indicates the reduction of Ag+ to Ag0 by reducing agent present in the aqueous fungal extract as metabolites and proteins.

Previous research on nanoparticles has shown that the observed change in color in a water-based solution is caused by the vibrations of the nanoparticles' surface plasmons (Khan *et al.*, 2018). The extracts consist of many biomolecules, including enzymes, proteins, amino acids, exopolysaccharides, and vitamins, that

facilitate the transformation of silver ions into silver nanoparticles. The synthesis of AgNPs is typically ascribed to the existence of the nitrate reductase enzyme in the microbial extract, as regarded by Anil *et al.* (2007) and Nahar *et al.* ,(2020). The AgNPs exhibited an absorbance peak at a wavelength of 400 nm, as illustrated in Figure 3. The intensity of the absorbance peak increased proportionally with the duration of the reaction, which ranged from 24 to 96 hours, whereas the peak at 400 nm remained unchanged.

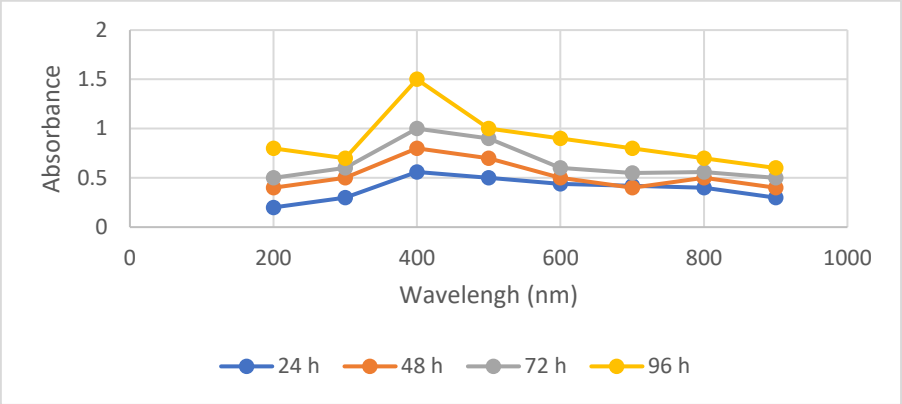

Fig. 3 UV–visible absorption spectrum of AgNPs biosynthesized by the reduction of AgNO3 solution with the cell filtrate of *T. harzianum* with different time intervals

The maximum absorbance of the silver nanoparticles that were created was seen at a wavelength of 400 nm. The present study employed UV-visible spectroscopy to detect silver nanoparticles, which exhibited a positive correlation with reaction time. This correlation suggests a gradual reduction of AgNO3 into AgNPs over time. The researchers Muthukrishnan and colleagues (2015) have declared that the evaluated incubation times allow for the highest level of absorbance to be recorded. This indicates the highest concentration of artificially manufactured AgNPs. Furthermore, this study clearly showed that there was no alteration in the highest point at 400 nm following two weeks of maintenance. This finding highlights the exceptional durability of the nanoparticles that were created by Krishnaraj *et al*. (2012).

Pereira *et al.*, (2014) also documented the same findings utilizing *Penicillium chrysogenum*. The ongoing work entails utilizing *Trichoderma harzianum* OR258299 as an eco-friendly approach for the manufacture of silver nanoparticles.

Out of the various concentrations of silver nitrate examined for the synthesis of AgNPs, the 1.0mM concentration was shown to be crucial in enabling the synthesis of AgNPs with a high concentration (Fig. 4). Hence, a concentration of 1.0mM was selected for the remaining tests in this investigation because to its superior absorbance in comparison to other substrate concentrations.

