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Antibacterial activity of nanosilver against *Escherichia coli* and *Pseudomonas aeruginosa*

Nada H. Ghazala [1*], Ashraf Elsayed [1], Attiya Mohamedin[1], Mohamed O. Abdel-Monem[2]

[1] *Botany Department, Faculty of Science, Mansoura University, Egypt*

[2] *Botany Department, Faculty of Science, Banha University, Egypt*

**** Correspondence to:** Nada89_hosni@yahoo.com*

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Abstract: Multidrug resistance bacteria are a major health concern so new strategies for controlling bacterial activity such as nanoparticles are urgently needed. *Fusarium oxysporum*-based biological approaches for nanoparticle synthesis have been proposed as promisingly green processes. By using UV-visible spectroscopy, which demonstrated a maximum absorption at 434 nm, biosynthesized silver nanoparticles (AgNPs) were characterized. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) techniques showed that monodispersed spherical shapes with mean diameters of 6-50 nm were formed and zeta potential analysis displayed (-16.7) mV with a single. The MIC of biosynthesized AgNPs for each bacteria *(Escherichia coli* and *Pseudomonas aeruginosa*) was determined. The concentration of 10 µg/ml of nano-silver completely inhibited the growth of all tested bacteria. The antibacterial effect of AgNPs on *E. coli and P. aeruginosa* showed a great potential effect of AgNPs than Ceftriaxone, while an increase of the inhibition zone diameter in the case of AgNPs antibiotic mixture had been investigated.

In conclusion, future therapeutic mechanism for multi-drug resistant pathogenic bacterial have a lot of potential for using silver nanoparticles as an efficient antibacterial agent.

keywords: MDR bacteria, *F. oxysporum*, Silver nanoparticles, Antibacterial activity**.**

1.Introduction

The expansion of antibiotic resistance is a growing menace today due to the indiscriminate use of antibiotics. It has resulted in the proliferation of selective pathogenic bacteria which are resistant to multiple drugs **[1].** There is a urgent demand for novel medications and alternatives because antibiotic resistance currently affects all types of natural and manmade chemicals. But due to the expense and complexity of drug discovery and development, very few novel antibiotics have been created in the last 40 years **[2]**. Numerous ways exist by which bacteria become resistant to antibacterial drugs, needing a novel strategy to create new bactericidal agent. Finding new antimicrobial agents or improving current ones to increase their antibacterial activity becomes essential **[3]**.

Nanotechnology provides a useful platform for modifying the physico-chemical properties of various materials compared to their bulk counterpart that can be harnessed for bio-applications **[4, 5].** Nanomedicine an offshoot of nanotechnology has taken a stride in the diagnosis, monitoring, drug delivery and control of diseasesNanoparticles have been created using physical and chemical processes **[6, 7]**. Basically, low yields have been a result of physical approaches, while environmental harm has been created by chemical methods due to the use of toxic solvents and the regeneration of dangerous byproducts **[8]**. The use of biosynthetic techniques as an alternative to chemical and physical techniques has been studied. Nanoparticles produced through biosynthesis are inexpensive, dependable, and biocompatible. There have been reports of many fungus genera producing metal nanoparticles **[9]**.

Silver nanoparticles could prove to be an alternative to antibiotics and to control microbial infections caused by MDR organisms **[10].** The usage of silver nanoparticles (AgNPs) is widespread in a wide range of industries, including biolabeling, sensors, antimicrobial agents, filters, microelectronics, and catalysis. This is a result of their distinct physico-chemical and biological characteristics **[11]**. The growth of bacteria, viruses, and other eukaryotic microorganisms is inhibited by AgNPs **[12]**. In addition to their unique qualities, their production costs are relatively modest **[13].** The toxicities of humans and animals may be lower for silver nanoparticles than for synthetic fungicides **[14, 15].**