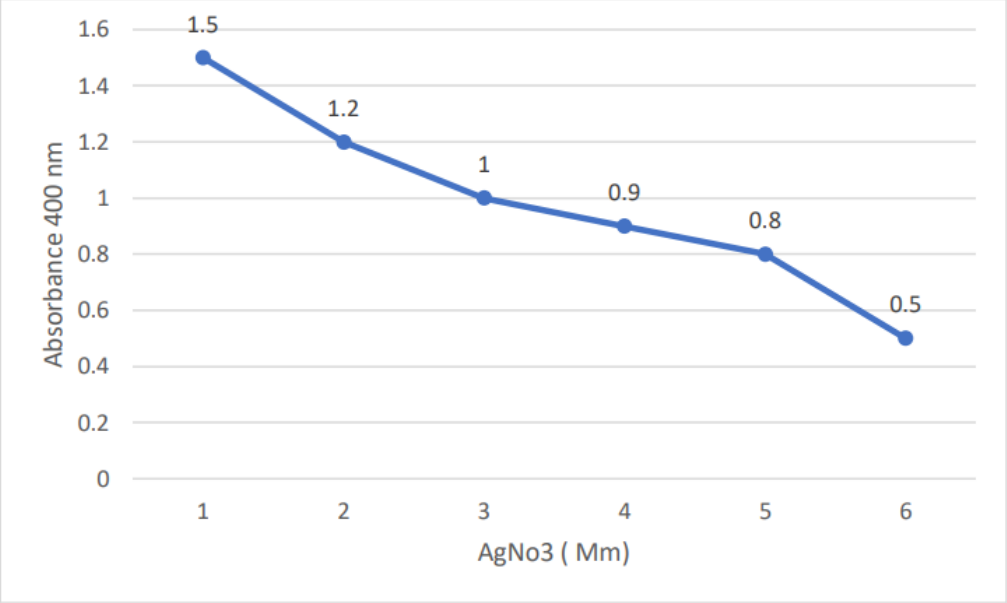

Fig. 4. Effect of different concentrations of AgNO3 (mM) on myco-synthesis of AgNPs by the mycelial-free extract of *T. harzianum*

Ahmad *et al*. (2003) and Ingle *et al.* (2009) found that a substrate concentration of 1.0 mM AgNO3 was the most effective for the biosynthesis of AgNPs in their respective studies. The presence of excessive silver ions in the reaction media can lead to the creation of large nanoparticles with irregular shapes due to competition with functional groups from the fungal filtrate (Abdel-Rahim *et al.*, 2017; Shahzad *et al*., 2019).

The study investigated the impact of temperature by subjecting the reaction mixture to incubation at specific temperatures: 30, 40, 50, 60, and 80 degrees Celsius. The findings demonstrated that raising the reaction temperature enhanced the biosynthesis of AgNPs, with the optimal temperature for AgNPs synthesis activity being 50°C.The duration required to reach peak production of AgNPs was decreased., as shown in Figure 5. A clearly defined peak emerged after 24 hours and was further amplified by extending the timeframe to 96 hours. Nevertheless, there was no noteworthy modification thereafter.

The synthesis and stability of nanoparticles were also impacted by the reaction temperature. The synthesis rate, as well as the size and stability of the resulting nanoparticles, may be affected by the temperature utilized in the process (Elamawi *et al*., 2018). The reaction time was shortened and the absorbance of the AgNPs was increased at higher temperatures. Possible explanation: faster kinetics at higher temperatures. So, it stands to reason that at low temperatures, not only will more time be needed to complete the initial synthesis of AgNPs, but also that the labile components necessary for the reaction will be maintained without denaturation for a considerable length of time. The transfer of electrons from free amino acids to silver ions has been seen during the creation of nanoparticles by certain fungi at high temperatures (Guilger-Casagrande and de Lima, 2019).

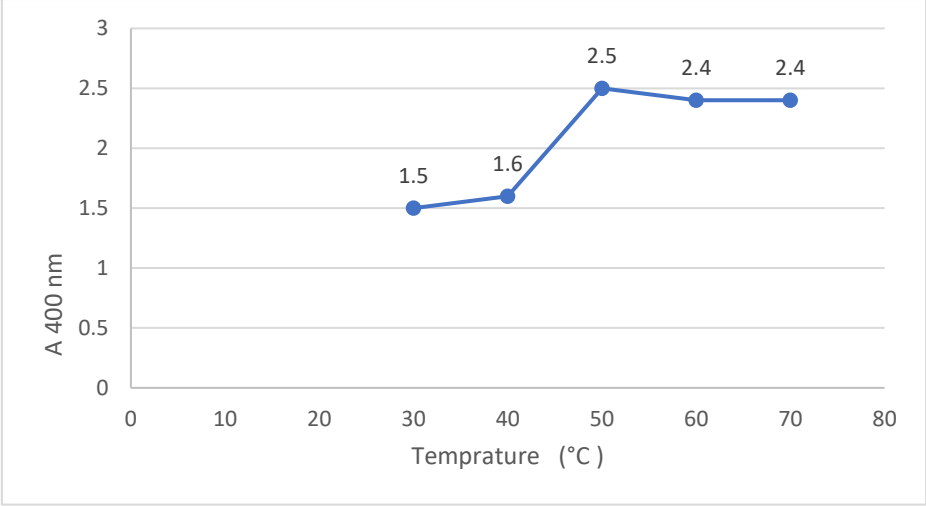

Fig. 5. The manufacture of silver nanoparticles (AgNPs) using the mycelial free extract of *T. harzianum* was conducted at various temperatures of the reaction mixture.

The pH of the reaction mixture was the last variable investigated in this study's attempt to optimize the synthesis of AgNPs (Fig.6). The optimal pH for AgNPs production has been reported to change depending on the kind of microbe used. In this investigation, we evaluated pH ranges from 2 to 12, finding that the most active range for AgNPs generation was between 8 and 10. At acidic pH (2-4), no color change was seen in AgNPs produced by *T. harzianum* (OR258299). Brown color creation started at pH 7, 9, and 11; and the intensity of the brown color rose as the pH value increased. Nanoparticle production and stability were significantly influenced by the reaction mixture's pH (Mishra *et al.*, 2011). In the present study, we found that the alkaline PH 10 value resulted in the highest activity. Similarly (Birla *et al.*, 2013) found that the optimal pH for AgNPs generation by *F. oxysporum* filtrates was 9. This might be because there are more OH- ions in the environment to donate electrons to the reduction of Ag+ to Ag0. According to research by Gurunathan *et al.* (2009), OH- ions have a significant impact on the adsorption, stability, and aggregation of AgNPs. According to Birla *et al.* (2013), proteins in fungal filtrates may bind to Ag+ more quickly at higher pH values, making the myogenesis of AgNPS quicker in an alkaline environment compared to an acidic one.

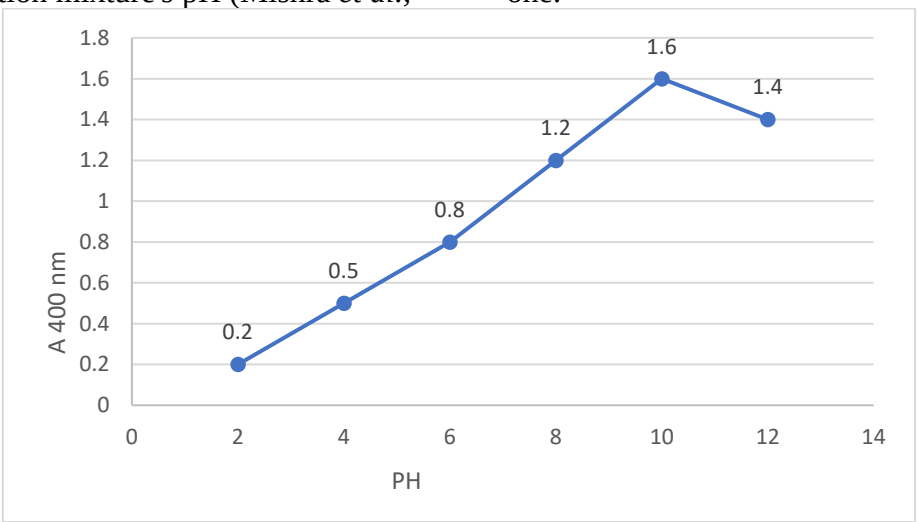

Fig. 6. Mycosynthesis of AgNPs by the mycelial free extract of *T. harzianum* at different pH of the reaction mixture.

Characterization Techniques of Silver Nanoparticles (AgNPs):

FTIR Analysis :

The utilization of Fourier Transform Infrared (FTIR) analysis facilitated the identification of the diverse biomolecules present in the silver nanoparticles (AgNPs**)** that are responsible for the capping and effective stabilization of the silver nanoparticles (AgNPs). In Figure (**7**), FTIR analysis of the biologically produced silver nanoparticles (AgNPs) exhibited many absorption peaks in which the FTIR absorption bands of biosynthesized AgNPs are depicted. The results indicated the presence of an alcohol group as evidenced by a large and robust peak observed a **3275**cm^{-1}, which corresponds to the stretching (O-H) functional group. As reported by Devaraj *et al. (*2014); Fatima *et al*. (2020); Vinodhini *et al*., (2022) and Attri *et al.*, 2023). The presence of the alkane group was observed at 2921 cm^{-1}, which corresponds to the (C-H) functional group (Vinodhini *et al.*, (2022); Fatima *et al*.,2020). A prominent peak, attributed to the (C-H)bonding, in the aromatic compound was seen at a wavenumber of 1744 cm-^{1} Additionally, notable peaks were identified at 1627cm^{-1}, corresponding to(C=C) stretching in unsaturated ketone, 1536 6 cm^{-1}, corresponding to(N-O) stretching in a nitro compound, Moreover, the observed absorption peaks at wavenumbers 1402cm^{-1} and 1048 cm^{-1} can be attributed to the functional groups (S=O) stretching vibrations in Sulfonate) and (C-F stretching) in fluoro compound respectively. by Devaraj *et al. (*2014); Fatima *et al*. (2020); Vinodhini *et al*., (2022) and Attri *et al.* (2023). The FTIR spectra were detected the provided evidence of exist of a diverse range of functional groups located at different locations. These biochemical constituents may be responsible for the encapsulation and stability of the synthesized Ag ions in the solution. These groups included alcohol, alkanes, and aromatic. Additionally, the reduction of AgNO3 to AgNPs was facilitated by a diverse range of bioactive compounds. (Patel ,2013).