The green methods for the synthesis of AgNPs include extracts from algae, plants, and microorganisms like bacteria and fungi **[16]**. It is also acknowledged that, in contrast to the use of plant extracts and bio macromolecules as reducing and capping agents, the synthesis of AgNPs by microbes necessitates a great deal of care and attention primarily due to the difficulty in microbial growth, maintaining the microbial culture, and standardising the inoculum sizes. The incubation of isolated or genetically altered bacteria in a culture medium made up of Luria-Bertani Broth (LB), malt extract, glucose, yeast extract, and peptone (MGYP) is a common procedure for the manufacture of AgNPs from microbes. The biomolecules included in the culture, including peptone, yeast extract, dextrose, and other essential growth agents, also have strong stabilising and reducing abilities. The synthesis of the AgNPs might be carried out using the microbial biomass removed after being discarded the used media from the cell culture, or alternatively, by using the used media (with or without the microbes) treated with silver salts **[17]**. Numerous researchers have examined the antibacterial activity of silver nanoparticles against pathogenic, MDR, and multidrug susceptible strains of bacteria. It has been demonstrated that silver nanoparticles are effective weapons against MDR bacteria like *Pseudomonas aeruginosa* and *Escherichia coli*.

With *Fusarium oxysporum*, silver nitrate was biologically reduced to form silver nanoparticles (AgNPs), which were then characterised. Testing the stability and antibacterial activity of the generated AgNPs against multidrug resistant bacteria was one of the our items.

2. Materials and methods

Isolation of *Fusarium oxysporum:*

Endophytic fungus *F. oxysporum* was provided from Mycological unit, Faculty of science, Mansoura University to test for the synthesis of AgNPs. Isolate was cultured and purified on PDA medium (potato dextrose agar). Place (5–6 disc) on PDA media (potato extract 200 ml, glucose 20g, agar 15g, distilled water up to 1L) at 25°C for five days to allow the fungus to form, then move fungus to new PDA media for seven days at 25°C. In order to use the isolated fungus in the biosynthesis process then it was kept at 4 °C **[18]**.

Collection samples of multidrug resistant bacteria:

Multidrug-resistant bacteria ,i.e., (Escherichia coli and Pseudomonas aeruginosa) employed in this work, were donated by the Clinical Microbiology Laboratories, Faculty of Medicine, Mansoura University, Eygpt. In clinical situations, the isolates were tested using the disc diffusion method against various classes of antibiotics to determine their antibiotic resistance **[19]**.

Biosynthesis of AgNPs by *Fusarium oxysporum:*

Biomass of *F. oxysporum* was obtained by culturing 6 mm fungal discs in 500 ml Erlenmeyer flasks, which contains 3 fungal discs in 100 ml of MGYP media (Malt extract 3g, Glucose 10g, Yeast extract 3g and Peptone 5g) at 28°C and 180 rpm for 72 hr. Filtration was used to remove the fungal mycelia, which were then thoroughly cleaned 3 to 5 times using sterilized distilled water. 20g of the fungal biomass in 100 ml sterilized water was incubated at the same previous condition for 24 h. after filtration with Whitman filter paper. The cell-free filtrate is collected then 50 ml of $AgNO_3$ solution (1 mM $AgNO_3$ concentration) was added to cell-free filtrate and stirred constantly. Control (without silver ions) was run along with the experimental flasks. The ability of the fungi to produce silver nanoparticles was assessed visually based on the reaction mixture's colour change **[20, 21]**.

Characterization of AgNPs

Ultravioletvisiblespectrophotometeranalysis:

The formation of the reduced AgNPs in colloidal solution was detected using a UV-vis spectrophotometer, and baseline correction was done using distilled water (Unicam UV-VIS. Spectrometer UV2, U.S.A). The supernatants' absorption spectra were measured between 300 and 700 nm, with the nano-silver-specific absorption band 400-450 nm included **[22]**.

Scan Electron microscope (SEM):

AgNPs fine powder was utilised. Using a SEM microscope (Nova Nano-SEM 450**, USA)** and an accelerating voltage of 20 kV, the images were captured. Energy dispersive analysis of X-ray (EDAX) spectrometer equipped with a SEM was used to analyse the elemental composition of AgNPs **[21]**.

Transmission electron microscopy (TEM):

Samples of the synthesized nano-silver were characterized by TEM. Standard copper with a carbon coating TEM grid was used (Type G 200, 3.05 µ diameter, TAAP, U.S.A). The sample was using TEM (JEOL JEM-2100) working at 160 k.v. Several images were taken to obtain sufficient representations of shape and size of the synthesized nano-silver particles**[23]**.

Zeta potential study:

To determine the stability of nano-colloidal solutions, a zeta potential analyzer **(Malvern Zeta size Nano-zs90, U.S.A.)** was utilised. The magnitude of the zeta potential represents the strength of the electrostatic attraction between neighbouring, similarly charged particles and the surface charge of AgNPs. Zeta potential analysise is used to describe the stability of colloidal particles in solution. Size distribution homogeneity is measured by the poly dispersity index (PDI), which has a value between 0 and 1. As the homogeneity of the nanoparticle size distribution decreases, the PDI value increases **[24]**.

Determination of Minimum inhibitory concentration (MIC) of biosynthesized AgNPs:

To determine the lowest concentration that can completely inhibit the visible growth of Gram-negative bacteria *(E. coli* and *P. aeruginosa)* were treated with increasing concentrations of AgNPs (2.5 µg/ml, 5 µ/ml and 10 µ/ml) and incubated for 24 h at 37 ◦C. The effect of different concentrations of nanosilver precipitated on the growth (OD_{600}) of these bacteria was estimated.

AntibacterialactivityofbiosynthesizedAgNPs:

The antibacterial assays were done against multidrug-resistant bacteria such as *E. coli* and *P. aeruginosa* by disc diffusion method to check the antibacterial activity of the AgNPs according to minimum inhibitory concentration (MIC) assays. Nutrient agar medium plates were prepared, sterilized, and solidified. After solidification, bacterial cultures were swabbed on these plates. Filter paper discs of silver nanoparticles solution only, Ceftriaxone antibiotic only and mixture AgNPs with Ceftriaxone antibiotic were placed in the nutrient agar plate and kept for incubation at 37◦C for 24 h. Zones of inhibition were measured.

3. Results

Microorganism and cultural conditions:

The tested fungus for producing nano-silver was identified morphologically as ***F. oxysporum***. This fungus was cultivated and maintained on Potato Dextrose Agar (PDA) at 25°C for 7 days and then kept at 4°C. **Fig.1.** Microscopic examination of ***F. oxysporum*** grown on PDA showing the macro-and microspores. Macroconidia (spores) of a ***F. oxysporum*** showing the characteristic foot cell. Macrospore bearing conidiophore **Fig.1.**

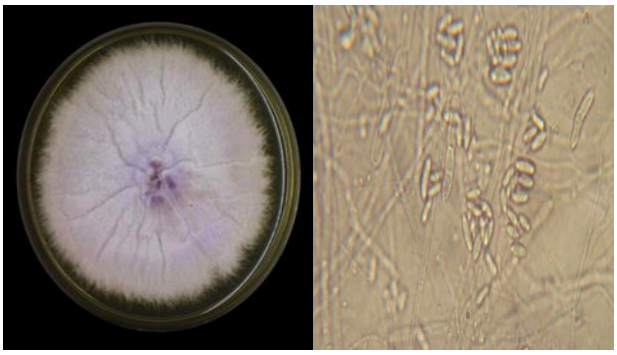

Fig. 1: ***F. oxysporum*** grown on Potato Dextrose Agar medium and its picture under the microscope.

Biosynthesized of AgNPs:

The tested endophytic *F. oxysporum* was cultured in Maltose Glucose Yeast Peptone (MGYP) broth medium for production of

fungal biomass. Comparing the fungal biomass's growth to that of the control flask's (MGYP growth medium), which is yellowish, reveals that the fungus is growing in a reddish color in **Fig.2 (before and after).** After incubation of *F. oxysporum* biomass with dionized water and further incubation of cell free filtrate with 1 mM silver nitrate ($AgNO_3$), the filtrate changed to dark brown color after 72 h **in Fig.3.** This indicates the gradual reduction of silver ions into silver nanoparticles. Then to make characterization of silver nanoparticles, we must convert the filtrate to powder by centrifugation at different speeds (4000 and 14000) rpm by using acetone as shown in **(Fig.4).**

Fig. 2: The growth of *F. oxysporum* in MGYP medium before (B) and after (A) development of mycelial mats at 72 h with continuous agitation then the fungus have a characteristic reddish color. **(B)** is the control flask (MGYP medium without inoculum)**, (A)** is the flask with biomass.