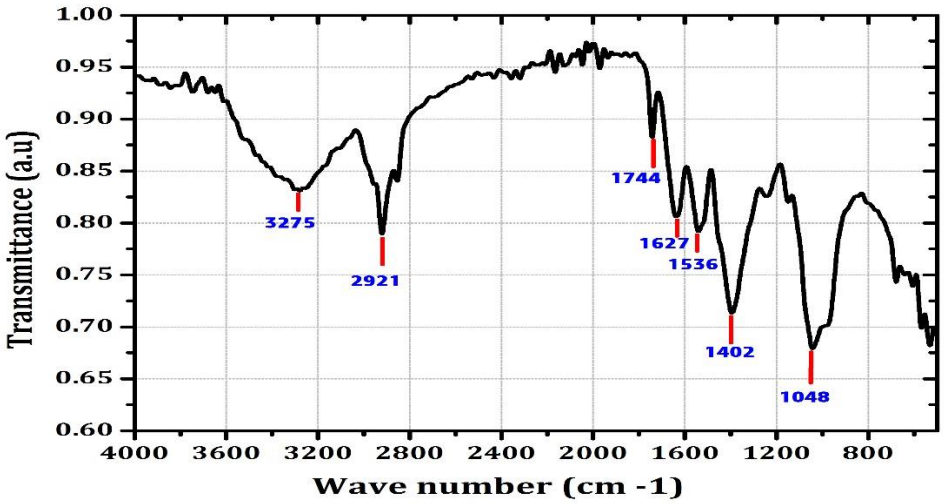

Fig. 7. FTIR spectrum of synthesized AgNPs.

Transmission Electron Microscopy (TEM):

The high-resolution transmission electron microscopy (HRTEM) was used to analyze the particle characteristics, including form, size, and morphological distribution of the silver nanoparticles (AgNPs) that were generated using the

mycelial free extract of *Trichoderma harzianum*. The TEM micrographs depicted in Figure 8, that illustrated a significant proportion of the particles have a spherical or ovate shape. The resulting images in our results produced by TEM revealed the presence of nanoparticles with spherical, and ovate shapes. The biosynthesized AgNPs were mostly round, with diameters between 9.15 and 32.5 nm. The particle shapes discovered in this investigation exhibit similarities to those reported by Devaraj *et al*. (2014) and Attri *et al*. (2023) In each of these experiments, the researchers examined the morphology of produced silver nanoparticles (AgNPs) using leaf extracts from *T. divaricata*, which demonstrated a mostly spherical particle form. In contrast, Vinodhini *et al*. (2022) reported that the presence of rod-shaped structures, with an average particle size ranging from 40 to 57 nm. Furthermore, in our finding the transmission electron microscopy (TEM) pictures provided evidence of a thin layer enveloping the silver nanoparticles (AgNPs). Similarly, Mallikarjuna *et al*. (2011) suggested that the observed films may potentially consist of functional groups that act as capping agents for the produced AgNPs. Moreover, it has been proposed that the application of capping functional groups could provide additional stability to the silver nanoparticles (AgNPs) present in the solution (Mittal *et al*., 2013). Furthermore, there have been reported that the characteristics of silver nanoparticles (AgNPs), including their size being less than 100 nm and their various shapes such as spherical, ovate, and triangular, make them very suitable for effectively targeting and inhibiting biological processes within microorganisms (Safavi, 2012).

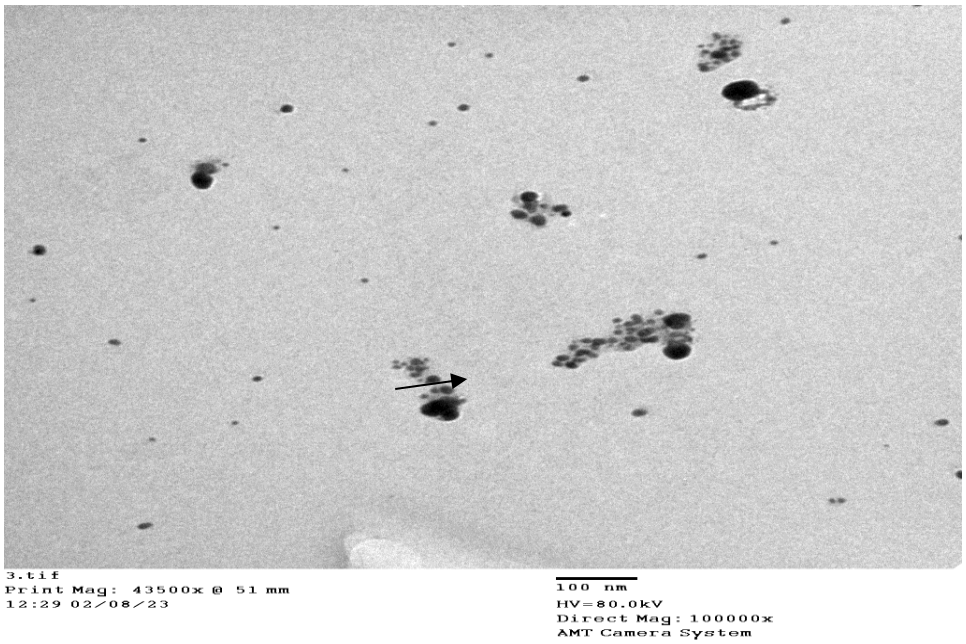

Fig. 8. Resolution Transmission Electron Microscopy images of synthesized AgNPs from *T. harzianum*