Fig. 3: The fungal cell-free filtrate after incubation with silver nitrate for 72 h changed to dark brown color which indicated to present silver nanoparticles.

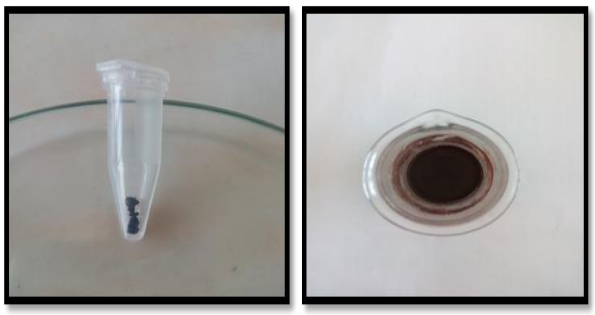

Fig.4: Purification of Silver nanoparticles

Characterization of AgNPs:

1. Ultraviolet-Visible Spectral Studies:

The characteristic peak at 434 nm was observed in **Fig. 5** after the nano solution was subjected to UV-VIS spectrum investigations for screening to the best absorbance peak in the nano-silver specific range (300 - 500 nm). Conduction band electrons create significant oscillations at this distinctive wave-length, which gives the brown colour that distinguishes nanoparticles from macroparticles.

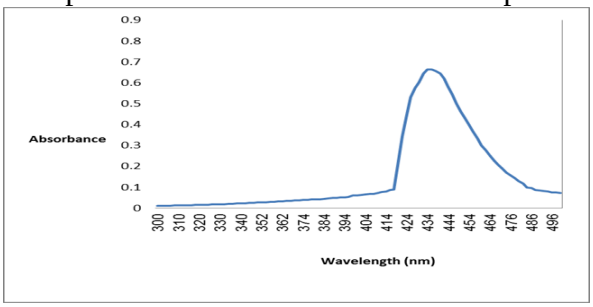

Fig. 5: UV-Vis spectrum scan (from 300 -500 nm) of AgNPs indicate the characteristic peak at 434 nm.

2. Scanning Electron Microscopy of AgNPs:

SEM was used to determine the crystallinity, size, and form of the nanoparticles; the silver nanoparticles were spherical to oval in shape and less than 100 nm in size, as shown in **Fig6**.

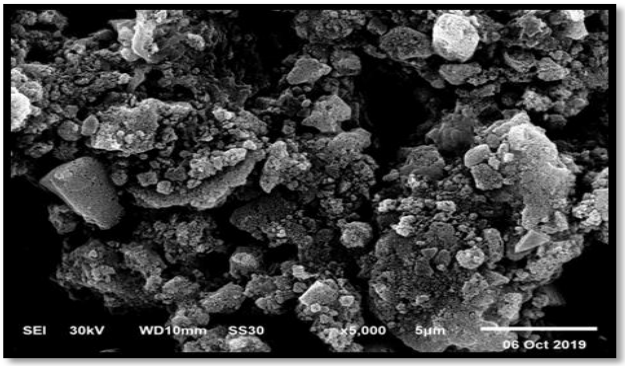

Fig. 6: SEM micrograph of AgNPs were spherical or oval shape size (less than 100 nm).

3. Transmission Electron Microscopy (TEM) :

TEM image demonstrates the presence of both individual and tiny aggregated particles in the range (6 - 50 nm); the capping agent that surrounds protein-made nanoparticles keeps them from contacting one another even within aggregates. The repulsion force between the particles is caused by this capping agent. Most of the AgNPs visible in the micrograph were spherical or oval in shape as shown in **Fig. 7.**