X-ray Diffraction (XRD):

Research was conducted to validate the crystalline character of the complex formed by the Ag NPs. In these results, A total of seven distinct instances of deviation were identified at the following values : 1.35938, 1.96175, 2.04762, 2.35792, 2.62036, 2.77221, and 2.95877 (Theta 10.000). The X-ray diffraction (XRD) pattern is depicted in Figure 9. The presence of silver nanoparticles indicated a cubic structure (fcc) centered on the face.

The findings shown here align with the results given by (Wiley *et al.* (2006); Mohanpuria *et al*.(2008); Schrand *et al.* (2008); Bansal *et al.* (2010); Bansal *et al.* (2011) and Elgorban *et a.l*(2016).

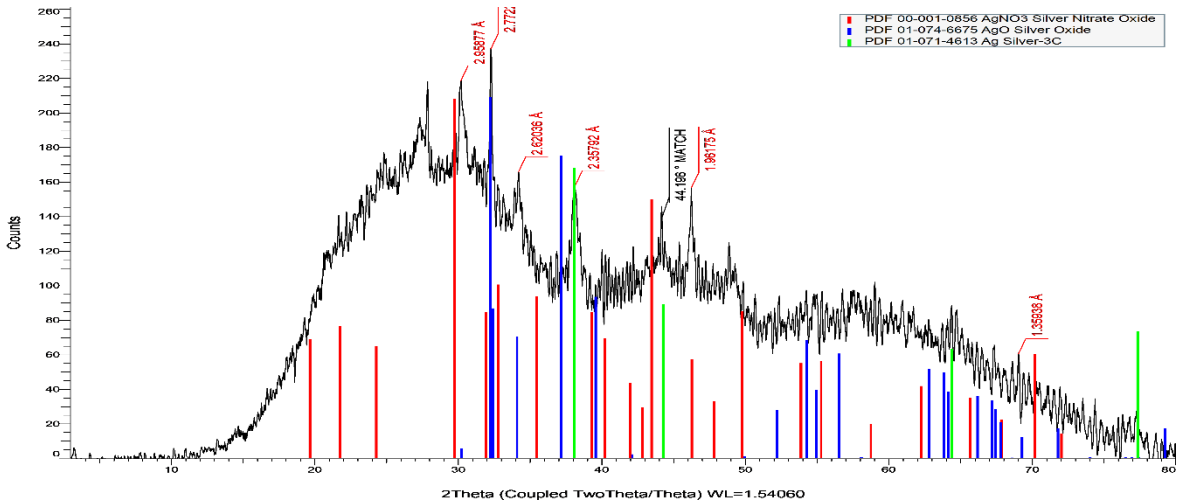

Fig.9: Scale values XRD of major synthesized bio-nanoparticles produced by selected *Trichoderma harzianum*

Antimicrobial Assay of Biosynthesized AgNPs :

The present work exhibited the antimicrobial properties of silver nanoparticles synthesized by *Trichoderma harzianum* OR258299 (via extracellular synthesis) using an Agar well-diffusion assay against four human pathogenic bacteria (two gram-positive and two gram-negative) including *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Proteus vulgaris* respectively, as well as two human pathogenic fungi, *Aspergillus fumigatus* and *Candida albicans*. The study focused on analyzing the growth kinetics and determining the minimum inhibitory concentration (MIC).

The mean inhibition zone against *Proteus vulgaris* was determined to be 19±0.577 mm, which was significantly greater than the mean inhibition zones observed for *Staphylococcus aureus*, *Bacillus subtilis*, and *Escherichia coli*, which were 15±2.309, 15±2.309, and 15±0.577 mm, respectively, for each respective species. Conversely, silver nanoparticles had a detrimental antifungal effect against *Aspergillus fumigatus* and *Candida albicans*. The minimum inhibitory concentration (MIC) of the produced silver nanoparticles on *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Proteus vulgaris* bacterial species were 312.5, 2500, 312.7, and 156.25 µg/ml, respectively, as indicated in (Table 1,and Figure 10), Prior research has investigated the antibacterial properties of different biosynthesized silver nanoparticles (AgNPs) and their potential to effectively hinder the growth of tested bacteria when produced by microorganisms or medicinal plants as explained by Vahabi *et al*.(2011); Devi *et al*.(2012) ; Pantidos and Horsfall, (2014); Alghuthaymi *et al.*, (2015); Vijayan *et al.*, (2016); Guilger *et al.*, (2017); Kamil *et al.*, (2017); Liang *et al*.(2017) and Mohamed *et al*. (2017).

Table.1. Antimicrobial activities of biosynthesized AgNPs

Tested microorganism	Fungal filtrate	AgNo3	Nano	Gentamycin	Terbinafine hydrochloride	Nano MIC(µg/ ml)
Mean values of inhibition in mm						
Aspergillus fumigatus	Nil	30±1.732 ^{b}	Nil	-	40±1.155 ^{c}	Nil
Candida albicans	Nil	30±2.309 ^{c}	Nil	-	16±1.443 ^{b}	Nil
Staphylococcus aureus	Nil	13±1.617 ^{b}	15±2.309 ^{b}	28±1.155 ^{c}	-	312.5
Bacillus subtilis	Nil	14±1.732 ^{b}	15±2.309 ^{b}	43±0.577 ^{c}	-	2500
Escherichia coli	Nil	13±2.367 ^{b}	15±0.577 ^{b}	25±1.155 ^{c}	-	312.7
Proteus vulgaris	Nil	23±2.309 ^{b}	19±0.577 ^{b}	37±1.732 ^{c}	-	156.25

Data are presented as mean ± standard error based on triplicate measurements. Values in the same column marked with different superscript letters (a, b, c) are significantly different ($p < 0.05$)."

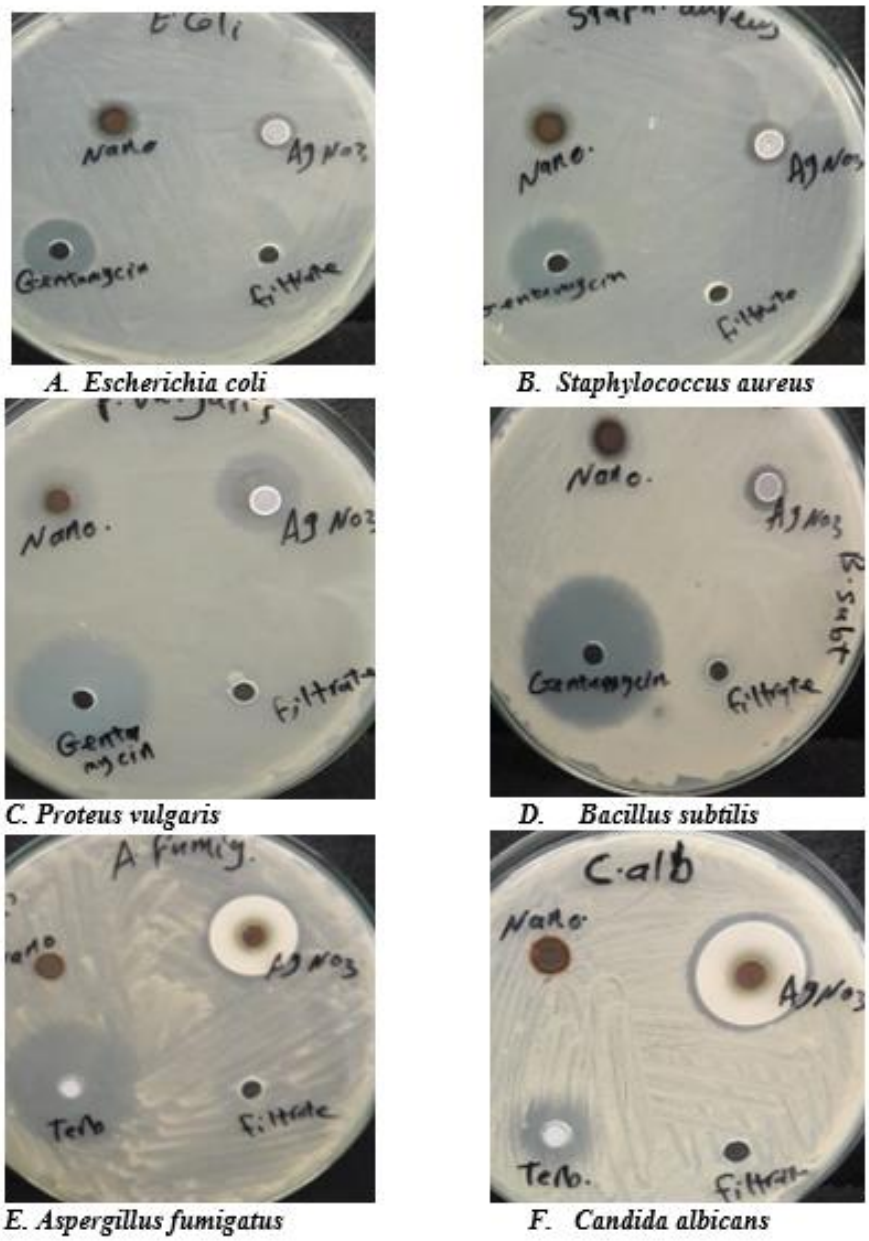

Fig. 10. Antimicrobial activity of silver nanoparticles synthesized using *Trichoderma harzianum* filtrate, as determined by diffusion method. (A*) Escherichia coli* ; (B) *Staphylococcus aureus* ; (C) *Proteus vulgaris* ; (D) *Bacillus subtilis*; (E) *Aspergillus fumigatus*; (F) *Candida albicans*