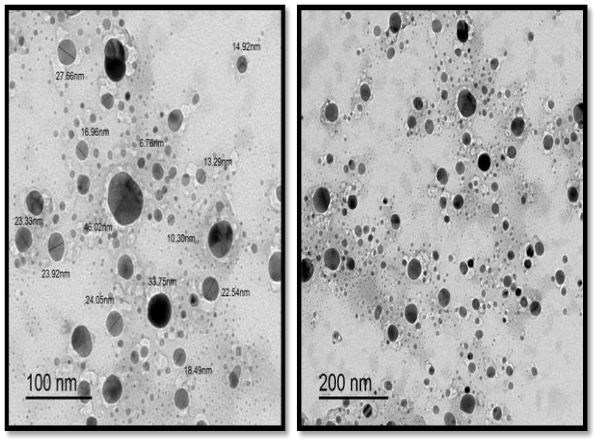

Fig. 7: TEM micrograph of AgNPs, this image showed spherical or oval shapes of AgNPs with average size (6 - 46 nm).

4. Zeta potential analysis:

Zeta potential analysis is used to measure the stability and surface charge of a nano-colloidal solution, the zeta potential measurement of the synthesized AgNPs in **Fig. 8**. The value of zeta potential of AgNPs -16.7 mV indicates the stability of the nano-silver. In addition, the conductivity, which is 0.169, indicates the stability and homogeneity of the resulting AgNPs.

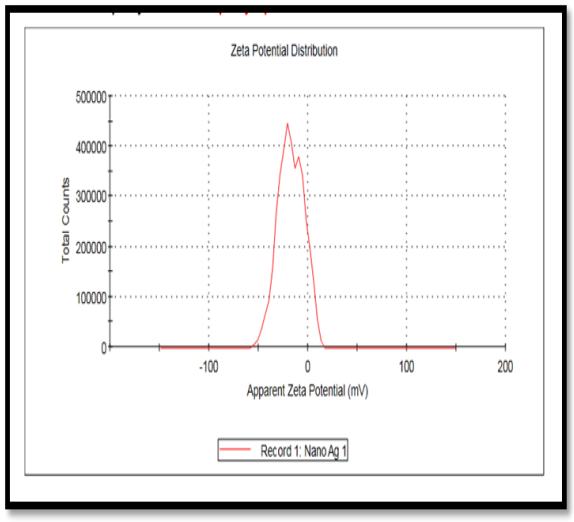

Fig. 8: Zeta potential of AgNPs

4. Energy dispersive analysis of X-ray (EDAX):

The EDAX spectrum recorded in the spot-profile mode provides the qualitative and quantitative status of elements that may be implicated in the formation of AgNPs **(Fig. 9)**. The optical absorption peak is noticed at 3.0 KeV, which is typical for the absorption of metallic AgNPs. The silver atoms are showing strong signals. From the EDAX spectrum, it is clear that nano-silver reduced by *F. oxysporum* has the weight percentage of silver as 67.7 % as shown in **Table (2).**

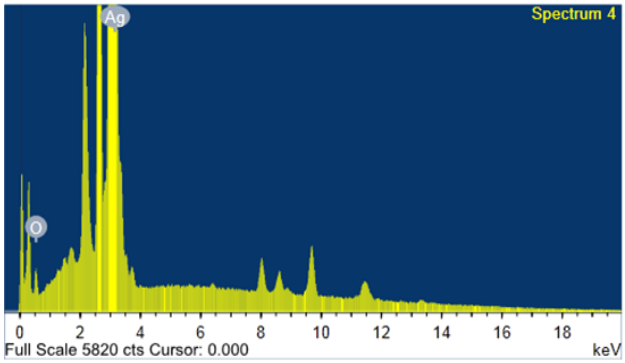

Fig. 9: EDAX spectrum of AgNPs.

Table 2: The element composition of the AgNPs of EDAX spectra

Elment	Weight%	Atomic%
O	7.04	42.82
Ag	78.4	70.76

Determination of AgNPs MIC its and antibacterial activity:

The multidrug-resistant bacteria ,i.e., (*E. coli* and *P. aeruginosa*) which used in this study. The antibiogram, often known as an antibiotic sensitivity test, was carried out using the Kirby Baure disc diffusion method **[19].** The isolates, recorded resistance to tested antibiotics ,i.e., (Ceftazidim, Cefaclor, Meropenem, Trimethoprim, Ampicillin/sulbactam, Imipenem, Ciprofloxacin and Amikacin) as shown in **Fig. 10**. The effect of different concentrations of AgNPs precipitated on the growth (OD_{600}) of these bacteria is represented in **Table (3).** All investigated bacteria' growth was fully inhibited by the AgNPs precipitate at a concentration of 10 µg/ml. On the other hand, the two other tested concentrations of AgNPs (5 µg/ml and 2.5 µg/ml) did not completely

stop the growth of the examined organisms **Fig. (11 and 12)**. By disc diffusion method to check the antimicrobial activity of the AgNPs according to the result of MIC assays and Comparison of Ceftriaxone antibiotic. The results of the inhibition zone study showed in **Table (4) and Fig. 13.** The AgNPs have a great potential effect than Ceftriaxone as an antibacterial agent for *E. coli* and *P. aeruginosa*. It was found that the increased inhibition zone diameter in the case of AgNPs with an antibiotic mixture.

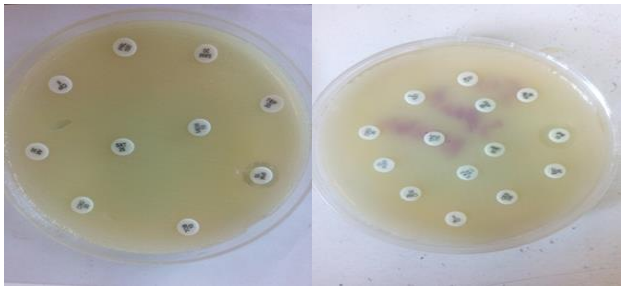

Fig. 10: Multidrug-resistant bacteria (*E. coli* and *P. aeruginosa*) with different antibiotic disks.

Table 3: Showed the MIC of AgNPs on microbial growth.

MDR bacteria	(MIC) µg/ml
Pseudomonas aeruginosa	**10 µg/ml**
Escherichia coli	**10 µg/ml**

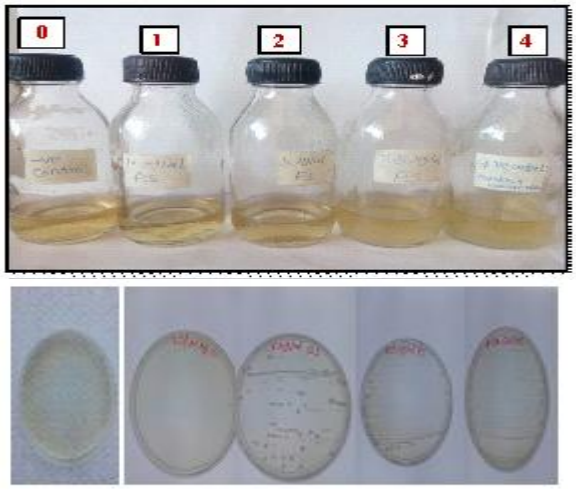

Fig. 11: MIC of nano-silver on *P. aeruginosa* grown in LB broth media and LB medium plates : number (0) is media only (-ve control), number (1) is the concentration of nanosilver 10µg/ml , number (2) is the concentration of nano-silver 5 µg/ml , number (3) is the concentration of nano-silver 2.5 µg/ml and number (4) is the media with *Pseudomonas aeruginosa* only without nano-silver.

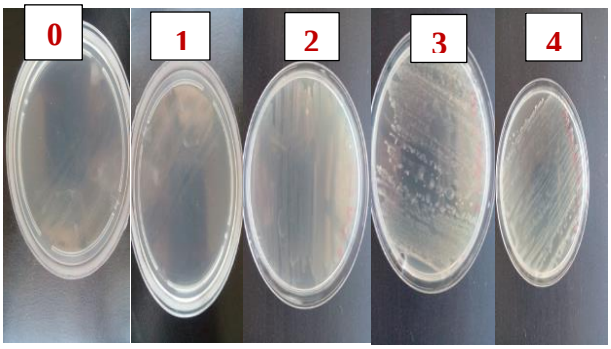

Fig. 12: MIC of nano-silver on *E. coli* grown in LB broth media and LB medium plates: number (0) is media only (-ve control), number (1) is the concentration of nano-silver 10µg/ml , number (2) is the concentration of nano-silver 5 µg/ml , number (3) is the concentration of nano-silver 2.5 µg/ml and number (4) is the media with *E. coli* only without nano-silver.