Positive control streptomycin and Terbinafine hydrochloride. AgNPs: silver nanoparticles; AgNO3 silver nitrate; Negative control (*Trichoderma harzianum* culture filtrate).

Antioxidant Activities :

The percentage of DPPH radical scavenging activity exhibited by biosynthesized AgNPs at various concentrations (ranging from 0.5 to 1000 µg/ml) when compared to Ascorbic acid as the reference, varied between 3.18% and 80.93%**.** The DPPH scavenging activity of the synthesized silver nanoparticles (AgNPs) was assessed by observing the color change from violet to yellow, which indicated the creation of diphenyl picryl hydrazine. The standard Ascorbic acid demonstrated the highest level of reducing power, with a scavenging efficacy of 98.65% at a concentration of 1000 µg/ml (Fig 11). The IC_{50} values were derived by analyzing the regression graph.

The antioxidant activity of silver nanoparticles (AgNPs) was tested in this study using the DPPH free radical scavenging strategies. DPPH is a chemically stable molecule that undergoes reduction through the acquisition of electrons or hydrogen, making it a commonly employed method for evaluating antioxidant properties. The DPPH scavenging activity of silver nanoparticles (AgNPs) was measured, yielding an IC_{50} value of 108.07±3.83µg/ml. In comparison, the IC_{50} value of Ascorbic acid was found to be 10.21 ± 0.77 µg/ml. The inverse relationship between IC_{50} values and DPPH scavenging activity has been observed. Metal-derived free radicals induce oxidative stress, wherein reactive oxygen species (ROS) lead to the degradation of bacterial cell walls, DNA, and mitochondria, ultimately culminating in cell death. the antioxidant activity of silver nano-particles was reported by previous researchers such as Tamboli and Lee, (2013). The silver nanoparticles (AgNPs) displayed much greater DPPH scavenging ability in a concentration-dependent manner, as evidenced by an IC_{50} value of 108.07±3.83µg/ml This value exhibits a notable increase compared to the values reported in prior studies (Mohanta *et al.* *(*2017); Patra and Baek, (2016). Hence, the bio-synthesized silver nanoparticles (AgNPs) exhibited concentration-dependent antioxidant activity, indicating potential as a supplementary natural antioxidant." aiding in the regulation of antioxidant, pro-oxidant, and reactive oxygen species (ROS) levels.

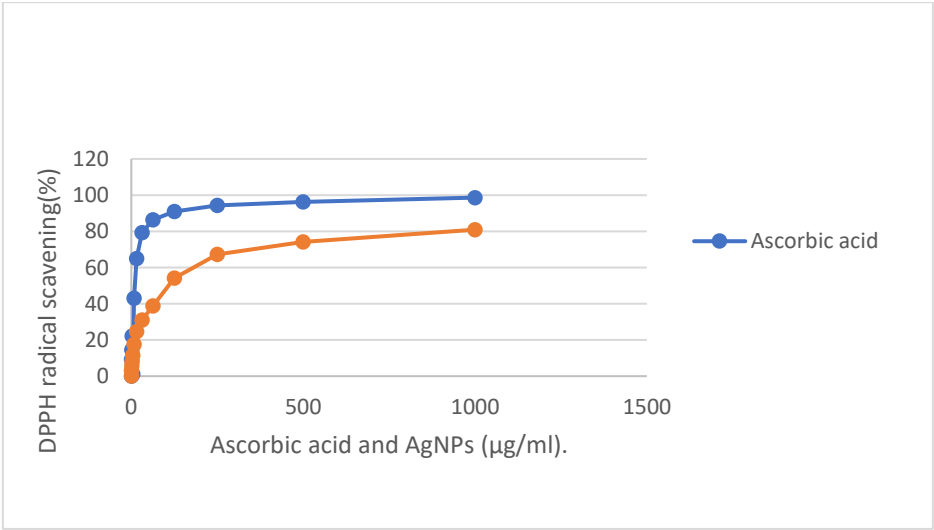

Fig. 11. Antioxidant activity of Ascorpic acid and *Trichoderma harzianum* based silver nanoparticles. IC_{50} of Ascorbic acid sample and Ag NPs under these experimental conditions equal 10.21 ± 0.77 µg/ml and 108.07±3.83µg/ml respectively.