Table (4): The inhibition zone of AgNPs and antibiotic

Strain	Mean zone of inhibition (mm)		
	AgNPs	Antibiotic (Ceftriaxone)	AgNPswith Antibiotic
Pseudomonas aeruginosa	15	5	22
Escherichia coli	13	1	16

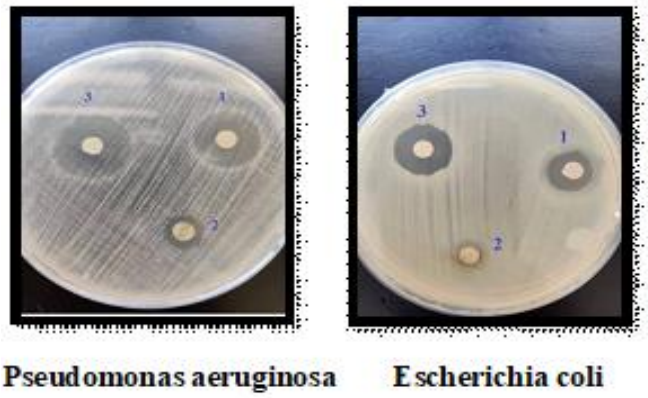

Fig. 13: Antibacterial activity of AgNPs in comparison to Ceftriaxone antibiotic on muller hinton agar by disc diffusion method for multidrug-resistant bacteria (*P. aeruginosa* and *E. coli*). Number (1) is silver nanoparticles (AgNPs), number (2) is Ceftriaxone and

number (3) is a mixture of (AgNPs) with Ceftriaxone.

4. Discussion

Multidrug-resistance has emerged as a severe problem that affects the treatment of infectious diseases brought on by pathogenic bacteria globally **[25].** This is mostly because antibiotics are used without restriction in animal medicine, agriculture, and human healthcare. **[26].**

Researchers are concentrating on the creation or discovery of new agents in light of these issues. Metal nanoparticles have been shown to have potential as antibacterial agents in recent literature. However, using a green production method can enhance the functional characteristics of metal nanoparticles. Given that it is more environmentally benign than other methods of nanoparticle synthesis, biological synthesis is attracting enormous attention **[27].** Although physical and chemical procedures can create nanoparticles of a specific size and form, their use is restricted due to the use of dangerous materials and their lower economic viability **[28].** Numerous researchers have examined the bactericidal efficacy of silver nanoparticles against pathogenic, MDR, and multidrug-susceptible types of bacteria. It has been demonstrated that silver nanoparticles are effective weapons against MDR pathogens including *Pseudomonas aeruginosa* and *Escherichia coli* **[29].**