Cytotoxicity Assay :

The toxicity of silver nanoparticles AgNPs depends on their size and stability, as reported by Lankveld *et al.* (2010). The present study revealed that the AgNPs exhibited inhibitory or damaging effects on cancer cells that varied with concentration and had no impact on normal cells, as evidenced by the results presented in Figure 12. The silver nanoparticles (AgNPs) significantly affected the viability of human breast cancer cells (MCF-7 cell line) following 24 hours of exposure to different doses of AgNPs.The cell viability diminished in a concentration-dependent way with sample exposure. Treatment of MCF-7 cells with 62.5 µg/mL AgNPs for 24 hours drastically reduced cell viability from 100% (untreated cells) to 39.02%. The IC_{50} value of the sample was 85.97 ± 3.65 µg/ml.

The research examined the anisotropic characteristics of silver nanoparticles (AgNPs), previously studied by Bharathiraja *et al*. (2016), who found that anisotropically structured AgNPs induce cell death at a higher rate than gold nanoparticles (AuNPs). The effects of silver nanoparticles were documented in MCF-7 human breast cancer cells and IMR 90, U251, and A549 lung cells by El-Kassas and El-Sheekh (2013), and in MDA-MB-231 cells by Asharani *et al*. (2009), Ahmad *et al.* (2008), and Gurunathan *et al.* (2013). The prospective anti-cancer efficacy of AgNPs may be ascribed to the surface coating of phytoconstituents and their diminutive size. Que *et al.* (2019) found that smaller nanoparticles exhibit enhanced anticancer activity due to their extensive specific surface area, resulting in more active sites. A plausible reason is that little nanoparticles can infiltrate cells through endocytosis or direct diffusion.

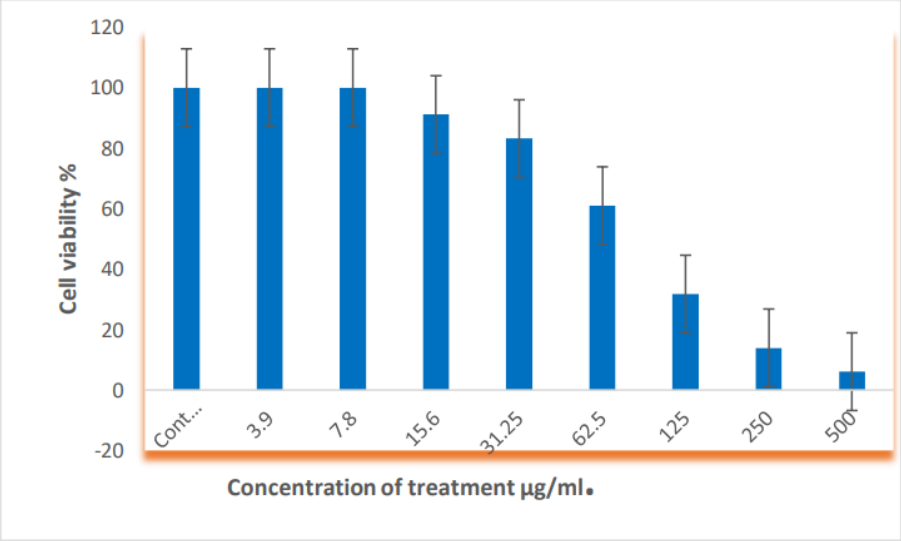

Fig.12.: Cytotoxicity of AgNPs on the viability of MCF7 cancer cells after 24 h exposure compared with control (untreated cells). IC50 = 85.97 ± 3.65 µg/ml.

Conclusion

The field of nanotechnology has had significant expansion in recent years and has been widely applied in the realms of healthcare, industry, and the environment. The silver nanoparticles produced from the culture filtrate of *T. harzianum* were determined to be stable and exhibited significant bioactivities. Hence The synthezied AgNPs demonstrated a wide range of antibacterial effectiveness against both Gram-positive and Gram-negative bacteria. Moreover, they demonstrated remarkable antioxidant properties.

Additionally, the present investigation demonstrated the existence of various biological activities, including the cytotoxicity potentiality. The methodology employed in this work for the eco-friendly production of AgNPs is efficient, cost-effective, environmentally sustainable, non-hazardous, and suitable for mass production. However, further investigation is required to confirm the existence of other biological functions, such as antidiabetic and anti-inflammatory properties, and to comprehend the underlying mechanism of action.

Abbreviations

AgNPs : Silver Nanoparticles
CFE : Cell-free water extract
TEM : Transmission Electron Microscopy
XRD: X-ray diffraction

Declarations:

Ethical Approval: This research was approved by URAF-IACUC reviewers (NO URAF I n.V. 1 24)

Funding: No funding was received.

Availability of Data and Materials: All datasets analyzed and described during the present study are available from the corresponding author upon reasonable request.

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