In this research work, the biosynthesized AgNPs as the activity of *F. oxysporum* extract was thoroughly investigated. Visual observations revealed a change in colour to dark brown in the fungal cell-free filtrate with silver nitrate solution as the silver nitrate ions were reduced during exposure to the cell filtrate of *F. oxysporum*. In culture supernatant treated with silver nitrate, a brownish color suggested the development of silver nanoparticles. Three Trichoderma species, *T. longibrachiatum, T. viride*, and *T. harzianum*, were employed in another study to produce stable AgNPs through the biosynthetic process. Each fungal isolate's filtrates were incubated with $AgNO_3$ and kept stirring at 28 °C in the dark. With *T. harzianum* and *T. viride*, a colour change from pale yellow to brown appeared after a 72h incubation period, but there was no colour change with *T. longibrachiatum*. The emergence of the brown hue was a sign that AgNPs were forming in the media **[30] [31]**. UV-visible spectroscopy was used in this study to analyse the generated AgNPs and their stability; this method has been demonstrated to be particularly effective for the investigation of nanoparticles. A high, broad peak was observed between 300 and 500 nm in the UV-VIS absorption spectra, with the distinctive peak being measured at 434 nm. Another study found a peak at 420 nm in the UV-visible spectrum of the production of silver nanoparticles utilising the fungus *T. harzianum* and *T. viride* **[32].** Also, a significant, broad peak was seen in the UV-vis absorption spectra between 430 and 450 nm and 400 and 430 nm for the silver nanoparticles made from *Kluyveromyces marxianus* and *Candida utilis*, respectively. This is very specific for silver nanoparticles **[33, 34].** The surface morphology of produced nanoparticles was examined using SEM. SEM image of silver nanoparticles created with *F. oxysporum* at 5,000x magnification. Silver nanoparticle is almost spherical shape of size (less than 100 nm). There are several magnifications of SEM images displayed. It is clear that the core particles had spherical forms and varied in size **[35]**. Recent results from a transmission electron-micrograph of nanoparticles revealed their distinct size and form. In this micrograph, individual and aggregated particles are present in the range (6 - 50 nm). The black field was illuminated by the AgNPs particles' diffraction pattern. This diffraction pattern confirms the synthesis of AgNPs because it reflects the crystalline structure of AgNPs and its diffraction rings. In another study, The TEM picture makes it abundantly evident that the AgNPs were spherical in shape. The image supports the SEM findings by displaying clusters of tiny grains and a few dispersed nanoparticles. The synthesized AgNPs were in the range of 10–30 nm. Also ,the selected area diffraction pattern confirms the face-centered cubic crystalline structure of AgNPs **[36] [37].**

To determine the stability and surface charge of a nano-colloidal solution, a zeta potential analyzer is used. In this study, the value of zeta potential of AgNPs -16.7 mV indicates the stability of the AgNPs. Additionally, the

conductivity, which is 0.173, shows that the produced AgNPs are stable and homogeneous. In a different study, it was shown that the zeta potential of the biosynthesized AgNPs had a high peak at -7.66 mV. It is assumed that the surfaces of the nanoparticles are negatively charged and have spread throughout the fluid. Its negative result reveals that the particles are stable and are drawn to one another **[38] [39]**. EDAX spectrum gives the optical absorption peak is observed at 3.0 KeV, which is typical for the absorption of AgNPs. High signals from the silver atoms were observed, from the EDAX spectrum, it is clear that nano-silver reduced by *F. oxysporum* have the weight percentage of silver as 67.7%. Similar findings have previously been published, and the creation of silver nanoparticles was in the 3.0 keV range and displays a typically large signal peak **[40, 41].**

In the current experiment, the antibacterial activity of synthesized AgNPs was examined on bacteria such *E. coli* and *P. aeruginosa* that are multi-drug resistant. According to Minimum inhibitory concentration (MIC) assays; all investigated bacterial growth was fully stopped at a concentration of 10 g/ml of nano-silver precipitate. However, the growth of the bacteria could not be completely inhibited by the two other tested concentrations of nano-silver (5 g/ml and 2.5 g/ml). The results of the inhibition study showed that AgNPs have great potential effect than Ceftriaxone and it was found that the increased of the inhibition zone diameter in the case of AgNPs with antibiotic mixtures. According to other studies, the synergistic effect that produces AgNPs and ampicillin complex causes the ampicillin molecule to pair on the surface of AgNPs. The AgNPs improved the inhibitory impact against all ampicillin-resistant bacteria, which made it clear that the test bacteria were resistant to the antibiotic when ampicillin alone had minimal inhibitory effect on them. However, it was discovered that all bacteria were, to variable degrees, sensitive to AgNPs up to a level of 20 mg/ml. A small increase in the inhibition zone of 2-3 mm caused by the addition of ampicillin in the presence of AgNPs improved the inhibitory activity against all the tested bacteria. When compared to *P. aeruginosa*, *K. pneumoniae*, and *E. coli*, among the examined bacteria, *Enterobacter sp*. was found to be more sensitive and have a bigger inhibition zone **[42, 43]**.

In conclusion, a future strategically therapy for multidrug resistant pathogenic bacterial diseases have a lot of potential for using silver nanoparticles as an efficient antibacterial agent

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